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ARTICLE

Development and Comparison of Two Multiresidue Methods for the Analysis of 17 Mycotoxins in Cereals by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

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Two multiresidue methods based on different extraction procedures have been developed and compared for the liquid chromatography electrospray ionization tandem mass spectrometry analysis of 17 mycotoxins including ochratoxin A, aflatoxins (B_1, B_2, G_1, and G_2), zearalenone, fumonisins (B_1 and B_2), T-2 toxin, HT-2 toxin, nivalenol, deoxynivalenol, 3- and 15-acetyldeoxynivalenol, fusarenon-X, diacetoxyscirpenol, and neosolaniol in cereal-based commodities. The extraction procedures considered were a QuEChERS-like method and one using accelerated solvent extraction (ASE). Both extraction procedures gave similar performances in terms of linearity ($r^2 > 0.98$) and precision (both RSD_r and $RSD_{iR} < 20\%$). Trueness was evaluated through participation in four proficiency tests and by the analysis of two certified reference materials and one quality control material. Satisfactory Z scores ($|Z| < 2$) and trueness values (73−130%) were obtained by the proposed procedures. Limits of quantification were similar by both methods and were within the 1.0−2.0 μg/kg range for aflatoxins, 0.5 μg/kg for ochratoxin A, and the 5−100 μg/kg range for all other mycotoxins tested. The QuEChERS-like method was found to be easier to handle and allowed a higher sample throughput as compared to the ASE method.

KEYWORDS: Mycotoxins; multiresidue methods; QuEChERS; accelerated solvent extraction; liquid chromatography tandem mass spectrometry; LC-MS/MS

INTRODUCTION

Mycotoxins are toxic metabolites produced by filamentous fungi belonging mainly to the genera *Aspergillus*, *Penicillium*, and *Fusarium*. Fungal infection can occur in a wide range of agricultural commodities, under varying climatic conditions, before, during, and after harvest. Variable patterns of contamination can be observed since some molds can produce more than one toxin, while some mycotoxins can be produced by more than one fungal species. Consequently, when contamination occurs, often more than one toxin is produced (*1*). Several hundreds of mycotoxins, characterized by a multitude of chemical structures, have been identified so far. Knowing that toxicity mechanisms are structure-dependent, numerous acute toxic and chronic carcinogenic, mutagenic, teratogenic, or estrogenic effects have been linked to mycotoxin exposure in humans and animals (*2*). Besides toxic effects, mycotoxins can cause tremendous economic losses deriving from the contamination of the world's crop production (*3*). Therefore, maximum levels in food commodities have been set up by the European Union (EU) for several mycotoxins (*4*, *5*).

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Because of the chemical diversity among mycotoxins, their analysis is usually performed through single compound determination or for certain classes of mycotoxins by high-performance liquid chromatography (HPLC) coupled to nonconfirmatory UV or fluorescence detectors or by gas chromatography (GC) using electron capture detection after specific extraction procedures and extensive cleanup (*6*, *7*). Such methodologies, although efficient at detecting low contamination levels, are often time-consuming. Rapid screening methods like enzyme-linked immunosorbent assays (ELISAs), fluorescence polarization immunoassays, dipsticks, or biosensors represent nowadays an attractive tool to lighten the sample preparation while increasing the sample throughput. However, these methods are qualitative or semiquantitative, still requiring positive results around the maximum limits to be both quantified and confirmed by confirmatory procedures. Besides, as synergistic or additive toxic effects can appear due to mycotoxins co-occurrence in food- and feedstuffs (*8*), information on the mycotoxin pattern for commodities prone to contain more than one mycotoxin (e.g., cereals) can be of importance. Consequently, the development of fast and easy but also precise analytical methods for mycotoxins analysis is highly desirable. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) became a prominent tool in multiresidue analysis, enabling the selective detection of analytes without, in principle, important cleanup upstream. Nevertheless, several published LC-MS/MS

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multiresidue methods kept intensive cleanup with sequential solid-phase extraction (SPE) steps, allowing the analysis of *Fusarium* toxins (trichothecenes, zearalenone and its metabolites) sometimes detected with fumonisins, aflatoxins, and ochratoxin A (*9*−*15*). These extensive sample pretreatments limited the number of analytes surveyed and were hardly compatible with a high-throughput routine analysis. The next generation of the "dilute and shoot" type methods (*16*−*22*) gave the opportunity to reduce or even circumvent the sample cleanup while extending the number of mycotoxins surveyed. However, LC-MS/MS methods with very basic sample preparation are prone to matrix effects, which, if not carefully considered, can compromise the quantification.

The aim of this study was to develop a fast and easy multiresidue method for the quantitative analysis of mycotoxins in cereal matrices by LC-MS/MS. A total of 17 mycotoxins were selected for this study including all relevant EU-regulated mycotoxins [aflatoxin B_1 (AFLA B1), aflatoxin B_2 (AFLA B2), aflatoxin G_1 (AFLA G1), aflatoxin G_2 (AFLA G2), fumonisin B_1 (FB1), fumonisin B_2 (FB2), ochratoxin A (OTA), deoxynivalenol (DON), and zearalenone (ZON)] and an additional selection of trichothecenes [T-2 toxin (T-2), HT-2 toxin (HT-2), nivalenol (NIV), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), fusarenon-X (Fus-X), diacetoxyscirpenol (DAS), and neosolaniol (NEO)]. Two extraction procedures were considered for this purpose: (a) a QuEChERS (acronym for quick, easy, cheap, effective, rugged, and safe)-like method originally developed for pesticides analysis (*23*) and recently adapted to mycotoxins analysis (*24*, *25*) and (b) a method using accelerated solvent extraction (ASE), already successfully applied to the simultaneous extraction of FB1, DON, and ZON in our laboratory (*9*). After their respective set up, both methods were compared by carrying out a full validation on corn, wheat, and rice flours. Their applicability was then extended to other commodities including other cereal flours (rye, oat, barley, and soya), one pet food ingredient (corn gluten), and one baby food product (infant cereals). Different quantification approaches are discussed. The standard addition procedure was retained and tested through participation in four proficiency tests and by the analysis of two certified reference materials (CRMs) and one quality control material (QCM).

MATERIALS AND METHODS

Chemicals and Reagents. The following chemical and reagents were obtained commercially: HPLC-grade LiChrosolv Water (H_2O), acetonitrile (MeCN), *n*-hexane, methanol (MeOH), 100% acetic acid, 98−100% formic acid, sodium chloride (NaCl) (Merck, Darmstadt, Germany); ammonium formate and magnesium sulfate ($MgSO_4$) (Sigma Aldrich, Buchs, Switzerland); Diacetomaceous Earth-Hydromatrix (Varian, Harbour City, CA); and C_{18}-modified silica material (Supelco, Bellefonte, PA). Mycotoxin standards, provided with their certificate of analysis, were all purchased from Sigma-Aldrich in ready-to-use ampules and were DON, 3- and 15-AcDON, NIV, NEO, T-2, HT-2, Fus-X, DAS, and ZON, each obtained at a 100 μg/mL concentration; OTA at 10 μg/mL; AFLA B1 and AFLA G1 each at 2 μg/mL; AFLA B2 and AFLA G2 each at 0.5 μg/mL; and FB1 and FB2 each at 50 μg/mL. All mycotoxins were dissolved in MeCN except FB1 and FB2, obtained in MeCN:H_2O (1:1; v/v).

CRMs and QCMs. Two CRMs, one containing FB1 and FB2 in maize flour (CRM 32923) and one containing ZON in maize flour (CRM 32921) were supplied by Sigma Aldrich. A QCM containing ZON in baby food (QCM 2236) was obtained from FAPAS (Sand Hutton, York, United Kingdom).

Standard Solutions. Four composite working standard solutions (solutions 1−4) were prepared by diluting the above-mentioned stock solutions either in MeCN:H_2O (1:1; v/v) for FB1 and FB2 or in MeCN for all other mycotoxins. The concentrations of each mycotoxin in working solution 1 were as follows: OTA, 0.05 μg/mL; aflatoxins B1, B2, G1, and G2, 0.1 μg/mL, respectively; T-2, 0.5 μg/mL; ZON, 2.0 μg/mL; NEO, DAS, HT-2, and Fus-X, 2.5 μg/mL, respectively; 15-AcDON and DON, 5.0 μg/mL, respectively; and NIV, 10 μg/mL. Working standard solution 2 contained only FB1 and FB2 (5 μg/mL, respectively). Working standard solutions 3 and 4 were obtained by a 10-fold dilution of working standard solutions 1 and 2 in MeCN and in MeCN:H_2O (1:1; v/v), respectively. Individual stock standard solutions and stock standard mixtures were stored at −18 °C and brought to room temperature before use.

Samples. Blank or low contaminated flour samples of corn, wheat, rice, oat, rye, barley, soya, corn gluten, and infant cereals were collected from local suppliers. Flour samples were kept at room temperature in airtight containers until analysis. All samples were already available as a finely ground powder and did not require any further comminution. For validation purposes, samples were fortified and kept overnight at room temperature to allow an optimal integration of the analytes into the respective matrix.

Sample Preparation. *QuEChERS-Like Method.* For the first extraction step, a 5.00 ± 0.01 g test portion was weighed into a 50 mL Falcon polypropylene tube (Becton Dickinson, Le Pont de Claix, France) to which H_2O (10 mL) and 0.5% acetic acid in MeCN solution (10 mL) were added. The resulting slurry was vigorously hand-mixed after each solvent addition, ensuring there was no aggregate in the sample, and was placed onto an automated shaker at 300 rpm for 5 min. For the second extraction step, a $MgSO_4$:NaCl salt mixture (4:1, w/w) (5.0 ± 0.2 g) was added to the slurry, which was immediately and vigorously hand-shaken for a few seconds before centrifugation (4000*g* at room temperature for 15 min). For clean-up, the resulting MeCN-based supernatant (5 mL) was transferred into a 15 mL Falcon polypropylene tube and further defatted with *n*-hexane (5 mL) under agitation using the automated shaker (200 rpm, 5 min). After centrifugation (4000*g* at room temperature for 1 min), the supernatant (1 mL, equivalent to 0.5 g of matrix) was pipetted into a new 15 mL Falcon polypropylene tube and evaporated to dryness at 40 °C under a stream of nitrogen. In the final treatment of the extract, the residue was reconstituted in MeOH (75 μL) and sonicated for a few seconds until complete resuspension. Water (75 μL) was added, and the suspension again mixed. The whole extract was then transferred into a 1.5 mL Eppendorf tube and centrifuged at 8500*g* for 10 min at room temperature. The resulting supernatant (60 μL) was then further diluted with water (140 μL) and recentrifuged (8500*g* for 10 min at room temperature), and the clear supernatant was transferred into a HPLC amber glass vial for further LC-ESI-MS/MS analysis.

ASE Method. Extraction was carried out on a ASE 200 System (Dionex, Sunnyvale, CA) equipped with an autosampler carrousel and a collection tray, allowing up to 24 samples to be extracted sequentially. For ASE extraction cell preparation, a 33 mL stainless steel ASE extraction cell was prepared by successively inserting (from bottom to top) a cellulose filter, C_{18}-modified silica material (2.0 ± 0.1 g), a cellulose filter, a homogeneous mixture made of the cereal test portion (5.00 ± 0.01 g) and hydromatrix (7.0 ± 0.5 g), a cellulose filter, and finally hydromatrix to completely fill the cell. Between the different steps, the filled material was compressed by means of a pole. For the ASE extraction step, the following settings were used: ASE extraction solvent, MeCN:H_2O:glacial acetic acid (80:19:0.5; v/v/v); extraction time, 3 min; extraction at room temperature; extraction pressure, 2000 psi; flush volume, 85%; purge time, 1 min; number of static cycles, 3; and preheating time, 0 min. For clean-up, the extract (40 mL) was collected into a glass collecting vial and transferred quantitatively into a 50 mL volumetric flask, which was then filled to the mark with the ASE extraction solvent. An aliquot (25 mL) of this extract was transferred into a 50 mL Falcon polypropylene tube already containing the QuEChERS salt mixture mentioned above. The tube was vigorously hand shaken for a few seconds, and the slurry was defatted with *n*-hexane (10 mL) for 5 min. After centrifugation (4000*g*, 15 min), a 4 mL aliquot (equivalent to 0.5 g of matrix) of MeCN phase was pipetted, transferred into a 15 mL Falcon polypropylene tube, and evaporated to dryness at 40 °C under a stream of nitrogen before the final treatment of the extract, which was identical to the one described in the QuEChERS-like sample preparation.

Preparation of Calibration Curves. Matrix-matched calibration curves were of two types: (a) Matrix-matched calibration curves (MMCCs) were built by spiking mycotoxins after extraction into blank

sample extracts. To build MMCCs, aliquots of the defatted MeCN phase obtained after extraction of blank samples (1 and 4 mL aliquots for the QuEChERS-like and ASE methods, respectively, representing in both cases an equivalent of 0.5 g of matrix) were fortified with 0, 35, 50, 75, 100, 125, and 150 μL of working solutions 3 and 4. Spiked aliquots were then evaporated and reconstituted sequentially in MeOH:H_2O (50:50; v/v) and then in MeOH:H_2O (15:85; v/v) according to the procedures described before. Spiking levels were equivalent to 0-, 0.7-, 1-, 1.5-, 2-, 2.5-, and 3-fold the limits of quantification (LOQs), reported in **Table 1**. (b) Method matrix-matched calibration curves (MMMCCs) were built by spiking mycotoxins before extraction into blank samples. For this, 5.00 ± 0.01 g of matrix was fortified with 0, 35, 50, 75, 100, 125, and 150 μL of working solutions 1 and 2 before being run through the extraction procedures. Spiking levels corresponded to 0-, 0.7-, 1-, 1.5-, 2-, 2.5-, and 3-fold the LOQs (**Table 1**). Both MMCCs and MMMCCs were constructed by plotting peak area against concentration (in μg/kg), and a linear function was applied to the calibration curves.

Table 1. LOQs and Linearity Ranges of MMMCCs Obtained by the QuEChERS-Like and ASE Methods

	maize, wheat, rye, rice, oat, barley, soya, and infant cereals		corn gluten	
	LOQ[a]	linearity range[a]	LOQ[a]	linearity range[a]
AflB1	1 (2)[b]	0−12	10	0−60
AflB2	1 (2)[b]	0−12	10	0−60
AflG1	1 (2)[b]	0−12	10	0−60
AflG2	1 (2)[b]	0−12	10	0−60
DON	50	0−2000	250	0−10000
NIV	100	0−600	500	0−3000
15-AcDON	50	0−300	250	0−1500
DAS	25	0−150	125	0−750
Fus-X	25	0−150	125	0−750
NEO	25	0−150	125	0−750
HT-2	25	0−500	125	0−2500
T-2	5	0−500	25	0−2500
FB1	50	0−1000	250	0−5000
FB2	50	0−1000	250	0−5000
ZON	20	0−400	100	0−2000
OTA	0.5	0−15	2.5	0−75

[a] LOQ and linearity range values are given in μg/kg. [b] For aflatoxins in soya.

LC-ESI-MS/MS. HPLC analysis was performed on a Zorbax Bonus-RP column 150 mm × 2.1 mm i.d., 3.5 μm, equipped with a Zorbax RB C_8 guard column 12.5 mm × 2.1 mm i.d., 5 μm (both from Agilent Technologies, Geneva, Switzerland), using an Agilent 1100 binary pump system. The mobile phase was constituted by solvent A, formic acid 0.15% (v/v) in water containing 10 mM ammonium formate, and solvent B, 0.05% formic acid (v/v) in MeOH. A linear gradient program was setup with 0−0.5 min 15% B, 0.5−9 min 100% B, then hold at 100% B for 6 min before coming back to 15% B in 1 min (the HPLC column was reconditioned at 15% B for an additional 9.5 min). The flow rate was 0.25 mL/min, and 40 μL of the extract was injected onto the column. The HPLC flow was directed into the MS detector between 2 and 16.5 min using a VICI diverter (Valco Instrument Co. Inc., Houston, TX).

MS detection was performed using an Applied Biosystems 4000 QTrap (Foster City, CA) equipped with a TurboIonSpray ionization source. MS tuning was performed in both positive and negative electrospray ionization (ESI) for all mycotoxins, by syringe-infusing separately a solution of each analyte (at a concentration of 10 μg/mL) at a flow rate of 10 μL/min mixed with a HPLC flow made of solvents A and B (50:50, v/v; 0.25 mL/min) using a T-connector. The block source temperature was maintained at 550 °C, and the gas set values were as follows: curtain gas, 40 psi; nebulizer gas, 50 psi; turbo gas, 30 psi; and collision gas, 1.2×10^{-4} psi. In the final method, all compounds were analyzed within the same HPLC run by switching from the positive ionization mode to the negative one at time $t = 12$ min for ZON acquisition and switching again at $t = 13$ min for OTA acquisition in positive ionization mode. Quantitative analysis was performed using tandem MS in selected reaction monitoring (SRM) mode alternating two transition reactions for each compound (**Table 2**). Data processing was carried out using Analyst software 1.5.

Methods Evaluation. To compare the efficiency of each extraction procedure, absolute recoveries were determined at three fortification levels on corn, wheat, and rice and under intermediate reproducibility (iR) conditions (*26*). Blank matrices were spiked before workup at 1, 1.5, and 2 times the respective LOQ, and absolute recoveries were calculated by means of MMCCs according to the following equation:

$$\text{absolute recovery}\ (\%) = \left(\frac{\text{area} - b_{\text{MMCC}}}{a_{\text{MMCC}}}\right) \times \frac{100}{C_{\text{spiked}}}$$

where area is the peak area of the analyte under survey, b_{MMCC} is the *y*-intercept of the calibration curve, a_{MMCC} is the slope of the calibration curve, and C_{spiked} is the spiked concentration (μg/kg) of the analyte under survey.

Three operators were involved in these experiments, each performing two replicates of each fortification level on two occasions. Thus, a total of $n = 12$ separate experiments per fortification level were thus obtained over $k = 6$ different days. Within-laboratory precision (relative standard deviation, RSD_{iR}) data were calculated from these trials (**Table 3**). Applicability of both extraction methods was then extended to oat, rye, barley, soya, infant cereals, and corn gluten where absolute recoveries were determined at 1, 1.5, and 2 times the respective LOQ, under repeatability conditions (r) (1 operator, $n = 6$; $k = 1$) (*26*). Within-day precisions (RSD_r) were calculated from these experiments (**Table 3**).

For the QuEChERS-like method only, apparent recoveries were determined on corn, wheat, and rice and under repeatability conditions (*26*). Blank matrices were spiked before workup at 1, 1.5, and 2 times the respective LOQ, and apparent recoveries were calculated by means of MMMCCs according to the following equation:

$$\text{apparent recovery}\ (\%) = \left(\frac{\text{area} - b_{\text{MMMCC}}}{a_{\text{MMMCC}}}\right) \times \frac{100}{C_{\text{spiked}}}$$

where area is the peak area of the analyte under survey, b_{MMMCC} is the *y*-intercept of the calibration curve, a_{MMMCC} is the slope of the calibration curve, and C_{spiked} is the spiked concentration (μg/kg) of the analyte under survey.

One operator was involved in these experiments performing two replicates of each fortification level on two occasions ($n = 4$; $k = 2$). Between-day precisions (RSD_r) were calculated from these experiments (**Table 4**).

LOQs were arbitrarily set at the lowest validated level. Preliminary trials showed that all analytes were detected with a chromatographic peak producing a signal-to-noise (S/N) ratio > 10 for SRM 1 and S/N > 3 for SRM 2. These limits were thus defined as "working LOQs".

The linearity of MMMCCs was checked for both methods in all matrices over the concentration ranges indicated in **Table 1** by calculating the RSD of the average of response factors (RF), which should be $RSD_{RF} < 15\%$ (*27*).

Proficiency Tests, CRMs, and QCM. Trueness by both QuEChERS-like and ASE procedures were obtained through participation in four FAPAS proficiency tests (P-tests) for (a) OTA (FAPAS No. 1779); (b) DON (FAPAS No. 2256); (c) T-2 and HT-2 (FAPAS No. 2252); and (d) aflatoxins B1, B2, G1, and G2 (FAPAS No. 04138) and by the analysis of two CRMs [one for ZON (CRM 32921) and one for FB1 and FB2 (CRM 32923)] and one QCM for ZON (QCM 2236). Quantification was performed using a two-point standard addition procedure. In that case, each sample was first divided into three 5 g test portions. One portion was analyzed as such, whereas the two other portions were spiked before sample workup with two distinct and increasing concentrations of analytes. The resulting concentration in the unspiked portion was then calculated as follows:

$$\text{concentration}\ (\mu\text{g/kg}) = \left|\frac{b}{a}\right|$$

where *a* is the slope and *b* is the y-intercept of the related analyte calibration curve.

Table 2. Transition Reactions Monitored by LC-ESI-MS/MS for the Analysis of Mycotoxins and Peak Area Ratios with their Limits of Acceptance According to Reference *26*

time	analyte	precursor ion	adduct	declustering potential (V)	product ions	collision energy (eV)	peak area ratio Q/C[a] ± limit (%)
0−12 min	AFLA B1	313.2	$[M + H]^+$	80	Q: 285.2	34	0.62 ± 20
				80	C: 269.1	43	
	DON	297.2	$[M + H]^+$	55	Q: 231.2	20	0.88 ± 20
				55	C: 249.2	23	
	AFLA B2	315.2	$[M + H]^+$	90	Q: 287.1	37	0.95 ± 20
				90	C: 259.0	41	
	AFLA G1	329.1	$[M + H]^+$	100	Q: 243.1	38	0.63 ± 20
				100	C: 200.2	57	
	NIV	330.1	$[M + NH_4]^+$	30	Q: 247.3	12	0.61 ± 20
				30	C: 229.2	20	
	AFLA G2	331.2	$[M + H]^+$	80	Q: 313.2	42	0.41 ± 25
				80	C: 245.2	43	
	3, 15-AcDON[b]	356.2	$[M + NH_4]^+$	40	Q: 231.2	32	0.39 ± 25
				40	C: 213.3	26	
	DAS	384.2	$[M + NH_4]^+$	50	Q: 307.3	16	0.50 ± 25
				50	C: 247.2	20	
	Fus-X	372.2	$[M + NH_4]^+$	35	Q: 247.3	10	0.66 ± 20
				35	C: 277.2	18	
	NEO	400.2	$[M + NH_4]^+$	55	Q: 245.2	17	0.94 ± 20
				55	C: 215.3	24	
	HT-2	442.2	$[M + NH_4]^+$	45	Q: 215.3	19	0.43 ± 25
				45	C: 323.2	13	
	T-2	484.3	$[M + NH_4]^+$	50	Q: 305.2	19	0.60 ± 20
				50	C: 215.2	27	
	FB2	706.4	$[M + H]^+$	70	Q: 336.7	53	0.79 ± 20
				70	C: 688.6	40	
	FB1	722.4	$[M + H]^+$	70	Q: 334.6	56	0.75 ± 20
				70	C: 704.6	41	
12−13 min	ZON	317.1	$[M - H]^-$	−80	Q: 174.9	−34	0.95 ± 20
				−80	C:131.0	−42	
13−15 min	OTA	404.1	$[M + H]^+$	55	Q: 239.0	34	0.96 ± 20
				55	C: 358.2	21	

[a] Q, transition reaction used for quantification; C, transition reaction used for confirmation. [b] 15- and 3-AcDON could not be chromatographically separated. Consequently, only their sum was considered.

Confirmation Criteria. The analytes were considered to be positively identified when the following criteria were met simultaneously: (a) the chromatographic retention time of the analyte in the sample corresponded to that of a calibration standard injected in the same run within a ±2.5% tolerance; (b) the peak area ratio from the two transition reactions recorded for each analyte; that is, the one used for quantification and the one used for peak confirmation, was similar to the one of a calibration standard injected in the same run within the tolerances fixed by the EU criteria (*26*), as shown in **Table 2**.

RESULTS AND DISCUSSION

LC-ESI-MS/MS Optimization. Mycotoxins were first analyzed in both positive and negative ESI-MS mode (ESI^+/ESI^-) to optimize the MS conditions. Using the ESI^+ mode, the ammonium adduct $[M + NH_4]^+$ ion was selected for all type A and B trichothecenes (NIV, Fus-X, 3- and 15-AcDON, DAS, NEO, T-2, and HT-2) but not for DON. For this latter analyte and for the aflatoxins B1, B2, G1, and G2, OTA, and FB1 and FB2 as well, predominance of $[M + H]^+$ ions was observed. Tuning experiments favored the choice of the ESI^+ mode since the sensitivity of critical compounds with low maximum levels (i.e., aflatoxins B1, B2, G1, and G2 and OTA) was clearly enhanced. In contrast, an acceptable sensitivity for ZON, as $[M - H]^-$ ion, was only obtained in the ESI^- mode. A better ionization yield for type B trichothecenes using ESI^- was not obtained, contrary to previous experiments (*28*). Collision-induced dissociation (CID) experiments were then conducted to select at least two SRMs per analyte (**Table 2**).

HPLC mobile phases commonly used for mycotoxins analysis are usually composed of water, MeCN, MeOH, with or without addition of salts, acids, or bases (*6*). MeOH is usually favored rather than MeCN for sensitivity reasons (*13*, *14*), and this fact was confirmed in our study. The addition of ammonium formate to the aqueous mobile phase clearly enhanced the sensitivity for both type A and B trichothecenes detected under their ammonium adduct $[M + NH_4]^+$, whereas formic acid in both mobile phases increased the overall sensitivity, giving better peak shape for the acidic compounds, i.e., FB1, FB2 and OTA (*14*). Chromatographic separation trials were performed with a Zorbax Bonus RP column and were essentially focused on the separation of ZON (detected in the ESI^- mode) from the other mycotoxins (detected in the ESI^+ mode) within one single run rather than two (*15*). Using the LC described conditions, more than 2000 sample extracts were injected without showing any notable shift of retention times. **Figure 1** shows SRM chromatograms of a spiked oat flour extract. However, we were not able to obtain a baseline separation for 3- and 15-AcDON under our LC conditions. Additionally, these positional isomers could not be characterized by specific product ions in the ESI^+ mode, contrary to previous findings (*29*−*31*). Consequently, both isomers were not quantified individually but rather as their sum by selecting transition reactions (m/z 356.2 → 231.2 and m/z 356.2 → 213.3) for which a comparable response was observed for both isomers.

Optimization of Extraction Methods. For the QuEChERS-like method, the main deviation compared to the original QuEChERS method (*23*) concerned essentially the cleanup step. Indeed, the use of dispersive SPE using primary secondary amine (PSA) salts to remove polar matrix components was not adapted

Table 3. Absolute Recovery and Precision Data Obtained by the QuEChERS-Like and ASE Extraction Methods

		absolute recovery ± RSD_{IR} (under intermediate reproducibility conditions, n^b = 12, k^c = 6)						absolute recovery ± RSD_r (under repeatability conditions, n^b = 6, k^c = 1)												
		corn		wheat		rice		rye		oat		barley		infant cereal		soya[d]		corn gluten		
analyte	spike levels[a]	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	spike levels[a]	QuEChERS	ASE
AFLA B1	1	92 ± 8	87 ± 9	97 ± 12	77 ± 4	98 ± 13	84 ± 11	99 ± 9	100 ± 7	99 ± 5	86 ± 7	96 ± 5	74 ± 8	94 ± 4	93 ± 11	98 ± 11	93 ± 17	10	89 ± 8	88 ± 5
	1.5	96 ± 11	78 ± 8	94 ± 14	75 ± 7	98 ± 9	86 ± 12	100 ± 7	99 ± 9	92 ± 5	85 ± 6	91 ± 5	85 ± 13	92 ± 4	85 ± 11	105 ± 9	83 ± 10	15	89 ± 6	83 ± 4
	2	98 ± 8	80 ± 7	93 ± 7	72 ± 9	101 ± 9	84 ± 13	92 ± 12	100 ± 6	90 ± 7	89 ± 4	91 ± 5	103 ± 8	92 ± 5	94 ± 3	100 ± 11	89 ± 15	20	91 ± 4	94 ± 3
AFLA B2	1	90 ± 10	90 ± 10	100 ± 12	80 ± 3	102 ± 9	78 ± 17	105 ± 8	110 ± 7	114 ± 10	78 ± 6	94 ± 7	82 ± 7	92 ± 2	93 ± 18	98 ± 16	87 ± 9	10	96 ± 3	78 ± 5
	1.5	93 ± 8	94 ± 10	99 ± 12	77 ± 5	95 ± 10	76 ± 22	102 ± 7	100 ± 9	105 ± 6	77 ± 4	90 ± 5	91 ± 6	90 ± 6	89 ± 12	96 ± 6	92 ± 10	15	95 ± 6	79 ± 2
	2	89 ± 7	87 ± 9	101 ± 9	80 ± 6	94 ± 5	72 ± 19	98 ± 10	98 ± 4	107 ± 4	81 ± 4	90 ± 6	98 ± 6	93 ± 4	96 ± 10	95 ± 4	103 ± 17	20	99 ± 1	86 ± 4
AFLA G1	1	99 ± 9	91 ± 13	97 ± 9	77 ± 6	101 ± 12	84 ± 11	99 ± 6	106 ± 5	105 ± 11	75 ± 4	95 ± 3	80 ± 5	91 ± 2	89 ± 7	113 ± 9	81 ± 7	10	92 ± 8	90 ± 9
	1.5	93 ± 10	85 ± 11	95 ± 12	69 ± 7	97 ± 7	82 ± 15	92 ± 6	104 ± 5	99 ± 6	74 ± 4	92 ± 6	91 ± 4	94 ± 3	85 ± 9	109 ± 9	89 ± 7	15	102 ± 5	86 ± 6
	2	97 ± 7	86 ± 12	95 ± 11	68 ± 9	99 ± 8	82 ± 15	89 ± 10	98 ± 4	99 ± 9	80 ± 4	89 ± 8	91 ± 6	95 ± 4	95 ± 10	116 ± 12	92 ± 10	20	105 ± 4	91 ± 6
AFLA G2	1	102 ± 11	87 ± 10	92 ± 9	77 ± 10	101 ± 9	80 ± 15	110 ± 8	107 ± 7	90 ± 12	78 ± 6	93 ± 7	87 ± 6	89 ± 5	89 ± 14	102 ± 7	77 ± 7	10	98 ± 10	81 ± 7
	1.5	93 ± 9	88 ± 8	95 ± 13	67 ± 11	97 ± 7	83 ± 14	96 ± 9	102 ± 4	90 ± 17	74 ± 3	89 ± 7	89 ± 6	91 ± 10	92 ± 10	113 ± 9	86 ± 7	15	98 ± 5	81 ± 3
	2	93 ± 6	94 ± 9	94 ± 5	71 ± 8	101 ± 3	83 ± 11	101 ± 7	99 ± 6	99 ± 7	80 ± 4	91 ± 7	88 ± 5	93 ± 4	97 ± 12	99 ± 9	93 ± 9	20	96 ± 7	85 ± 6
DON	50	88 ± 9	74 ± 5	112 ± 22	65 ± 6	81 ± 5	78 ± 11	99 ± 12	94 ± 9	84 ± 11	95 ± 3	81 ± 5	83 ± 3	81 ± 2	89 ± 12	95 ± 10	93 ± 1	250	98 ± 5	78 ± 5
	75	87 ± 8	82 ± 8	104 ± 17	69 ± 15	79 ± 7	77 ± 12	102 ± 4	92 ± 5	81 ± 7	97 ± 4	79 ± 5	83 ± 2	77 ± 5	86 ± 4	88 ± 5	88 ± 7	375	98 ± 6	80 ± 4
	100	83 ± 8	78 ± 3	97 ± 11	77 ± 9	78 ± 5	80 ± 11	99 ± 8	92 ± 7	83 ± 7	100 ± 5	75 ± 5	81 ± 3	82 ± 7	85 ± 3	87 ± 3	78 ± 2	500	98 ± 4	78 ± 3
NIV	100	**61 ± 8**	81 ± 3	**57 ± 12**	76 ± 4	**53 ± 9**	72 ± 10	**56 ± 5**	92 ± 2	**57 ± 10**	77 ± 5	**58 ± 6**	79 ± 6	**37 ± 4**	90 ± 10	**60 ± 3**	72 ± 4	500	73 ± 5	79 ± 2
	150	**58 ± 6**	79 ± 3	**58 ± 11**	72 ± 5	**55 ± 10**	73 ± 17	**61 ± 1**	90 ± 2	**53 ± 6**	79 ± 3	**58 ± 4**	82 ± 2	**44 ± 9**	86 ± 4	**60 ± 6**	73 ± 6	750	75 ± 5	80 ± 2
	200	**60 ± 8**	77 ± 4	**55 ± 7**	73 ± 7	**57 ± 6**	71 ± 11	**61 ± 5**	98 ± 4	**61 ± 10**	82 ± 1	**54 ± 8**	77 ± 6	**47 ± 8**	75 ± 8	**58 ± 4**	70 ± 5	1000	75 ± 3	79 ± 4
15-AcDON	50	99 ± 8	88 ± 4	83 ± 6	81 ± 9	103 ± 6	81 ± 11	84 ± 6	105 ± 5	97 ± 10	85 ± 5	92 ± 7	95 ± 5	93 ± 2	95 ± 6	97 ± 7	82 ± 4	250	111 ± 1	87 ± 5
	75	98 ± 6	85 ± 3	79 ± 13	77 ± 10	96 ± 7	82 ± 11	81 ± 9	101 ± 2	102 ± 6	82 ± 2	90 ± 3	95 ± 2	95 ± 3	86 ± 8	95 ± 2	82 ± 4	375	104 ± 3	86 ± 2
	100	96 ± 4	80 ± 4	73 ± 9	75 ± 8	100 ± 4	80 ± 8	80 ± 4	91 ± 3	102 ± 5	83 ± 2	89 ± 5	86 ± 5	97 ± 4	84 ± 14	94 ± 2	80 ± 5	500	97 ± 3	89 ± 2
DAS	25	99 ± 5	87 ± 6	86 ± 9	82 ± 5	97 ± 13	86 ± 9	88 ± 7	105 ± 2	104 ± 8	86 ± 4	95 ± 4	88 ± 3	97 ± 1	97 ± 9	107 ± 6	71 ± 4	125	101 ± 2	84 ± 3
	37.5	96 ± 6	85 ± 9	73 ± 15	80 ± 6	97 ± 10	85 ± 11	89 ± 8	103 ± 2	106 ± 4	87 ± 3	94 ± 2	93 ± 4	96 ± 3	84 ± 11	91 ± 11	78 ± 4	187.5	104 ± 4	83 ± 2
	50	98 ± 5	86 ± 7	77 ± 9	80 ± 2	101 ± 4	87 ± 7	88 ± 13	101 ± 2	103 ± 7	90 ± 3	93 ± 3	96 ± 5	101 ± 3	83 ± 9	84 ± 8	88 ± 8	250	113 ± 1	87 ± 3
FUS-X	25	99 ± 6	79 ± 8	71 ± 21	70 ± 13	100 ± 12	85 ± 20	83 ± 3	111 ± 11	95 ± 9	86 ± 5	92 ± 6	86 ± 3	87 ± 2	81 ± 10	94 ± 7	75 ± 4	125	97 ± 3	72 ± 1
	37.5	93 ± 6	81 ± 8	66 ± 24	69 ± 11	99 ± 9	87 ± 20	82 ± 1	104 ± 4	95 ± 5	87 ± 3	92 ± 5	88 ± 3	87 ± 2	80 ± 4	92 ± 2	73 ± 4	187.5	100 ± 1	73 ± 2
	50	95 ± 5	84 ± 6	68 ± 19	72 ± 5	93 ± 1	83 ± 15	81 ± 4	91 ± 4	97 ± 7	91 ± 3	89 ± 5	84 ± 5	90 ± 7	86 ± 10	89 ± 2	73 ± 4	250	101 ± 4	73 ± 2
NEO	25	99 ± 6	89 ± 5	81 ± 11	82 ± 5	96 ± 7	85 ± 7	92 ± 4	101 ± 2	100 ± 7	84 ± 5	98 ± 4	86 ± 3	92 ± 2	84 ± 11	96 ± 6	80 ± 4	125	101 ± 2	85 ± 3
	37.5	96 ± 6	86 ± 6	80 ± 16	81 ± 5	93 ± 8	90 ± 9	94 ± 4	98 ± 2	99 ± 1	86 ± 3	95 ± 4	88 ± 2	92 ± 3	80 ± 9	93 ± 2	77 ± 3	187.5	100 ± 2	83 ± 4
	50	96 ± 5	85 ± 4	76 ± 13	81 ± 3	98 ± 5	86 ± 5	91 ± 4	96 ± 3	100 ± 5	86 ± 2	91 ± 6	83 ± 4	95 ± 5	87 ± 14	90 ± 2	74 ± 7	250	96 ± 2	86 ± 3
HT-2	25	97 ± 7	88 ± 6	100 ± 7	85 ± 5	96 ± 10	84 ± 7	104 ± 7	103 ± 2	116 ± 5	82 ± 6	93 ± 5	87 ± 6	95 ± 3	88 ± 11	108 ± 8	72 ± 4	125	97 ± 5	86 ± 3
	37.5	97 ± 5	84 ± 5	100 ± 12	82 ± 5	96 ± 8	82 ± 9	105 ± 7	98 ± 2	115 ± 6	83 ± 2	90 ± 2	85 ± 8	94 ± 5	91 ± 9	99 ± 7	78 ± 5	187.5	94 ± 3	85 ± 5
	50	95 ± 4	82 ± 3	101 ± 9	80 ± 3	101 ± 4	85 ± 5	102 ± 8	95 ± 3	109 ± 9	84 ± 3	89 ± 4	91 ± 5	93 ± 3	88 ± 2	93 ± 7	76 ± 7	250	103 ± 4	92 ± 2

Table 3. Continued

		absolute recovery ± RSD_{IR} (under intermediate reproducibility conditions, n^b = 12, k^c = 6)						absolute recovery ± RSD_r (under repeatability conditions, n^b = 6, k^c = 1)												
		corn		wheat		rice		rye		oat		barley		infant cereal		soya[d]		corn gluten		
analyte	spike levels[a]	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS	ASE	spike levels[a]	QuEChERS	ASE
T-2	5	98 ± 7	91 ± 6	80 ± 14	79 ± 7	99 ± 12	86 ± 9	95 ± 7	104 ± 5	103 ± 14	78 ± 6	103 ± 4	87 ± 3	101 ± 2	87 ± 11	117 ± 10	66 ± 7	25	99 ± 6	76 ± 5
	7.5	95 ± 6	91 ± 6	69 ± 5	81 ± 6	95 ± 9	80 ± 12	96 ± 5	100 ± 3	108 ± 18	76 ± 5	97 ± 2	87 ± 2	106 ± 3	104 ± 12	104 ± 6	65 ± 6	37.5	103 ± 8	79 ± 3
	10	100 ± 3	89 ± 5	74 ± 11	80 ± 3	99 ± 4	81 ± 6	94 ± 8	97 ± 3	104 ± 12	79 ± 3	92 ± 5	89 ± 3	106 ± 2	100 ± 9	96 ± 5	72 ± 9	50	102 ± 4	79 ± 3
FB1	50	79 ± 14	*77 ± 32*	**64 ± 7**	**49 ± 8**	73 ± 13	85 ± 30	**61 ± 4**	118 ± 5	**32 ± 10**	76 ± 11	**67 ± 7**	76 ± 4	66 ± 5	103 ± 8	**12 ± 10**	84 ± 4	250	*72 ± 27*	125 ± 9
	75	90 ± 20	94 ± 17	**62 ± 11**	**52 ± 11**	71 ± 10	81 ± 8	**64 ± 3**	111 ± 7	**33 ± 11**	76 ± 6	**65 ± 3**	82 ± 1	69 ± 9	108 ± 8	**12 ± 7**	95 ± 3	375	84 ± 17	124 ± 6
	100	93 ± 10	97 ± 6	**60 ± 10**	**52 ± 16**	70 ± 10	82 ± 17	**63 ± 6**	103 ± 4	**38 ± 9**	76 ± 13	**62 ± 5**	81 ± 8	74 ± 9	99 ± 7	**12 ± 17**	81 ± 4	500	80 ± 9	102 ± 9
FB2	50	108 ± 9	94 ± 14	79 ± 4	**52 ± 13**	88 ± 12	85 ± 25	75 ± 4	100 ± 4	**58 ± 15**	109 ± 8	78 ± 7	83 ± 7	**39 ± 3**	98 ± 11	**5 ± 9**	69 ± 8	250	72 ± 12	126 ± 8
	75	95 ± 17	93 ± 10	80 ± 5	**55 ± 12**	86 ± 12	78 ± 13	76 ± 6	111 ± 7	**58 ± 4**	113 ± 12	70 ± 9	84 ± 4	**43 ± 8**	113 ± 17	**6 ± 9**	76 ± 8	375	89 ± 6	121 ± 8
	100	97 ± 6	94 ± 12	83 ± 6	**54 ± 23**	76 ± 10	87 ± 12	77 ± 8	103 ± 4	**61 ± 5**	118 ± 9	76 ± 10	86 ± 7	**47 ± 11**	81 ± 9	**6 ± 15**	74 ± 4	500	109 ± 9	107 ± 5
ZON	20	100 ± 6	93 ± 7	95 ± 10	76 ± 8	98 ± 13	82 ± 16	97 ± 10	110 ± 7	91 ± 8	89 ± 5	93 ± 5	89 ± 5	91 ± 3	78 ± 4	115 ± 13	**49 ± 9**	100	113 ± 3	52 ± 9
	30	96 ± 7	87 ± 12	91 ± 14	70 ± 8	95 ± 12	80 ± 16	105 ± 4	113 ± 3	89 ± 11	86 ± 6	87 ± 3	86 ± 6	86 ± 3	87 ± 8	105 ± 10	**58 ± 8**	150	101 ± 6	65 ± 3
	40	95 ± 8	89 ± 7	89 ± 8	69 ± 7	96 ± 11	74 ± 12	103 ± 8	108 ± 5	96 ± 7	89 ± 5	83 ± 2	89 ± 5	86 ± 5	103 ± 3	99 ± 9	**56 ± 8**	200	113 ± 4	74 ± 5
OTA	0.5	100 ± 6	94 ± 13	112 ± 6	73 ± 23	104 ± 9	74 ± 18	96 ± 9	102 ± 3	87 ± 7	87 ± 23	91 ± 7	87 ± 23	87 ± 2	81 ± 4	67 ± 15	74 ± 5	2.5	93 ± 3	88 ± 3
	0.75	97 ± 8	85 ± 15	97 ± 15	88 ± 6	99 ± 11	82 ± 13	98 ± 13	99 ± 6	80 ± 16	92 ± 6	85 ± 9	92 ± 6	88 ± 6	83 ± 4	77 ± 7	77 ± 8	3.75	95 ± 3	88 ± 3
	1	100 ± 7	88 ± 14	97 ± 10	83 ± 9	110 ± 7	82 ± 11	102 ± 21	94 ± 3	88 ± 8	94 ± 4	90 ± 11	94 ± 4	91 ± 6	89 ± 3	76 ± 21	73 ± 3	5	92 ± 3	82 ± 1

[a] Spike levels are given in μg/kg. [b] *n* = number of replicates per level. [c] *k* = number of days. [d] Levels of fortification for AFLAs in soya were 2−3 and 4 μg/kg. Quantification was performed by MMCC. Absolute recovery values significantly outside the 70−120% range are written in bold, while precision data (RSD_r and RSD_{IR}) above the analytical requirements established in ref *34* are written in bold and italic.

Table 4. Absolute (ABS) and Apparent (APP) Recovery Data (%) by the QuEChERS-Like Method[a]

		corn		wheat		rice	
analyte	spike levels (μg/kg)	ABS	APP	ABS	APP	ABS	APP
AFLA B1	1	96 ± 10	98 ± 4	99 ± 18	105 ± 7	98 ± 3	102 ± 4
	1.5	100 ± 14	93 ± 3	96 ± 7	100 ± 6	94 ± 1	99 ± 1
	2	98 ± 3	108 ± 6	94 ± 2	95 ± 1	93 ± 5	99 ± 6
AFLA B2	1	86 ± 11	85 ± 4	98 ± 4	89 ± 5	105 ± 5	99 ± 5
	1.5	89 ± 7	99 ± 18	107 ± 8	102 ± 10	95 ± 3	99 ± 4
	2	87 ± 8	103 ± 10	106 ± 0	105 ± 3	91 ± 3	97 ± 3
AFLA G1	1	91 ± 11	87 ± 8	93 ± 7	101 ± 5	97 ± 3	98 ± 2
	1.5	93 ± 7	97 ± 25	98 ± 6	104 ± 5	97 ± 3	93 ± 6
	2	101 ± 9	97 ± 2	97 ± 3	102 ± 5	98 ± 5	99 ± 4
AFLA G2	1	103 ± 6	94 ± 6	91 ± 6	99 ± 8	97 ± 2	101 ± 5
	1.5	91 ± 9	89 ± 7	92 ± 6	105 ± 6	96 ± 2	105 ± 6
	2	94 ± 7	99 ± 7	97 ± 4	101 ± 5	91 ± 2	95 ± 2
DON	50	89 ± 6	89 ± 4	105 ± 4	102 ± 17	77 ± 5	99 ± 4
	75	93 ± 4	87 ± 4	96 ± 2	104 ± 2	73 ± 6	98 ± 6
	100	91 ± 3	90 ± 3	94 ± 8	102 ± 4	72 ± 0	97 ± 3
NIV	100	**64 ± 3**	86 ± 4	**59 ± 1**	99 ± 9	**53 ± 3**	94 ± 2
	150	**60 ± 0**	85 ± 1	**61 ± 5**	101 ± 3	**54 ± 1**	96 ± 0
	200	**70 ± 4**	95 ± 6	**57 ± 3**	98 ± 5	**51 ± 4**	93 ± 3
15-AcDON	100	98 ± 3	95 ± 4	72 ± 2	100 ± 4	72 ± 2	103 ± 3
	150	97 ± 3	98 ± 7	75 ± 2	104 ± 5	75 ± 2	99 ± 3
	200	106 ± 1	103 ± 1	69 ± 2	99 ± 1	69 ± 2	101 ± 10
DAS	25	101 ± 1	93 ± 3	**59 ± 2**	99 ± 1	93 ± 4	96 ± 4
	37.5	100 ± 3	95 ± 4	**60 ± 6**	98 ± 4	94 ± 1	95 ± 1
	50	103 ± 3	100 ± 2	**61 ± 5**	99 ± 3	95 ± 3	97 ± 3
FUS-X	25	92 ± 9	90 ± 9	**64 ± 2**	95 ± 2	86 ± 3	102 ± 4
	37.5	92 ± 1	88 ± 8	**71 ± 2**	96 ± 3	93 ± 2	99 ± 3
	50	95 ± 3	96 ± 4	**65 ± 6**	101 ± 1	89 ± 1	101 ± 2
NEO	25	102 ± 3	92 ± 2	81 ± 3	97 ± 3	91 ± 3	101 ± 5
	37.5	102 ± 2	94 ± 5	83 ± 4	98 ± 4	87 ± 3	97 ± 3
	50	103 ± 2	95 ± 2	82 ± 2	101 ± 4	91 ± 1	98 ± 1
HT-2	25	99 ± 2	97 ± 6	100 ± 0	101 ± 1	94 ± 3	96 ± 3
	37.5	101 ± 1	98 ± 6	100 ± 2	104 ± 2	95 ± 1	99 ± 1
	50	103 ± 3	104 ± 2	100 ± 0	102 ± 2	92 ± 3	99 ± 5
T-2	5	99 ± 3	92 ± 8	68 ± 6	97 ± 1	92 ± 5	100 ± 3
	7.5	100 ± 1	102 ± 4	65 ± 7	100 ± 1	91 ± 4	96 ± 6
	10	104 ± 2	103 ± 4	64 ± 4	101 ± 4	90 ± 2	94 ± 2
FB1	50	75 ± 12	79 ± 11	**63 ± 3**	98 ± 3	77 ± 5	95 ± 5
	75	83 ± 16	93 ± 17	**61 ± 4**	98 ± 3	78 ± 5	96 ± 4
	100	93 ± 6	106 ± 11	**61 ± 9**	100 ± 2	79 ± 3	96 ± 3
FB2	50	108 ± 5	88 ± 4	74 ± 3	98 ± 3	104 ± 3	97 ± 4
	75	101 ± 3	93 ± 6	77 ± 1	97 ± 8	97 ± 4	96 ± 4
	100	97 ± 1	89 ± 4	82 ± 5	98 ± 3	104 ± 5	101 ± 6
ZON	20	103 ± 2	94 ± 1	83 ± 3	101 ± 18	95 ± 4	96 ± 4
	30	99 ± 6	98 ± 9	87 ± 5	108 ± 1	93 ± 5	93 ± 5
	40	103 ± 0	102 ± 3	86 ± 2	103 ± 0	94 ± 3	95 ± 3
OTA	0.5	105 ± 8	97 ± 4	119 ± 5	94 ± 4	124 ± 4	93 ± 5
	0.75	99 ± 10	101 ± 4	110 ± 4	100 ± 3	117 ± 3	92 ± 1
	1	102 ± 3	106 ± 4	99 ± 7	102 ± 4	117 ± 7	95 ± 3

[a] Values are means ± RSD_r (n = 4, k = 2 days) under repeatability conditions. Absolute recovery values significantly outside the (70−120%) range are written in bold. Quantification was performed by MMMCC.

in our case since it leads to the loss of the acidic FB1 and FB2. No cleanup steps at all, as proposed elsewhere (*16*, *18*, *22*), was not conclusive as well. Indeed, significant matrix effects and insufficient sensitivity (especially for the aflatoxins) were noticed when analyzing extracts obtained directly after the $MgSO_4$:NaCl partitioning step. Among the several cleanup procedures investigated (SPE on either Oasis HLB, Carbograph-4, and C_{18} cartridges or dispersive-SPE with both PSA and C_{18}-modified silica material), a simple defatting step with *n*-hexane followed by a two-step sequential reconstitution in MeOH:H_2O was shown to be adapted to all analyte/matrix combinations. Another deviation from the QuEChERS protocol was the addition of 0.5% of acetic acid to the extracting solution, which was found mandatory to reach satisfactory absolute recovery (> 80%) for FB1 and FB2 in corn, in which these mycotoxins predominantly occur.

For the ASE method, already considered for mycotoxins analysis in grain (*32*), the best absolute recoveries were obtained when the extraction medium was composed of MeCN, water, and acetic acid (80:19.5:0.5; v/v/v). As for the QuEChERS-like extraction, a good extraction yield for FB1 and FB2 was related to the addition of 0.5% of acetic acid. The influence of the temperature of the extraction medium was investigated as well, and extraction at room temperature was favored since higher temperatures (above 60 °C) led to the extraction of more matrix components and consequently more interfering peaks. Direct injection of the resulting ASE extracts still led to high matrix effects, thus requiring a mandatory cleanup step, which

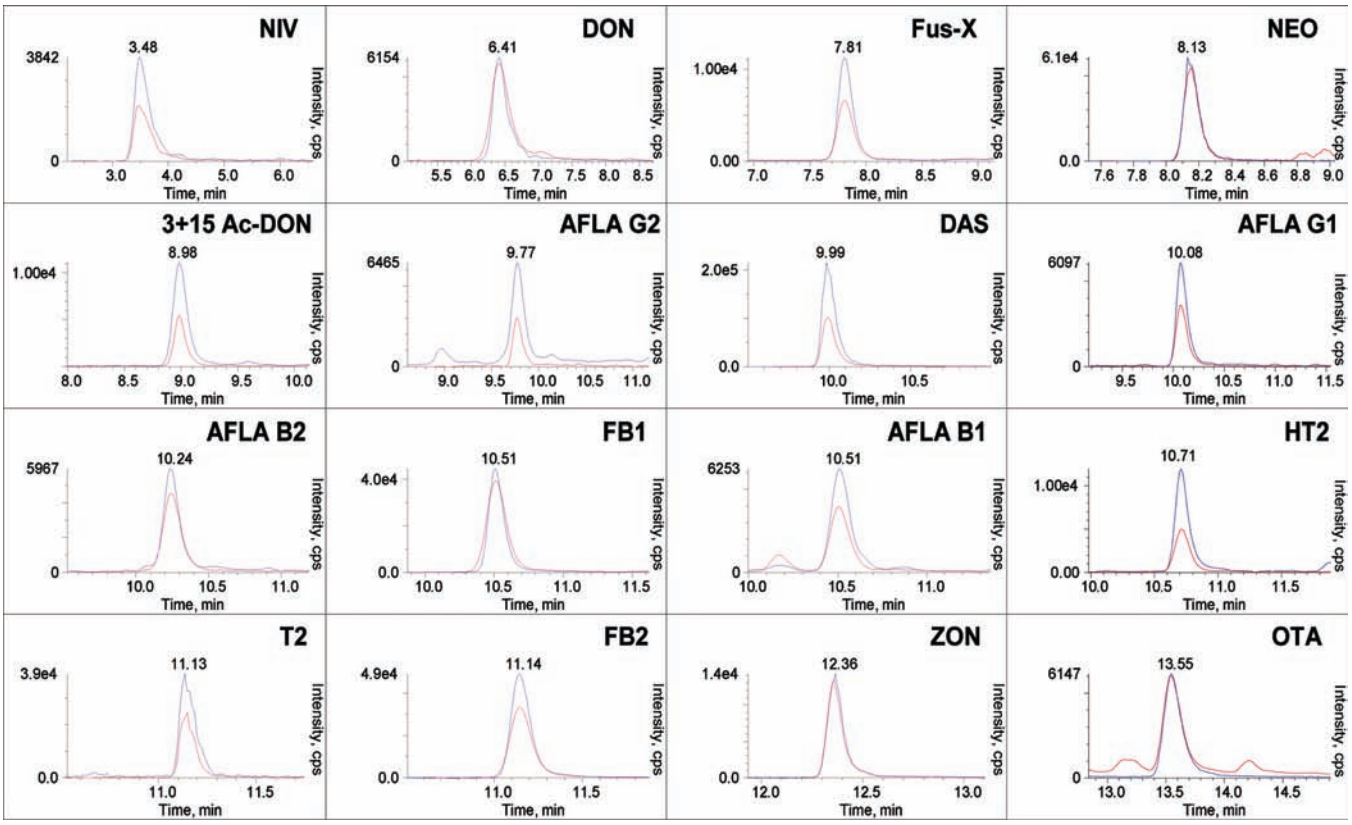

Figure 1. LC ESI-MS/MS chromatograms of mycotoxins from an extract of oat flour. Spiking levels: 75 (DON, 15-AcDON, FB1, and FB2), 150 (NIV), 37.5 (Fus-X, HT-2, NEO, and DAS), 7.5 (T-2), 3 (AFLA B1, B2, G1, and G2), 20 (ZON), and 3 μg/kg (OTA).

was similar to the one described for the QuEChERS-like procedure.

Methods Performance Characteristics. Absolute recovery values assessed for the nine matrices are presented in **Table 3**. By the QuEChERS-like method, values fall within the 70−120% range [as recommended in pesticide residues analysis (*33*)] with some exceptions, that is, NIV (around 55%) in all matrices but corn gluten, FB1 (12−67%) in wheat, rye, oat, barley, and soya, and finally FB2 (5−61%) in oat, infant cereal, and soya. By the ASE method, absolute recovery values were also scored as satisfactory, except for FB1 and FB2 (around 52%) in wheat and ZON (around 54%) in soya. These data were generally consistent for both extraction procedures (n = 432) with average RSD_r and RSD_{IR} values < 20% (QuEChERS: min = 1%, max = 27%, and median = 7%; ASE: min = 1%, max = 32%, and median = 6%). Only one value for each method (QuEChERS: RSD_r = 27% for FB1 in corn gluten at the 250 μg/kg level; ASE: RSD_{IR} = 32% for FB1 in corn at the 50 μg/kg level) was outside the analytical requirements for precision established in ref *34*. On the basis of these experiments, one would conclude that the extraction efficiencies achieved by both methods were high and consistent despite values were more comparable from matrix to matrix for the ASE method than for the QuEChERS-like method. Nevertheless, in rare cases for both methods, and notably for FB1 and FB2, absolute recoveries were dependent on the mycotoxin/matrix combination. In contrast, the use of MMMCCs used for the analysis of corn, wheat, and rice (tested only for the QuEChERS-like extraction) gave all apparent recoveries within the 70−120% range (min = 79%, max = 108%, and median = 98%), with good related precision values (min = 0%, max = 25%, and median = 4%), whatever the mycotoxin/matrix combination (**Table 4**).

Both extraction procedures in all tested matrices gave LOQs below the maximum levels settled in refs *4* and *5* except for AFLA B1 in infant cereals (maximum level = 0.1 μg/kg, LOQ = 1 μg/kg). Matrix effects were nevertheless more important in soya (LOQ for the aflatoxins B1, B2, G1, and G2 = 2 μg/kg) and even more in corn gluten (pet food material). For this latter, the extent of the matrix effect rendered mandatory to decrease the test portion at 1.0 g, all other parameters remaining constant. Higher LOQs were thus obtained in corn gluten (pet food ingredient) for which no regulatory limits have been set yet (**Table 1**).

Selection of the Quantification Approach. When no isotopically labeled internal standard is available (or too expensive to be considered for routine analysis) for each analyte under survey, quantification by means of MMMCCs is usually considered to be the best option to compensate for both losses during extraction and matrix effects generated during the ionization of the analytes (*35*). This quantification procedure, although successfully tested by the QuEChERS-like method (**Table 4**), still requires the availability of matrices free of any mycotoxin surveyed, which can be a difficult prerequisite in cereals analysis. Additionally, one MMMCC per matrix type is necessary, as proposed by Frenich et al (*22*), leading to a considerable workload when different types of cereals have to be monitored simultaneously. Moreover, the use of one single MMMCC for different samples of the same matrix type but of different origins may not efficiently compensate matrix effects, as demonstrated by Sulyok (*16*) during the monitoring of different rice samples.

Quantification by the standard addition procedure may represent a better alternative. When the absolute recovery of extraction for each analyte/matrix combination is known in advance (i.e., from the validation process, as done in this study), quantification of a positive sample can be performed directly using the final extract solution. Thus, this one is first divided in several portions and further supplemented with increasing concentrations of standard solutions before LC-ESI-MS/MS analysis and quantification.

Table 5. Comparison of Trueness Data Obtained by both Extraction Methods during the Analysis of Four FAPAS P-Tests, Two CRMs, and One QCM[a]

			QuEChERS-like			ASE		
matrix	analyte	assigned value (μg/kg)	results (μg/kg)	trueness (%)	Z score	results (μg/kg)	trueness (%)	Z score
oat (FAPAS 2252)	T-2	194	211	109	0.4	213	110	0.5
	HT-2	125	144	115	0.7	116	93	−0.3
wheat (FAPAS 2256)	DON	774	717	93	−0.5	802	104	0.2
barley (FAPAS 1779)	OTA	3.52	3.34	95	−0.2	3.21	91	−0.4
	AFLA B1	1.87	1.90	102	0.1	1.33	71	−1.4
	AFLA B2	0.51	0.47	92	−0.4	0.51	100	0.0
maize (FAPAS 04138)	AFLA G1	0.96	0.89	93	−0.3	1.25	130	1.3
	AFLAG2	0.52	0.55	106	−0.3	0.60	115	0.7
	Total AFLA	3.79	3.79	100	0	3.69	97	−0.1
maize (CRM 32923)	FB1	2406	2493	104	NA[b]			
	FB2	630	594	94	NA[b]			
maize (CRM 32921)	ZON	60	52	87	NA[b]	44	73	NA[b]
baby food (QCM FAPAS 2236)	ZON	20.2	20.1	100	NA[b]	14.9	74	NA[b]

[a] The quantification method used was the 2-point standard addition. [b] Values not available.

As such, results are corrected for matrix effects but not for recovery, meaning that its previous knowledge is mandatory to obtain precise results. This procedure is currently used in pesticide residue analysis, as explained in European Norm EN 15662 (*36*). However, the validation process clearly showed the complexity of such an approach since some absolute recoveries were varying, depending on the mycotoxin/matrix combination (**Table 3**). Additionally, variable absolute recovery can be obtained when dealing with samples of the same matrix type but with different properties (e.g., comminution degree, moisture content, etc.) compromising thus the use of this "absolute recovery based standard addition".

Another approach of the standard addition procedure is to perform spiking experiments at the beginning of the sample workup. Each routine sample is divided in several test portions of identical mass, which are then spiked at different fortification levels. By spiking samples before workup, results are thus automatically compensated for both matrix effects and extraction recovery as shown during apparent recovery experiments (**Table 4**). This "apparent recovery based standard addition" procedure was tested within the frame of four FAPAS P-tests and by the analysis of two CRMs and one QCM, involving all EU-regulated mycotoxins. The two-point standard addition procedure was used for these experiments and for both procedures, and results are summarized in **Table 5**. All Z scores obtained during P-tests were $|Z| < 2$ for both procedures, proving the suitability of this quantification approach. Nevertheless, trueness values obtained during P-tests, CRM, and QCM analysis were better for the QuEChERS-like method (within the 92−115% range) as compared to those from the ASE method (within the 71−130% range).

Method Comparison and Applicability in Routine. Both methods showed high extraction efficiency in a broad range of cereal-based products and with a comparable sensitivity. Nevertheless, the easiness-to-handle of these extraction methods was definitely in favor of the QuEChERS-like procedure, since not asking for any tedious preparation of extraction cells, requiring less reagents and glassware and involving less intermediate steps. Consequently, a higher sample throughput was possible, with up to 40 individual samples extracted over one working day as compared to the 24 individual samples processed over a one and a half working days by the ASE procedure. On a routine basis, the QuEChERS-like method constitutes undeniably the best option.

For the applicability of the QuEChERS-like method in routine analyses, when many different cereal-based foodstuffs have to be screened, the two-point standard addition approach constitutes the best option, combining a good sample throughput with reliable quantitative results. Each routine sample is analyzed in triplicate, one as such, whereas the second and the third ones are fortified with increasing concentrations of mycotoxins, for example, at 2- and 4-fold the LOQ. When the routine sample is free of any mycotoxin, looking at the chromatogram of the spiked sample at 2-fold the LOQ allows to verify that all mycotoxins are present and that the method is still capable of detection around the LOQ concentrations. When the routine sample is contaminated, the standard addition procedure permits the quantification provided that the concentrations of the added analytes are consistent with linearity and are ideally between one and five times the original concentration of the analyte (*33*). In case of results around/above the maximum level or not fulfilling the prerequisites cited above, a confirmation using a four- or five-point standard addition procedure will be performed.

Safety. Various mycotoxins are mutagenic, teratogenic, and immunosuppressive and should be handled with appropriate caution. The handling or preparation of standards, working solutions, and samples must be performed in a fume hood with appropriate protective attire (laboratory coat, mask, and gloves). Prior to their disposal, the contaminated glassware should be decontaminated with sodium hypochlorite (5%) and then with acetone (5% of the total volume) for at least 30 min in both cases.

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