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The Development of a Sensitive Multi-Residue LC-MS/MS Method for the Quantitative Determination of Mycotoxins in Animal Feedstuffs and Silage Using Xevo TQ-S

Sara Stead,[1] Dominic Roberts,[1] Antonietta Gledhill,[1] Theo de Rijk,[2] Christof Van Poucke,[3] and Sarah De Saeger[3]
[1] Waters Corporation, Manchester, UK; [2] RIKILT Institute of Food Safety, Wageningen, The Netherlands
[3] Ghent University, Ghent, Belgium

APPLICATION BENEFITS

- Provides a quantitative LC-MS/MS method for the simultaneous determination of 33 mycotoxins in animal feedingstuffs and silage.
- Suitable for use as sensitive screening assay for the determination of low level mycotoxin contamination in animal feedingstuffs.
- Reduces complex matrix effects by incorporating a simple extract dilution step prior to analysis.

WATERS SOLUTIONS

ACQUITY UPLC® I-Class System

Xevo® TQ-S

TargetLynx™ Application Manager

Quanpedia™ Database

KEY WORDS

mycotoxin, feedingstuffs, silage, tricothecene, beauvercin, enniatin, fumonisin, ochratoxin, T2, HT-2, alternaria, tandem quadrupole,

INTRODUCTION

There are now over 400 recognized mycotoxins that may be found in animal feedings materials and it has been reported that as much as 25% of the world's cereal grains may be contaminated with mycotoxins.[1]

The analysis of animal feedingstuffs including silage represents a major technical challenge due to the complexity and in-homogeneity of these matrices. Although permitted limits for mycotoxins are set at relatively high (µg kg^{-1}) concentrations in the EU,[2,3] toxic effects such as immunotoxicity and feed uptake problems in certain species (poultry and porcine) are often observed at sub µg kg^{-1} concentrations.[4] For this reason there is often a requirement to achieve low detection limits in feedingstuffs. There is also a potential for co-contamination due to pre- and post- harvest infestation resulting in the occurrence of tricothecenes, beauvercin and enniatins, fumonisins, ochratoxin, T2, HT-2, and alternaria toxins for example within a single feed sample.[5]

In this application note, we report the development of a quantitative method for the determination of 33 relevant mycotoxins in a variety of animal feed and silage extracts. A Waters® ACQUITY UPLC I-Class System coupled to a Xevo TQ-S was used for rapid, high quality, and ultra-sensitive analysis of multiple mycotoxins in feed extract. Our goal was to investigate the effect of matrix dilution and enhanced instrument sensitivity to overcome common analytical challenges such as ion suppression and to reduce the effects of matrix variability.

EXPERIMENTAL

Extract preparation

The extracts of different animal feedingstuffs and silage were kindly provided by RIKILT, The Netherlands and Ghent University for the purposes of this study. A generic and simplified sample extraction protocol based on 84:16 (v/v) acetonitrile: acidified water for the recovery of mycotoxins from the variety of feedingstuffs and silage was used.[6] Briefly, the feed samples were mechanically homogenized in the presence of the extraction solvent followed by a centrifugation step. An aliquot of the supernatant was removed and placed in autosampler vial for subsequent LC-MS/MS analysis.

UPLC conditions

LC system:	ACQUITY UPLC I-Class
Column:	BEH C_{18}, 2.1 x 100 mm, 1.7 µm
Temp.:	40 °C
Injection volume:	5 µL
Flow rate:	0.4 mL/min
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid

MS conditions

MS System:	Xevo TQ-S
Ionization mode:	ES +/- switching
Capillary voltage:	3.4 kV
Source temp.:	150 °C
Desolvation temp.:	400 °C
Cone gas flow:	150 L/hr
Desolvation gas flow:	800 L/hr

Table 1. UPLC gradient.

Time	A	B	Curve
0	90	10	
3	90	10	
10	30	70	
10.1	10	90	6
12	10	90	
12.1	90	10	
15	90	10	

Table 2. MS/MS conditions optimized using Quanpedia for the multi-mycotoxin analysis method for feedingstuffs and silage using the Xevo TQ-S.

Mycotoxin	MRM transition	Dwell time (s)	Cone voltage (V)	Collision energy (eV)	Polarity
Aflatoxin B1	Q 313.1 > 241.1 q 313.1 > 285.1	0.02	25	50 30	Pos
Aflatoxin B2	Q 315.1 > 259.1 q 315.1 > 287.1	0.02	25	40 35	
Alfatoxin G1	Q 329.1 > 243.1 q 329.1 > 311.1	0.02	20	33 20	
Alfatoxin G2	Q 331.1 > 245.1 q 331.1 > 313.1	0.037	25	18 19	
DON	Q 297.1 > 231.1 q 297.1 > 249.1	0.103	20	30 36	
Enniatin A	Q 682.6 > 99.9 q 682.6 > 210.3	0.108	70	30 34	
Enniatin A3	Q 668.5 > 99.9 q 668.5 > 210.0	0.052	64	30 36	
Enniatin B	Q 640.5 > 85.9 q 640.5 > 195.9	0.052	64	30 36	
Enniatin B1	Q 654.5 > 85.9 q 654.5 > 195.9	0.02	70	55 50	
Fumonisin B1	Q 722.4 > 334.3 q 722.4 > 352.3	0.023	25	55 50	
Fumonisin B2	Q 706.4 > 336.2 q 706.4 > 318.2	0.023	50	5 12	
HT-2	Q 425.2 > 245.1 q 425.2 > 263.1	0.021	14	32 30	
OTA	Q 404.2 > 245.1 q 404.2 > 263.1	0.021	10	5 5	
T-2	Q 467.3 > 245.1 q 467.3 > 305.1	0.052	20	23 19	
Zearalenone	Q 319.1 > 185 q 319.1 > 187	0.038	78	70 28	
Beauvericin	Q 784.5 > 133.9 q 784.5 > 244.0	0.052	32	43 30	
Tentoxin	Q 415.2 > 131.9 q 415.2 > 312.1	0.038	17	12 7	
Pencillic acid	Q 171.1 > 125.0 q 171.1 > 153.0	0.163	28	25 25	
Citrinin	Q 251.1 > 191.0 q 251.1 > 205.1	0.023	25	30 25	
Alternariol	Q 259.0 > 185.1 q 259.0 > 213.2	0.02	10	20 8	
NIV	Q 313.7 > 175.1 q 313.7 > 295.1	0.163	14	15 7	
α- Zearalenone	Q 323.0 > 277.1 q 323.0 > 305.2	0.023	14	15 7	
β- Zearalenone	Q 323.0 > 277.1 q 323.0 > 305.2	0.023	20	39 36	
Sterigimatocystin	Q 325.1 > 253.1 q 325.1 > 281.1	0.05	22	20 26	
Cyclopiazonic acid	Q 337.1 > 182.0 q 337.1 > 196.0	0.163	18	19 12	
3-acetyl-DON	Q 339.1 > 137.0 q 339.1 > 231.0	0.03	14	12 10	
15-acetyl-DON	Q 339.1 > 261.0 q 339.1 > 279.1	0.03	20	20 13	
Fusarenon-X	Q 355.2 > 229.1 q 355.2 > 247.1	0.03	14	10 10	
Diacetoxyscirpenol	Q 367.2 > 289.1 q 367.2 > 307.2	0.02	17	27 20	
Neosolaniol	Q 383.1 > 185.1 q 383.1 > 215.1	0.03	27	28 19	
Roquefortin	Q 390.2 > 193.0 q 390.2 > 322.2	0.032	25	42 30	
Ergotamine	Q 587.3 > 208.1 q 587.3 > 187.5	0.02	35	40 30	
Alternariol monomethyl ether	Q 271.2 > 228.2 q 271.2 > 256.2	0.031	42	30 10	Neg

Figure 1 shows the MRM transitions and automated time window scheduling functionality to obtain a minimum of 12 points across each chromatographic peak generated by Quanpedia. This method is available in the Quanpedia database.

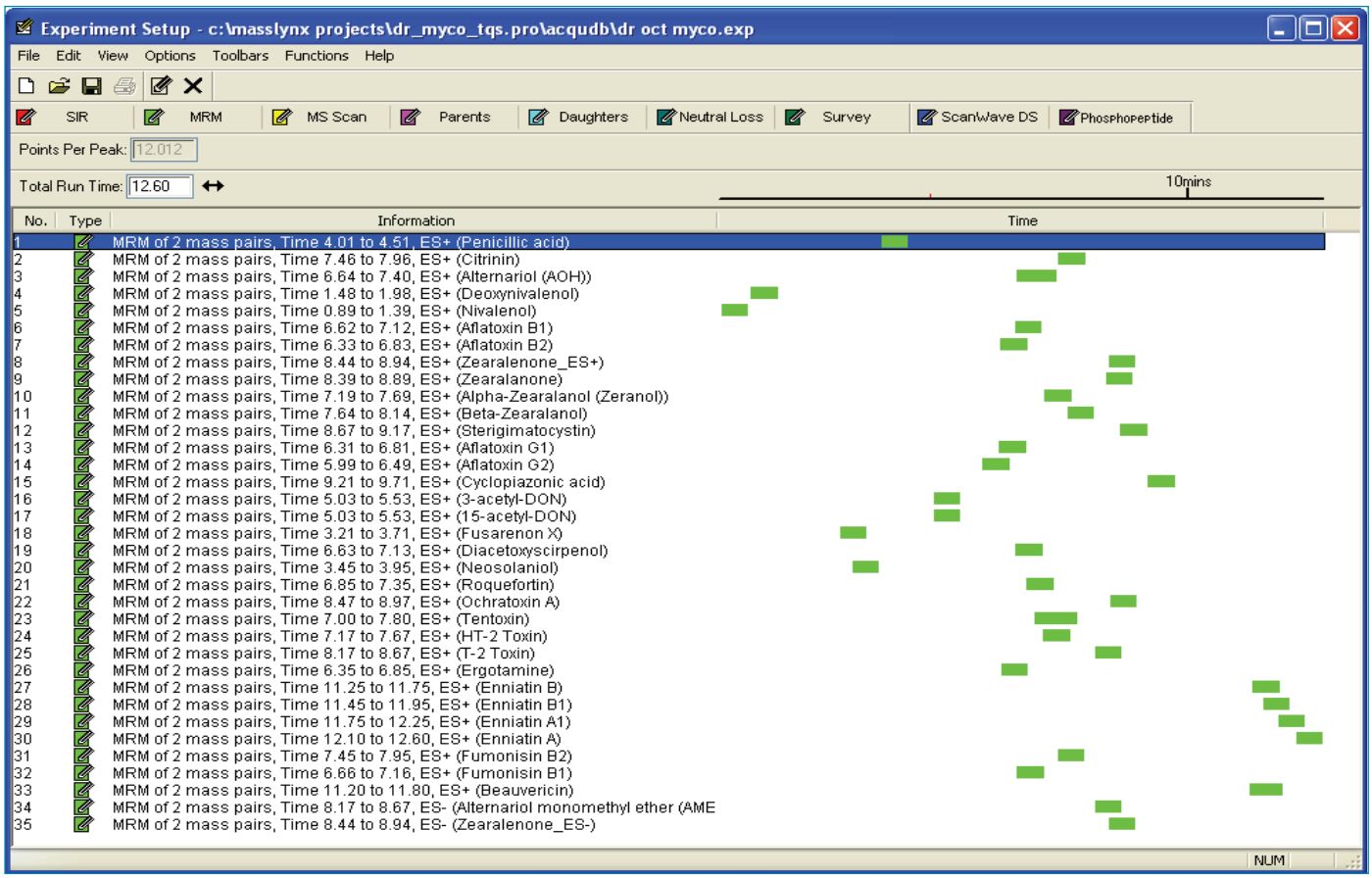

Figure 1. MRM transitions for 33 mycotoxins (in ESI + and - modes) and automated time window scheduling functionality generated by the Quanpedia Database.

RESULTS AND DISCUSSION

The feed and silage samples were analyzed as neat and diluted extracts and quantified against either solvent or matrix matched standards (as available). As anticipated, the matrix interference profile was found to be highly complex and variable between samples as determined via the RADAR functionality. Figure 2 shows the LC-MS/MS spectra obtained for a neat extract of a porcine feed. The Base Peak Intensity (BPI) spectrum obtained in full scan mode and the simultaneously acquired MRM transitions for five mycotoxins identified in the sample (displayed in the Figure inset) were found to elute in a region of high matrix background. The presence of high concentrations of matrix background can result in variable ion suppression effects for the analytes of interest and can affect the overall analytical performance. For this reason, matrix matched calibrants, standard addition, and isotopically labelled internal standards are approaches typically used for the quantitative analysis of mycotoxins in complex matrices to overcome the matrix effects and improve the quantitative accuracy and precision.

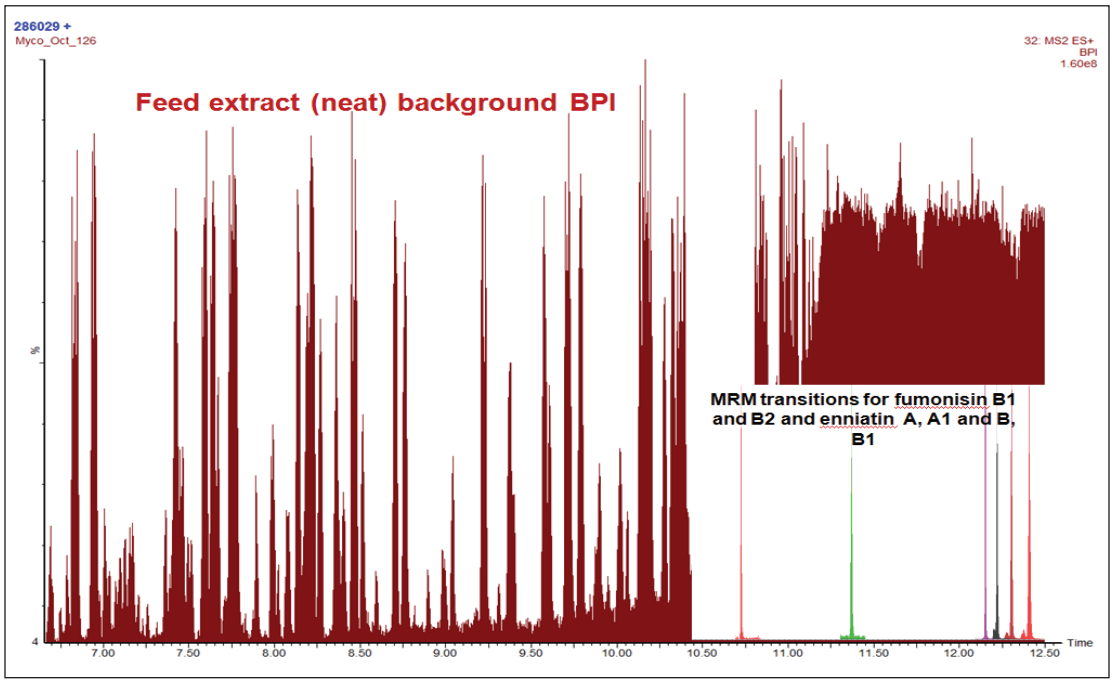

Figure 2. Base peak intensity (BPI) chromatogram obtained for naturally contaminated porcine feed extract showing the background matrix profile monitored in full scan mode (MS function 2, RADAR) and the MRM transitions (MS function 1) for fumonisins B1 and B2 and enniatins A, A1, B, and B2.

In this study, we investigated the use of a simple dilution step coupled to the enhanced sensitivity of the Xevo TQ-S to reduce the matrix contribution and improve measurement repeatability and accuracy between different sample types. Table 3 shows the repeatability data obtained for six different extracts of feed spiked with six mycotoxins diluted 1:10 prior to injection. The intra-day method precision (expressed as %RSD) was found to be good at less than 23% for all the spiked mycotoxins.

The same dilution factor (1:10) was also applied to a wider selection of feedingstuffs (12) and silage (10) sample extracts and analyzed according to the optimized Xevo TQ-S conditions monitoring for 33 mycotoxins and quantified against an appropriate calibration series. Figure 3 shows the TargetLynx report generated showing the linearity and sensitivity of the method and calculated concentrations for the unknown samples.

Table 3. The repeatability data generated for a selection of six mycotoxins spiked into to a variety of different animal feeds (n=6), the extracts were diluted 1:10 prior to analysis on the Xevo TQ-S.

Feed Type	Measured concentration in animal feed extract diluted 1:10 (ng/g)*					
	Enniatin A	Enniatin A1	Enniatin B	Enniatin B1	Fumonisin B1	Fumonisin B2
Maize Gluten	305.9	165.6	48.0	86.1	14.0	17.1
Pig feed	275.5	160.5	43.6	56.5	17.0	17.5
Rye	258.5	136.1	61.8	62.1	18.7	19.3
Oats	221.9	115.5	41.4	62.0	22.7	20.1
Sunflower seed	197.4	117.7	48.2	77.1	13.0	13.2
Cattle feed	193.9	102.2	32.0	51.3	14.0	14.8
Mean	242.2	133.0	45.8	65.9	16.6	17.0
SD	45.1	25.7	9.8	13.1	3.7	2.6
%RSD	19	19	21	20	22	15

**Mycotoxins spiked into feed samples prior to extraction*

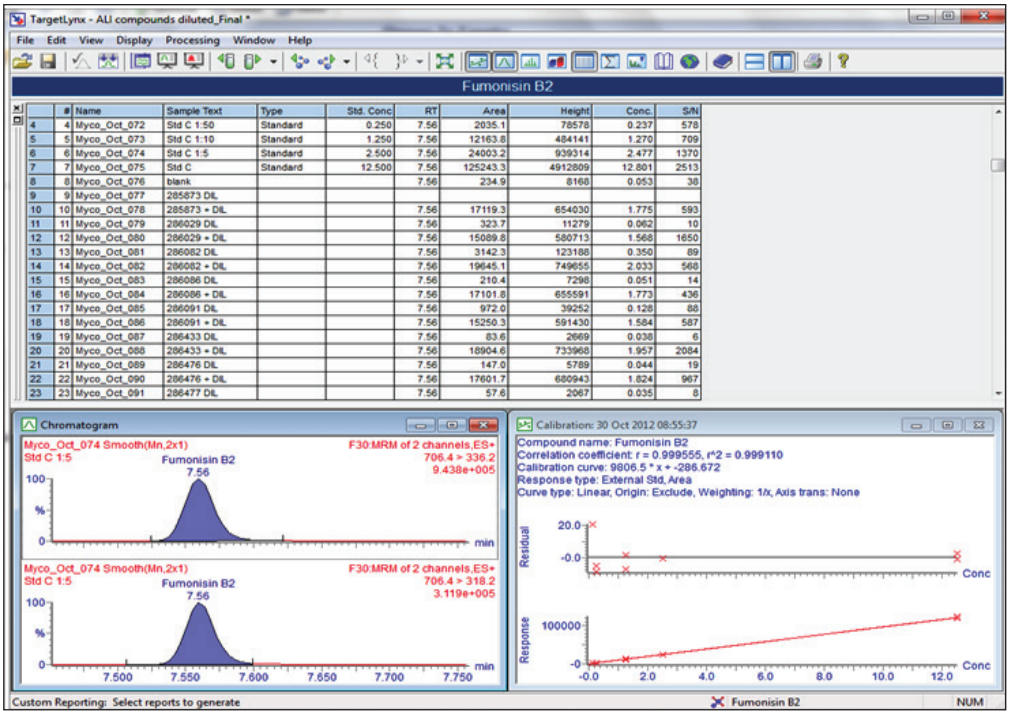

Figure 3. TargetLynx report showing linearity and sensitivity of standards down to 0.25 µg kg for fumonisin B2 using the Xevo TQ-S.

Tables 4 and 5 show the measured concentrations of the mycotoxins identified (monitoring two MRM transitions) in naturally contaminated feeds and silage samples, respectively along with the calculated LODs (S:N of ≥1:3). The LOD values were found to be in the ppt to low ppb range in both solvent and matrix for all the mycotoxins identified. The naturally contaminated feed samples were found to contain multiple mycotoxins ranging from 3 to 12 of the 33 potential mycotoxins monitored for and estimated to be present at concentrations equivalent sub-1 to circa 300 µg kg^{-1}. The method was considered to be suitable for use as a highly sensitive presence/absence screen as the contaminant concentrations were determined against a solvent standard calibration series, therefore matrix effects may still affect the quantitative performance.

Table 4. The measured concentrations for a range of mycotoxins identified using two MRM transitions in 12 different samples of animal feedingstuffs diluted 1:10 prior to analysis on the Xevo TQ-S.

Mycotoxin	LOD (ng/g)	Measured concentration in animal feed extract diluted 1:10 (ng/g)*											
		Animal feed sample identity and type											
		U1 / cattle feed	U2 / pig feed	U3 / maize gluten	U4 / Diva L vital pig feed	U5 /Alpha maximal pig feed	U6 / rye	U7 / barley	U8 / wheat	U9 / oats	U10 / maize	U11 / sunflower oil	U12 / pig feed
15-acetyl-deoxynivalenol	0.50	*nd*	*nd*	152.8	*nd*	*nd*	*nd*	13.2	33.4	*nd*	*nd*	*nd*	*nd*
Aflatoxin B1	0.05	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	0.2	*nd*
Aflatoxin B2	0.05	*nd*	*nd*	0.8	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	0.1	*nd*
Aflatoxin G1	0.05	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	0.1	*nd*
Aflatoxin G2	0.05	0.3	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Alternariol	0.06	*nd*	3.2	*nd*	*nd*	*nd*	5.3	*nd*	*nd*	7.6	2.6	10.0	*nd*
DON	0.13	*nd*	21.2	283.6	13.2	18.4	*nd*	*nd*	*nd*	4.8	*nd*	0.3	*nd*
Enniatin A	0.10	59.3	6.3	1.4	15.7	39.9	9.7	11.7	0.4	3.2	*nd*	*nd*	50.5
Enniatin A1	0.10	148.6	17.1	3.2	40.1	19.0	14.2	34.1	0.5	4.9	*nd*	*nd*	122.4
Enniatin B	0.10	125.2	43.3	5.8	65.3	53.3	92.8	52.9	0.4	9.0	*nd*	*nd*	116.1
Enniatin B1	0.10	263.0	41.8	5.5	72.1	32.3	42.8	64.0	0.5	9.9	*nd*	*nd*	238.2
Fumonisin B1	0.10	0.3	0.7	18.9	*nd*	4.0	*nd*	*nd*	*nd*	*0.4*	92.8	*nd*	1.7
Fumonisin B2	0.10	0.1	*nd*	3.1	*nd*	0.8	*nd*	0.2	*nd*	*nd*	16.0	*nd*	0.3
HT-2 Toxin	0.25	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	3.9	*nd*	*nd*	*nd*
Ochratoxin A	0.06	0.1	*nd*	*nd*	0.1	*nd*	0.2	2.8	*nd*	*nd*	*nd*	*nd*	0.1
Roquefortine	0.10	*nd*	0.3	0.3	0.2	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Sterigmatocystin	0.10	*nd*	0.1	0.4	0.2	*nd*	10.7	*nd*	*nd*	*nd*	*nd*	0.1	0.2
Zearalenone	0.20	*nd*	1.6	84.0	*nd*	4.9	31.2	*nd*	6.1	*nd*	*nd*	*nd*	*nd*
Number of mycotoxins found		*8*	*10*	*12*	*8*	*9*	*8*	*7*	*6*	*8*	*3*	*6*	*8*

**Concentration determined against a solvent calibration series.*

Fewer mycotoxins were detected in the silage samples (ranging from 0 to 3 of the 33 potential mycotoxins monitored for) by comparison with the compound feed samples. Figure 4 shows the chromatographic separation achieved for an extract of spiked silage following 1:10 dilution. The calculated concentrations ranged from circa 20 to 260 µg kg^{-1} The recovery values determined from the spiked control sample ranged from 89% to 108% for 17 mycotoxins with a mean value of 96%.

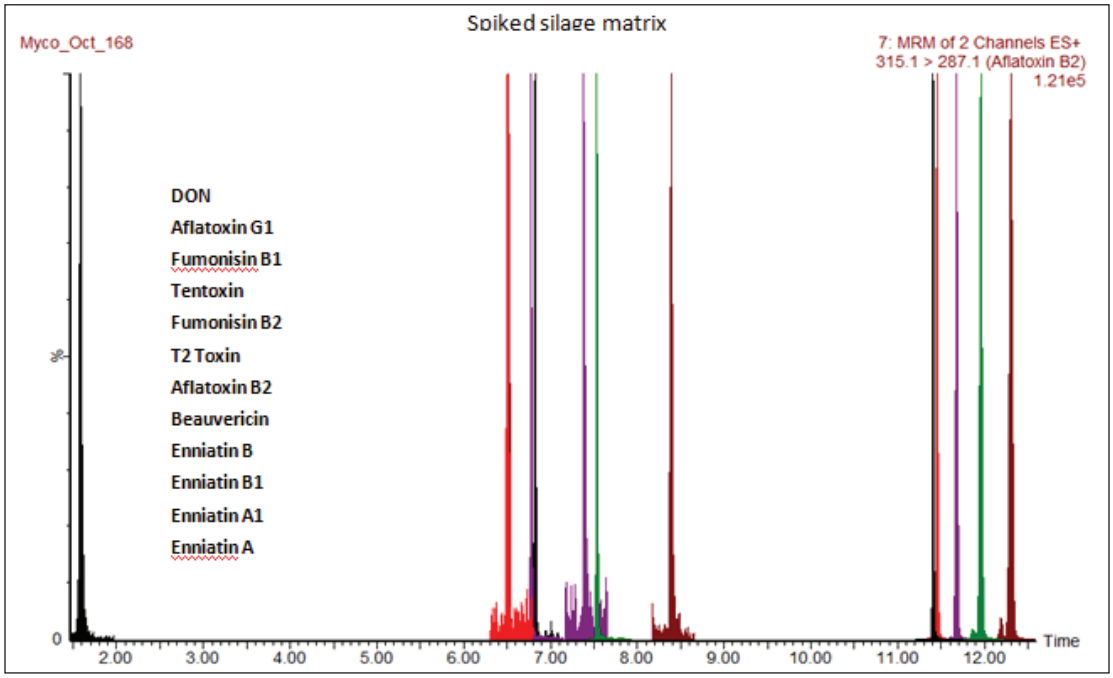

Figure 4. Chromatographic separation achieved for a spiked silage extract using the ACQUITY UPLC I-Class System with Xevo TQ-S.

Table 5. The measured concentrations and %recovery values obtained for a range of mycotoxins identified using two MRM transitions in 10 different samples of silage diluted 1:10 prior to analysis on the Xevo TQ-S.

Mycotoxin	LOD (ng/g)	Measured concentration in silage samples diluted 1:10 (ng/g)*										
		Silage sample identity										
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	Spike control %recovery
Aflatoxin B1	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	95
Aflatoxin B2	0.2	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	104
Aflatoxin G1	0.6	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	93
Aflatoxin G2	0.4	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	94
DON	0.9	958.1	1310.0	158.4	550.8	435.4	359.8	216.4	*nd*	261.7	*nd*	100
Enniatin A	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	94
Enniatin A1	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	94
Enniatin B	0.1	*nd*	*nd*	64.0	72.4	50.9	108.4	204.1	*nd*	82.7	*nd*	89
Enniatin B1	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	94
Fumonisin B1	2.0	*nd*	*nd*	*nd*	256.3	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	93
Fumonisin B2	0.4	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	95
HT-2 Toxin	0.8	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	36.2	*nd*	*nd*	*nd*	97
Ochratoxin A	0.8	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	103
T2 Toxin	5.0	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	96
Zearalenone	2.5	170.2	140.2	108.4	42.5	*nd*	84.2	76.1	*nd*	76.3	*nd*	108
Beauvericin	0.2	*nd*	72.5	23.2	*nd*	*nd*	28.4	22.5	*nd*	*nd*	*nd*	96
Tentoxin	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	92
Number of mycotoxins found		2	3	4	4	2	4	5	0	3	0	17

CONCLUSIONS

- A quantitative LC-MS/MS method applicable for the simultaneous determination of 33 mycotoxins in animal feedingstuffs and silage has been developed with the Waters ACQUITY UPLC I-Class System coupled to the Xevo TQ-S using the Quanpedia database.
- Due to the enhanced sensitivity of the TQ-S instrument it has been possible to incorporate a simple extract dilution step prior to analysis to reduce the complex matrix effects associated with these challenging samples and achieve good method repeatability and accuracy.
- The observed intra-day repeatability (%RSD) was found to be ≤22%, and the mean recovery for 17 mycotoxins in silage was found to be 96%.
- The optimized method has been used to identify the presence and determine the concentrations of a range of mycotoxins present in a wide variety of samples.

Acknowledgments

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Milford, MA 01757 U.S.A.
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F: 1 508 872 1990
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