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Ultrahigh-Performance Liquid Chromatographic−Tandem Mass Spectrometric Multimycotoxin Method for Quantitating 26 Mycotoxins in Maize Silage

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ABSTRACT: A multianalyte method was developed to identify and quantitate 26 mycotoxins simultaneously in maize silage by means of ultrahigh-performance liquid chromatography−tandem mass spectrometry (UHPLC-MS/MS). The extraction and cleanup procedure consists of two extraction steps followed by purification on a Waters Oasis HLB column. The method developed was validated with the requirements of Commission Decision 2002/657/EC taken into account. The limit of detection and quantitation ranges were 5−348 and 11−695 ng/g, respectively. Apparent recovery varied between 61 and 116%, whereas repeatability and reproducibility were within the ranges of 3−45 and 5−49%, respectively. The method developed was successfully applied for maize silage samples taken at the cutting surface and 1 m behind that surface. Mainly *Fusarium* toxins (beauvericin, deoxynivalenol, enniatins, fumonisins, fusaric acid, and zearalenone) were detected, but postharvest toxins such as mycophenolic acid and roquefortine C were identified as well.

KEYWORDS: multimycotoxin analysis, UHPLC-MS/MS, maize silage, validation

INTRODUCTION

Ensiled whole maize crop constitutes a major part of the feed ration for cattle in many parts of the world. Use of silage is increasing, in winter as well as during the summer. The main advantages of maize silage are the ease of mechanization from harvest to feed-out, the prolonged preservation of the feed, its use in seasons when fresh feed is no longer available, and its high digestibility and energy value for ruminants.[1] Good-quality silage depends on creating anaerobic conditions and reaching a stable, low pH. Disruption of these anaerobic conditions can result in mold growth. Such deterioration can lead to a reduction of the nutritional value of the feed and allergic reactions due to fungal spores, but, most importantly, it can lead to the presence of mycotoxins. In addition to mycotoxins produced by molds at the postharvest stage (during storage), mycotoxins may also originate from molds contaminating the crop in the field, that is, at the preharvest stage. As a result of their carcinogenic, teratogenic, estrogenic, nephrotoxic, neurotoxic, hepatotoxic, and/or immunosuppressive effects, mycotoxins present in feed commodities might imply important health risks for farm animals.[2] Specifically, their presence may lead to economic losses and veterinary costs due to the negative effects on animal production and welfare of ruminants such as reduced feed intake or even feed refusal, disruption of the immune system, increased incidence of mastitis, and reproductive problems.[3,4] Due to their very high chemical and physical resistance, some mycotoxins are known to be transmitted into animal-derived products such as milk and meat.[3−5] This feature of indirect exposure of humans to mycotoxins present in contaminated feed commodities may jeopardize human health, although further research to estimate the degree of such exposure is needed.

The main mycotoxigenic postharvest mold genera reported in silage are *Penicillium*, *Aspergillus*, *Byssochlamys*, and *Monascus*, whereas *Fusarium* appears to be the most important preharvest contaminant in maize. In particular, the presence of species such as *Aspergillus fumigatus* Fresen., *Aspergillus parasiticus* Speare, *Byssochlamys nivea* Westling, *Fusarium verticillioides* (Sacc.) Nirenberg, *Fusarium graminearum* Schwabe, *Monascus ruber* Tiegh., *Paecilomyces variotii* Bainier, *Penicillium carneum* Frisvad, *Penicillium expansum* Link, *Penicillium paneum* Frisvad, and *Penicillium roqueforti* Thom has been described.[6−13] Not surprisingly, mycotoxins such as fumonisins, aflatoxin B_1, citrinin, gliotoxin, zearalenone, deoxynivalenol, roquefortine C, mycophenolic acid, andrastin A, festuclavine, patulin, and others have been detected in maize silage.[7,8,11,12,14,15] The co-occurrence of different toxic compounds in one matrix implies a potential risk for additional or even synergistic toxic effects. Additionally, it points to the need to develop a multimycotoxin analysis method that will enable correct assessment of the degree of mycotoxin contamination of maize silage and, indirectly, the possible correlated animal health risks. Such multimethods allow detecting many compounds in one analytical run, thus reducing analysis time and costs. Unfortunately, the high compound diversity of the mycotoxins considered in these methods creates the need for compromises concerning extraction solvents, sample cleanup, and chromatographic separation. Several multimycotoxin liquid

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chromatographic−tandem mass spectrometric (LC-MS/MS) methods have been described for different kinds of feed and food commodities, most of them concerning high-performance liquid chromatography (HPLC) methods. Use of HPLC is now giving way to UHPLC because the latter combines high sample throughput, fast detection, and quantitation with higher sensitivity, selectivity, and resolution. This is a result of the smaller particle size of the column packing material compared with HPLC. The number of literature reports of UHPLC-MS/MS multimycotoxin methods is expanding. From the LC-MS/MS studies focusing on the occurrence of several mycotoxins in maize silage, only a few have been validated,[8,16,17] but none used UHPLC in combination with tandem mass spectrometry. Several literature papers give an overview of the analytical methods used for the determination of mycotoxins.[18−21]

The aim of this study is the development of an UHPLC-MS/MS method for the simultaneous determination of 26 mycotoxins from maize silage, focusing on postharvest (e.g., roquefortine C) and preharvest mycotoxins (e.g., deoxynivalenol, zearalenone, and fumonisins as well as emerging *Fusarium* mycotoxins such as enniatins and beauvericin). Method performance characteristics such as specificity, linearity, possible matrix effects, apparent recovery (R_A), repeatability (RSD_r), intralaboratory reproducibility (RSD_R), and limits of detection (LOD) and quantitation (LOQ) were evaluated. This method was successfully applied for maize silage samples taken at the cutting surface of the silo and 1 m behind that surface. The method developed is the first report of an UHPLC-MS/MS method for maize silage and is an excellent tool to conduct further research on the true burden of mycotoxins in this type of feed commodity. Data on the co-occurrence of mycotoxins in maize silage can give indications about the exposure degree of cattle to these toxins, the risk of animal health concerns, and the possibility of transmission into milk and meat.

MATERIALS AND METHODS

Maize Silage Samples. Two types of maize silage samples were collected on five farms in Belgium in the period from November 2009 to January 2010 approximately within 1 month after the silo had been opened. Silage samples were taken in transects by means of a metal, cleanable core sampler. The first type of sample was maize silage taken approximately 20 cm from the silo cutting surface, whereas the second sample type was taken 1 m behind this first sampling spot (as a reference of the fermented material not yet exposed to air). One kilogram of each sample type was collected in duplicate in a sterile bag and thoroughly homogenized. All samples were kept on ice during transport to the laboratory. Fifty grams of silage material was used to isolate and enumerate the contaminating molds.[10] One kilogram was used for analyzing pH, lactic acid,[22] and acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid.[23] The remaining part of silage was stored at −20 °C until UHPLC-MS/MS analysis for the presence of mycotoxins.

Mold Identification. The molds isolated after dilution plating of silage samples on dichloran Rose-Bengal chloramphenicol agar (DRBC, Oxoid Ltd., Basingstokes, Hampshire, U.K.) medium were purified by subsequent incubation on malt extract agar (MEA) medium (Oxoid Ltd.).[10] Afterward, the isolates were stored both as a MEA agar slant collection and as a −80 °C glycerol collection. Isolates were identified on the basis of morphological features as well as molecularly identified on the basis of the internal transcribed spacer (ITS) region.[10] Yeast isolation and identification were not included in this study.

UHPLC-MS/MS Method. *Reagents and Chemicals.* Methanol (MeOH, LC-MS absolute), acetonitrile (MeCN, ULC-MS), and formic acid (99%, ULC-MS) were supplied by Biosolve B.V. (Valkenswaard, The Netherlands). Ammonium acetate and isopropanol were purchased from Merck (Darmstadt, Germany). Water (H_2O) was of HPLC grade (generated by a Milli-Q Gradient purification system, Millipore, Bedford, MA).

The standards of aflatoxins (**1**−**4**), beauvericin (**5**), citrinin (**6**), cyclopiazonic acid (**7**), deoxynivalenol (**8**), fusaric acid (**13**), fumonisins (**14**, **15**), gliotoxin (**16**), HT-2 toxin (**17**, 0.1 mg/mL in MeCN), mycophenolic acid (**18**), nivalenol (**19**), ochratoxin A (**20**), patulin (**21**), salinomycin, sterigmatocystin (**23**), T-2 toxin (**24**), verrucarol, α-zearalenol (**25**), β-zearalenol (**26**), and zearalenone (**27**) were supplied by Sigma-Aldrich (Bornem, Belgium) (Figure 1). Roquefortine C (**22**) was obtained from IRIS Biotech GmbH (Marktredwits, Germany). Enniatin standards (**9**−**12**) were purchased from Alexis Biochemicals (Farmingdale, NY). Stock solutions of **1**−**5**, **19**, **23**, **24**, and **27** were prepared in MeCN at a concentration of 1 mg/mL. Methanol was used to dissolve the standards of **6**−**16**, **18**, **20**−**22**, **25**, and **26** at 1 mg/mL. All stock solutions were stored at −20 °C. Working solutions were prepared fresh and protected from light prior to each experiment.

Instrumental Conditions. An Acquity Ultra Performance liquid chromatograph (Waters, Milford, MA) was used. The column used was a 100 mm × 2.1 mm i.d., 1.7 μm, UPLC BEH C_{18}, with an Acquity UPLC column in-line filter (Waters). The column was held at 45 °C for chromatographic separation. The injection volume was set at 10 μL and the eluent flow at 800 μL/min. This eluent consisted of a gradually changing amount of H_2O + 0.1% formic acid + 0.1% isopropanol + 1 mM ammonium acetate (solvent A) and MeCN + 0.1% formic acid + 0.1% isopropanol (solvent B). This gradient was initiated with 10% eluent B, continued with a linear increase to 12% B in 1.50 min, subsequent linear increase to 95% B in 5.25 min, held at 100% B for 1 min, and finally re-equilibrated at 10% B for 1.25 min prior to the next injection.

The MS equipment consisted of a Xevo TQ mass spectrometer (Waters) equipped with a Z-spray system. Mass spectrometric characteristics such as cone voltage and collision energy were optimized by continuously infusing pure standards (1 μg/mL, 15 μL/min) into the mass spectrometer in combination (T-device) with a solvent flow consisting of MeCN/H_2O (50:50, v/v) + 0.1% formic acid from the LC column (flow rate of 250 μL/min). The precursor ion and the two product ions with the highest signal-to-noise (S/N) value and the highest peak intensity were selected for each analyte. The product ion with the highest S/N ratio and highest intensity was selected for validation experiments, whereas the second specific product ion was used for confirmation purposes. The sum of the relative peak areas of both ions was used for quantitation. Depending on the compound, the electrospray ionization (ESI) interface operated in both negative and positive mode in each run. Source temperature and desolvation temperature were set at 150 and 600 °C, respectively. Analyte-specific detection parameters are listed in Table 1. The data obtained were analyzed using MassLynx software version 4.1 (Waters).

Extraction and Cleanup Procedure. The extraction procedure described by Garon et al.[8] was used as a starting point. Prior to extraction, these samples were spiked with the internal standard salinomycin (250 ng/g) and verrucarol (1000 ng/g) and were left to equilibrate for 30 min. For each sample, an extraction procedure based on two separate steps was executed. In the first step, 15 mL of MeOH was added to 2 g of maize silage (wet weight) in a Falcon tube protected from light and put on a horizontal shaker for 30 min. After centrifugation at 4000*g* for 10 min, the supernatant was collected and kept apart in a light-protected Falcon tube. A second extraction step with 15 mL of MeCN/H_2O (84:16, v/v) was introduced. After 30 min of shaking and centrifugation for 10 min at 4000*g*, the supernatant was combined with the MeOH layer. An extra

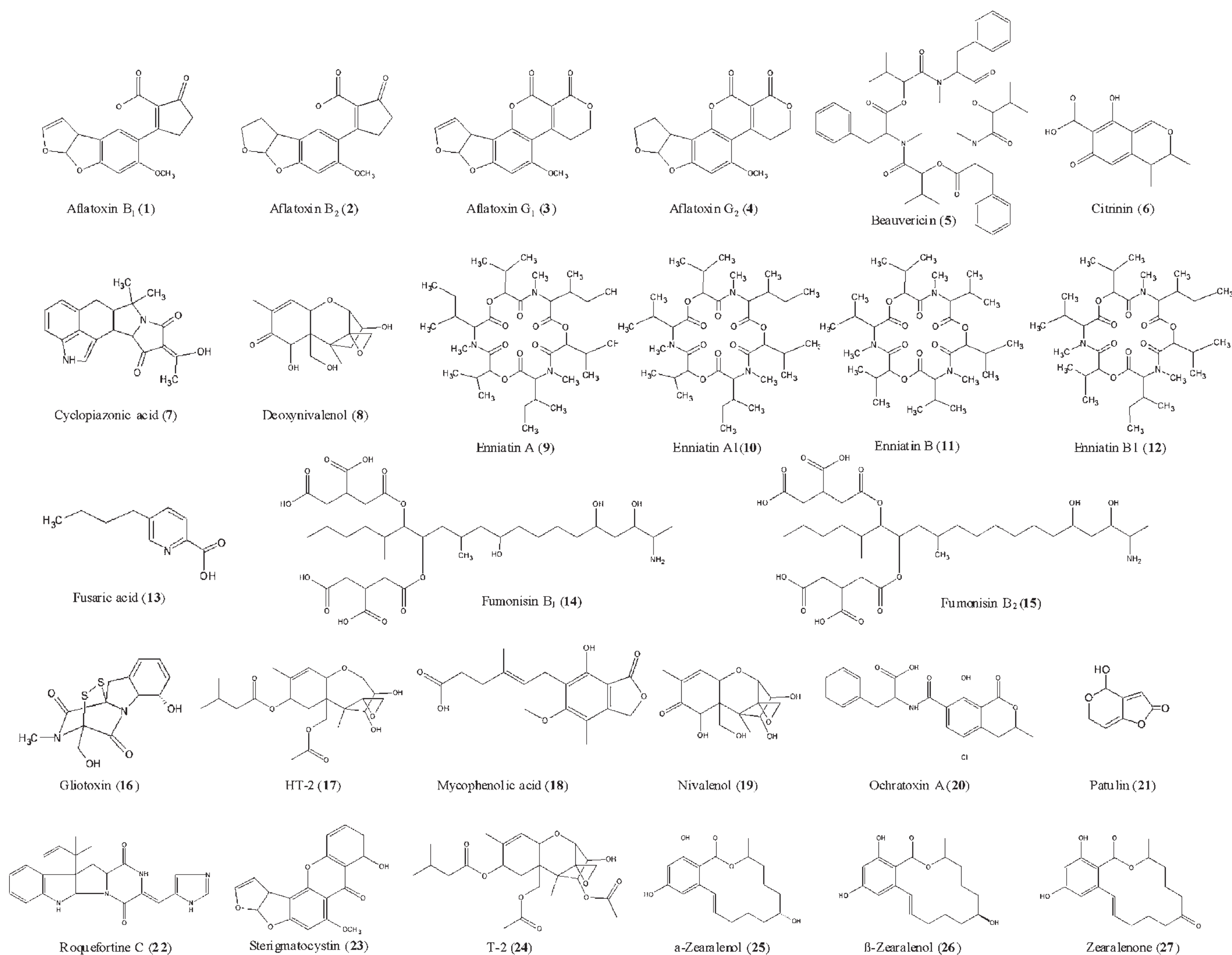

Figure 1. Chemical structures of mycotoxins considered in the present study.

centrifugation step at 4000*g* for 5 min was introduced. The supernatant was collected and evaporated to 5 mL by nitrogen gas at 60 °C. Subsequently, this was 1:10 diluted by adding HPLC/H_2O. This total volume of 50 mL was brought on an Oasis HLB column (Waters), which was equilibrated before starting cleanup by subsequently passing 4 mL of MeOH and 4 mL of HPLC H_2O over the column. After the total extract had been applied to this column, the column was washed with 2 mL of HPLC H_2O. Subsequent elution was performed with a volume of 5 mL of MeOH followed by 5 mL of MeCN. The total eluate was evaporated to dryness with nitrogen gas at 60 °C. Afterward, this residue was redissolved in a volume of 1 mL of H_2O/MeCN (90:10, v/v) and vortexed for 1 min. This extract was transferred to a microvial and stored at 4 °C prior to injection into the UHPLC-MS/MS system.

Method Validation. The analyte-dependent characteristics of the multimycotoxin method optimized in this study concern specificity, linearity, possible matrix effects, apparent recovery, repeatability, intralaboratory reproducibility, and limits of detection and quantitation. These parameters were validated with the guidelines of Commission Decision 2002/657/EC taken into account. Calculations were based on relative peak areas, that is, the peak area of the compound of interest divided by the peak area of the internal standard added to the same sample. Different Belgian maize silage samples were tested for mycotoxin presence. One specific sample of low mycotoxin content was chosen as "blank" and used for spiking experiments. Because of the difficulty of finding noncontaminated maize silage, to define "blank", the term "cutoff level" was introduced.[24] For aflatoxin B_1 (**1**), deoxynivalenol (**8**), fumonisin B_1 (**14**), fumonisin B_2 (**15**), ochratoxin A (**20**), and zearalenone (**27**), this cutoff level is based on the current regulatory limits (Commission Directive 2003/100/EC and Commission Recommendation 2006/576/EC), whereas such a cutoff level was chosen arbitrarily for the other mycotoxins. A sample was considered to be blank if the mycotoxin content was below one-fourth of this cutoff level.

Specificity was checked by analyzing 1 μg/mL of each pure liquid standard separately and searching for signal interference between the different MRMs. Nonspecific peaks due to silage compounds were also searched for. To evaluate linearity, possible matrix effects, R_A, RSD_r, and RSD_R, a total of three series blank samples were spiked with pure mycotoxin standards prior to extraction. Each series included one specific spiking level (1, 1.5, and 2 times the concentration of the cutoff chosen, Table 2) in six replicates. A matrix-matched calibration curve of at least five spiking concentrations was considered as well (Table 2). Prior to extraction, these samples were left to equilibrate for 30 min. Linearity of the matrix-matched calibration curves was evaluated on the basis of graphical interpretation of the residual plots, R^2 ($\geq$0.99) and the *F* statistic (goodness-of-fit). A minimum of five data points for each calibration curve was used within the concentration range of 0−250 ng/g for **1**−**4**, **7**, **9**−**15**, **18**, **20**, and **22**−**24**, a range of 0−2000 ng/g for **5** and **21**, a range of 0−3000 ng/g for **6**, **16**, **17**, **25**−**27**, and a range of

Table 1. Optimized ESI-MS/MS Conditions

mycotoxin	precursor ion (m/z)		cone voltage (V)	product ions (m/z)	collision energy (eV)	retention time (min)
aflatoxin B_1 (**1**)	$[M + H]^+$	313.03	35	241.09[a]/285.10	35/22	3.32
aflatoxin B_2 (**2**)	$[M + H]^+$	315.12	40	259.06[a]/287.06	25/25	3.17
aflatoxin G_1 (**3**)	$[M + H]^+$	329.12	35	215.14/243.12[a]	25/32	3.15
aflatoxin G_2 (**4**)	$[M + H]^+$	331.15	40	245.06[a]/313.12	29/24	2.99
beauvericin (**5**)	$[M + H]^+$	784.45	40	244.13[a]/262.18	24/24	6.14
citrinin (**6**)	$[M + H]^+$	251.08	22	205.16/233.09	24/16	3.62
cyclopiazonic acid (**7**)	$[M + H]^+$	337.19	27	182.20/196.19[a]	18/24	4.70
deoxynivalenol (**8**)	$[M + H]^+$	297.15	20	230.99/248.89[a]	14/10	0.81
enniatin A (**9**)	$[M + H]^+$	682.72	35	210.03[a]/228.31	25/30	6.57
enniatin A1 (**10**)	$[M + H]^+$	668.31	35	100.09/210.22[a]	50/25	6.37
enniatin B (**11**)	$[M + H]^+$	640.40	38	196.16[a]/527.13	25/22	5.96
enniatin B1 (**12**)	$[M + H]^+$	654.47	35	196.16[a]/214.19	28/25	6.17
fusaric acid (**13**)	$[M + H]^+$	180.13	18	134.12[a]/162.11	16/10	1.78
fumonisin B_1 (**14**)	$[M + H]^+$	722.25	40	334.13[a]/352.30	38/36	3.38
fumonisin B_2 (**15**)	$[M + H]^+$	706.40	35	318.17[a]/336.15	37/36	3.81
gliotoxin (**16**)	$[M + H]^+$	327.22	12	245.23/263.07[a]	20/12	3.14
HT-2 (**17**)	$[M + Na]^+$	447.20	30	285.10/345.15[a]	20/18	3.65
mycophenolic acid (**18**)	$[M + H]^+$	321.09	15	159.13/207.11[a]	34/22	3.85
nivalenol (**19**)	$[M + H]^+$	313.10	13	175.10[a]/177.20	20/13	0.53
ochratoxin A (**20**)	$[M + H]^+$	404.15	30	238.99[a]	23	4.32
	$[M + Na]^+$	426.14		261.20	20	
patulin (**21**)	$[M - H]^-$	152.90	15	81.10[a]/108.91	13/10	0.57
roquefortine C (**22**)	$[M + H]^+$	390.22	32	193.07[a]/322.17	26/19	3.43
sterigmatocystin (**23**)	$[M + H]^+$	325.16	35	281.10[a]/310.09	35/25	4.42
T-2 (**24**)	$[M + NH_4]^+$	484.29	18	245.10/304.98[a]	14/14	4.17
α-zearalenol (**25**)	$[M - H]^-$	319.09	35	159.95/275.24[a]	30/20	3.94
β-zearalenol (**26**)	$[M - H]^-$	319.09	35	159.95/275.24[a]	30/20	3.69
zearalenone (**27**)	$[M - H]^-$	317.14	35	131.14[a]/175.01[a]	30/24	4.29
salinomycin	$[M + Na]^+$	773.65	54	432.26[a]	50	6.80
verrucarol	$[M + H]^+$	267.20	18	249.10[a]	10	2.06

[a] Product ion used in screening program.

0−4000 ng/g for **8** and **19**. R_A percentages at the three spiking levels were calculated by using the matrix-matched calibration curves for quantitation. Specifically, for each spiking level, the observed concentration levels were calculated by using the relative peak area and the matrix-matched calibration curve. Subsequently, the apparent recovery was expressed as a percentage by comparing these observed values to the actual spiked levels. The data obtained from these experiments conducted on a single day were used to study the intraday precision or repeatability by calculating the relative standard deviation. For the interday precision, these experiments were carried out on three different days. For each analyte, the limit of detection and limit of quantitation were calculated by 3 and 6 times, respectively, the standard error of the intercept divided by the slope of the calibration curve. The calibration curves of spiked extracts were used to determine possible matrix effects by comparing them to the corresponding calibration curves of the pure standards. These effects were expressed in terms of signal suppression/enhancement (SSE) and calculated as follows: SSE (%) = 100 × $slope_{extract\text{-}matched\ standard}/slope_{pure\ standard}$.[25] All calculations were executed in Excel.

Mycotoxin Contamination of Maize Silage Samples. First, samples were screened for the presence of mycotoxins. Subsequently, these mycotoxins were quantitated by means of the standard addition approach in which the corresponding mycotoxin standards were added in increasing volumes, assuring linear calibration curves of at least six data points. The extraction and cleanup procedure was performed as described above. For each mycotoxin detected in the samples, correct compound identification was verified on the basis of the ion ratio (i.e., the peak area of product $ion_{lowest\ peak\ area}$ divided by the peak area of product $ion_{highest\ peak\ area}$) and relative retention time (RR_t = retention $time_{mycotoxin}$/retention $time_{IS}$). These ion ratios and relative retention times were compared with those of the calibration standard of comparable concentration. Maximum tolerable limits for the ion ratio and the relative retention time according to Commission Decision 2002/657/EC were applied.

Statistical Analysis. For evaluation of the linearity of the matrix-matched calibration curves, an F statistic (goodness-of-fit) using the Linest function was performed in Excel (α = 0.05). A two-sided t test was used to compare sets of contamination levels of each mycotoxin for the samples from the cutting surface and those taken 1 m behind this first sampling surface. The significance level was set at 0.05. These analyses were performed using the software program Statistica 9.0 (StatSoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Method Development. *UHPLC-MS/MS.* Mass spectrometric parameters of both the precursor ion and the product ions were tuned in their most sensitive ESI mode (+/−), with most of them by preference detected in ESI+. The analyte-dependent tuning results are given in Table 1. For each compound, two specific

Table 2. Overview of the Percentage Apparent Recovery (R_A), Repeatability (RSD_r), and Interday Precision over 3 Days (RSD_R) at the Three Concentrations Used for Validation and Limits of Detection and Quantitation (LOD and LOQ) and Signal Suppression/Enhancement (SSE) for Each Mycotoxin

		low concentration[a]			medium concentration			high concentration					
mycotoxin	concentrations (ng/g)	R_A (%)	RSD_r (%)	RSD_R (%)	R_A (%)	RSD_r (%)	RSD_R (%)	R_A (%)	RSD_r (%)	RSD_R (%)	LOD (ng/g)	LOQ (ng/g)	SSE (%)
1	20−30−40	100	11	12	101	10	12	101	10	10	7	14	5
2	20−30−40	91	9	17	95	7	8	97	7	8	7	14	30
3	20−30−40	96	7	8	94	5	14	97	7	7	5	11	13
4	20−30−40	97	6	7	101	5	11	101	7	8	7	13	10
5[b]	500−750−1000	93	3	8	90	6	8	94	6	10	22	44	190
6	1000−1500−2000	69	37	44	61	45	43	80	34	40	221	443	[c]
7	20−30−40	105	10	23	99	10	18	99	11	15	13	25	5
8	1000−1500−2000	82	18	18	96	11	13	80	12	18	50	99	200
9[b]	100−150−200	95	14	19	85	12	13	89	11	25	24	48	94
10[b]	100−150−200	87	18	20	84	12	22	77	13	29	21	43	78
11[b]	100−150−200	76	25	26	74	29	30	77	25	49	26	52	99
12[b]	100−150−200	91	23	27	80	20	21	80	16	31	24	47	98
13	50−75−100	93	9	9	93	5	6	95	5	5	30	59	77
14	20−30−40	96	7	9	94	4	7	91	4	6	8	15	74
15	40−60−80	86	4	6	80	4	8	82	7	12	14	28	83
16	750−1125−1500	92	6	9	92	9	16	92	7	13	160	320	273
17	500−750−1000	106	12	13	105	11	14	103	9	11	122	244	2
18	40−60−80	97	5	9	101	5	8	102	5	6	7	14	22
19	1500−2250−3000	110	19	22	98	22	29	113	18	26	348	695	78
20	50−75−100	101	12	17	99	8	8	94	11	12	9	17	6
21	750−1125−1500	110	7	8	104	6	9	99	5	8	135	270	15
22	20−30−40	99	9	9	97	6	6	94	4	5	13	25	18
23	40−60−80	108	14	18	106	10	14	107	11	13	11	21	6
24	20−30−40	104	7	10	100	7	7	104	5	6	6	13	40
25	500−750−1000	111	10	20	104	10	16	107	14	14	44	88	4
26	500−750−1000	100	8	17	100	8	11	103	9	13	32	64	6
27	500−750−1000	116	17	32	105	12	15	113	18	24	11	23	12

[a] Cutoff level. [b] Salinomycin used as internal standard instead of verrucarol. [c] Could not be determined.

product ions were selected from the mass spectrum after fragmentation. The product ion with the highest S/N ratio and highest intensity was selected for validation experiments, whereas the second specific product ion was used for confirmation purposes. The sum of the relative peak areas of both ions was used for quantitation. To reduce the chromatographic run time of each sample, the mass spectrometer was forced to switch between the positive and negative ESI modes in the final optimized multimycotoxin MS/MS method instead of analyzing each sample separately as done previously.[16,25] Chromatographic separation was performed by means of reversed phase gradient elution within a time frame of 9 min. Fifteen data points per peak was set as a minimal value for each compound. The column temperature and flow rate were calculated according to the Van Deemter curve and were set at values allowing optimal UHPLC chromatographic separation corresponding to a column pressure of about 10000−12000 psi. Different mobile phases, column temperatures, and flow rates were tested and evaluated in terms of resolution, peak intensity, peak shape, and S/N ratio (data not shown). From these experiments, H_2O + 0.1% formic acid + 0.1% isopropanol + 1 mM ammonium acetate was chosen as solvent A, whereas MeCN + 0.1% isopropanol + 0.1% formic acid was chosen as solvent B. The presence of ammonia and formic acid in the mobile phase promotes ionization and pH stability. However, as the enniatins (**9**−**12**) and beauvericin (**5**) seemed to suffer from intersample carry-over, 0.1% isopropanol was added to both solvents to reduce this phenomenon. Another option would be to run additional injections of different blanks after each sample.[16] Rasmussen et al. (2010) applied a postrun cleaning procedure with injection of formic acid in MeCN, in MeOH, and in water. However, this approach was not chosen as this would create a 4-fold increase in the total run time per sample. Instead, only one postrun cleaning step was introduced by injection of H_2O/MeCN (50:50, v/v). Coeluting peaks did not form any problem because MS/MS fragmentation and detection have the intrinsic characteristic of differentiation between coeluting peaks based on the ion ratio. Combined UHPLC-MS/MS chromatograms for blank maize silage and for maize silage spiked at a specific concentration for each mycotoxin are shown in Figure 2.

Extraction Procedure. After optimization of the LC-MS/MS conditions, development of a suitable extraction procedure for 27 mycotoxins from maize silage was aimed for. Although, theoretically, cleanup of raw extracts can be kept to a minimum when analytical methods as sensitive and specific as UHPLC-MS/MS

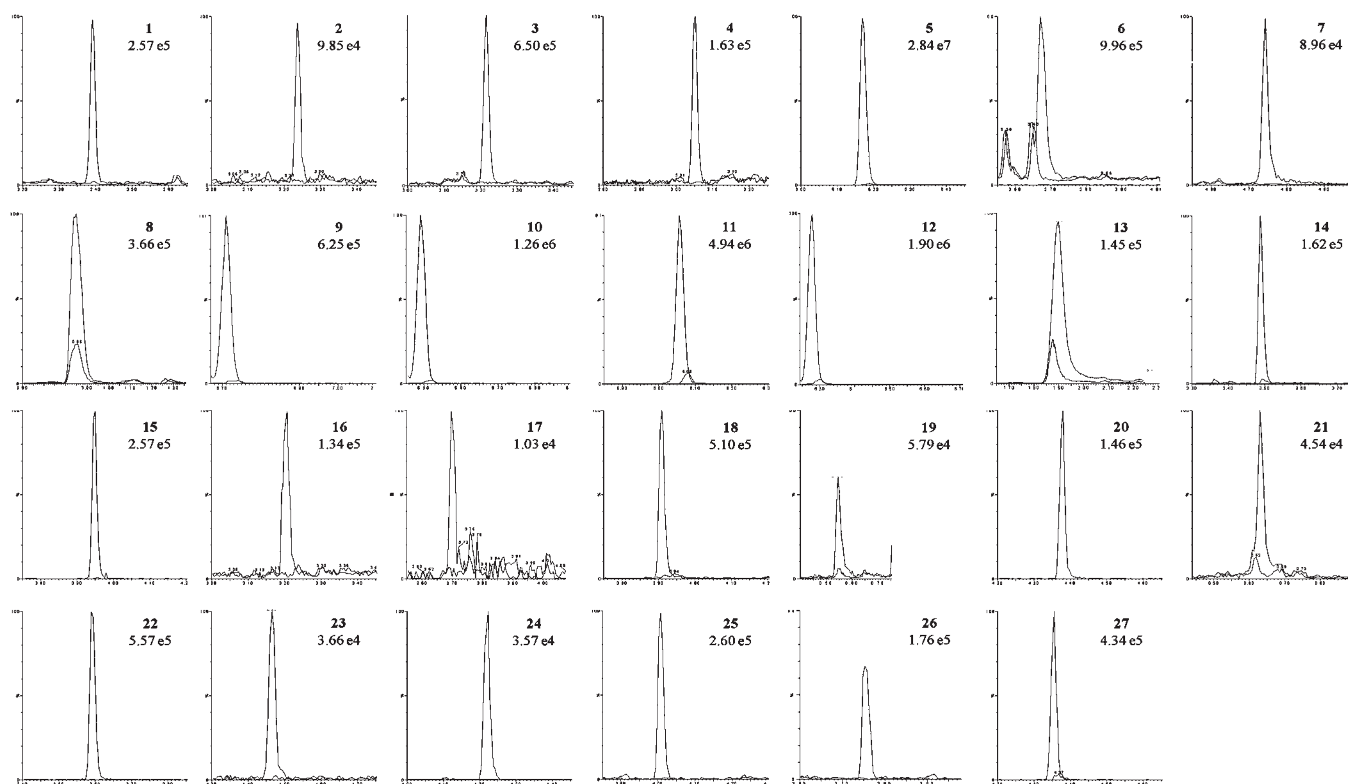

Figure 2. Combined chromatogram obtained with the multimycotoxin UHPLC-MS/MS method for blank maize silage and for maize silage spiked at a concentration of 20 ng/g (**1**−**4**, 7, **14**, **22**, **24**), 40 ng/g (**15**, **18**, **23**), 50 ng/g (**13**, **20**), 100 ng/g (**9**−**12**), 500 ng/g (**5**, **17**, **25**−**27**), 750 ng/g (**16**, **21**), 1000 ng/g (**6**, **8**), or 1500 ng/g (**19**).

are applied, the "dilute-and-shoot" principle was not tested in this study. Given the complexity of silage commodities and the yet to be proven long-term suitability of such methods without cleanup step(s) in terms of minimally polluting the MS equipment, a more time-consuming cleanup method was tested. Oasis HLB, C_{18}, and silica cartridges were tested for several extraction solvents. From these tests (data not shown), it was decided to perform cleanup with Oasis HLB columns. As for the extraction solvents, $MeCN/H_2O$ (84:16, v/v) seemed to be a suitable compromise. However, apparent recovery percentages for **8**, **19**, and **21** were insufficient. Because MeOH-based extraction solvents yielded better recovery percentages for these mycotoxins, an extraction step with 100% MeOH was introduced prior to the $MeCN/H_2O$ (84:16, v/v) extraction step. Although the initial extraction procedure was based on literature data,[8] the final procedure in this study is somewhat different because of the higher diversity of mycotoxins considered. In particular, no extra filtering step prior to transferring the purified extracts to the vial was applied because of loss of the enniatins (**9**−**12**) and beauvericin (**5**). The method developed in this study is different from the one described by Rasmussen et al.[16] for maize silage. They used a pH-buffered extraction procedure based on the quick, easy, cheap, effective, rugged and safe (QuEChERS) method used for pesticide residues. Briefly, they performed extractions using MeCN + 1% acetic acid, H_2O, and sodium acetate trihydrate followed by a phase separation step based on the addition of salts. This approach was tested in the present study for both magnesium sulfate and sodium chloride as salt, although without satisfactory recovery results, especially for fusaric acid (**13**) and the fumonisins (**14**, **15**).

Method Validation. With the exception of the enniatins (**9**−**12**) and beauvericin (**5**), the trichothecene verrucarol was used as internal standard in this study to compensate for matrix effects, losses during extraction as well as cleanup, and analysis errors. Its use was previously reported.[26−29] For the ionophoric enniatins (**9**−**12**) and beauvericin (**5**), the ionophoric coccidiostat compound salinomycin was used as internal standard because of its similar structure (complex formation with Na^+). Theoretically, the use of an isotopically labeled internal standard for every single mycotoxin included in the multimycotoxin method is preferable,[30,31] although not feasible in practice. Financial limitations as well as lack of commercial availability of each compound force the search for one or more structurally related standards instead. If internal standards are not available, the standard addition approach is another option.

LC-MS/MS runs of single mycotoxin standards indicated that the different compounds did not interfere with each other. In addition, running silage samples did not yield chromatographic peaks interfering with the mycotoxin-specific peaks within the different measuring windows. For patulin (**21**), special care has to be taken for possible interference with nonspecific peaks. For each sample, possible false patulin-positive outcomes can be detected on the basis of the ion ratio (peak area of product $ion_{lowest\ peak\ area}$/peak area of product $ion_{highest\ peak\ area}$) and relative retention time (RR_t = retention time mycotoxin/retention time internal standard) for each extract and their comparison with the ion ratio and relative retention time of the calibration standard of comparable quantity.

Calibration curves of matrix-matched standards were used to evaluate linearity in terms of residual scatter plots, R^2 values, and

goodness-of-fit testing. For all compounds, linearity was found to be adequate. The calibration curves of spiked extracts were used to determine SSE by comparing them to the corresponding calibration curves of the pure standards. The results shown in Table 2 indicate that most of the mycotoxins seemed to be subject to signal suppression, with the aflatoxins (**1**−**4**), cyclopiazonic acid (**7**), HT-2 (**17**), mycophenolic acid (**18**), ochratoxin A (**20**), patulin (**21**), roquefortine C (**22**), sterigmatocystin (**23**), zearalenone (**27**), and its metabolites (**25**, **26**) being the most suppressed (≤30% SSE). Whereas the enniatins **9**, **11**, and **12** did not seem to suffer from a marked signal influence (SSE approximately 100%), the signals of beauvericin (**5**), deoxynivalenol (**8**), and gliotoxin (**16**) were strongly enhanced. The observed SSE emphasized the need to quantitate mycotoxins in silage commodities by means of matrix-matched calibration curves, that is, mycotoxins spiked in the silage matrix. The standard addition approach can be used as well.

Table 2 gives an overview of the overall percentage apparent recovery, repeatability, interday precision over three days, and LOD and LOQ (ng/g) for each mycotoxin. The overall R_A was calculated as a mean of the three different concentrations extracted. With the exceptions of citrinin (**6**), enniatin B (**11**), and zearalenone (**27**) (70, 76, and 111%, respectively), R_A percentages (average over the three days and the three concentrations) were within the range 80−110%, that is, the strictest limits set in Commission Decision 2002/657. However, the recovery percentages for citrinin (**6**), enniatin B (**11**), and zearalenone (**27**) were still considered to be acceptable from an analytical point of view (70−120%). Noteworthy is the excellent average recovery (97%) for roquefortine C (**22**), an important mycotoxin in maize silage due to the high prevalence of *P. roqueforti* and *P. paneum* in this type of feed commodity,[12] compared with the recovery obtained for the multimycotoxin method developed by Rasmussen et al.[16] (>200%). With the exception of citrinin (**6**), deoxynivalenol (**8**), the enniatins (**9**−**12**), nivalenol (**19**), α-zearalenol (**25**), and zearalenone (**27**), intralaboratory reproducibility and repeatability were considered to be acceptable according to the guidelines stipulated in the performance criteria of Commission Decision 2002/657/EC. This is why only for citrinin (**6**) and enniatin B (**11**) were the average RSD values over the three concentrations considered to be unsatisfactory from an analytical point of view (>25%). Although no regulatory limits or recommendations of maximum allowed levels are specifically set for mycotoxins in maize silage, one can conclude that the values for the limits of detection and quantitation obtained with the multimycotoxin method developed in this study were below the regulations for aflatoxin B_1 (**1**), deoxynivalenol (**8**), fumonisins B_1 (**14**) and B_2 (**15**), ochratoxin A (**20**), and zearalenone (**27**) in feed (Table 2): 20 ng/g for aflatoxin B_1 (all feed materials, Commission Directive 2003/100/EC), 12000 ng/g for deoxynivalenol (maize byproducts, Commission Recommendation 2006/576/EC), 60000 ng/g for fumonisins B_1 and B_2 (maize and maize-based products, Commission Recommendation 2006/576/EC), 250 ng/g for ochratoxin A (cereal and cereal products, Commission Recommendation 2006/576/EC), and 3000 ng/g for zearalenone (maize byproducts, Commission Recommendation 2006/576/EC). For most of the nonregulated mycotoxins, LOD and LOQ are far below such limits. For gliotoxin (**16**), HT-2 (**17**), patulin (**21**), citrinin (**6**), and nivalenol (**19**) the limits of detection and quantitation are somewhat higher, although still low for feed matrices for cattle.

Molds and Mycotoxins Present in Maize Silage Samples. Detection of naturally occurring mycotoxins in maize silage was investigated through examination of a limited set of maize silage samples. The samples were first screened for the presence of mycotoxins. As each silage commodity starts from different material and the silage process is not a controlled industrial process, silage samples will always be different. This is why the mycotoxins detected were quantitated by means of the standard addition approach instead of matrix-matched calibration curves of the blank sample. For some silage samples, the signal of the internal standard salinomycin was not optimal. Using the standard addition approach allowed quantitation based on absolute peak areas instead for the enniatins (**9**−**12**) and beauvericin (**5**). The mycotoxin quantitation results and an overview of the molds isolated from the maize silage silos are shown in Table 3. These data indicate that the most frequently found mycotoxins in these samples are the preharvest *Fusarium* toxins beauvericin (**5**), deoxynivalenol (**8**), enniatins B (**11**) and B1 (**12**), fusaric acid (**13**), and fumonisin B_1 (**14**). No statistically significant difference ($P > 0.05$) could be detected between the concentration for each mycotoxin measured at the cutting surface of the silo and 1 m behind that surface. Adding salt at ensiling (silo 4) did not seem to lead to lower contamination levels of postharvest mycotoxins mycophenolic acid (**18**) and roquefortine C (**22**). In general, the concentrations observed in all silo samples were rather low and below the maximum contents in feed recommended by the European Commission. Deoxynivalenol (**8**) was detected in all samples examined, with 262 ng/g being the highest concentration. The amounts of this toxin detected are in line with those previously reported (100−213 ng/g).[8] The amounts detected are far below 12000 ng/g as stipulated for maize byproducts. Beauvericin (**5**) and fusaric acid (**13**) were also present in all samples, but generally at concentrations around or below their LOQ. In few cases, trace amounts of fumonisin B_2 (**15**) and zearalenone (**27**) were detected, the latter confirming the results for zearalenone (**27**) obtained by Garon et al. (23−41 ng/g).[8] In contrast, fumonisin B_1 (**14**) was detected more often, but always in a concentration lower than 15 ng/g silage. Of the enniatins, mainly enniatins B (**11**) and B1 (**12**) were observed. However, concentrations of these mycotoxins did not exceed 52 ng/g maize silage. The presence of enniatin B (**11**) at a mean concentration of 44 ng/g in visibly unspoiled silage was already reported.[16] Roquefortin C (**22**) and mycophenolic acid (**18**) were the only postharvest mycotoxins encountered. In most silos, their presence was correlated with the presence of *P. paneum* (roquefortine C producer) and/or *P. roqueforti* (roquefortine C/mycophenolic acid producer). Remarkably, the postharvest molds *P. roqueforti* and/or *P. paneum* were also detected for the type-b silage samples taken 1 m behind the cutting surface of silos 2, 4, and 5. This finding was correlated with visible mold growth on the silage just beneath the plastic cover of the silo (silos 2 and 4). In contrast, their presence in silo 5 was correlated with the occurrence of lumps of blue mold scattered throughout the silo. Detection of the postharvest mycotoxin citrinin in maize silage,[8,15] was not confirmed by our data. Although *A. niger* Tiegh. is known to be able to produce ochratoxin A (**20**) and fumonisin B_2 (**15**), its occurrence in three of the five silos was not correlated with detectable amounts of these mycotoxins. Although the presence of *A. fumigatus* in silage was found to be correlated with the presence of gliotoxin (**16**) and detected up to 800 ng/g in mature maize silage[15,32] and up to 906 ng/g silage in fungal hot spots,[16] no gliotoxin could be

Table 3. Overview of the Different Fungal Species Identified and the Amount of Secondary Metabolites Detected by Means of UHPLC-MS/MS in Five Maize Silos with Samples Taken at the Cutting Surface (a) and 1 m Behind This Surface (b)

			fungal species identified									mycotoxins detected (ng/g)											
silo	sample type	CFU/g silage	*A. fumigatus*	*A. niger*	*P. roqueforti*	*P. paneum*	*P. radicum*	*P. crustosum*	*Trichoderma* spp. *Rhizomucor*/*Mucor* spp.	*Trichoderma gamsii*	*Glonium*/*Sphaeriothyrium*/*Epicoccum*	*beauvericin (5)* LOQ = 44	*deoxynivalenol (8)* LOQ = 99	*enniatin A (9)* LOQ = 48	*enniatin A1 (10)* LOQ = 421	*enniatin B (11)* LOQ = 52	*enniatin B1 (12)* LOQ = 47	*fusaric acid (13)* LOQ = 59	*fumonisin* B_1 *(14)* LOQ = 28	*fumonisin* B_2 *(15)* LOQ = 154	*mycophenolic acid (18)* LOQ = 14	*roquefortine C (22)* LOQ = 25	*zearalenone (27)* LOQ = 23
1	a	$<10^2$			+							53	<LOQ	nd[a]	ta[b]	<LOQ	<LOQ	<LOQ	t.a.	nd	nd	nd	nd
	b	$<10^2$			+		+					<LOQ	<LOQ	nd	ta	ta	<LOQ	<LOQ	<LOQ	nd	nd	nd	nd
2	a	2×10^4									+	<LOQ	262	ta	ta	<LOQ	<LOQ	<LOQ	<LOQ	ta	<LOQ	ta	ta
	b	4×10^4	+		+	+			+			<LOQ	140	ta	ta	<LOQ	<LOQ	180	ta	ta	194	90	nd
3	a	2×10^2		+	+	+		+				<LOQ	<LOQ	<CCα	<LOQ	<LOQ	<LOQ	<LOQ	nd	nd	nd	<LOQ	nd
	b	$<10^2$										59	125	nd	nd	ta	<LOQ	<LOQ	nd	nd	nd	nd	nd
4	a	2×10^3		+	+	+				+		<LOQ	<LOQ	nd	ta	<LOQ	<LOQ	<LOQ	<LOQ	nd	nd	<LOQ	nd
	b	$<10^2$				+						<LOQ	<LOQ	nd	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	nd	nd	nd	nd
5	a	3×10^5		+	+	+						ta	133	ta	<LOQ	<LOQ	<LOQ	<LOQ	nd	nd	29	52	ta
	b	2×10^5			+	+						<LOQ	135	nd	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	nd	18	57	ta

[a] Not detected (mycotoxin not present in this sample). [b] Trace amount (mycotoxin detected but very low concentration (≪LOQ)).

detected in our study for the maize silage sample contaminated with *A. fumigatus*. It has already been described that not all *A. fumigatus* isolates produce this toxin under the same conditions.[33] In addition, it has already been reported that not all or even none of the *A. fumigatus* metabolites produced on agar are detected in laboratory-inoculated silage hot spots.[34]

As all maize silage samples were contaminated with more than one mycotoxin, this emphasizes the need for multimycotoxin methods as well as the risk for additive or synergistic negative health effects for cattle. Variation in the type and amount of mycotoxins detected may occur as a result of environmental, geographical, and weather conditions as well as whole-season storage.[13] For example, it has already been reported that the risk of fungal spoilage is highest 5−7 months after ensiling and lowest after 11 months.[13]

The fermentation products of both types of silage samples were analyzed for each silo. The ratio of lactic acid/acetic acid was around ≥3 (data not shown), indicating adequate fermentation in these silos.[35] The pH of all silage samples was approximately 4.0, which is said to be an indication for silage of good quality, that is, no microbial spoilage.[35] Although pH was expected to be higher for the silage samples with visible fungal spoilage, this was not observed. The data from this study point out that the parameters described above do not permit any assumptions to be made about whether a certain silage commodity is contaminated with mycotoxins or not.

In conclusion, the method developed in this study is the first report of an UHPLC-MS/MS method for 26 mycotoxins in maize silage with an analysis time of 9 min. The extraction itself concerns a user-friendly procedure in combination with an SPE cleanup that reduces pollution of the MS equipment. The use of the standard addition approach for quantitation is recommended after screening because a real blank silage matrix that can be used for matrix-matched calibration curves in practice does not really exist in the in-house produced character of silage. The standard approach implies that using an internal standard can be omitted. The method described fulfills the strictest performance criteria required by Commission Decision 2002/657/EC for most of the mycotoxins considered. Only for citrinin were results not satisfactory with the method developed. It was successfully applied for analyzing maize silage samples, in which mainly *Fusarium* toxins (enniatins, beauvericin, deoxynivalenol, fumonisins, fusaric acid, and zearalenone) were detected. From the postharvest mycotoxins, only mycophenolic acid and roquefortine C were encountered.

Although only low contamination levels were observed in this study and a rather limited amount of samples were analyzed, these data as well as other literature sources indicate that mycotoxin-free maize silage is hard to find. In addition, factors such as climate change, seasonal changes, long-term preservation, and others may imply higher mycotoxin contamination levels at specific time points and thus present higher health risks for the animals. The method developed is an excellent tool to conduct further research on the true burden of mycotoxins in this type of feed commodity. For instance, it could be applied for studying possible shifts in the presence of different mycotoxins from harvest until last feed-out or to examine the effect of silage additives on the degree of mycotoxin contamination of this type of animal farm forage. In addition, data on the contamination degree and the co-occurrence of mycotoxins in maize silage will give indications of the risk for chronic exposure of cattle and possible synergistic effects. It would be interesting to correlate such data with data on the transmission of mycotoxins into milk or meat, a topic that has been insufficiently explored up to now.

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■ ABBREVIATIONS USED

ITS, internal transcribed spacer; QuEChERS, quick, easy, cheap, effective, rugged and safe; R_A, apparent recovery; RR_t, relative retention time; RSD_r, repeatability; RSD_R, reproducibility; UHPLC-MS/MS, ultrahigh-performance liquid chromatography—tandem mass spectrometry.

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