A quantitative LC-MS/MS method for 15 mycotoxins in corn-based animal feed

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Goals

To develop a comprehensive quantitation method for diagnostically relevant mycotoxins in corn-based animal feed using LC-MS/MS.

Introduction

Mycotoxins are toxic compounds produced by fungi. They are commonly found in corn, wheat, and other grains during growth or storage.[1] These products are used to produce food for human consumption and mixed into animal feed. Testing of animal feed is important in managing animal nutrition, preventing toxicosis, and preventing financial loss in the animal production industry.[1,2] The different types of mycotoxins result in a variety of toxic effects in horses, cattle, poultry, and swine.[1,2,3] Veterinary Diagnostic Labs are often requested to analyze animal feed for mycotoxins before the feed is distributed to livestock for consumption.[2] The most common analytical method used by Veterinary Diagnostic Labs for feed analysis is LC-MS/MS. The method presented here quantifies 15 mycotoxins that are monitored in corn-based animal feed using a liquid-liquid extraction and LC-MS/MS. This method uses a dilute-and-shoot approach to quickly obtain accurate veterinary diagnostic information.

Experimental

Target analytes

A total of 15 mycotoxin compounds were analyzed with this method. Table 1 shows a list of all target analytes along with their retention time, RF lens values, and quantifier and qualifier ions. All standard analytes were prepared at 1 mg/mL in 100% acetonitrile except for fumonisins and nivalenol were prepared in 50/50 acetonitrile/water due to solubility. All analytical standards were purchased from Cayman Chemical (Ann Arbor, MI). All standards were mixed into a total volume of 100 mL. All 8 internal standards were purchased pre-made from Romer Labs GmbH (Tulln, Austria).

Table 1. Target analytes and SRM table

Compound	Retention Time (min)	Polarity	Precursor *(m/z)*	Product Ion #1 *(m/z)*	Product Ion #2 *(m/z)*	Product Ion #3 *(m/z)*	RF Lens (V)
Nivalenol	2.74	[M+H]+	313.1	175.1	177.1	205.0	68
Deoxynivalenol	3.09	[M+H]+	297	177.1	203.2	249.0	55
3-Acetyl-Deoxynivalenol	4.06	[M+H]+	339	203.1	213.1	231.1	51
Aflatoxin B1	5.21	[M+H]+	313	213.0	241.1	285.1	124
Aflatoxin B2	5	[M+H]+	315	243.1	259.1	287.1	133
Aflatoxin G1	4.72	[M+H]+	329	200.1	243.1	311.0	123
Aflatoxin G2	4.5	[M+H]+	331.1	245.1	285.1	313.1	133
T-2	6.52	[M+NH4]+	484.2	185.1	215.1	305.2	59
HT-2	5.98	[M+NH4]+	442.2	215.1	263.2	323.2	49
Fumonisin B1	6.43	[M+H]+	722.4	334.3	352.3		118
Fumonisin B2	7.3	[M+H]+	706.3	336.2	512.3		107
Fumonisin B3	6.89	[M+H]+	706.3	318.3	354.3		113
α-Zearalenol	6.95	[M-H]-	319	160.0	187.9	275.2	96
Zearalenone	7.04	[M-H]-	316.9	131.1	175.0	273.2	99
Ochratoxin A	6.97	[M+H]+	404	221.0	239.0	358.1	66
$^{13}C_{34}$ Fumonisin B1	6.43	[M+H]+	756.4	356.3	374.3		114
$^{13}C_{34}$ Fumonisin B2	7.3	[M+H]+	740.5	358.3	722.5	340.4	110
$^{13}C_{34}$ Fumonisin B3	6.89	[M+H]+	740.5	376.4	540.3		133
$^{13}C_{15}$ Deoxynivalenol	3.09	[M+H]+	312.1	245.2	263.2	276.2	42
$^{13}C_{17}$ Aflatoxin B1	5.21	[M+H]+	330	255.1	284.1	301.2	147
$^{13}C_{24}$ T-2	6.52	[M+NH4]+	508.2	198.2	229.2	322.2	62
$^{13}C_{20}$ Ochratoxin A	6.97	[M+H]+	424.2	145.2	203.1	232.1	82
$^{13}C_{18}$ Zearalenone	7.04	[M-H]-	335	140.1	185.1	290.2	112

Sample preparation

Corn on the cob was purchased at a local store and was shelled and ground. It was analyzed prior to use as a control to ensure it was free from mycotoxin contamination. 5 g (± 1%) was weighed into a 50 mL conical tube for each calibration point, quality control (QC), and matrix blank. An eight-point calibration curve was prepared along with a set of three QC samples. Table 2 lists the effective linear range along with the concentration of QC samples for each analyte. All calibration points and QCs were pre-spiked with a mix of mycotoxin standards and internal standards. Spiking was based on a screening target concentration (STC) level specific to each analyte. A mixture of 70/30 methanol/water + 0.1% formic acid solution was added to each sample to bring the total volume to 20 mL. A solvent blank spiked with internal standards was prepared in a total of 20 mL.

All samples were placed on a multi-tube vortex mixer (Fisher Scientific) to shake at 2000 rpm for 30 minutes. The samples were centrifuged at 3000 rpm for 15 minutes (Sorvall ST40 Thermo Fisher Scientific). A 200 µL Eppendorf® micropipette was used to transfer a 150 µL aliquot of extract to a 1.5 mL flip top tube which was then diluted 1:5 with extraction solvent blank. This was repeated for all calibration points, QC samples, and the matrix blank.

All flip top tubes were centrifuged at 6500 rpm for 10 minutes (Eppendorf 5417C). A 1000 µL Eppendorf micropipette was used to transfer 800 µL of extract from the flip top tube to a 1.5 mL glass amber LC vial. This was repeated for all calibration samples, QCs, and matrix blanks. All vials were placed into the LC autosampler for analysis. All data was processed and acquired using Thermo Scientific™ Xcalibur™ software.

Table 2. Linear range and LLOQ of target analytes

Compound	Linear Range (ng/g)	QC Samples (ng/g)	R2 Value	LLOQ (ng/g)
3-Acetyl Deoxynivalenol	30-150	36, 90, 132	0.996	30
Aflatoxin B1	0.5-2.5	0.6, 1.5, 2.2	0.999	0.5
Aflatoxin B2	0.5-2.5	0.6, 1.5, 2.2	0.997	0.5
Aflatoxin G1	0.5-2.5	0.6, 1.5, 2.2	0.999	0.5
Aflatoxin G2	0.5-2.5	0.6, 1.5, 2.2	0.999	0.5
α-Zearalenol	30-150	36, 90, 132	0.988	30
Deoxynivalenol	40-200	48, 120, 176	0.992	40
Fumonisin B1	30-150	36, 90, 132	0.987	30
Fumonisin B2	30-150	36, 90, 132	0.990	30
Fumonisin B3	30-150	36, 90, 132	0.988	30
HT-2	10-50	12, 30, 44	0.997	10
Nivalenol	75-375	90, 225, 330	0.994	75
Ochratoxin A	5-25	6, 15, 22	0.986	5
T-2	5-25	6, 15, 22	0.999	5
Zearalenone	30-150	36, 90, 132	0.983	30

Liquid chromatography

LC: Thermo Scientific™ Vanquish™ Flex system, binary pump, autosampler, switching valve, and dual column compartments. The column heater was set at 40 °C using forced air.

LC Conditions	
Column	Thermo Scientific™ Hypersil GOLD™ aQ, 100 x 2.1 mm (1.9 µm)
Mobile phase A	Water + 2% Methanol + 5 mM Ammonium Formate + 0.1% Formic Acid + 0.1% Acetic Acid
Mobile phase B	Methanol + 2% Water + 5 mM Ammonium Formate + 0.1% Formic Acid + 0.1% Acetic Acid
LC gradient	At a flow rate of 0.3 mL/min start at 0% B and hold for 0.5 minutes, switch to 30% B and start a linear gradient to 100% B for 8 minutes, hold for one minute, drop to 0% B and equilibrate for three minutes for a total run time of 12 minutes

Mass spectrometry

A Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer was used for all analysis. These data were acquired in timed-SRM mode.

Source settings	
Spray voltage positive mode	3500 V
Spray voltage negative mode	2000 V
Sheath gas	42
Auxiliary gas	17
Sweep gas	1
Ion transfer tube temperature	350 °C
Vaporizer temperature	300 °C

MS settings	
Resolution of Q1 and Q3	0.7 FWHM
CID gas pressure	2 mTorr
Chromatographic peak width	4 s
Cycle time	0.4 s

Results and discussion

Figure 1 is an overlay of the chromatograms of all the mycotoxins in the method. The number at the base of each peak identifies which analyte corresponds to the peak.

All data were analyzed using internal calibration with a weighting factor of 1/x. Table 2 lists the effective linear range and LLOQ of each analyte in ng/g. The LLOQ was defined as the lowest calibration standard for which a bias of ± 20% and a coefficient of variation (CV) of ≤ 20% was achieved. All calibration curves have excellent linearity as evidenced by the R^2 values shown in Table 2. Figure 2 shows representative calibration curves of four analytes in the method.

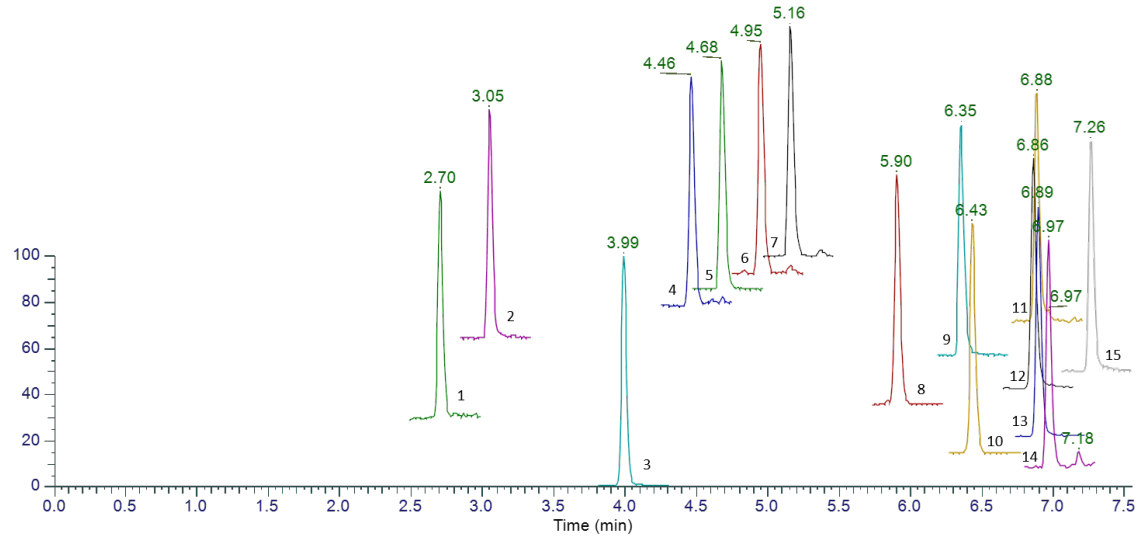

Figure 1. Chromatogram containing all 15 compounds in the mycotoxin method. Each number corresponds to the following analytes: 1. Nivalenol 2. Deoxynivalenol 3. 3-Acetyl-Deoxynivalenol 4. Aflatoxin G2 5. Aflatoxin G1 6. Aflatoxin B2 7. Aflatoxin B1 8. HT-2 9. Fumonisin B1 10. T-2 11. α-Zearalenol 12. Fumonisin B3 13. Ochratoxin A 14. Zearalenone 15. Fumonisin B2

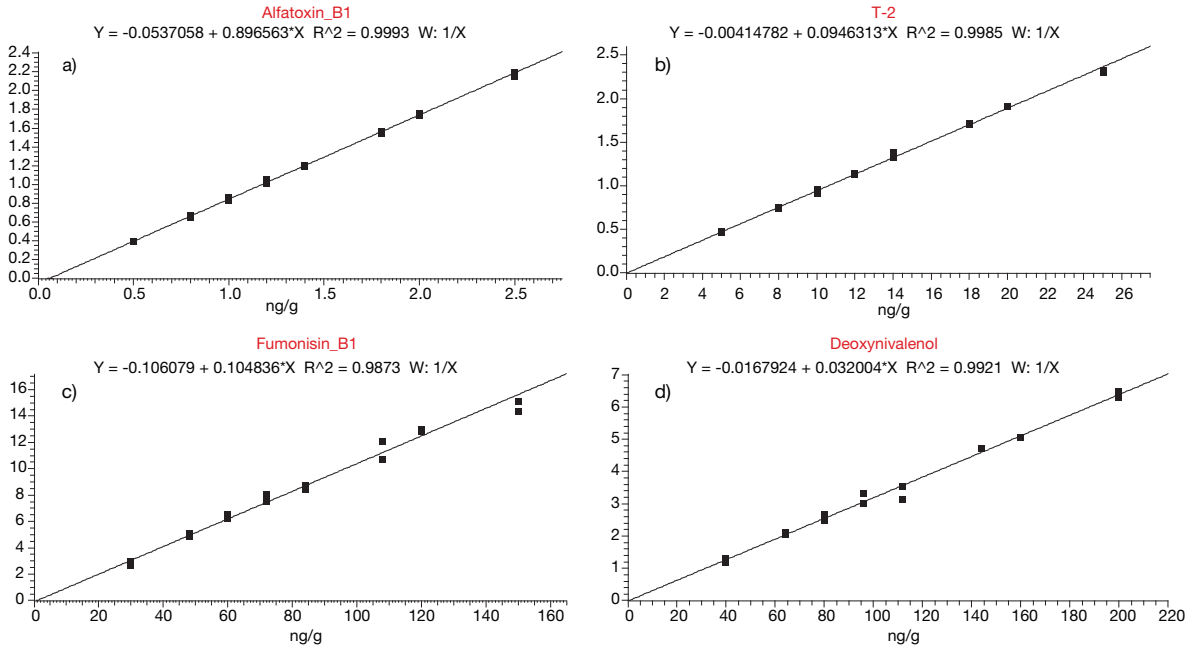

Figure 2. Representative calibration curves of a) Aflatoxin B1 b) T-2 Toxin c) Fumonisin B1 d) Deoxynivalenol

Table 3 shows the average recovery (%) and matrix effect (ME %) for each analyte in corn feed. The recovery was determined at three different calibration levels for all analytes. The average of the recovery at the three levels is shown in Table 3. The recovery of all analytes is 86-100%. The matrix effect was calculated by dividing the mean signal area of the compound spiked in matrix by the mean signal area in spiked solvent and multiplying by 100. This was done for the signal of each compound at three different calibration levels. The average matrix effect at all three levels is reported in Table 3. The matrix effect ranges between 31-173%. Fumonisin B1, B2, and B3 show signal enhancement while all other analytes are suppressed by at least 68%.

The bias and precision for five replicates of each analyte at a low, medium, and high QC level along with the LLOQ is given in Table 4. All analytes show a bias of less than 15% at the low, medium, and high QC levels except Aflatoxin B2 and Nivalenol. The bias at the LLOQ for all analytes is below 20%. The values in Table 4 show that the method has accurate measurements and reliable precision. All analytes have a CV less than 15% at all levels including the LLOQ. The precision of Aflatoxin B1 at the mid-level QC (1.5 ppb) over the course of three analytical runs is 11.3%. This value is below 15% and illustrates the robustness of the method with a total of N=15 replicates over multiple analytical runs.

Table 3. Recovery and matrix effect

Compound name	Average recovery (%)	Average ME (%)
3-Acetyl-Deoxynivalenol	93	46.4
Aflatoxin B1	86	41.0
Aflatoxin B2	90	41.0
Aflatoxin G1	91	43.9
Aflatoxin G2	90	46.6
α-Zearalenol	99	31.2
Deoxynivalenol	92	42.5
Fumonisin B1	94	154.8
Fumonisin B2	88	173.1
Fumonisin B3	100	144.3
HT-2	100	58.8
Nivalenol	95	46.3
Ochratoxin A	89	65.8
T-2	95	67.5
Zearalenone	93	32.6

Table 4. Accuracy and precision

Compound name	Level	Bias (%)	CV (%)
3-Acetyl-Deoxynivalenol	LLOQ	-0.50	2.10
	Low	-2.09	6.17
	Medium	0.31	1.93
	High	2.05	3.91
Aflatoxin B1	LLOQ	-2.17	2.83
	Low	2.19	8.99
	Medium	8.10	1.75
	High	10.63	1.60
Aflatoxin B2	LLOQ	-3.26	3.51
	Low	>15	9.00
	Medium	>15	2.19
	High	>15	4.21
Aflatoxin G1	LLOQ	-5.96	4.39
	Low	-4.87	5.96
	Medium	-0.20	1.59
	High	2.05	1.55
Aflatoxin G2	LLOQ	-2.74	4.07
	Low	-8.46	3.16
	Medium	-1.46	3.16
	High	-0.15	3.02
α-Zearalenol	LLOQ	15.17	8.40
	Low	6.00	3.16
	Medium	2.55	5.83
	High	3.09	11.43
Deoxynivalenol	LLOQ	-0.30	5.04
	Low	-2.22	2.83
	Medium	-1.04	2.21
	High	-0.22	1.94
Fumonisin B1	LLOQ	-3.27	3.87
	Low	-4.17	7.04
	Medium	-4.86	1.62
	High	0.06	2.94
Fumonisin B2	LLOQ	7.56	6.46
	Low	-5.93	7.06
	Medium	-2.17	2.38
	High	-0.75	6.05
Fumonisin B3	LLOQ	3.30	12.44
	Low	-10.41	5.56
	Medium	0.65	4.07
	High	1.14	6.51
HT-2	LLOQ	-4.18	4.18
	Low	-5.09	5.20
	Medium	-1.53	3.39
	High	1.85	1.86

Table 4: (Cont.)

Compound name	Level	Bias (%)	CV (%)
Nivalenol	LLOQ	2.10	4.33
	Low	>15	5.54
	Medium	>15	2.31
	High	>15	2.82
Ochratoxin A	LLOQ	-0.91	5.41
	Low	-3.40	9.49
	Medium	-2.71	4.90
	High	-5.31	3.79
T-2	LLOQ	-3.42	3.21
	Low	-4.13	4.25
	Medium	0.52	1.79
	High	-0.38	1.97
Zearalenone	LLOQ	4.47	7.93
	Low	7.46	4.49
	Medium	2.51	10.65
	High	3.40	9.01

Conclusion

This is a fast and reliable method to quantitate 15 mycotoxin compounds of interest in corn-based mixtures of animal feed. The method uses a simple liquid-liquid extraction and a dilute-and-shoot approach that results in high analyte recovery and reliable quantitation. Robustness of the method was demonstrated by full validation in the laboratory which consisted of four analytical runs with duplicate calibration curves along with 5 quality control sample replicates at low, medium, and high concentrations. Excellent % RSDs for the target analytes were obtained over the course of the analytical runs. This method is important for veterinary diagnostic applications in food animal production medicine.

References and acknowledgements

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