SPECIAL GUEST EDITOR SECTION

Multimycotoxin Analysis by LC-MS/MS in Cereal Food and Feed: Comparison of Different Approaches for Extraction, Purification, and Calibration

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Twelve different approaches commonly used for the simultaneous LC tandem MS (MS/MS) determination of mycotoxins (deoxynivalenol, aflatoxins, ochratoxin A, T-2 and HT-2 toxins, fumonisins, and zearalenone) were tested in cereals and feed materials. They comprised different extraction solvents, types of cleanup [solid-phase extraction, QuEChERS, and immunoaffinity (IMA)], and calibration approaches (external or matrix-matched). The percentage of mycotoxins with acceptable recovery, according to Regulation (EC) No. 401/2006, ranged from 9 to 100%. The approach giving the highest percentage of acceptable results was selected and further tested for corn, rice, and feed spiked at three different mycotoxin levels (low, medium, and high). The method is based on extraction with MeOH–water (70 + 30, v/v) and cleanup with two multiantibody IMA columns. For corn and rice spiked at low mycotoxin levels, a significant matrix effect was observed and was compensated by using ^{13}C calibration. At higher mycotoxin levels (medium and high), matrix effects were negligible as no significant differences were observed for the majority of recovery results calculated by ^{13}C calibration and external calibration. Although the proposed method still needs improvement in terms of accuracy and, to a lesser extent, precision, it was successfully tested with four proficiency tests in buckwheat, corn, rice, and feed, giving acceptable *z*-scores for 97% (34 out of 35) of results.

The role of mycotoxins in the life cycle of fungal species that produce these toxic secondary metabolites is not completely known, but their effects on human and animals health are serious and pose a major concern when present in food and feed. Moreover, they are often responsible for financial losses in food production and livestock breeding (1, 2).

Among the regulated mycotoxins, aflatoxins B_1 (AFB_1), G_1 (AFG_1), B_2 (AFB_2), and G_2 (AFG_2); ochratoxin A (OTA); fumonisins B_1 (FB_1) and B_2 (FB_2); deoxynivalenol (DON); T-2 and HT-2 toxins (T-2 and HT-2); and zearalenone (ZEN) are those that are most commonly found in cereals and cereal products (3). Considering the different physicochemical characteristics of the different groups of mycotoxins, LC coupled with MS is the only technique that can analyze all of them in a single run. Despite this positive characteristic, this analytical instrumentation is particularly prone to the so-called "matrix effect," which consists in the suppression or enhancement of the signal of an analyte due to alteration of ionization efficiency as a result of the presence of coeluting substances (4). Matrix effect can affect quantitative results because of the different signal intensities of mycotoxins in unknown samples compared with the calibration solution in the solvent (5). To compensate for this effect, a matrix-matched (MM) calibration or ^{13}C calibration can be used for quantitation. MM calibration is frequently questioned because of the difficulty to find a perfect blank matrix representative of each commodity. This problem is particularly important in animal feed analysis due to the large inhomogeneity in the composition of such matrixes. No official or standard methods for the simultaneous determination of all regulated mycotoxins in food and feed are currently available, although numerous LC tandem MS (MS/MS) methods have been published in the last decade (4–13). The first aim of this work was the evaluation, according to Regulation (EC) No. 401/2006 (14), of currently available methods for the simultaneous determination of regulated mycotoxins in cereals. For this purpose, 12 analytical methods were compared that differed in (*1*) extraction, i.e., the solvent used and the protocol (single/repeated extraction); (*2*) purification strategy, including immunoaffinity (IMA), solid-phase extraction (SPE), and QuEChERS, as well as "extract and shoot" and "extract, concentrate, and shoot" approaches; and (*3*) calibration, i.e., the exploitation of external (E-) and MM calibration. The second aim of this work was to select and in-house validate an LC-MS/MS method for the determination of regulated mycotoxins in cereal food and feed by using E- and/or ^{13}C calibration.

Experimental

Chemicals

Acetonitrile (ACN), methanol (MeOH), glacial acetic acid, formic acid, and *n*-hexane were purchased from VWR

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International srl (Milan, Italy). Ultrapure water was produced with a Milli-Q system (Millipore, Bedford, MA). The standard solutions were prepared as follows: DON (100.5 μg/mL) in ACN (Sigma-Aldrich srl, Milan, Italy); T-2 (100.6 μg/kg) and HT-2 (100.4 μg/kg) in ACN; FB_1 (50.3 μg/mL), FB_2 (50.1 μg/mL), and FB_3 (50.1 μg/mL) in ACN–water (50 + 50, v/v; Romer Laboratory Diagnostic, Tulln, Austria); AFB_1 (0.9 μg/mL), AFG_1 (0.9 μg/mL), AFB_2 (0.3 μg/mL), and AFG_2 (0.3 μg/mL) in benzene–ACN (98 + 2, v/v); OTA (52 μg/mL) in benzene–acetic acid (99 + 1, v/v; Sigma-Aldrich srl); and ZEN (100.2 μg/mL) in ACN (Libios SARL, Pontcharra-sur-Turdine, France). ^{13}C standard solutions were purchased from Romer Laboratory Diagnostic. In particular, solutions of [$^{13}C_{15}$]–DON (25 μg/mL), [$^{13}C_{17}$]–AFB_1 (0.5 μg/mL), [$^{13}C_{17}$]–AFG_1 (0.5 μg/mL), [$^{13}C_{17}$]–AFB_2 (0.5 μg/mL), [$^{13}C_{17}$]–AFG_2 (0.5 μg/mL), [$^{13}C_{24}$]–T-2 (25 μg/mL), [$^{13}C_{22}$]–HT-2 (25.5 μg/mL), [$^{13}C_{20}$]–OTA (10 μg/mL), and [$^{13}C_{18}$]–ZEN (25 μg/mL) were prepared in ACN, whereas [$^{13}C_{34}$]–FB_1 (25 μg/mL), [$^{13}C_{34}$]–FB_2 (10 μg/mL), and [$^{13}C_{34}$]–FB_3 (10 μg/mL) were prepared in ACN–water (50 + 50, v/v). Filter paper and a GF/A glass microfiber filter were purchased from Whatman (Maidstone, United Kingdom). Ammonium acetate (for MS) and phosphate-buffered saline (PBS) tablets were purchased from Sigma-Aldrich srl. For QuEChERS extraction, sodium chloride and magnesium sulfate were obtained from Sigma-Aldrich srl, trisodium citrate dehydrate was purchased from VWR (Dublin, Ireland), disodium hydrogen citrate sesquihydrate was obtained from Acros (Milano, Italy), and primary secondary amine (PSA) and C18 were purchased from Agilent Technologies (Waldbronn, Germany). Myco6in1 IMA columns were purchased form VICAM LP (Watertown, MA). AOF-MS-PREP and DZT-MS-PREP IMA columns were purchased from R-Biopharm AG (Darmstadt, Germany). Isolute Myco 60 mg/3 mL SPE columns were purchased from Biotage Italy srl (Milan, Italy). PTFE filters (0.45 μm) were purchased from Sartorius Stedim Biotech (Goettingen, Germany).

LC-MS/MS Equipment and Parameters

LC-MS/MS analyses were performed with a triple quadrupole API 3200 QTrap system (Applied Biosystems, Foster City, CA), equipped with an electrospray ionization (ESI) interface and a 1200 series HPLC system comprising a quaternary pump and an autosampler (Agilent Technologies). Chromatographic separations were performed with a Hypersil GOLD column (100 × 2.1 mm; 5 μm particle size; Thermo Fisher Scientific) thermostated at 30°C. The flow rate of the mobile phase was 500 μL/min, and the injection volume was 20 μL.

Mobile phase A and B were water and MeOH, respectively, both containing 0.5% acetic acid and 1 mM ammonium acetate. A 7 min linear gradient program was set up as follows: 1.2 min at 15% eluent B, linearly increasing the proportion to 65% in 0.8 min, and maintained isocratic for 5 min. The column was re-equilibrated with 15% eluent B for 5 min. The ESI interface was used in positive-ion mode and the system operated in multiple-reaction monitoring mode by monitoring two transitions (one quantifier and one qualifier). The optimized MS/MS conditions and retention time for each analyte are listed in Table 1. Each analyte was identified by the retention time and the ion ratio that were within the tolerance limits, i.e., ±0.2 min and ±30% (relative), respectively.

Samples

For preliminary experiments, the following materials were used: commercial cereal products based on corn and wheat, certified corn materials (blank Trilogy TR-F100 LOT F-C-400 and contaminated Trilogy TR-MT100 LOT MTC-9999E naturally contaminated with AFB_1, AFB_2, AFG_1, DON, OTA, ZEN, FB_1, FB_2, FB_3, T-2, and HT-2 and a blank), and cereal-based animal feed (FAPAS T04249 naturally contaminated with AFB_1, OTA, DON, and ZEN).

For full validation of the method based on the tandem IMA columns, the following materials were used: commercial cereal product based on rice; certified corn material (blank Trilogy TR-F100 LOT F-C-400); commercial feed; and proficiency testing materials for buckwheat (BIPEA 31b-146), corn (BIPEA 31b-147), rice (BIPEA 31b-150), and animal feed (BIPEA 31b-149) spiked with 8–12 mycotoxins.

Multitoxin Extraction Procedures

For the preliminary evaluation of the available methodologies for the analysis of the main mycotoxins (DON, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, FB_3, HT-2, T-2, OTA, and ZEN) in cereal food and feed, the 12 methodologies summarized in Table 2 were tested.

Methods Based on Solid-Phase Extraction Cleanup (Methods A, B, and C)

The methods based on Biotage application notes (15, 16) were tested. Briefly, 5 g sample were extracted with 20 mL ACN–water (50 + 50, v/v) for 30 min and centrifuged; 8 mL supernatant were diluted with 32 mL water and purified with an SPE column (Isolute Myco 60 mg/3 mL; Biotage). Three milliliters of diluted extract were purified in the Isolute Myco column. The SPE column was washed with 3 mL water and 3 mL ACN–water (10 + 90, v/v) and eluted with 2 mL ACN 0.1% formic acid and 2 mL MeOH. The purified extract was evaporated to dryness, and the residue was reconstituted with 250 μL MeOH–water (50 + 50, v/v), filtered, and analyzed by LC-MS/MS (method A). DON was not included in the panel of mycotoxins analyzed with this method according to Application Note AN782 (15). This procedure was modified by consecutively extracting the sample with water and ACN–water (50 + 50, v/v), and the two extracts were separately purified on two SPE columns (Isolute Myco 60 mg/3 mL; Biotage). The purified extracts were collected from the two SPE columns, combined, and analyzed by LC-MS/MS (method B) or collected separately from the SPE column and analyzed separately by LC-MS/MS (method C). Methods B and C were derived by merging the protocols described in two Biotage application notes (15, 16). E-calibration was used for the three methods.

Methods Based on "Extract and Shoot" (Methods D and L)

A method based on ref. 17 was tested. Briefly, 5 g sample were extracted with 20 mL ACN–water (80 + 20, v/v), and 1 mL filtered extract was evaporated to dryness, reconstituted with 250 μL ACN–water (20 + 80, v/v), filtered, and analyzed by LC-MS/MS (method D). Both E- and MM calibrations were used.

Table 1. LC-MS/MS conditions for the detection of mycotoxins by the multiple-reaction monitoring method

Analyte	RT, min	*m/z* precursor ion (Q1)[a]	*m/z* product ions (Q3)[b]	DP, V[c]	EP, V[d]	CE, V[e]	CXP, V[f]
Deoxynivalenol	2.09	297	249,[g] 203	43	4.3	15, 18	3.7, 2.4
[$^{13}C_{15}$]–deoxynivalenol	2.09	312	263,[g] 216	45	5.3	14, 20	5.4, 2.1
Aflatoxin G_2	4.15	331	313,[g] 245	72	6.5	27, 40	4.3
[$^{13}C_{17}$]–aflatoxin G_2	4.15	348	330,[g] 259	72	6.5	27, 40	4.3
Aflatoxin G_1	4.15	329	243,[g] 215	64	6.0	35, 40	3.1, 3.7
[$^{13}C_{17}$]–aflatoxin G_1	4.15	346	257,[g] 226	64	6.0	35, 40	3.1, 3.7
Aflatoxin B_2	4.31	315	259,[g] 287	70	5.0	37, 34	3.4, 4.2
[$^{13}C_{17}$]–aflatoxin B_2	4.30	332	273,[g] 301	70	5.0	37, 34	3.4, 4.2
Aflatoxin B_1	4.39	313	241,[g] 213	72	3.8	48, 56	2.5, 2.6
[$^{13}C_{17}$]–aflatoxin B_1	4.38	330	255,[g] 227	72	3.7, 3.8	48, 56	2.5, 2.6
HT-2 toxin	4.94	442	263,[g] 215	33	4.0	17, 23	2.2
[$^{13}C_{22}$]–HT-2 toxin	4.94	464	278,[g] 229	33	4.0	17, 23	2.2
Fumonisin B_1	4.98	723	334,[g] 352	87	11.0	56, 48	3.5, 3.9
[$^{13}C_{34}$]–fumonisin B_1	4.97	757	375,[g] 356	87	11.0	48, 56	3.9, 3.5
T-2 toxin	5.37	484	215,[g] 305	37	3.9	26, 23	2.3, 4.7
[$^{13}C_{24}$]–T-2 toxin	5.38	508	229,[g] 322	37	3.9	26, 23	2.3, 4.7
Fumonisin B_3	5.73	707	337,[g] 355	92	7.2	45	2.4
[$^{13}C_{34}$]–fumonisin B_3	5.71	741	359,[g] 377	92	7.2	45	2.4
Zearalenone	6.02	319	283,[g] 187	40	5.1	18, 26	4.6, 2.4
[$^{13}C_{18}$]–zearalenone	6.02	337	215,[g] 301	42	5.6	32, 17	2.4, 6.0
Ochratoxin A	6.15	404	239,[g] 221	43	5.1	30, 48	3.9, 2.9
[$^{13}C_{20}$]–ochratoxin A	6.13	424	250,[g] 232	43	5.1	30, 48	3.9, 2.9
Fumonisin B_2	6.94	707	337,[g] 319	92	7.2	45, 52	2.4
[$^{13}C_{34}$]–fumonisin B_2	6.95	741	359,[g] 341	92	7.2	45, 52	2.4

[a] Q1 = First quadrupole.

[b] Q3 = Third quadrupole.

[c] DP = Declustering potential.

[d] EP = Entrance potential.

[e] CE = Collision energy.

[f] CXP = Collision cell exit potential.

[g] Product ion used for quantitation.

A method based on ref. 17 was tested using MM calibration. Briefly, 5 g sample were extracted with 30 mL MeOH–water (80 + 20, v/v), and the extract was filtered and analyzed by LC-MS/MS (method L). Two laboratories (Laboratories 12 and 41) successfully used this method in the first LC-MS/MS proficiency test (18, 19).

Methods Based on QuEChERS (Methods E, F, G, H, and I)

A method based on EN 15662 (20) was tested for mycotoxins. Briefly, 5 g sample were extracted with 10 mL water; after a few minutes, 10 mL ACN were added and mixed for 20 min. Successively, sodium chloride, magnesium sulfate, trisodium citrate dehydrate, and disodium hydrogen citrate sesquihydrate were added and mixed. Successively, 0.5 mL dried ACN extract was purified by adding PSA, C18 material, and more magnesium sulfate. The purified extract was dried, reconstituted with 250 µL water–ACN (80 + 20, v/v), and analyzed by LC-MS/MS (method E). This procedure was modified by excluding PSA and C18 and maintaining sodium chloride, magnesium sulfate, trisodium citrate dehydrate, and disodium hydrogen citrate sesquihydrate (method F). In a further modification, 5 g sample were extracted with 10 mL water, followed by 10 mL ACN after a few minutes. After mixing for 20 min and centrifugation, 0.5 mL was evaporated to dryness, reconstituted with 250 µL water–ACN (80 + 20, v/v), and analyzed by LC-MS/MS (method G).

A method based on ref. 21 was also tested. Briefly, 2 g sample were extracted with 10 mL of 0.1% formic acid in deionized water. After 10 min, 10 mL ACN were added and shaken for 3 min. Four grams of magnesium sulfate and 1 g sodium chloride were added, and the mixture was shaken again and centrifuged. Subsequently, 1.25 mL organic phase was evaporated to dryness, reconstituted with 250 µL water–ACN (80 + 20, v/v), filtered, and analyzed by LC-MS/MS (method H).

A method based on ref. 22 was then tested. Briefly, 5 g sample were extracted with 10 mL water and, after a few minutes, 10 mL ACN 0.5% acetic acid were added, and the sample was shaken for 20 min. Four grams of magnesium sulfate and 1 g sodium chloride were added, and the sample was hand-shaken and then centrifuged. Five milliliters of supernatant were extracted by liquid–liquid extraction with 5 mL *n*-hexane. After centrifugation, the upper *n*-hexane phase was removed and 1 mL purified extract was evaporated to dryness, reconstituted

Table 2. Results of the recovery experiments using different LC-MS/MS methods for the determination of DON, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, and OTA in cereals (n = 2)

Method ID	Extraction	Purification	Calibration	Matrix	Toxins	No. of acceptable recoveries	Ref.
A	Extract with ACN–water (50 + 50, v/v) by shaking[a]	Isolute Myco polymeric SPE column, concentrate[b]	E[c]	Spiked corn	AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	6/11	Based on refs. 15 and 16
				Spiked wheat	AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	7/11	
B	Extract consecutively with water and ACN–water (50 + 50, v/v) by shaking	Clean up separately with 2 Isolute Myco polymeric SPE columns, inject the combined extracts	E	Spiked corn	DON, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	1/12	
				Spiked wheat	DON, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	5/12	
C	Extract consecutively with water and ACN–water (50 + 50, v/v) by shaking	Clean up separately with two Isolute Myco polymeric SPE columns, inject the two extracts separately	E	Spiked corn	DON, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	5/12	
D	Extract with ACN–water (80 + 20, v/v) by shaking, concentrate	No	MM and E[d]	NC corn (Trilogy)[e]	DON, AFB_1, AFB_2, AFG_1, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	2/11 4/11	Based on ref. 17
E	Extract with water and ACN by shaking, add four different salts	Clean up with PSA + C18 + $MgSO_4$, concentrate	MM and E	NC corn (Trilogy)	DON, AFB_1, AFB_2, AFG_1, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	1/11 1/11	Based on ref. 20
F	Extract with water and ACN by shaking, add four different salts, concentrate	No	MM and E	NC corn (Trilogy)	DON, AFB_1, AFB_2, AFG_1, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	4/11 1/11	
G	Extract with water and ACN by shaking, concentrate	No	E	NC corn (Trilogy)	DON, AFB_1, AFB_2, AFG_1, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	2/11	
H	Extract with acidic water and ACN by shaking, add $MgSO_4$ and NaCl, concentrate	No	MM and E	NC corn (Trilogy)	DON, AFB_1, AFB_2, AFG_1, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	6/11[f] 3/11	Based on ref. 21
I	Extract with water and acidic ACN by shaking, add $MgSO_4$ and NaCl	Clean up by partitioning with n-hexane, concentrate	MM and E	NC corn (Trilogy)	DON, AFB_1, AFB_2, AFG_1, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	3/11[g] 2/11[g]	Based on ref. 22
L	Extract with MeOH–water (80 + 20, v/v) by shaking[h]	No	MM	NC corn (Trilogy)	DON, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, T-2, HT-2, ZEN, OTA	9/10[g]	Based on ref. 17
M	Extract with PBS and MeOH by shaking[i]	Clean up with Myco6in1 multiantibody IMA column, concentrate[j]	MM	NC corn (Trilogy)	DON, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, T-2, HT-2, ZEN, OTA	6/10[g]	Based on ref. 23
N	Add water and slurry, extract with MeOH and NaCl by shaking	Clean up with AOF-MS-PREP and DZT-MS-PREP multiantibody IMA columns in tandem	E	NC corn (Trilogy)	DON, AFB_1, AFB_2, AFG_1, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	6/11[g]	Based on ref. 24
				Spiked blank corn (Trilogy)	DON, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, T-2, HT-2, ZEN, OTA	10/11[g]	
				Spiked NC animal feed (FAPAS)[k]	DON, AFB_1, FB_1, FB_2, ZEN, OTA	6/6[g]	

Table 2. (*continued*)

Method ID	Extraction	Purification	Calibration	Matrix	Toxins	No. of acceptable recoveries	Ref.
					DON, AFB_1, FB_1, FB_2, FB_3, T-2, HT-2, ZEN, OTA	8/9[g]	

[a] ACN = Acetonitrile.

[b] SPE = Solid-phase extraction.

[c] E = External calibration.

[d] MM = Matrix-matched calibration.

[e] NC = Naturally contaminated.

[f] n = 4.

[g] n = 5.

[h] MeOH = Methanol.

[i] PBS = Phosphate-buffered saline.

[j] IMA = Immunoaffinity.

[k] FAPAS = Food Analysis Performance Assessment Scheme.

in 150 µL MeOH–water (50 + 50, v/v), and centrifuged. Sixty microliters of extract were diluted with 140 µL water, mixed, centrifuged, and injected into the LC-MS/MS (method I). Both, E- and MM calibrations were used for these methods.

Methods Based on Multiantibody Immunoaffinity Column Cleanup (Methods M and N)

The method based on ref. 23 was tested. Briefly, 10 g sample were first extracted with 50 mL PBS. After centrifugation, 35 mL PBS extract (extract A) were filtered through a glass microfiber filter. Subsequently, 35 mL MeOH were added to the remaining solid material containing 15 mL PBS, and the sample was extracted again and centrifuged. After centrifugation, 10 mL MeOH–PBS extract were diluted with 90 mL PBS and filtered through a glass microfiber filter (extract B). Fifty milliliters of extract B were passed through a multiantibody IMA column (Myco6in1) and the column washed with 20 mL PBS, loaded with 5 mL extract A, and then washed with 10 mL water. The mycotoxins were eluted with 2× 1.5 mL MeOH. The methanolic eluates were combined, dried, and reconstituted with 200 µL MeOH–water (20 + 80, v/v) containing 1 mM ammonium acetate and 0.1% acetic acid, and then analyzed by LC-MS/MS (method M). MM calibration was used.

A method based on ref. 24 was also tested. Briefly, 5 g sample were slurried with 6 mL water, extracted with 14 mL MeOH and 1 g sodium chloride, and then centrifuged. Five milliliters of the supernatant were combined with 60 mL PBS, filtered through glass microfiber filter paper, and 20 mL passed through AOF-MS-PREP and DZT-MS-PREP IMAs columns connected in tandem. The columns were washed with 2× 10 mL water and the mycotoxins eluted with 1 mL MeOH, followed by 1 mL water, which was collected, combined, dried, and dissolved in 1 mL MeOH–water (50 + 50, v/v) and then filtered and analyzed by LC-MS/MS (method N). This method was preliminary tested on blank certified material spiked with 11 mycotoxins (Trilogy TR-F100), a certified material naturally contaminated with 11 mycotoxins (Trilogy TR-MT100), and a certified feed material (Food Analysis Performance Assessment Scheme) naturally contaminated with four mycotoxins (AFB_1, DON, ZEN, and OTA) and spiked with FB_1 and FB_2 or with FB_1, FB_2, FB_3, T-2, and HT-2. E-calibration was used.

This method was further validated for the 12 principal mycotoxins in three different matrixes (corn, rice, and feed) spiked at different mycotoxin levels (Table 3) and by participating in four proficiency tests organized by the Bureau Interprofessionnel des Etudes Analytiques for multimycotoxin determination in buckwheat, corn, rice, and feed (Table 4). For calibration, ^{13}C standards were used. In particular, 100 µL ^{13}C standard solutions containing [$^{13}C_{15}$]–DON (1.5 µg/mL), [$^{13}C_{17}$]–AFB_1 (0.025 µg/mL), [$^{13}C_{17}$]–AFG_1 (0.025 µg/mL), [$^{13}C_{17}$]–AFB_2 (0.006 µg/mL), [$^{13}C_{17}$]–AFG_2 (0.006 µg/mL), [$^{13}C_{24}$]–T-2 (0.006 µg/mL), [$^{13}C_{22}$]–HT-2 (0.006 µg/mL), [$^{13}C_{20}$]–OTA (0.012 µg/mL), and [$^{13}C_{18}$]–ZEN (0.127 µg/mL) were prepared in ACN, whereas [$^{13}C_{34}$]–FB_1 (0.253 µg/mL), [$^{13}C_{34}$]–FB_2 (0.250 µg/mL), and [$^{13}C_{34}$]–FB_3 (0.250 µg/mL) were prepared in ACN–water (50 + 50, v/v) and added to each calibration solution and to the sample extracts prior to LC-MS/MS analysis to compensate for any matrix effect. In addition, E-calibration was also used for quantitation, and the results obtained by using the two calibration approaches were statistically compared.

A SigmaPlot for Windows statistical software package (Version 12.0; Systat Software, Inc., Chicago, IL) was used for *t*-test calculation, and analyses were performed in quintuplicate. LOD and LOQ values were calculated from the calibration curves according to Miller and Miller (25): LOD = a + $3S_{y/x}$ and LOQ = a + 10 $S_{y/x}$, where a = intercept of the regression line; and $S_{y/x}$ = random errors in the *y*-direction.

The linearity of E-calibration and ^{13}C calibration curves obtained in the concentration ranges reported in Table 5 was assessed according to point C17 in Document No. SANTE/11945/2015 (26) and the *Eurachem Guide* (27).

Results and Discussion

Preliminary Study

The results obtained by using different approaches for the simultaneous determination of regulated mycotoxins in cereal

Table 3. Recovery and repeatability values (n = 5) for corn, rice, and feed spiked with mycotoxins at three different levels and quantitated using ^{13}C calibration

	DON	AFB_1	AFB_2	AFG_1	AFG_2	FB_1	FB_2	FB_3	T-2	HT-2	ZEN	OTA
Corn low level, µg/kg	101	0.50	0.17	0.47	0.14	201	200	200	5.0	5.0	20	1.25
Recovery, %	93[a]	74[a]	ND[b]	107[a]	ND	48	55	74[a]	70[a]	98[a]	53	89[a]
RSD_r, %[c]	6.3[a]	8.9[a]	ND	12.1[a]	ND	32.1	19.8[a]	8.6[a]	7.6[a]	16.3[a]	22.6[a]	24.6
Corn medium level, µg/kg	503	2.05	0.68	1.93	0.58	402	401	400	25.2	25.1	50	3.02
Recovery %	89[a]	66	77[a]	92[a]	95[a]	58	103[a]	122	60[a]	94[a]	63[a]	63
RSD_r, %[c]	5.3[a]	13.2[a]	9.9[a]	11.0[a]	19.5[a]	3.1[a]	12.8[a]	5.1[a]	23.6[a]	12.6[a]	20.5[a]	14.5[a]
Corn high level, µg/kg	1507	8.02	2.66	7.53	2.28	1609	1603	1600	100.6	100.4	200	11.99
Recovery, %	70[a]	73[a]	76[a]	66	75[a]	86[a]	89[a]	104[a]	73[a]	100[a]	69	80[a]
RSD_r, %[c]	4.1[a]	9.2[a]	11.3[a]	8.5[a]	4.6[a]	3.2[a]	6.8[a]	5.4[a]	9.7[a]	11.5[a]	6.0[a]	9.2[a]
Rice low level, µg/kg	101	0.50	0.17	0.47	0.14	201	200	200	5.0	5.0	20	1.25
Recovery %	92[a]	85[a]	ND	96[a]	ND	70[a]	60[a]	71[a]	66[a]	81[a]	75[a]	53
RSD_r, %[c]	7.5[a]	8.6[a]	ND	27.5[a]	ND	10.2[a]	10.8[a]	11.1[a]	16.3[a]	23.9[a]	13.0[a]	20.0[a]
Feed low level, µg/kg	181	2.05	0.68	1.93	0.58	1258	1253	1253	60.4	60.2	20	20.86
Recovery %	88[a]	66	96[a]	62	113[a]	89[a]	95[a]	115	76[a]	101[a]	46	83[a]
RSD_r, %[c]	7.6[a]	5.7[a]	15.1[a]	18.5[a]	25.5[a]	3.8[a]	1.4[a]	4.7[a]	17.9[a]	12.6[a]	32.3[a]	6.9[a]
Feed medium level, µg/kg	905	5.03	1.67	4.73	1.43	2515	2505	2505	120.7	120.5	100	52.14
Recovery %	79[a]	78[a]	62	86[a]	95[a]	89[a]	94[a]	117	76[a]	106[a]	63[a]	82[a]
RSD_r, %[c]	8.1[a]	6.3[a]	33.4	7.9[a]	20.7[a]	3.7[a]	5.9[a]	7.7[a]	19.8[a]	15.3[a]	10.4[a]	3.7[a]
Feed high level, µg/kg	1809	20.5	6.8	19.27	5.83	4024	4008	4008	251.5	251	1002	312.83
Recovery, %	63	74[a]	79[a]	76	82[a]	93[a]	90[a]	95[a]	78[a]	100[a]	68	82[a]
RSD_r, %[c]	6.9[a]	2.1[a]	15.9[a]	21.8[a]	11.1[a]	4.7[a]	4.0[a]	2.5[a]	11.9[a]	11.8[a]	9.7[a]	1.8[a]

[a] Results that are acceptable according to Regulation (EC) No. 401/2006 as subsequently amended and supplemented.

[b] ND = Not detected.

[c] RSD_r = Within-laboratory RSD.

materials are reported in Table 2. The calibration approaches for the methods tested in Table 2 followed these criteria: E-calibration was used for methods involving IMA and SPE cleanup; both E- and MM calibrations were used for methods that did not use IMA and SPE cleanup, but used QuEChERS, "extract and shoot" and "extract, concentrate, and shoot" approaches. An exception was the use of MM calibration for method M, which uses IMA cleanup.

Recovery results were used to evaluate and compare the performance of each method tested. For each method, matrix, and type of calibration used, we calculated the recovery obtained for each mycotoxin. The numbers of mycotoxins that gave acceptable values of recovery with respect to the total number of mycotoxins analyzed are reported in Table 2 for each method. The table also summarizes the methods used, the matrixes analyzed, and the type of calibration used. For six methods (D, E, F, G, H, and I), we used both E- and MM calibrations. Each result was considered acceptable if the calculated recovery was within the acceptability range tabulated in Regulation (EC) No. 401/2006 for each mycotoxin (14). For the mycotoxins analyzed that had acceptable recoveries, values ranged between 9 and 100%, depending on the method and calibration approach used. In general, a higher percentage of acceptable recovery was obtained by using MM calibration, with the exception of method D. However, for these methods, the use of MM calibration was not sufficient to obtain acceptable results for most analytes probably because, in addition to matrix effects, other method characteristics were not under control. Concerning the influence of extraction solvent mixture on recovery results, the methods that gave higher acceptable recovery results (55–100%) were based on the use of a MeOH–water mixture (L, M, and N). Two of these methods (M and N) were also based on multiantibody IMA column cleanup. Although method L gave 90% of acceptable results and did not require sample cleanup, it required MM calibration and the injection of a low amount of matrix equivalent (1.6 mg). The injection of a low amount of matrix equivalent requires a powerful/sensitive LC-MS/MS apparatus to obtain acceptable values for LODs and LOQs. The analytical methods presented in this preliminary study were tested using the same LC-MS/MS apparatus, but different extraction/purification approaches and calibration (MM or E-). Usually the simplest one ("extract and shoot") is less expensive in terms of costs and time needed, but tends to be less effective in terms of performance. Often, it is necessary to use MM calibration to improve performance with a consequent increase of costs and analytical time. The SPE and QuEChERS methods have intermediate performances with costs and times generally higher than those of the previous techniques. Methods using IMA cleanup generally have better performance but higher costs and are more time-consuming.

It was, therefore, decided to further test a method based on MeOH–water (70 + 30, v/v) for mycotoxin extraction and multiantibody IMA cleanup. Between the two methods based on multiantibody columns (methods N and M), we selected the one based on tandem IMA columns (method N; 24) because the percentage of acceptable recovery results was higher, and the

Table 4. Results of mycotoxin levels and *z*-scores obtained by participating in four proficiency tests for four matrixes

	Buckwheat			Corn			Rice			Feed		
Toxin	Assessed value, µg/kg	Lab value, µg/kg	*z*-Score	Assessed value, µg/kg	Lab value, µg/kg	*z*-Score	Assessed value, µg/kg	Lab value, µg/kg	*z*-Score	Assessed value, µg/kg	Lab value, µg/kg	*z*-Score
DON	174	191	0.38[a]	1015	1085	0.31[a]	212	182	–0.55[a]	962	1026	0.29[a]
AFB_1	3.0	3.1	0.11[a]	1.3	1.2	–0.50[a]	28.1	23.9	–0.58[a]	14.7	13.9	–0.20[a]
AFB_2	1.4	1.7	0.75[a]	3.3	4.1	0.80[a]	31.9	34.4	0.30[a]	ND[b]	ND	ND
AFG_1	6.6	6.2	–0.21[a]	14.3	17.6	0.83[a]	30.3	31.8	0.19[a]	ND	ND	ND
AFG_2	8.9	11.1	0.84[a]	3.2	7.3	4.21	28.6	39.2	1.43[a]	ND	ND	ND
FB_1	ND	ND	ND	1239	986	–0.92[a]	ND	ND	ND	412	340	–0.69[a]
FB_2	ND	ND	ND	325	245	–0.96[a]	ND	ND	ND	126	120	–0.18[a]
FB_3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T-2	99	100	0.04[a]	103	111	0.29[a]	54	51.8	–0.20[a]	18	28	0.83[a]
HT-2							55	39.4	–1.03[a]	28	36	0.94[a]
ZEN	53	43	–0.67[a]	170	225	1.25[a]	76	75	–0.05[a]	140	164	0.66[a]
OTA	4.3	4.0	–0.23[a]	3.3	3.8	0.50[a]	7.2	7.9	0.34[a]	3.6	4.9	1.18[a]

[a] Acceptable *z*-scores.

[b] ND = Not detected.

extraction, cleanup, and calibration procedures were simpler compared with those of the other method (method M; 23). Although the use of IMA columns represented an additional and important cost, we selected this approach because they provide a better cleanup and good method sensitivity in addition to good recoveries. To study the matrix effect on the recovery results, the quantitation of each mycotoxin was performed using two types of calibration curves that were prepared in pure solvent with and without the addition of ^{13}C standards.

Validation Study

The matrixes considered in the validation study of the selected method were cereals and animal feed. The selected method was based on extraction with MeOH–water (70 + 30, v/v) and cleanup with two multiantibody IMA columns coupled in tandem. Corn, rice, and feed materials were selected as representative matrixes of food and feed, which were spiked with DON, AFB_1, AFB_2, AFG_1, AFG_2, OTA, T-2, HT-2, FB_1, FB_2, FB_3, and ZEN. Corn and feed materials were spiked at three different levels (low, medium, and high), whereas rice was spiked only at low levels that are close to the naturally occurring levels for this cereal. Considering that the maximum permitted levels of mycotoxins in feed are higher than the maximum permitted levels in food, higher spiking levels were used for feed. In particular, the lower spiking levels in feed samples were comparable with the medium levels in corn.

For validation, we followed the approach provided in section 4.3.1.1 (performance criteria) of Regulation (EU) No. 519/2014 (28), which states that an in-house validated method must be tested on relevant matrixes belonging to the commodity group of interest. For this reason, corn and rice were used (as well as animal feed) to verify accuracy and precision. Participation, with excellent results, in different Proficiency Testings (34 out of 35

Table 5. LOD and LOQ values and calibration curve parameters for the selected method (method N)

			Linearity					
Toxin	LOD, µg/kg	LOQ, µg/kg	$(y - a)/x$	$(y_i - y_i')/y_i' \times 100$	Range, µg/kg	r^2	Slope (b)	Intercept (a)
DON	12.1	36.8	Yes	Yes	36.8–3262.3	0.9994	197.3404	709.9779
AFB_1	0.2	0.7	Yes	Yes	0.50–48.4	0.9997	13814.8121	599.3599
AFB_2	0.2	0.5	Yes	Yes	0.17–16.0	0.9987	9509.7835	104.1153
AFG_1	0.4	1.1	Yes	Yes	0.47–45.5	0.9991	11123.8026	–91.4095
AFG_2	0.1	0.3	Yes	Yes	0.14–13.8	0.9991	5727.3517	284.7035
FB_1	39.2	118.7	Yes	Yes	118.7–5226.0	0.9992	930.9928	–11428.5714
FB_2	28.0	84.9	Yes	Yes	84.9–5205.2	0.9996	1752.2595	–8357.1429
FB_3	24.6	74.5	Yes	Yes	74.5–5205.2	0.9997	1360.2951	–6657.1429
T-2	1.0	2.9	Yes	Yes	2.9–326.6	0.9999	1238.9037	61.1431
HT-2	2.2	6.6	Yes	Yes	5.0–331.2	0.9994	687.4552	498.0081
ZEN	14.7	44.5	Yes	Yes	20.0–3253.2	1.0000	811.5341	5192.2894
OTA	0.7	2.0	Yes	Yes	1.2–338.6	1.0000	3966.5809	501.2459

acceptable *z*-scores) in four different matrixes (buckwheat, rice, corn, and feed) confirmed the applicability of the method to the matrixes belonging to the "cereals grain and production thereof" commodity category in table A in Regulation (EU) No. 519/2014.

In Table 5, LOD and LOQ values and the parameters of the calibration curves obtained for the 12 mycotoxins are reported. The LOD and LOQ values for the 12 mycotoxins ranged from 0.1–39.2 to 0.3–118.7 μg/kg, respectively. The LOD and LOQ values were all well below the maximum limits (MLs) established for cereals and feed (29, 30) and complied with the method criteria proposed by the Codex Committee on Methods of Analysis and Sampling for all mycotoxins (31): for ML < 100 μg/kg, LOD ≤ ML × 1/5 and LOQ < ML × 2/5; and for ML > 100 μg/kg, LOD ≤ ML × 1/10 and LOQ < ML × 1/5. For example, for an ML of 2 μg/kg (AFB_1 in cereals and derived products), the LOD and LOQ should be <0.4 and <0.8 μg/kg, respectively. In our case, the values of LOD and LOQ for AFB_1 were 0.2 and 0.7 μg/kg, respectively.

When using LC-MS/MS methods, various factors should be adequately considered to achieve low LOD and LOQ values. These factors comprise the power/sensitivity of the MS/MS apparatus, as well as the purity and amount of injected matrix equivalent extract. The injection of a high amount of matrix equivalent does not automatically give low LOD and LOQ values because high amounts of matrix equivalent often produce ion suppression (i.e., matrix effect) that increases LOD and LOQ values. A suitable strategy to inject a high amount of matrix equivalent without producing strong ion suppression (i.e., matrix effect) is to purify the sample extract. The IMA cleanup permits the adequate purification and concentration of the sample extract, thus allowing the injection of high amounts of matrix equivalent and an acceptable matrix effect. Often, LC-MS/MS apparatuses are used for the analysis of sample extracts submitted to minimal or no cleanup (4, 6–13). This approach makes the method fast, but may produce higher LOD and LOQ values due to a matrix effect (ion suppression) and/or to low amounts of matrix equivalent injected. LOQ values up to 16, 50, 50, 80, 200, and 200 μg/kg for OTA, aflatoxins, DON, ZEN, T-2/HT-2, and fumonisins, respectively, were reported in a survey of analytical laboratories that used LC-MS/MS for mycotoxin analysis (32). Although these data are not very recent (i.e., 2009) and better sensitivity can be now obtained with modern apparatuses, these values do not fulfill the numeric values for LOD and LOQ criteria in relation to most of the maximum permitted levels for these mycotoxins in cereals (29, 31). In our case, the use of two IMA columns coupled in tandem permitted the efficient purification and concentration of the sample extract. The method gave good sensitivity and a reduced matrix effect. The LC-MS/MS apparatus used for this study (API 3200 system) belongs to a prior generation of LC-MS/MS, therefore, better sensitivity could be obtained using a newer and more powerful LC-MS/MS apparatus.

The results of the validation study conducted with corn, rice, and feed are reported in Table 3. These results were evaluated according to the method criteria parameters reported in Regulation (EC) No. 401/2006 (14). Recovery values were calculated with ^{13}C calibration, and the majority of recovery results (76%; 61 out of 80 results) fulfilled the recovery criteria. FB_3 was included in the evaluation even if no analytical criteria and no maximum levels are defined in legislation. Unfortunately, low recoveries were obtained for FB_1, FB_2, and ZEN at low spiking levels in corn; AFB_1, FB_1, and OTA at medium spiking levels in corn; AFG_1 and ZEN at high spiking levels in corn; OTA at low spiking levels in rice; AFB_1, AFG_1, and ZEN at low spiking levels in feed; AFB_2 and ZEN at medium spiking levels in feed; and DON and ZEN at high spiking levels in feed. The performance criteria in force for food were also adopted for feed materials, as no criteria have been defined yet for feed. It should be mentioned that for some unacceptable results, the recovery values were close to the criteria (Table 3).

The majority of repeatability results (96%; 77 out of 80 results) were acceptable according to Regulation (EC) No. 401/2006 (14). Some of the RSD_r values were higher than the criteria, i.e., FB_1 and OTA in corn at low spiking levels and AFB_2 in feed at medium spiking levels.

When the recoveries were calculated using E-calibration, the percentage of acceptable results decreased to 57% (Table S1 in Supplemental Information). In particular, low recoveries were observed at low spiking levels probably because ion suppression is significant at these levels as compared with medium and high spiking levels.

To compensate for matrix effects in LC-MS/MS determinations, two approaches are mainly used: MM or ^{13}C calibration. To reduce or eliminate matrix effects, two approaches are generally used: efficient purification of sample extract and/or reduction to minimum amount of matrix equivalent injected by diluting the sample extract or reduction of the injected volume (no need to purify the extract). The last approach was successfully used by a participant in the first proficiency test for the simultaneous determination of 11 mycotoxins in corn by LC-MS/MS. This laboratory injected 0.5 μL crude extract containing only 0.08 mg matrix equivalent (18). However, this approach requires a powerful and highly sensitive LC-MS/MS apparatus for the quantitative measurement of mycotoxins in food and feed at levels <1–5 μg/kg.

Efficient purification of a sample extract can be obtained by using IMA columns. This approach also allows a concentration of sample extract that permits the injection of high amounts of matrix equivalent. Amounts of matrix equivalent as high as 100–118 mg (23) and 19.3–50 mg (24) have been reported using multiantibody IMA columns for sample cleanup. However, in the first case, a matrix effect was evident, which required MM calibration, whereas in the second case, it seems that no matrix effect was observed because E-calibration (in pure solvent) was used. Other examples of LC-MS/MS methods dealing with matrix effects associated with multimycotoxin analysis and approaches used to compensate for it have been reported by Zhang et al. (5). From these papers, it was not clear if the use of IMA columns for sample cleanup and concentration of the extract were sufficient to eliminate the matrix effects in the LC-MS/MS determination of mycotoxins. To evaluate matrix effects by using multiantibody IMA columns (method N; *see* Table 2), we compared the recovery results obtained using ^{13}C calibration and E-calibration. The amount of matrix equivalent injected was 7.7 mg by injecting 20 μL purified sample extract. The recovery results were calculated using both E- and ^{13}C calibration. The results obtained for corn spiked at three different levels (low, medium, and high) are shown in Figure 1. From the statistical comparison of results obtained using the two calibration approaches, significant differences ($P < 0.05$) were observed for the majority of mycotoxins spiked at low levels (Figure 1a). In particular, recovery results obtained using ^{13}C calibration were significantly higher for DON, AFB_1, AFG_1,

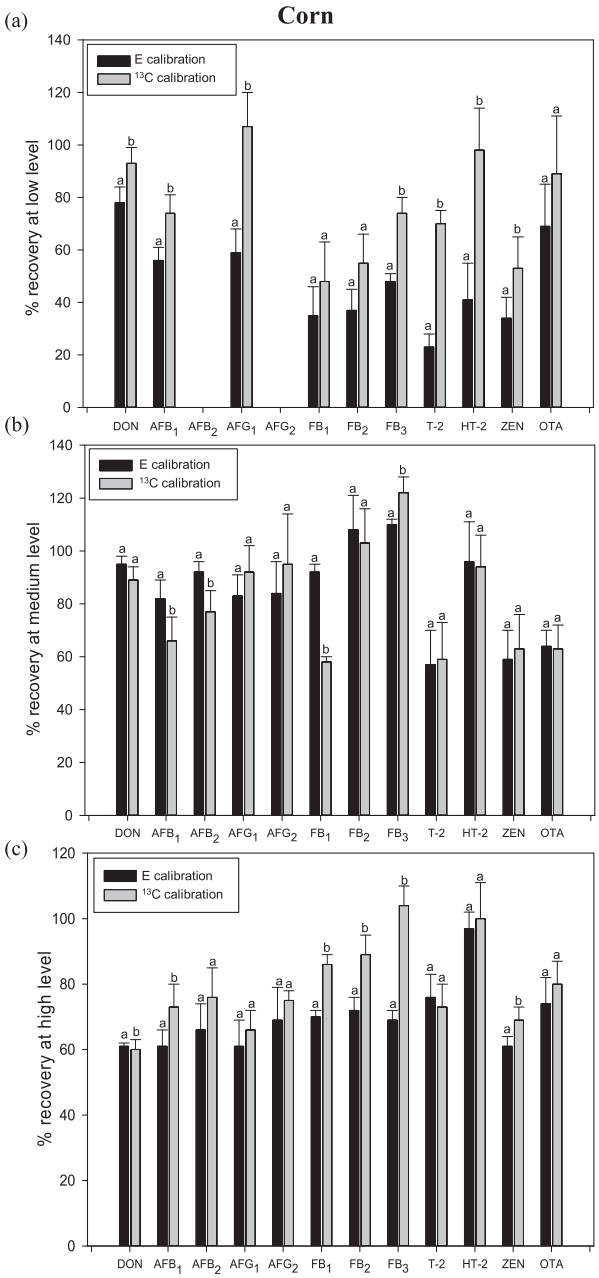

Figure 1. Recovery results ±SD obtained for corn spiked at (a) low, (b) medium, and (c) high mycotoxin levels. Comparison of results obtained by using E- and ^{13}C calibration curves. Spiking levels are reported in Table 3. Different letters at the top of the whisker bars indicate statistical differences within the pairs of data (P<0.05).

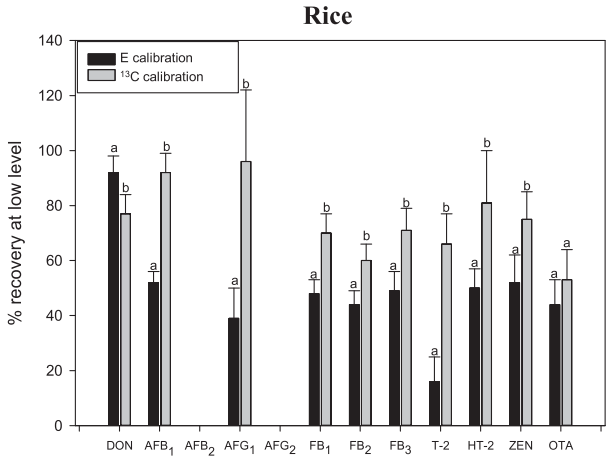

Figure 2. Recovery results ±SD obtained for rice spiked at low mycotoxin levels. Comparison of results obtained by using E- and ^{13}C calibration curves. Spiking levels are reported in Table 3. Different letters at the top of the whisker bars indicate statistical differences within the pairs of data (P<0.05).

FB_3, T-2, HT-2, and ZEN as compared with those obtained with E-calibration. Higher recovery values were also obtained for FB_1, FB_2, and OTA using ^{13}C calibration, although the differences were not statistically significant (Figure 1a). The same results were observed for rice spiked at low mycotoxin levels, as the recovery of 8 out of 10 mycotoxins was significantly higher using ^{13}C calibration as compared with E-calibration (Figure 2). The results of AFB_2 and AFG_2 were not evaluated because these mycotoxins were spiked at levels below their LOQ values and could not be quantitated. Instead, no significant differences were observed for corn spiked at medium and high levels (Figure 1b and c).

As shown in Figure 3, no significant differences were observed for the majority of mycotoxins in feed spiked at the three levels and quantitated with both calibration approaches. These results confirm those obtained for corn spiked at medium and high levels. In fact, the three spiking levels of feed are similar or higher than the medium and high spiking levels of corn.

Taken together, these results demonstrate that matrix effect compensation is not necessary at medium and high mycotoxin levels (*see* Figures 1b and c and 3) by injecting a relatively low amount of matrix equivalent (7.7 mg). On the other hand, matrix effect compensation is necessary at low mycotoxin levels (*see* Figures 1a and 2).

The method developed by Wilcox et al. (24) was tested in this study (method N; *see* Table 2) with minor modifications. In general, the recovery and repeatability results we obtained for corn confirmed those obtained by Wilcox et al. (24) for the same matrix. However, our study clearly showed that a residual matrix effect was still present. This effect should be compensated for if low mycotoxin levels are measured, whereas the effect is negligible at medium and high mycotoxin levels. In fact, the acceptable recovery results at low spiking levels in corn and rice were 80 and 12% using ^{13}C and E-calibration curves, respectively. The acceptable recovery results at medium and high spiking levels in corn and feed were 75 and 70% using ^{13}C and E-calibration curves, respectively. These results clearly demonstrate the need for matrix effect compensation in the determination of low mycotoxin levels. The matrix effect compensation does not seem to be necessary at medium and high mycotoxin levels, provided adequate sample cleanup is performed.

The analytical method tested herein was further validated by participating in four Proficiency Testings for the multiple determination of mycotoxins in buckwheat, corn, rice, and feed. As shown in Table 4, acceptable z-scores ($|z| \leq 2$) were obtained for 34 out of 35 results (97%), with only the AFG_2 value for corn returning an unacceptable result ($|z| = 4.21$).

The extraction and purification method extensively tested herein was similar to the method of Wilcox et al. (24) with some minor modifications, which are reported below. The test portion size and volume of extraction solvent were proportionally reduced from 25 to 5 g and from 100 to 20 mL, respectively. A minor modification was adopted for

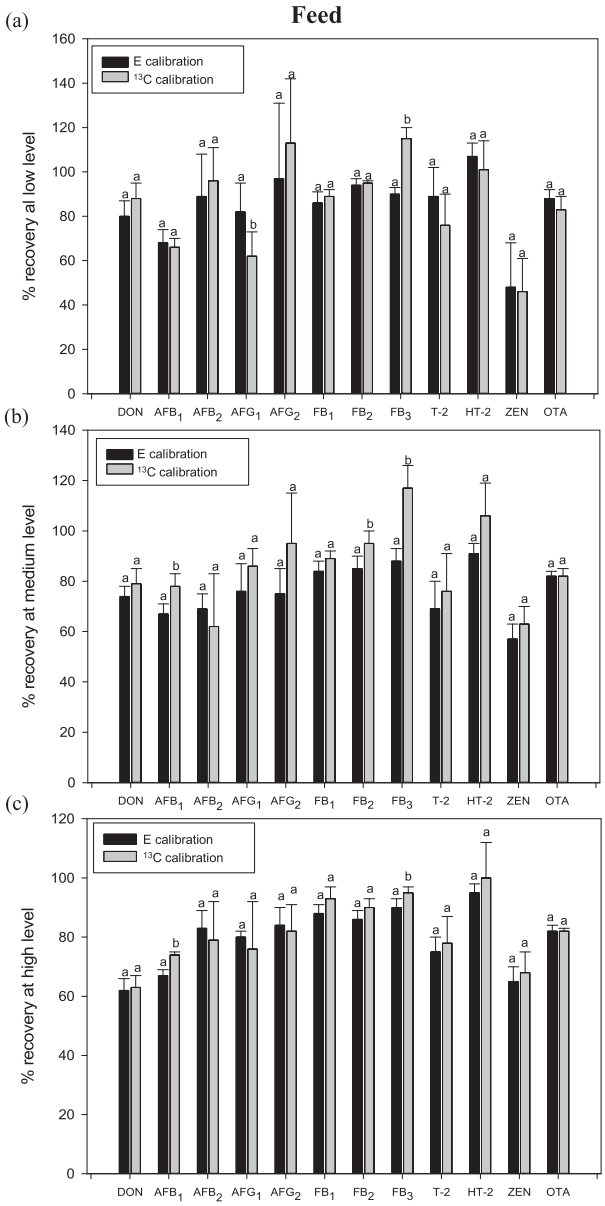

Figure 3. Recovery results ±SD obtained for feed spiked at (a) low, (b) medium, and (c) high mycotoxin levels. Comparison of results obtained by using E- and ^{13}C calibration curves. Spiking levels are reported in Table 3. Different letters at the top of the whisker bars indicate statistical differences within the pairs of data (P<0.05).

the extraction. In particular, 6 mL water were added to 5 g matrix and slurried for 30 min, then 1 g NaCl and 14 mL MeOH were added and blended for 15 min. In the procedure of Wilcox et al. (24), NaCl is added to the matrix, and mycotoxin extraction is performed with a MeOH–water mixture (70 + 30, v/v). Finally, we injected 20 µL purified extract (7.7 mg matrix equivalent) instead of 50 µL (19.2 mg matrix equivalent). For chromatographic separation, we used a Hypersil GOLD column (100 × 2.1 mm, 5 µm; Thermo Fisher Scientific) at a 500 µL/min flow rate instead of a Gemini C18 column (150 × 3 mm, 5 µm; Phenomenex) at a 300 µL/min flow rate. The composition of the mobile phase was the same, but we used ammonium acetate and acetic acid instead of ammonium formate and formic acid. Other modifications were the duration of linear gradient (7 min instead of 20 min) and linear gradient program (from 15 to 65% MeOH instead of from 20 to 90% MeOH). In our opinion, these modifications do not cause significant changes to the original method and should not have a negative impact on method performance.

Conclusions

The results of this study confirm that LC-MS/MS methods are a good option for multimycotoxin determination in cereals. However, for some mycotoxins, these methods still need improvements in terms of accuracy and, to a lesser extent, precision. IMA cleanup is a good approach to reduce matrix effects and to improve method sensitivity. Residual matrix effects are still present in cereal sample extracts purified with IMA columns. These effects should be compensated for if mycotoxins are measured at low levels. At medium and high mycotoxin levels, these effects are negligible, and the E-calibration approach (in pure solvent) is sufficient for accurate measurements. The method selected in this study is based on two multiantibody IMA columns and gave good performance in terms of accuracy and precision. With this method, expensive labeled internal standards should be used only for food analysis, which further reduces costs. Among the analytical methods considered, those with low cost and fast implementation are more suitable for surveying and research, whereas the one based on the multiantibody IMA column selected and validated herein has a performance that makes it suitable for regulatory purposes and research work that requires low LOD and LOQ values even when using less modern, lesser-performing LC-MS/MS apparatus.

References

(1) Pitt, J.I., & Miller, J.D. (2016) *J. Agric. Food Chem.* **65**, 7021–7033. doi: 10.1021/acs.jafc.6b04494

(2) Mitchell, N.J., Bowers, E., Hurburgh, C., & Wu, F. (2016) *Food Addit. Contam. Part A*Chem Anal Control Expo Risk Assess. **33**, 540–550. doi:10.1080/19440049.2016.1138545

(3) Lee, H.J., & Ryu, D. (2017) *J. Agric. Food Chem.* **65**, 7034–7051. 10.1021/acs.jafc.6b04847

(4) Berthiller, F., Brera, C., Iha, M.H., Krska, R., Lattanzio, V.M.T., MacDonald, S., Malone, R.J., Maragos, C., Solfrizzo, M., Stranska-Zachariasova, M., Stroka, J., & Tittlemier, S.A. (2017) *World Mycotoxin J.* **10.1**, 5–29. doi:10.3920/WMJ2016.2138

(5) Zhang, K., Wong, J.W., Krynitsky, A.J., & Trucksess, M.W. (2016) *J. AOAC Int.* **99**, 890–894. doi:10.5740/jaoacint.16-0116

(6) Shephard, G.S., Berthiller, F., Dorner, J., Krska, R., Lombaert, G.A., Malone, B., Maragos, C., Sabino, M., Solfrizzo, M., Trucksess, M.W., van Egmond, H.P., & Whitaker, T.B. (2009) *World Mycotoxin J.* **2**, 3–21. doi:10.3920/WMJ2008.1095

(7) Shephard, G.S., Berthiller, F., Dorner, J., Krska, R., Lombaert, G.A., Malone, B., Maragos, C., Sabino, M., Solfrizzo, M., Trucksess, M.W., van Egmond, H.P., & Whitaker, T.B. (2010) *World Mycotoxin J.* **3**, 3–23. doi:10.3920/WMJ2009.1172

(8) Shephard, G.S., Berthiller, F., Burdaspal, P., Crews, C., Jonker, M.A., Krska, R., MacDonald, S., Malone, B., Maragos, C., Sabino, M., Solfrizzo, M., van Egmond, H.P., & Whitaker, T.B. (2011) *World Mycotoxin J.* **4**, 3–28. doi:10.3920/WMJ2010.1249

(9) Shephard, G.S., Berthiller, F., Burdaspal, P.A., Crews, C., Jonker, M.A., Krska, R., MacDonald, S., Malone, R.J., Maragos, C., Sabino, M., Solfrizzo, M., van Egmond, H.P., & Whitaker,

T.B. (2012) *World Mycotoxin J.* **5**, 3–30. doi:10.3920/WMJ2011.1338

(10) Shephard, G.S., Berthiller, F., Burdaspal, P.A., Crews, C., Jonker, M.A., Krska, R., Lattanzio, V.M.T., MacDonald, S., Malone, R.J., Maragos, C., Sabino, M., Solfrizzo, M., van Egmond, H.P., & Whitaker, T.B. (2013) *World Mycotoxin J.* **6**, 3–30. doi:10.3920/WMJ2012.1492

(11) Berthiller, F., Burdaspal, P.A., Crews, C., Iha, M.H., Krska, R., Lattanzio, V.M.T., MacDonald, S., Malone, R.J., Maragos, C., Solfrizzo, M., Stroka, J., & Whitaker, T.B. (2014) *World Mycotoxin J.* **7**, 3–33. doi:10.3920/WMJ2013.1637

(12) Berthiller, F., Brera, C., Crews, C., Iha, M.H., Krsha, R., Lattanzio, V.M.T., MacDonald, S., Malone, R.J., Maragos, C., Solfrizzo, M., Stroka, J., & Whitaker, T.B. (2015) *World Mycotoxin J.* **8**, 5–35. doi:10.3920/WMJ2014.1840

(13) Berthiller, F., Brera, C., Crews, C., Iha, M.H., Krsha, R., Lattanzio, V.M.T., MacDonald, S., Malone, R.J., Maragos, C., Solfrizzo, M., Stroka, J., & Whitaker, T.B. (2016) *World Mycotoxin J.* **9**, 5–30. doi:10.3920/WMJ2015.1998

(14) Commission Regulation (EC) No. 401/2006 (2006) *Off. J. Eur. Union*. **L70**, 12–34

(15) Biotage Application Note AN782 (2013) Extraction of Multiple Mycotoxins from Grain using Isolute Myco prior to LC-MS/MS Analysis http://www.jasco.hu/4tgrdvxgv/uploads/2017/05/an782_extraction_of_multiple_mycotoxins_from_grain_using_isolute_myco_prior_to_lc-ms-ms_analysis.pdf (accessed on September 25, 2017)

(16) Biotage Application Note AN783 (2013) Extraction of Deoxynivalenol from Grain using Isolute Myco prior to LC-MS/MS Analysis, http://www.jasco.hu/4tgrdvxgv/uploads/2017/05/an783_extraction_of_deoxynivalenol_from_grain_using_isolute_myco_prior_to_lc-ms-ms_analysis.pdf (accessed on September 25, 2017)

(17) Spanjer, M.C., Rensen, P.M., & Scholten, J.M. (2008) *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **25**, 472–489. doi:10.1080/02652030701552964

(18) Solfrizzo, M., De Girolamo, A., Lattanzio, V.M.T., Visconti, A., Stroka, J., Alldrick, A., & van Egmond, H.P. (2013) *Qual. Assur. Saf. Crop* **5**, 15–48. doi:10.3920/QAS2012.0140

(19) De Girolamo, A., Solfrizzo, M., Lattanzio, V.M.T., Stroka, J., Alldrick, A., van Egmond, H.P., & Visconti, A. (2013) *World Mycotoxin J.* **6**, 317–334. doi:10.3920/WMJ2012.1538

(20) European Standard EN 15662 (2008), Food of plant origin – Determination of pesticides residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and cleanup by dispersive SPE – QuEChERS method http://www.chromnet.net/Taiwan/QuEChERS_Dispersive_SPE/QuEChERS_%E6%AD%90%E7%9B%9F%E6%96%B9%E6%B3%95_EN156622008_E.pdf (accessed on September 26, 2017)

(21) Rubert, J., James, K.J., Mañes, J., & Soler, C. (2012) *Food Chem. Toxicol.* **50**, 2034–2041. doi:10.1016/j.fct.2012.03.063

(22) Desmarchelier, A., Tessiot, S., Bessaire, T., Racault, L., Fiorese, E., Urbani, A., Chan, W.C., Cheng, P., & Mottier, P. (2014) *J. Chromatogr. A* **1337**, 75–84. doi:10.1016/j.chroma.2014.02.025

(23) Lattanzio, V.M.T., Solfrizzo, M., Powers, S., & Visconti, A. (2007) *Rapid Commun. Mass Spectrom.* **21**, 3253–3261. doi:10.1002/rcm.3210

(24) Wilcox, J., Donnelly, C., Leeman, D., & Marley, E. (2015) *J. Chromatogr. A* **1400**, 91–97. doi:10.1016/j.chroma.2015.04.053

(25) Miller, J.N., & Miller, J.C. (2005) *Statistics and Chemometrics for Analytical Chemistry*, 6th Ed., Pearson Education Ltd, London, United Kingdom

(26) European Commission Directorate-General for Health and Food Safety SANTE/11945/2015 30 November - 1 December 2015 rev. 0 (2016), Analytical quality control and method validation procedures for pesticide residues analysis in food and feed https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_11945.pdf (accessed on September 27, 2017)

(27) Magnusson, B., & Örnemark, U. (Eds) (2014) *Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics*, 2nd Ed., ISBN 9789187461590, http://www.eurachem.org

(28) Commission Regulation (EC) No. 519/2014 (2014) *Off. J. Eur. Union* **L146**, 29–43

(29) Commission Regulation (EC) No. 1881/2006 (2006) *Off. J. Eur. Union.* **L364**, 5–24

(30) Commission Recommendation (EC) No. 2006/576/EC (2006) *Off. J. Eur. Union*. **L229**, 7–9

(31) Codex Committee on Methods of Analysis and Sampling (2009) Guidelines for Establishing Methods Criteria for the Identification of Relevant Analytical Methods, 30th Session of the Joint FAO/WHO Food Standards Programme, Balatonalmadi, Hungary, 9–13 March 2009, Document CX/MAS 09/30/7, ftp://ftp.fao.org/codex/Meetings/CCMAS/ccmas30/ma30_07e.pdf (accessed on June 16, 2017)

(32) Solfrizzo, M., Alldrick, A.J., & van Egmond, H.P. (2009) *Qual. Assur. Saf. Crop* **1**, 121–132. doi:10.1111/j.1757-837X.2009.00021.x