APPLICATION NOTE 65867

Simultaneous analysis of mycotoxins in corn-based animal feed by quadrupole-Orbitrap LC-MS/MS

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Goal

To demonstrate the productivity, robustness, and accuracy of the Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer coupled with ultra-high-pressure liquid chromatography (UHPLC) for the identification and quantitation of mycotoxins in a highly complex matrix.

Introduction

Mycotoxins are toxic secondary metabolites produced by fungi that can grow on food products and crops[1]. These toxins account for the worldwide annual loss of millions of dollars in human health, animal health, and condemned agricultural products[2]. Many regulatory agencies have set up guidelines for the maximum tolerance levels

for mycotoxins in products intended for use as animal feed (Table 1). Animal producers often test their feed to determine the level of mycotoxin contamination. This information is then used to determine how much to dilute the feed to safely feed their production animals.

Liquid chromatography-mass spectrometry (LC-MS) has become a powerful tool for simultaneous analysis of mycotoxins due to their co-occurrence and complexity in feed. Thermo Scientific™ Orbitrap™ high-resolution accurate-mass (HRAM) technology provides excellent full-scan quantitation of target compounds. In addition, MS/MS confirmation is possible using a highly curated, comprehensive compound database and spectral library matching. In this application, we evaluated three different HRAM full-scan workflows to measure 15 mycotoxins in corn-based feed. The Orbitrap Exploris 120 mass spectrometer is designed to deliver consistently accurate data and enhanced analytical productivity for labs performing environmental and food safety analyses.

Experimental

Sample preparation and analysis

Corn feed for control samples was purchased from Trilogy Labs (Washington, MO) and then analyzed for background levels of mycotoxins. Approximately 5 g of corn feed powder was weighed into a 50 mL conical tube (Thermo Fisher Scientific, catalog #339653). Using a simple QuEChERS procedure, samples were solvent-extracted with 20 mL of a mixture of methanol: water (70:30 v/v) containing 0.1% formic acid. The mixture was vigorously mixed on a multi-tube vortexer for 30 minutes, followed by centrifugation at 4000 rpm for 5 minutes. The supernatants were collected and filtered through 0.45 µm Regenerate Cellulose filters (Thermo Fisher Scientific catalog #F2513-7). A 2 uL injection was made into a Thermo Scientific Vanquish Flex Binary UHPLC system coupled to an Orbitrap Exploris 120 mass spectrometer, capable of rapid polarity switching while producing full-scan and MS/MS data.

Calibration standards, quality control, and spike recovery preparation

Standards were purchased from Cayman Chemical (Ann Arbor, MI). A working standard was prepared in 50:50 acetonitrile: water. For calibration, seven concentration levels were prepared as Matrix Matched Standards (MMS) by post-spiking the control corn feed extracts with 15 mycotoxins (3-acetyldeoxynivalenol, Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2, α-zearalenol, Deoxynivalenol, Fumonisin B1, Fumonisin B2, Fumonisin B3, HT2-toxin, T2-toxin, Nivalenol, Ochratoxin A, and Zearalenone) at concentrations ranging from 0.5–1000 ng/mL (equivalent to 2–4000 ng/g). All calibrants were run in triplicate. Quality control samples (n=5) were prepared by spiking the extract with mycotoxins at the mid-level of the calibration curve. Additionally, a Matrix Extracted Spike (MES) sample was prepared using the control corn feed matrix. The target mycotoxins were spiked into the control sample at the mid-level of the calibration prior to extraction. Spike recovery was then estimated by the ratio of the peak area observed in the MMS (post-spiked sample) and MES (pre-spiked sample).

Separation

LC:	Thermo Scientific™ Vanquish™ Flex UHPLC System, consisting of a binary pump, autosampler, and column heater set at 40 ^{0}C
Column:	Thermo Scientific™ Hypersil™ GOLD aQ Column, 100 × 2.1 mm, 1.9 µm
Mobile phase:	A: Water with 5 mM ammonium formate, 0.1% formic acid, 0.1% acetic acid B: Methanol with 5 mM ammonium formate, 0.1% formic acid, 0.1% acetic acid
Gradient:	Start at 5% B, hold for 0.5 min and then use a linear gradient to 95% B for 10 mins, hold for 1.5 min, then drop to original 5% B and equilibrate for additional 2.5 mins for a total run time of 13 min with an HPLC flow rate of 0.300 mL/min

Orbitrap Exploris 120 Mass Spectrometer scan modes and settings

The Orbitrap Exploris 120 mass spectrometer was evaluated using three scan modes with polarity switching:

1. Data Independent Acquisition (DIA): Full-scan analysis at mass resolution 30,000 (FWHM) with resolution of 15,000 for DIA, with 4 precursor isolation windows across the scan range
2. Data Dependent Acquisition (ddMS^2): Full-scan analysis at mass resolution 30,000 (FWHM) and target mass list for MS^2 with resolution of 15,000
3. Targeted MS^2 (tMS^2), similar to a typical triple quadrupole mass analyzer SRM experiment at mass resolution of 30,000 (FWHM)

Mass Spectrometer settings

Instrumentation	Orbitrap Exploris 120 mass spectrometer
Spray voltage	3.5 kV POS/2.5 kV NEG
Sheath gas	35
Aux gas	5
Sweep gas	1
Capillary temp.	325 ^{0}C
Vaporizer temp.	300 ^{0}C
Ion polarity	POS/NEG switching

Data acquisition and processing

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software (version 5.1), to ensure full automation from instrument setup to raw data collection, data processing, and reporting. Data acquired from the three scan modes were analyzed with an extraction mass tolerance of 5 ppm for both precursor and product ions. Analytes were quantified based on full-scan information. In addition, confirmation of target mycotoxins was performed using MS^2 fragment matching and searches against a highly curated Thermo Scientific™ mzCloud™ mass spectral library.

Results and discussion

Chromatographic optimization

Chromatographic separation of the 15 mycotoxins was optimized to achieve good peak symmetry, especially for early-eluting compounds (e.g., 3-acetyldeoxynivalenol and deoxynivalenol at retention times 2.65 min and 3.58 min, respectively). Fumonisins, which are structurally similar to sphinganine containing the backbone precursor of sphingolipids, are less polar and hence elute later (retention time between 8-9 min) on the reversed-phased column. Good separation was obtained for all 15 mycotoxins on the analytical column (Figure 1).

Table 1. Global guidance levels (ng/g) for mycotoxins in feed products intended for animal use. The values have a broad range because the tolerance level for each mycotoxin varies widely for different animal species and stages of production.

Mycotoxin	Canada	US	EU	China
Aflatoxin	20	20–300	5–20	10–50 (B1)
DON	1000–5000	5000–10000	900–12000	1000–5000
Fumonisin (total)		5000–100000	5000–60000	5000–60000 (B1+B2)
Ochratoxin A	200–2000		50–100	100000
Zearalenone	250–5000	1000–3000	100–3000	100–1500
T-2	1000			500
HT-2	25–100			

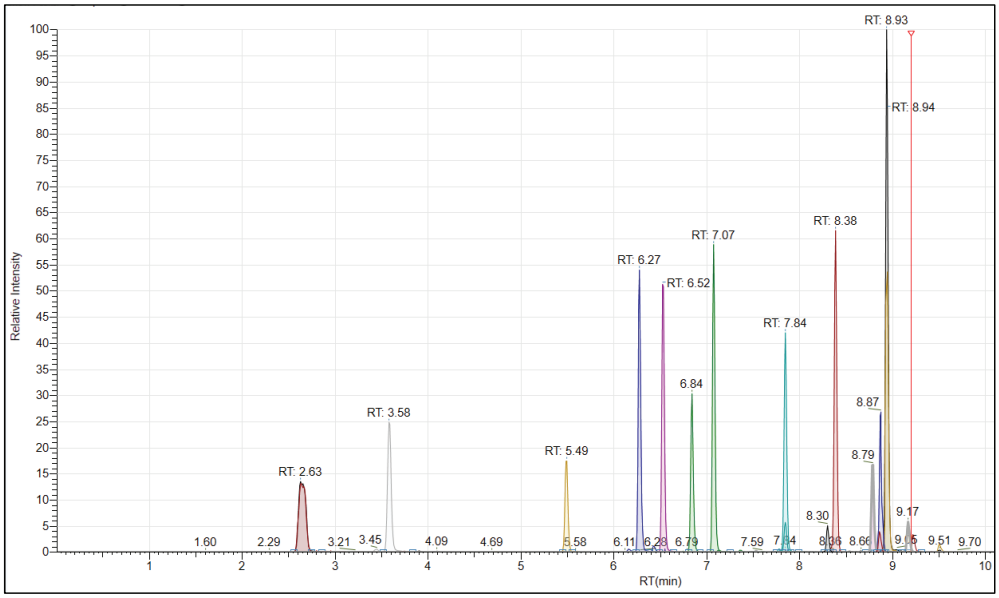

Figure 1. Chromatogram of 15 mycotoxins in spiked corn feed at 10 ppb for Aflatoxin and Ochratoxin, 40 ppb for Fumonisin and T2, 200 ppb for Zearalenone, HT2, and 3-acetyldeoxynavilenol, and 400 ppb for nivalenol, and α-Zearalenol.

Method linearity and recovery

Using the three acquisition modes described above, the linearity and spike recovery for the targeted compounds were compared (Table 2). Among the 15 mycotoxins studied, HT2 and T2 showed the highest signal intensities for the ammonium adduct. The majority of mycotoxins were easily ionized in positive ionization mode; however, α-Zearalenol and Zearalenone required use of negative ionization mode. The results showed comparable calibration linearity ($R^2 > 0.995$) and good spike recovery (80 – 120%) for all 15 mycotoxins in all three acquisition modes (Table 2).

The extraction procedure for this method was simple and effective. Excellent sensitivity and linearity (2 – 100 ng/g) were also achieved in each of three different scan modes for Aflatoxin B1 (Figure 2). This suggests that the method can quantitate at concentrations lower than the 5 – 100 ng/g levels set by China, the EU, and the US for aflatoxins in corn products. The results in both the DIA and $ddMS^2$ scan modes were confirmed with retention time confirmation, fragment matching, and spectral library matching using highly curated mass spectra within the Thermo Scientific™ mzVault™ application. In tMS^2, a full-scan product ion spectrum was acquired and used to search against the spectral library. In addition, ion ratio confirmation was used to provide further evidence in the identification.

Method precision

Excellent precision was achieved for all compounds studied in each scan mode. Table 3 shows the %RSD of five replicate samples of mycotoxins at the middle calibration level spiked into the control corn feed matrix. All regulatory requirements indicates a %RSD less than 15% with all three scan modes and all compounds having a

Table 2. Polarity, coefficient of determination for linear regression curves, and recovery (mean ± SD%) for mycotoxins spiked into control corn feed using three scan modes.

		DIA		$ddMS^2$		tMS^2	
	Adduct	r^2	Recovery	r^2	Recovery	r^2	Recovery
3-acetyl-deoxynivalenol	$[M+H]^+$	0.997	104.6 ± 4.0	0.997	106.5 ± 2.8	0.998	103.9 ± 1.3
Aflatoxin B1	$[M+H]^+$	0.998	90.9 ± 1.5	0.999	92.2 ± 0.9	0.998	92.1 ± 0.5
Aflatoxin B2	$[M+H]^+$	0.998	96.1 ± 2.4	0.999	96.0 ± 0.6	0.999	95.4 ± 1.3
Aflatoxin G1	$[M+H]^+$	0.998	95.2 ± 1.4	0.999	96.3 ± 0.9	0.998	95.1 ± 0.5
Aflatoxin G2	$[M+H]^+$	0.998	99.0 ± 1.8	0.999	100.7 ± 1.1	0.998	99.2 ± 0.9
α-Zearalenol	$[M+H]^+$	0.999	98.9 ± 1.3	0.999	101.8 ± 1.0	0.998	96.8 ± 1.5
Deoxynivalenol	$[M+H]^+$	0.996	98.2 ± 2.2	0.997	104.0 ± 1.0	0.993	99.5 ± 1.0
Fumonisin B1	$[M+H]^+$	0.998	112.1 ± 2.6	0.998	113.1 ± 1.2	0.998	108.5 ± 3.7
Fumonisin B2	$[M+H]^+$	0.997	116.3 ± 4.8	0.998	119.2 ± 3.9	0.998	112.6 ± 1.2
Fumonisin B3	$[M+H]^+$	0.999	107.8 ± 2.5	0.999	110.0 ± 1.4	0.997	107.2 ± 2.0
HT2-Toxin	$[M+NH_4]^+$	0.997	114.8 ± 3.2	0.998	111.5 ± 1.0	0.997	109.6 ± 0.9
Nivalenol	$[M+H]^+$	0.999	98.8 ± 1.1	0.999	98.6 ± 0.9	0.996	97.5 ± 2.5
Ochratoxin A	$[M+H]^+$	0.997	88.6 ± 1.7	0.997	92.6 ± 2.5	0.999	90.8 ± 1.9
T2-Toxin	$[M+NH_4]^+$	0.998	111.9 ± 2.3	0.999	115.3 ± 1.1	0.997	111.4 ± 0.8
Zearalenone	$[M-H]^-$	0.998	96.2 ± 0.4	0.999	99.6 ± 0.4	0.997	95.3 ± 1.5

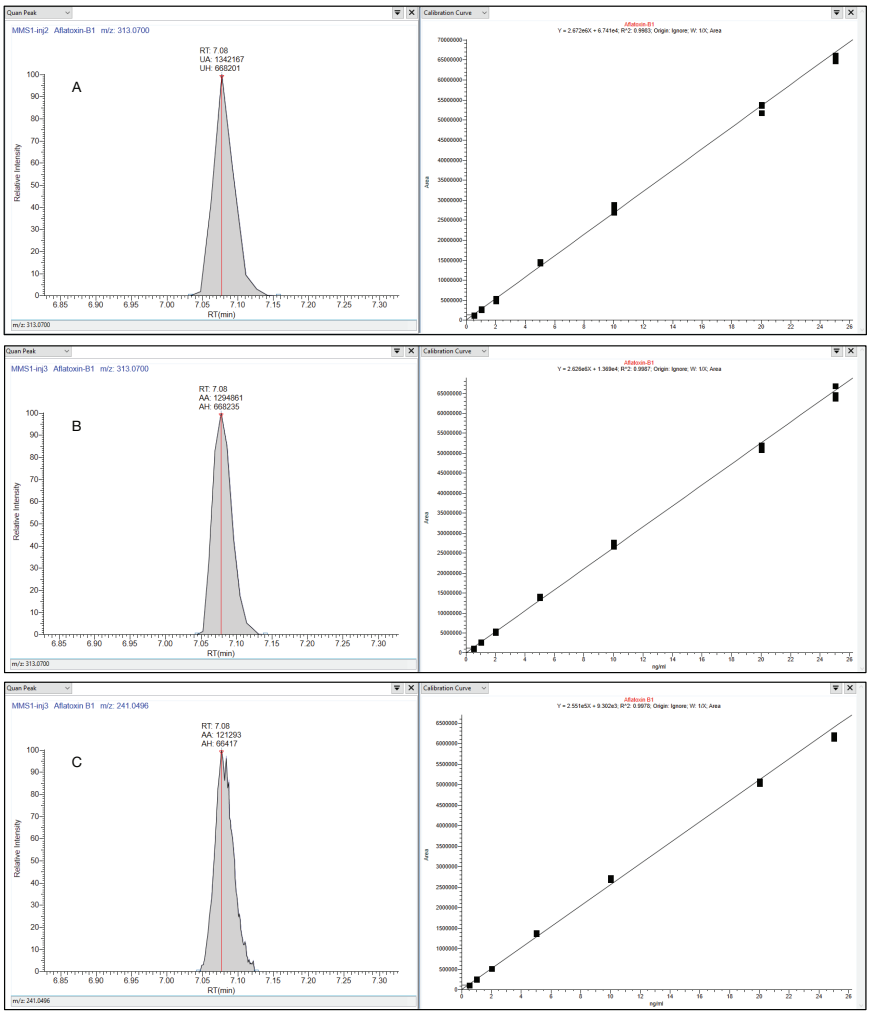

Fig 2. Comparison of extracted ion chromatograms for Aflatoxin B1 at 0.5 ng/ml (2 ng/g) and calibration linearity from 0.5 – 25 ng/mL (2 – 100 ng/g) for three scan modes: A) Full scan used for quantitation in the DIA experiment, B) Full scan used for quantitation in ddMS^2 experiment, and C) Primary MS^2 transitions used for quantitation in tMS^2 experiment.

Table 3. Precision (%RSD, n=5) for mycotoxins in spiked corn feed using three different scan modes.

		Precision (%RSD)		
	Concentration (ng/g)	DIA	ddMS^2	tMS^2
3-acetyl-deoxynivalenol	240	11.7	7.4	10.2
Aflatoxin B1	20	3.7	3.3	3.1
Aflatoxin B2	20	2.9	4.8	3.9
Aflatoxin G1	20	4.7	2.1	2.9
Aflatoxin G2	20	4.8	3.4	4.0
α-Zearalenol	480	11.3	9.4	9.1
Deoxynivalenol	480	7.6	7.9	10.7
Fumonisin B1	64	9.9	4.8	2.6
Fumonisin B2	64	9.2	7.1	3.2
Fumonisin B3	240	7.1	9.5	8.0
HT2-Toxin	240	6.0	9.7	6.5
Nivalenol	480	9.6	9.2	9.0
Ochratoxin A	20	8.1	7.7	3.3
T2-Toxin	64	4.6	2.5	3.1
Zearalenone	240	2.2	9.4	8.6

%RSD less than 12%.

Mass accuracy and quantitative results during polarity switching

The high field Thermo Scientific™ Orbitrap™ mass analyzer coupled with the fast polarity switching of the Thermo Scientific™ OptaMax™ NG ion source produced excellent mass stability and quantitative results during polarity switching. Table 5 lists the exact mass measurements across every scan in the peak for Zearalenone in both polarities. This compound had good response in both ionization modes and excellent mass accuracies of < 1 ppm maintained across the scans within the peaks (see Table 5a and 5b). In addition, the MS^2 fragments had < 5 ppm mass accuracy in both polarities. In all three scan modes, quantitation of the mycotoxins in the corn feed matrix was excellent. Figure 3 shows an example of calibration curves and fragment matches obtained for Zearalenone in the data dependent (DDA) acquisition experiment at each polarity.

Analysis of certified reference materials

Quantitation using the three scan modes was also evaluated using certified reference material (CRM). The measured concentrations for mycotoxins present in the CRM were not significantly different between the three scan modes and were comparable (percent difference < 20%) to the values specified in the CRM (Table 4). The mean measured concentration of Ochratoxin A was > 20% more than its nominal concentration, but there was no statistical difference between the measured and nominal concentrations.

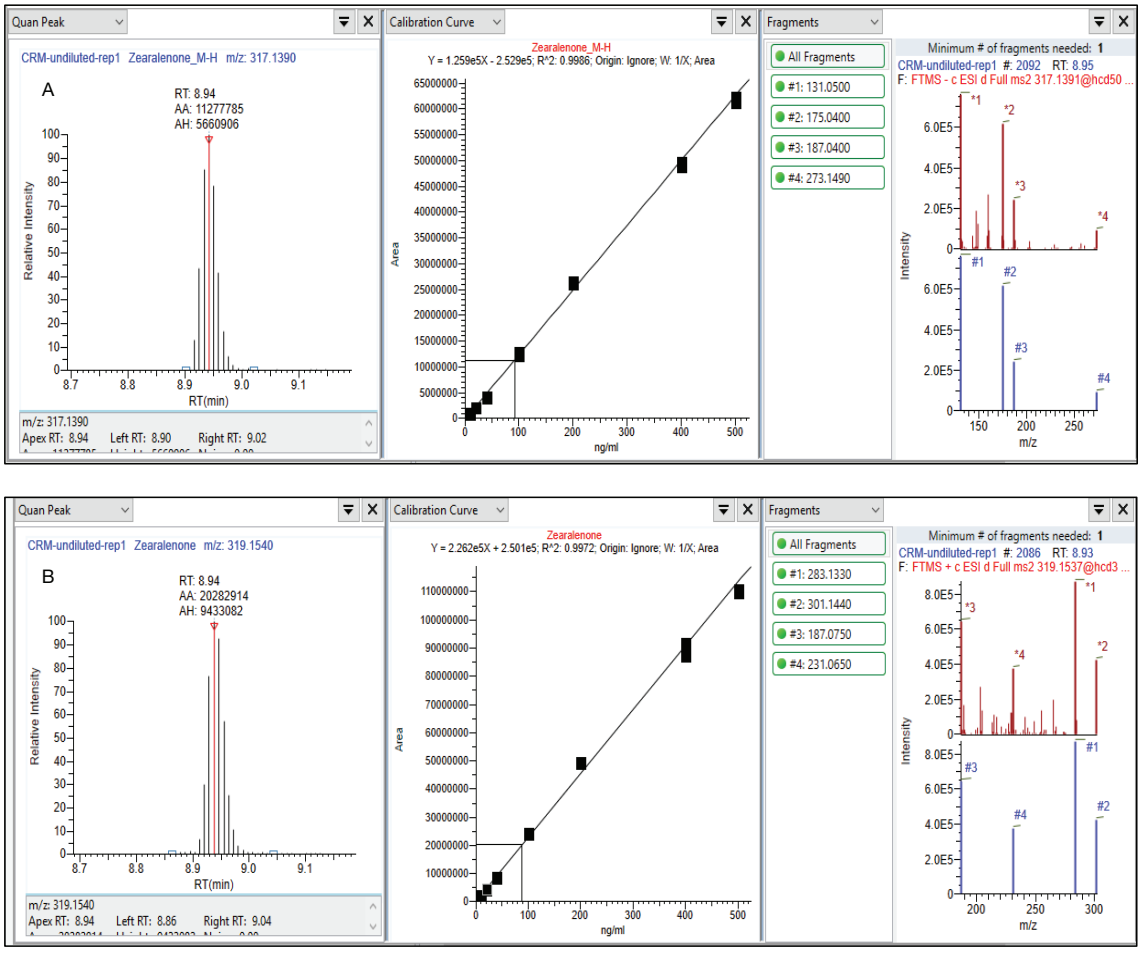

Figure 3: Quantitative results for Zearalenone in negative mode (A) and positive mode (B) taken from the data dependent acquisition (DDA) scan experiment. Plenty of scans are available across the chromatographic peak (left trace in the figures) with excellent calibration linearity and fragment matches obtained in both polarities.

Table 4. Nominal and measured concentrations (mean ± SD, ng/g) of mycotoxins in certified reference material using three different scan modes.

	Nominal concentration (ng/g)	Measured concentration (ng/g)		
		DIA	ddMS²	tMS²
Aflatoxin B1	12.1 ± 2.6	12.3 ± 2.9	14.1 ± 2.8	13.3 ± 3.8
DON	2200 ± 200	2258.2 ± 241.7	2333.7 ± 213.7	2156.3 ± 144.8
Total fumonisins*	10200 ± 1000	-	-	-
HT-2	199.9 ± 21.9	230.7 ± 18.0	219.8 ± 24.1	236.5 ± 22.3
Ochratoxin A	9.9 ± 1.9	12.9 ± 1.6	13.0 ± 1.3	12.9 ± 0.6
T-2	254.0 ± 18.6	273.0 ± 32.2	291.3 ± 20.5	277.3 ± 20.1
Zearalenone	347.4 ± 23.6	351.4 ± 15.5	367.4 ± 16.9	336 ± 38.3

*Results for total fumonisins were excluded due to very high concentrations in the samples, which were significantly outside of the calibration ranges.

Table 5 (5a and 5b): Mass accuracy calculated across the scans of Zearalenone during polarity switching. Table 5a: Positive ion mode. Table 5b: negative ion mode. Average mass accuracies across the peaks were < 1 ppm.

5a

Mass accuracy in positive mode with Zearalenone across the peak			
Scan	Exact mass	Observed mass	Delta ppm
1	319.1536	319.1526	-3.13
2	319.1536	319.1536	0.00
3	319.1536	319.1532	-1.25
4	319.1536	319.1535	-0.31
5	319.1536	319.1535	-0.31
6	319.1536	319.1534	-0.63
7	319.1536	319.1534	-0.63
8	319.1536	319.1534	-0.63
9	319.1536	319.1535	-0.31
Average			**-0.80**

5b

Mass accuracy in negative mode with Zearalenone across the peak			
Scan	Exact mass	Observed mass	Delta ppm
1	317.1390	317.1386	-1.26
2	317.1390	317.1389	-0.32
3	317.1390	317.1389	-0.32
4	317.1390	317.1390	0.00
5	317.1390	317.1389	-0.32
6	317.1390	317.1391	0.32
7	317.1390	317.1390	0.00
8	317.1390	317.1389	-0.32
9	317.1390	317.1388	-0.63
Average			**-0.32**

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Conclusions

The Orbitrap Exploris 120 mass spectrometer coupled to Vanquish Flex UHPLC provides an excellent platform for rapid screening and quantitation of multiple mycotoxin compounds in a single method. The method proved to be efficient and robust using a simple QuEChERS extraction procedure with no further cleanup in corn-based animal feed matrix. All three scan modes studied showed excellent quantitation performance with MS^2 fragment matching and library searching against highly curated databases. Fast polarity switching was very effective, with mass accuracies remaining stable across the peak in both polarities, providing plenty of scans across the peak for accurate quantitation. The ability to analyze a sample extract in both positive and negative ion modes, in a single analytical run instead of two, offers a huge productivity advantage.

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