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ARTICLE

Occurrence of Mycotoxins in Feed as Analyzed by a Multi-Mycotoxin LC-MS/MS Method

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Crops used for animal feed can be easily contaminated by fungi during growth, harvest, or storage, resulting in the occurrence of mycotoxins. Because animal feed plays an important role in the food safety chain, the European Commission has set maximum levels for aflatoxin B_1 and recommended maximum levels for deoxynivalenol, zearalenone, ochratoxin A, and the sum of fumonisin B_1 and B_2. A multimycotoxin LC-MS/MS method was developed, validated according to Commission Decision 2002/657/EC and EN ISO 17025 accredited for the simultaneous detection of 23 mycotoxins (aflatoxin-B_1, aflatoxin-B_2, aflatoxin-G_1, aflatoxin-G_2, ochratoxin A, deoxynivalenol, zearalenone, fumonisin B_1, fumonisin B_2, fumonisin B_3, T2-toxin, HT2-toxin, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, diacetoxyscirpenol, fusarenon-X, neosolaniol, altenuene, alternariol, alternariol methyl ether, roquefortine-C, and sterigmatocystin) in feed. The decision limits of the multimycotoxin method varied from 0.7 to 60.6 μg/kg. The apparent recovery and the results of the precision study fulfilled the performance criteria as set in Commission Decision 2002/657/EC. The analysis of three different feed matrices (sow feed, wheat, and maize) provided a good basis for the evaluation of the toxin exposure in animal production. In total, 67 samples out of 82 (82%) were contaminated; type B-trichothecenes and fumonisins occurred most often. The majority of the infected feed samples (75%) were contaminated with more than one type of mycotoxin.

KEYWORDS: LC-MS/MS; multimycotoxin; feed analysis; validation

INTRODUCTION

Food crops and feed materials can be easily infected by fungal species which may produce mycotoxins during growth, harvest, and storage. The ability of molds to produce mycotoxins is influenced by environmental factors such as temperature, relative humidity, and drought (*1*). Miraglia et al. (*2*) classified the occurrence of mycotoxins in food and feed as an emerging food safety issue within the framework of the evaluation of climate change and food safety. A shortage in the availability of farm crops as a result of the conversion of arable land to biofuel production and changing climatic conditions may lead to compensation by using different crops or crops with a lower quality grade, potentially containing mycotoxins of a different nature or with a higher prevalence (*2*).

Crops can be infected by different toxigenic molds, which potentially results in the co-occurrence of several mycotoxins. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive, or synergistic. The final toxic effects, appearing in consumers exposed to a mixture of mycotoxins, are related to the toxicokinetic behavior, the metabolism, and the toxicodynamic effects of mycotoxins. The issue of combined toxicity is very complicated, but generally it can be concluded that exposure to several classes of mycotoxins often results in an additive effect, with exceptions indicating a synergistic interaction (*3*−*7*).

Currently there is clearly an increased focus of the feed industry toward the reduction of mycotoxin levels in feed raw materials and finished feeds. This increased attention is mainly driven by the improved awareness about mycotoxins as well as by the intensifying legislative framework worldwide related to the maximum tolerable levels of mycotoxins in feed. In this environment there is a clear need for fast and efficient analytical methods to support the feed and food industry in the management of mycotoxins. High-performance techniques will endorse the strict implementation of legislation, will support the HACCP plans, and will allow broad screening of new harvests of commonly used feed ingredients in order to obtain a general assessment of the current risk of mycotoxin contamination. Because of the stricter regulations in many areas of the world, there is an increased demand for more information on all mycotoxins present in feed, even at levels well below the legal limits. Legislation clearly aims at improving food safety, while animal production also wants to eliminate subclinical effects of mycotoxins that may influence profitability.

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LC-MS is a highly reliable analyte confirmation tool and has become a routine technique in food analysis. This technical and instrumental progress also has an increasing impact on the expanding field of mycotoxin analysis, particularly in the development of multimycotoxin methods (*8*). The first developed multi methods were focused on the determination of several mycotoxins being part of one class of mycotoxins (*Aspergillus, Penicillium*, or *Fusarium* toxins) followed by methods for the simultaneous determination of *Fusarium, Aspergillus, Penicillium*, and *Alternaria* toxins (*9*−*12*). Further evolution led to new published multiple methods where also mycotoxin metabolites, masked mycotoxins, and ergot alkaloids were included (*10*, *13*−*15*). This study was undertaken to develop and validate a multimycotoxin LC-MS/MS method for the simultaneous detection of aflatoxin-B_1 (AF-B_1), aflatoxin-B_2 (AF-B_2), aflatoxin-G_1 (AF-G_1), aflatoxin-G_2 (AF-G_2), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), T2-toxin (T2), HT2-toxin (HT2), nivalenol (NIV), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), fusarenon-X (F-X), neosolaniol (NEO), altenuene (ALT), alternariol (AOH), alternariol methyl ether (AME), roquefortine-C (ROQ-C), and sterigmatocystin (STERIG) and its application in the analysis of naturally contaminated feed samples.

MATERIALS AND METHODS

Reagents and Chemicals. Methanol, acetonitrile, and hexane were high-performance liquid chromatography grade from VWR International (Zaventem, Belgium). *N*,*N*-Dimethylformamide was purchased from Acros Organics (Geel, Belgium). Water was obtained from a Milli-Q Gradient System (Millipore, Brussels, Belgium). Acetic acid, 100%, from Merck (Darmstadt, Germany) was used. Ammonium acetate, minimum 98%, was supplied by Grauwmeer (Leuven, Belgium).

Zearalanone (ZAN) 9.91 μg/mL, ochratoxin A 10.05 μg/mL, aflatoxin mix (aflatoxin-B_1, B_2, G_1, and G_2) respectively 19.9, 19.9, 20.1, and 20.1 μg/mL, de-epoxydeoxynivalenol (DOM) and sterigmatocystin 50.9 μg/mL, HT2-toxin 100.2 μg/mL, diacetoxyscirpenol 100.3 μg/mL, deoxynivalenol 100.4 μg/mL, 15-acetyldeoxynivalenol and zearalenone 100.5 μg/mL, fusarenon-X 100.6 μg/mL, 3-acetyldeoxynivaleon and T2-toxin 100.7 μg/mL, nivalenol 103.9 μg/mL, and neosolaniol 104.7 μg/mL were certified mycotoxin standard solutions in acetonitrile from Biopure (Coring System Diagnostix, Gernsheim, Germany). The fumonisin mix (fumonisin B_1 and B_2) respectively 50.3 and 49.3 μg/mL was a certified mycotoxin standard solution in acetonitrile/water (50/50, v/v) from Biopure (Coring System Diagnostix, Gernsheim, Germany). Altenuene, alternariol, and alternariol methyl ether were obtained from Sigma (Bornem, Belgium), and roquefortine-C was purchased from Alexis Biochemicals (Enzo Life Sciences BVBA, Zandhoven, Belgium). Fumonisin B_3 was obtained from Promec unit (Tygerberg, South Africa). Stock solutions of altenuene and roquefortine-C (1 mg/mL) were prepared in methanol. Alternariol and alternariol methyl ether stock solutions (1 mg/mL) were prepared in methanol/dimethylformamide (60/40, v/v). The stock solution of fumonisin B_3 was prepared in 1 mL acetonitrile/water (50/50, v/v). All stock solutions were stored for 1 year or until the expiration date at −18 °C, except for fumonisin B_3, which was stored at 4 °C. Working standard solutions were made by diluting the stock standard solutions in methanol and were stored at −18 °C for 3 months. From the individual stock standard solutions and working solutions a standard mixture was prepared with the following concentrations: diacetoxyscirpenol (0.5 ng/μL); roquefortine-C (1 ng/μL); aflatoxin-B_1, B_2, G_1, and G_2 (2 ng/μL); 15-acetyldeoxynivalenol (2.5 ng/μL); ochratoxin A, 3-acetyldeoxynivalenol, altenuene, and sterigmatocystin (5 ng/μL); zearalenone, T2-toxin, HT2-toxin, neosolaniol, and alternariol (10 ng/μL); nivalenol, fusarenon-X, and alternariol methyl ether (20 ng/μL); fumonisin B_3 (25 ng/μL); deoxynivalenol, fumonisin B_1 and B_2 (40 ng/μL).

Samples of Feed and Feed Raw Materials. A total of 29 wheat samples were collected in three EU countries: 8 from the Czech Republic, 14 from Denmark, and 7 from Hungary. Samples were collected in the period of July−October 2008 and stored at −18 °C until analysis. A total of 34 maize samples were collected in three EU countries: 8 from the Czech Republic, 14 from Spain, and 12 from Portugal. Samples were collected in the period of August−-October 2008 and stored at −18 °C until analysis. A total of 4 sow feed samples, 1 wheat sample, and 14 maize samples were obtained at the laboratory in the framework of a regular monitoring program.

Sample Preparation. Feed samples were blended using a blender (Moulinette, Moulinex, France). Five grams of feed sample was extracted with 20 mL of acetonitrile/water/acetic acid (79/20/1, v/v/v), tumbled on a end-over-end tumbler for 1 h, and centrifuged for 15 min at 3300*g*. Grace octadecyl (C_{18}), 500 mg/6 mL, SPE columns were purchased from Grace Discovery Sciences (Lokeren, Belgium). The C_{18}-SPE column was conditioned by passing through 2 × 5 mL acetonitrile/water/acetic acid (79/20/1, v/v/v) and washed using 5 mL of extraction solvent. The supernatant was passed through the C_{18}-SPE column and immediately collected in a volumetric flask of 25 mL. The content of the volumetric flask was adjusted with extraction solvent. The content of the volumetric flask was transferred into a plastic test tube of 50 mL. To this test tube was added 10 mL of hexane, and the tube was tumbled on a end-over-end tumbler during 10 min. After 15 min of centrifugation at 3300*g* the hexane layer was removed.

In order to recover the 23 mycotoxins, two cleanup pathways were necessary. In the first pathway 27.5 mL of acetonitrile/acetic acid (99/1, v/v) was added to 12.5 mL of the defatted extract. After homogenization 30 mL was passed through a Multisep226, Aflazon+ Multifunctional column, purchased from Coring System Diagnostics (Gernsheim, Germany), and 5 mL of acetonitrile/acetic acid (99/1, v/v) was used to wash the column.

In the second pathway 10 mL of defatted extract was filtered using a Whatman glass microfilter of 125 mm diameter, from VWR International (Zaventem, Belgium). Two milliliters of the filtered extract was combined with the Multisep226 eluate. After evaporation of the combined mixture, the residue was dissolved in 150 μL of mobile phase containing mobile phase A/mobile phase B (60/40, v/v) and 5 mM ammonium acetate and the mixture ultracentrifuged for 10 min at 14000*g* using Millipore ultrafree-MC centrifugal filter devices of 0.22 μm (Bedford, MA) before LC-MS/MS analysis.

Apparatus. A Waters Acquity UPLC system coupled to a Micromass Quatro Micro triple-quadrupole mass spectrometer (Waters, Milford, MA) was used to analyze the samples, equipped with Masslynx software for data processing. The column used was a 150 mm × 2.1 mm i.d. 5 μm Symmetry C_{18}, with a 10 mm × 2.1 mm i.d. guard column of the same material (Waters, Zellik, Belgium).

LC-MS/MS Analysis. The detailed analytical conditions were described by Monbaliu et al. (*11*). The column was kept at room temperature. The injection volume was 20 μL. The mobile phase consisted of variable mixtures of mobile phase A (water/methanol/acetic acid, 94/5/1 (v/v/v), and 5 mM ammonium acetate) and mobile phase B (methanol/water/acetic acid, 97/2/1 (v/v/v) and 5 mM ammonium acetate) at a flow rate of 0.3 mL/min with a gradient elution program. The gradient elution started at 95% mobile phase A with a linear decrease to 35% in 7 min. The next 4 min mobile phase A decreased to 25%. An isocratic period of 100% mobile phase B started at 11 min for 2 min. Initial column conditions were reached at 23 min using a linear decrease of mobile phase B, and over 5 min mobile phase A was used to recondition the column. The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode. The capillary voltage was 3.2 kV, and nitrogen was used as the spray gas. Source and desolvation temperatures were set at 150 and 350 °C, respectively. Mycotoxins were analyzed using selected reaction monitoring (SRM) channels.

Method Validation. The method was validated according to Commission Decision 2002/657/EC (*16*). Twelve blank feed samples were spiked with a known concentration of mycotoxin mixture at four different concentration levels, namely 0.5, 0.75, 1, and 1.5 times the cutoff (CO) level, and analyzed. Because no minimum required performance limits (MRPLs) were established for mycotoxins in feed, the term CO level was introduced in this study. For every mycotoxin a cutoff level was established. The CO level for aflatoxin-B_1, deoxynivalenol, zearalenone, ochratoxin A, and fumonisin B_1 and B_2 was based on the current regulatory limits (*17*, *18*). For the other described toxins the CO level was arbitrarily chosen. Because it is difficult to find noncontaminated feed

samples, the feed samples used for the validation study were considered to be blank if the concentration of the detected toxin was not below one-fourth of the CO level. Zearalanone and de-epoxydeoxynivalenol were added as internal standards (IS). This experiment was repeated on three different days. Matrix-matched calibration plots were constructed by applying the least-squares method and by plotting the relative peak area (peak area toxin/peak area IS) against the spiked concentration level of the feed sample. The decision limit (CCα) was defined as the concentration at "the *y*intercept plus 2.33 times the standard deviation of the within lab reproducibility" (α = 1%) in the case of substances for which no permitted limit has been established. In the case of substances with a maximum limit such as aflatoxin-B_1, CCα was established by fortifying blank material around the maximum limit. The corresponding concentration at the permitted limit plus 1.64 times the standard deviation of the within-laboratory reproducibility equals the decision limit (α = 5%). The detection capability (CCβ) was calculated as the concentration at "the decision limit plus 1.64 times the standard deviation of the within-lab reproducibility of the mean measured content at the decision limit" (β = 5%).

The linearity was tested graphically using a scatter plot, and the linear regression model was tested using a lack of fit test. The specificity was tested by analyzing 20 different feed samples. No interfering signals that can lead to nonconforming results were detected. At the four concentration levels the apparent recovery was calculated by quantifying the mycotoxins using the matrix-matched calibration plot. For each mycotoxin the observed value was divided by the spiked level. The precision of the method in terms of repeatability (intraday precision, the analysis of three replicates on the same day at four different concentration levels) and reproducibility (interday precision, the analysis of three replicates at four different concentration levels performed on three different days) was evaluated calculating the relative standard deviation (RSD). The validation parameters were calculated using the relative peak area, in which the peak area was divided by the peak area of the internal standard de-epoxydeoxynivalenol (used for 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and deoxynivalenol) and zearalanone (used for all other described toxins). The evaluation of the uncertainty of analytical results is compulsory for laboratories accredited according to ISO 17025 (*19*). The expanded measurement uncertainty (U) was calculated using the top-down approach (ISO/TS 21748), in which the validation data (repeatability, reproducibility, and trueness estimates) were used (*20*). The equation used to calculate the expanded measurement uncertainty (U) was the coverage factor (k) multiplied by the combined uncertainty (u_c): $U = ku_c$. A coverage factor of 2 was used to obtain a confidence interval of 95%. The combined uncertainty was determined by the square root of the sum of the square of the uncertainty of the bias (u_{bias}) and the square of the uncertainty of the precision (u(Rw)): $u_c = [u_{bias}{}^2 + u(\mathrm{Rw})^2]^{1/2}$. For the calculation of the uncertainty of the bias, the root-mean-square of the bias (rms_{bias}), the uncertainty on the reference value of the spike (u(Cref, spike)), and the uncertainty due to the spiking procedure from a spike (u(spiking)) was calculated: $u_{bias} = [RMS_{bias}{}^2 + u(\mathrm{spiking})^2 + u(\mathrm{Cref, spike})_2]^{1/2}$. The intralaboratory reproducibility (s_R) was used for the estimation of the precision: $u(\mathrm{Rw}) = s_R$. All calculations were executed in Excel2007 or in SPSS16.

RESULTS AND DISCUSSION

Method Development. A multimycotoxin LC-MS/MS method was developed for the analysis of sweet pepper by Monbaliu et al. (*11*). For feed samples, however, no clean extracts were obtained using the described procedure and the sensitivity was not satisfactory, due to matrix interferences. In this new sample cleanup procedure feed samples were extracted using an acetonitrile/water/acetic acid (79/20/1, v/v/v) mixture as reported by Sulyok et al. (*21*) and two pathways were required. In the first pathway a Multisep226 SPE column was used but it was not possible to recover the fumonisins. Therefore, a second pathway was necessary, using a glass microfilter. Although it was possible to recover all the selected mycotoxins by only using the glass microfilter, a dilution was necessary, to minimize matrix interferences, resulting in a nonsatisfactory sensitivity. By combination of the two pathways, a compromise was made to recover all the selected mycotoxins and to obtain satisfying sensitivity. After recombination of both purified parts of the sample extract, the combined solvents were evaporated and redissolved in the mobile phase. Consequently only one LC-MS/MS injection was necessary for the LC-MS/MS analysis.

Table 1. Summary of the Results of 67 Contaminated Feed Samples

mycotoxin	no. of contaminated samples	mean ± SD, μg/kg	minimum, μg/kg	maximum, μg/kg
AME	1		19	19
OTA	2	27.5 ± 7.8	22	33
DAS	3	5.1 ± 1.5	3.5	6,3
AOH	3	20.3 ± 4.2	17	25
ROQ-C	4	4.6 ± 6.1	1.3	14
T2	7	28.9 ± 37.1	10	112
HT2	7	47.0 ± 32.6	22	116
NIV	9	416.2 ± 807.3	70	2547
ZEN	12	157.2 ± 117.5	58	387
FB_3	23	95.8 ± 55.2	25	246
FB_2	29	292.5 ± 305.5	28	1527
15-ADON	31	118.3 ± 187.9	9.9	1047
3-ADON	35	35.8 ± 56.3	6.0	339
FB_1	36	913.6 ± 1225	36	5114
DON	52	948.6 ± 1772	74	9528

In the recently published multimycotoxin LC-MS/MS methods two different approaches for sample preparation can be distinguished. On the one hand, there are time-consuming cleanup methods using different types and combinations of solid-phase extraction columns, and on the other hand, there exist methods without a cleanup step where raw extracts are injected (*10*). Because animal feed can be a very complex matrix, as it is often a mixture of different kinds of crops, a cleanup step was necessary. The performance without cleanup was tested, but unsatisfactory sensitivity was obtained (results not shown). In addition, no cleanup resulted in unclear chromatograms and polluted MS equipment, which can cause MS damage. In general, the long-term influence of so-called "dilute-and-shoot" methods on LC-MS/MS equipment is questionable and still unknown.

Method Validation. Commission Directive 2003/100/EC sets maximum levels for aflatoxin-B_1, while recommended maximum levels were set in Recommendation 2006/576/EC for deoxynivalenol, zearalenone, ochratoxin A, and the sum of fumonisin B_1 and B_2 (*17*, *18*). For aflatoxin-B_1 the CCα and CCβ values at the 5 μg/kg level, which is the most strict regulation, were respectively 6.8 μg/kg and 8.1 μg/kg (*17*). For the toxins with recommended levels the CCα and CCβ values were below the most strict limits. Therefore, the sensitivity of the developed method complies with the maximum levels as stated in the existing regulations. The calculated expanded measurement uncertainty was for all the toxins between 8.0% and 40.2%. The apparent recovery varied from 97.0% to 104.8% and fulfilled the performance criteria of Commission Decision 2002/657/EC because all the results were within 80% and 110%. A precision study was performed by determining the repeatability and the reproducibility at the four concentration levels. The most strict performance criteria of Commission Decision 2002/657/EC of respectively 12% and 18% were fulfilled (*16*). The obtained RSD for repeatability was within 1.8% and 11.8%, and the obtained RSD for reproducibility was within 2.2% and 13.2%. The developed method was EN ISO 17025 accredited (*19*).

Analysis of Feed Samples. A total of 82 feed samples (sow feed (N = 4), wheat (N = 30), and maize (N = 48)) were analyzed with the newly developed multimycotoxin method. Of these, 67 samples (sow feed (N = 4), wheat (N = 18), and maize (N = 45)) or 82% were contaminated with at least one mycotoxin.

Table 2. Concentrations (μg/kg) of the Detected Toxins of the Co-contaminated Samples

		type B-trichothecenes					fumonisins			type A-trichothecenes						
sample	type/origin[a]	NIV	DON	3-ADON	15-ADON	ZEN	FB_1	FB_2	FB_3	DAS	HT2	T2	AOH	AME	ROQ-C	OTA
1	W/CZ		443		9.9	61										
2	W/CZ	200	8841	67	100	155										
3	W/CZ						78	28								
4	W/HU		1799		23											
5	W/HU		1997	18	35											
6	W/HU		2113	15	23											
7	W/HU														2.5	22
8	M/CZ		81	17	43		123	67	25							
9	M/CZ		9528	339	1047	387	82	38,1			33	18	25			
10	M/CZ	2547	457	34	82		56			6.3						
11	M/CZ			13	41										1.6	
12	M/CZ		910	56	192						22	13				
13	M/CZ		956	47	107		853	229	84	3.5	56	13	19			
14	M/CZ		469	31	84		739	329	66			10				
15	M		75												1.3	
16	M		171				55									
17	M			7.1			36								14	
18	M						295	58	26							
19	M			6			50									
20	M		899	14		147										
21	M		74	8.9												
22	M		320	21												
23	M		175	8.2			258	53					17			
24	M		163	8.3			511	82			116	112		19		
25	M/ES		930	33	92											
26	M/ES		537	36	151					5.6						
27	M/ES	94	1394	30	94	356	198	83	40							
28	M/ES		514	30	136		1010	298	63							
29	M/ES						5114	1527	192							
30	M/ES		1920	77	382		61									
31	M/ES						4048	723	89							
32	M/ES						345	80	41							
33	M/ES		105				126					10				
34	M/ES		877	44	134		1496	283	96							
35	M/ES		583		27		245	136	67							
36	M/ES		515	22	70		1356	481	117							
37	M/ES		734	9.4		58	429	134	85							
38	M/ES		959	16	59	86	2679	286	147							
39	M/PT		88		13		740	287	73							
40	M/PT	85	611	30	85	115	1149	308	105							
41	M/PT	144	274	21	63		2068	589	176							
42	M/PT		420	23	74		617	212	73							
43	M/PT		134	15	62		127	98								
44	M/PT		711	17	63	73	840	333	99							
45	M/PT	70	526	20	77		758	265	86							
46	M/PT		384		32	73	1858	611	155							
47	M/PT	85	777	11	33	281	224	122								
48	M/PT	435	3039	86	233	94	193	143	53							
49	M/PT						3761	600	246							
50	W	86	750								46	26				

[a] Legend: W = wheat, M = maize, CZ = Czech Republic, HU = Hungary, ES = Spain, PT = Portugal.

A summary of the results is shown in **Table 1**. Alternariol methyl ether, ochratoxin A, diacetoxyscirpenol, alternariol, roquefortine-C, T2-toxin, HT2-toxin, nivalenol, zearalenone, fumonisin B_3, fumonisin B_2, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, fumonisin B_1, and deoxynivalenol were detected in the analyzed feed samples. Type B-trichothecenes 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, and deoxynivalenol and fumonisins occurred most often in the contaminated samples (> 30%). The published legal maximum levels depend on the type of animal feed and do not take into account the possible co-occurrence of mycotoxins (except for the sum of fumonisin B_1 and B_2) (*17*, *18*). Only in one wheat sample (8841 μg/kg) and one maize sample (9528 μg/kg) was the EU recommended value for deoxynivalenol (8000 μg/kg in grain and grain products) exceeded, despite the high amount of contaminated feed samples. In 17 samples or 25% of the contaminated samples only one mycotoxin was detected: 3-acetyldeoxynivalenol ($N = 1$), fumonisin B_1 ($N = 1$), ochratoxin A ($N = 1$), HT2-toxin ($N = 2$), and deoxynivalenol ($N = 12$). In 50 samples or 75% more than one mycotoxin was detected. The individual results of the cocontaminated samples are shown in **Table 2**. This table shows that the toxins 15-acetyldeoxynivalenol ($N = 31$), fumonisin B_2 ($N = 29$), fumonisin B_3 ($N = 23$), zearalenone ($N = 12$), nivalenol ($N = 9$), T2-toxin ($N = 7$), roquefortine-C ($N = 4$), alternariol ($N = 3$), diacetoxyscirpenol ($N = 3$), and alternariol methyl ether ($N = 1$) always co-occurred in this study. The toxins

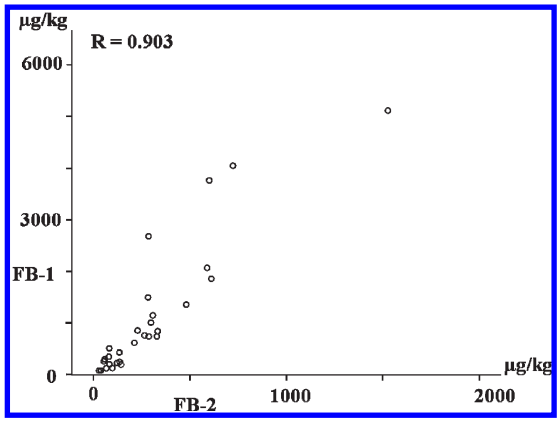

Figure 1. Linear correlation between FB_2 and FB_1 shown in a scatterplot.

3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol did not necessarily co-occur: in 6 samples (17.1%) 3-acetyldeoxynivalenol occurred without 15-acetyldeoxynivalenol and in 5 samples (16.1%) 15-acetyldeoxynivalenol occurred without 3-acetyldeoxynivalenol. Nivalenol and 15-acetyldeoxynivalenol always co-occurred with deoxynivalenol, while in 3 samples (8.6%) 3-acetyldeoxynivalenol occurred without deoxynivalenol. A statistically significant ($p < 0.01$) correlation in the detected concentration was obtained between fumonisin B_2 and fumonisin B_1 ($R = 0.903$), fumonisin B_3 and fumonisin B_1 ($R = 0.790$), and fumonisin B_3 and fumonisin B_2 ($R = 0.720$). **Figure 1** illustrates the positive linear correlation between fumonisin B_2 and fumonisin B_1 in a scatter plot. These data points were derived from 1 wheat sample and 28 maize samples. In 1999 Placinta et al. (*22*) reported the worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins, thereby demonstrating the co-occurrence of nivalenol, deoxynivalenol, zearalenone, and fumonisin B_1, B_2, and B_3. These findings were confirmed by our results: type B-trichothecenes and fumonisins are the main co-occurring mycotoxins detected in the 67 samples. The co-occurrence of these specific toxins can be taken into account in the design of future toxicity studies to examine the impact on animal health.

Interpretation of Results Based on Matrix and Origin. *Wheat*. In total, 5 out of 8 wheat samples originating from the Czech republic contained one or more mycotoxins produced by *Fusarium* species. In 2 samples a co-occurrence was observed of type B-trichothecenes with zearalenone. In 1 sample fumonisin B_1 and B_2 co-occurred. Only 5 out of 14 wheat samples collected in Denmark during the harvest season in 2008 contained a single mycotoxin, deoxynivalenol, HT2-toxin, or roquefortine-C, at relatively low levels. No contamination with more than one mycotoxin was observed. The Hungarian samples were more heavily contaminated. Out of 7 wheat samples, 6 contained one or more mycotoxins produced by *Fusarium* or *Penicillium* molds. In four samples the co-occurrence of multiple mycotoxins was confirmed.

Maize. In general, a much higher incidence of co-occurrence of mycotoxins was observed for the maize samples ($N = 41$). Only 8 out of the 48 samples (17%) contained only mycotoxins from one class: i.e., only type B-trichothecenes or only fumonisins. All other samples were contaminated with multiple mycotoxins from different classes. In the samples from Portugal and Spain the co-occurrence of type B-trichothecenes and fumonisins was very clear, as demonstrated in the correlation analysis. Also in samples from the Czech Republic this co-occurrence was observed, albeit to a somewhat lesser extent. However, the Czech samples showed a more frequent appearance of other classes of mycotoxins such as type A-trichothecenes, alternariol, and roquefortine-C.

The occurrence of mycotoxins in feed is an emerging issue, and therefore a multimycotoxin LC-MS/MS method was developed, validated, and EN ISO 17025 accredited. The analysis of 82 samples of 3 different feed matrices (sow feed, wheat, and maize) resulted in the detection of 67 contaminated samples (82%); type B-trichothecenes and fumonisins most often occurred (> 30%). Most contaminated feed samples (75%) were contaminated with more than one mycotoxin. The findings reported in this paper emphasize the importance of more research into the toxic effects of co-occurring mycotoxins. At this point no satisfactory toxicological data are available. It is expected that these more performant analytical methods will assist other research areas to increase the understanding of the toxicological risks and interactions between different mycotoxins. These findings will be required to re-evaluate the current legal maximum limits as well as the required control of mold growth in feeds as a part of Good Storage Practices (Commission Recommendation 2006/583/EC) (*23*).

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