Extraction of Multiple Mycotoxins From Animal Feed Using ISOLUTE® Myco SPE Columns prior to LC-MS/MS Analysis

This application note describes a Solid Phase Extraction (SPE) protocol for the extraction of a range of mycotoxins from animal feed using ISOLUTE® Myco SPE columns with LC-MS/MS analysis.

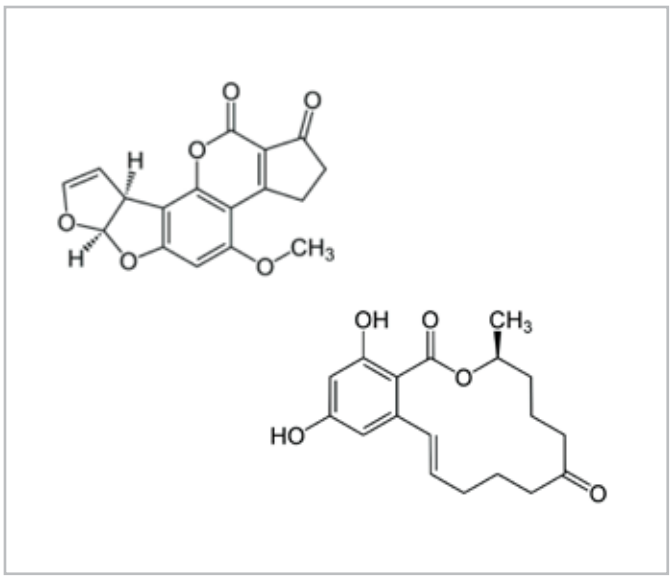


Figure 1. Structures of Aflatoxin B1 and Zearalenone

Introduction

Mycotoxins are toxic metabolites produced by fungal molds on food crops. Regulation and legislation for testing of mycotoxin contamination has established which mycotoxins are prevalent on a wide variety of food crops. This application note describes an SPE protocol appropriate for LC-MS/MS analysis of a range of mycotoxins found on animal feedstuffs.

The method described in this application note achieves high recoveries of a majority of relevant mycotoxins from differing animal feed matrices with %RSDs and LOQs that all meet the requirements set in European regulations for measurement of these analytes in animal feedstuffs.

ISOLUTE Myco solid phase extraction columns provide robust, reliable sample preparation for multiple mycotoxin classes from a wide range of foodstuffs.

Using a single, easy to use sample preparation product, along with optimized matrix specific application notes, scientists can prepare diverse food/crop samples for analysis by LC-MS/MS.

Analytes

Aflatoxin B1, ochratoxin A, fumonisin B1, zearalenone, T-2 mycotoxin, HT-2 mycotoxin, deoxynivalenol.

Column Configuration: ISOLUTE® Myco 60 mg/3 mL (Tabless), part number 150-0006-BG

Sample Pre-treatment

A) Mycotoxin classes excluding type B trichothecenes (e.g. deoxynivalenol)

1. **Sample processing:** Grind the sample (50 g) with a burr-grinder or equivalent device. Store ground sample in a sealed container at room temperature until required.
2. **Extraction:** Mix the ground sample (5 g) with 4% formic acid (aq) (10 mL) and shake vigorously by hand for 30 seconds. Add acetone (30 mL) and shake vigorously by hand for 30 seconds. Place the sample pre-treatment tube on a shaking table for 30 minutes. Transfer the extract to a 50 mL centrifuge tube and centrifuge at 4000 g for 10 minutes.
3. **Dilution:** Take the supernatant (6 mL), transfer to a new 50 mL centrifuge tube and dilute with water (39 mL). Centrifuge diluted extract at 4000 g for a further 10 minutes.

B) Type B trichothecene mycotoxins

1. **Sample processing**: Grind the sample (50 g) with a burr-grinder or equivalent device. Store ground sample in a sealed container at room temperature until required.
2. **Extraction:** Mix the ground sample (5 g) with 1% formic acid (aq) (40 mL) and shake vigorously by hand for 30 seconds. Place the sample pre-treatment tube on a shaking table for 30 minutes. Transfer the extract to a 50 mL centrifuge tube and centrifuge at 4000 g for 10 minutes.
3. **Dilution:** Take the supernatant (6 mL), transfer to a new 50 mL centrifuge tube and dilute with water (39 mL). Centrifuge diluted extract at 4000 g for a further 10 minutes.

Solid Phase Extraction

Use flow rates of 1 mL min^{-1} throughout.

A) Mycotoxin classes excluding type B trichothecenes

Condition:	Condition the column with acetonitrile (2 mL).
Equilibration:	Equilibrate column with water (2 mL)
Sample Loading:	Load pre-treated sample (3 mL) onto the column at a maximum flow rate of 1 mL min^{-1} (gravity load is recommended).
Interference Wash 1:	Wash the column with water (3 mL)
Interference Wash 2:	Wash the column with 10% acetonitrile (3 mL)
Drying:	Dry the column for 30 seconds at maximum vacuum, 2 bar/29 psi
Elution 1:	Elute with 0.1% formic acid in acetonitrile (2 mL)
Elution 2:	Elute with 0.1% formic acid in methanol (2 mL)
Post Elution:	Dry the combined eluate in a stream of air or nitrogen using a SPE Dry (35 °C, 20 to 40 L min^{-1}) or TurboVap LV (1.5 bar at 35 °C for 40 min). Reconstitute with 0.1 % acetic acid in 20 % acetonitrile : methanol (1 mL, 1:1, v/v). Syringe-filter using a 0.2 µm PTFE membrane prior to analysis.

Type B trichothecene mycotoxins

Condition:	Condition the column with acetonitrile (2 mL)
Equilibration:	Equilibrate column with water (2 mL)
Sample Loading:	Load pre-treated sample (3 mL) onto the column at a maximum flow rate of 1 mL min^{-1} (gravity load is recommended).
Interference Wash:	Wash the column with water (3 mL)
Elution:	Elute with 10% acetonitrile (3 mL)
Post Elution:	Dry the combined eluate in a stream of air or nitrogen using a SPE Dry (35 °C, 20 to 40 L min^{-1}) or TurboVap LV (1.5 bar at 35 °C for 40 min). Reconstitute with 0.1 % acetic acid in 20 % acetonitrile : methanol (1 mL, 1:1, v/v). Syringe-filter using a 0.2 µm PTFE membrane prior to analysis.

HPLC Conditions

Note: Extracts from extraction method A (mycotoxin classes excluding type B tricothecenes) and extraction method B (type B tricothecenes) were analyzed separately using the conditions shown below.

Instrument:	Shimadzu Nexera UHPLC (Shimadzu Europe Gmbh)
Column:	Kinetex XB-C18 50 x 2.1 mm 2.6 µm dp (Phenomenex, Macclesfield UK)
Mobile Phase:	A: 1 mM ammonium acetate, 0.5% acetic acid B: 1mM ammonium acetate, 0.5% acetic acid in 95% methanol (aq)
Flow rate:	0.45 mL min^{-1}
Injection:	20 µL
Gradient	Initial 20 % B, hold 1.0 min linear ramp to 73 % B in 6 min linear ramp to 100 % B in 0.2 min, hold 2.3 min linear ramp to initial conditions in 0.2 min hold 2.3 min, total run time 10.0 min
Column temperature:	40 °C
Sample temperature	15 °C

Table 1. Typical retention times for a range of mycotoxins using the LC-MS/MS method described.

Compound	Retention time (min)
deoxynivalenol	0.7
aflatoxin B1	4.1
HT-2	5.0
T-2	5.6
fumonisin B1	5.4
zearalenone	5.9
ochratoxin A	6.1

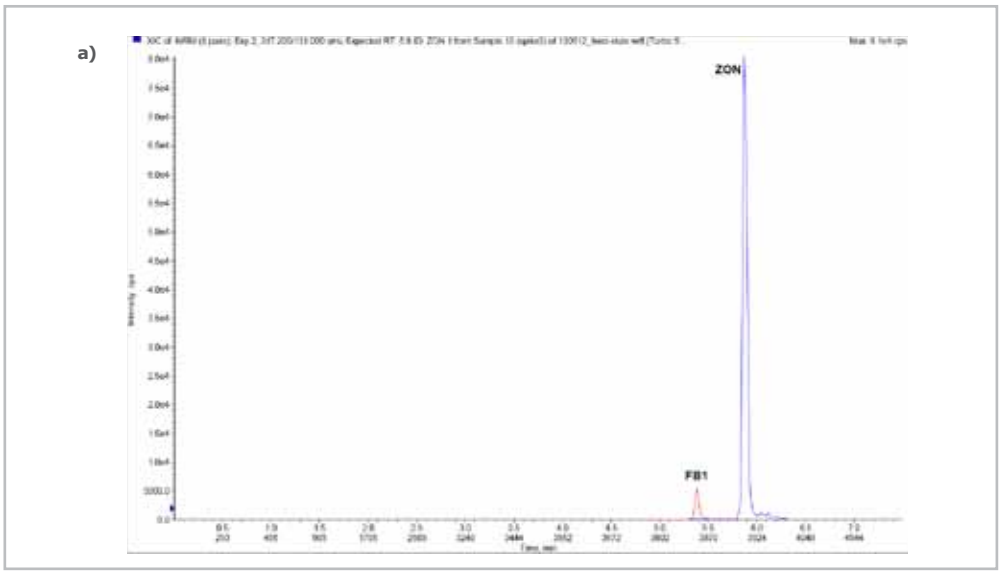
MS Conditions

Ions were selected in order to achieve maximum sensitivity, the MS was operated in dual polarity (+ve/-ve switching) mode, using multiple reaction monitoring.

Instrument:	AB Sciex Triple Quad 5500 (Warrington, UK)
Source:	Turbo-V ESI
Desolvation temperature:	500 °C
Curtain gas:	30 psi
Spray voltage:	+5.0 kV / -4.5 kV
Gas 1:	60 psi
Gas 2:	60 psi
Collision gas:	7 psi

Table 2. Negative Ion Mode - MRM Parameters

MRM transition	RT	Compound ID	DP, V	EP, V	CE, V	CXP, V
355.1>59.0	0.7	deoxynivalenol 1	-50	-10	-45	-15
335.1>295.1	0.7	deoxynivalenol 2	-50	-10	-13	-15
335.1>265.1	0.7	deoxynivalenol 3	-50	-10	-20	-15
720.2>157	5.4	fumonisin B 1	-160	-12	-45	-15
720.2>562.3	5.4	fumonisin B1 2	-160	-12	-36	-15
317.2>131	5.9	zearalenone 1	-40	-4	-38	-15
317.2>175	5.9	zearalenone 2	-40	-4	-30	-15
317.2>255.1	5.9	zearalenone 3	-40	-4	-20	-15
MRM detection window 60 s / target scan time 0.1 s / settling time 50 ms / scan pause 5 ms						



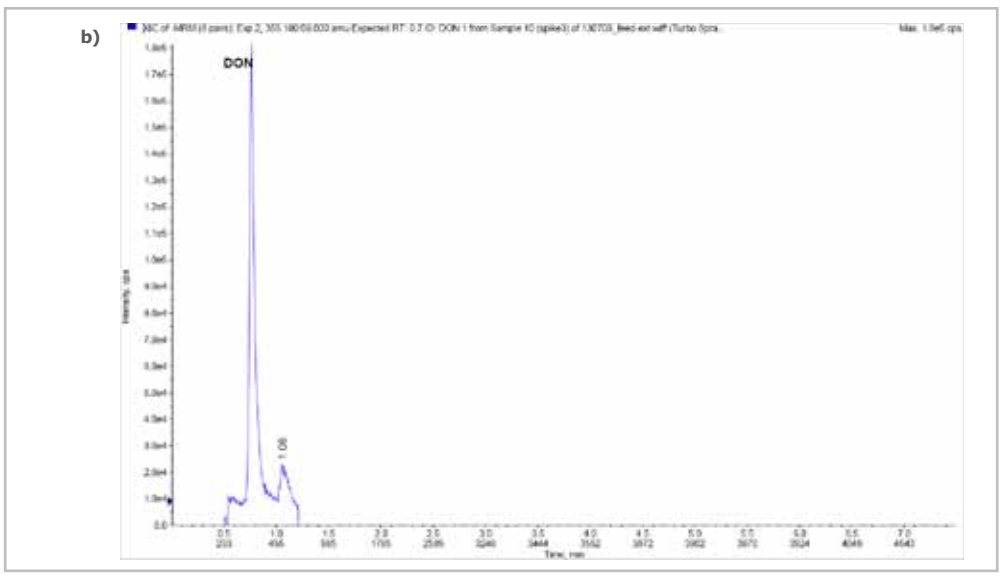


Figure 2 a) and 2 b). Extracted ion chromatograms in negative ion mode using ISOLUTE® Myco protocol at 100 µg kg^{-1} from composite horse feed: a) FB1 and ZON using extraction conditions for mycotoxins excluding type B trichothecenes; b) DON using extraction conditions for type B trichothecenes.

Table 3. Positive Ion Mode - MRM Parameters

MRM transition	RT	Compound ID	DP, V	EP, V	CE, V	CXP, V
313.1>285	4.1	aflatoxin B1 1	100	10	31	18
313.1>241.1	4.1	aflatoxin B1 2	100	10	49	18
313.1>185	4.1	aflatoxin B1 3	100	10	65	18
442.2>263.1	5.0	HT-2 toxin 1	50	12	18	12
442.2>215.1	5.0	HT-2 toxin 2	50	12	18	12
484.2>305.1	5.6	T-2 toxin 1	60	10	18	12
484.2>215.1	5.6	T-2 toxin 2	60	10	17	12
484.2>185.1	5.6	T-2 toxin 3	60	10	28	12
404.1>239	6.1	ochratoxin A 1	165	10	32	12
404.1>221	6.1	ochratoxin A 2	165	10	47	12
404.1>102	6.1	ochratoxin A 3	165	10	84	12
MRM detection window 60 s / target scan time 0.1 s / settling time 50 ms / scan pause 5 ms						

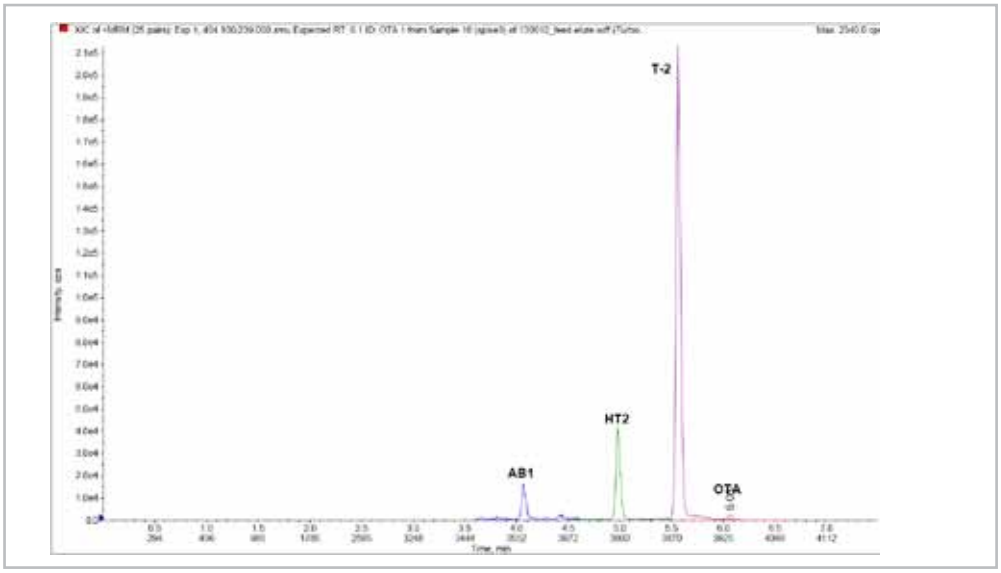


Figure 3. Extracted ion chromatograms in positive ion mode using ISOLUTE Myco protocol at 5 µg kg^{-1} (aflatoxin B1, ochratoxin A and T 2 toxin) and 100 µg µg kg^{-1} (HT-2 toxin) from composite horse feed.

Validation criteria

Method linearity was determined using matrix-matched calibration standards in six replicates over eight levels; the ranges are shown below.

Analytes	Working Range, µg kg^{-1} (pg µL^{-1} on column)
aflatoxin B1, ochratoxin A, T-2 toxin	0.4 to 40.0 (0.02 to 2.0)
fumonisin B1, zearalenone, HT-2 toxin	40 to 4000 (2 to 200)
deoxynivalenol	40 to 4000 (2 to 200)

LOQ was determined from the lowest matrix-matched standard meeting EU repeatability and recovery criteria. Where no criteria were specified the LOQ were estimated by correlation to similar analytes.

Repeatability (%RSD^r) was determined from single acquisitions of 5 SPE replicates of a single sample extraction. The RSDs generated gave close agreement when a single sample was extracted and processed using ISOLUTE® Myco from three separate sorbent batches.

Recovery was determined as a % of ISOLUTE Myco extract spike before sample prep to spike after close to the analytical LOQ.

Results

The extracted ion chromatograms in figures 2 and 3 demonstrate chromatography at 5 µg kg^{-1} (aflatoxin B1, ochratoxin A and T 2 toxin) and 100 µg kg^{-1} for all other analytes from a spiked extraction of 5 g ground feed substrate. Good linearity was achieved for all analytes in all the different matrices as demonstrated in the example charts shown in **Figures 4 and 5**.

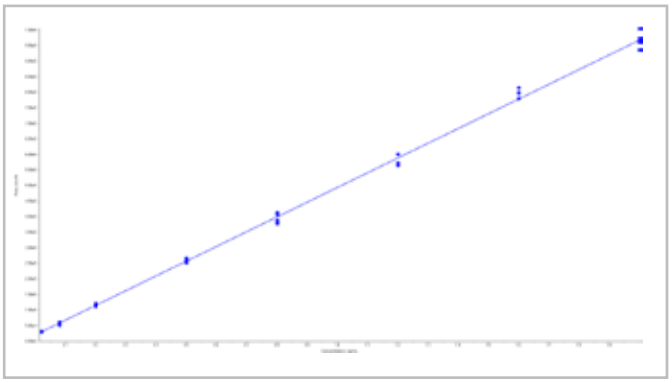


Figure 4. Calibration curve for aflatoxin B1 from ground composite horse feed using the ISOLUTE Myco protocol from 0.02 – 2.0 ng mL^{-1}

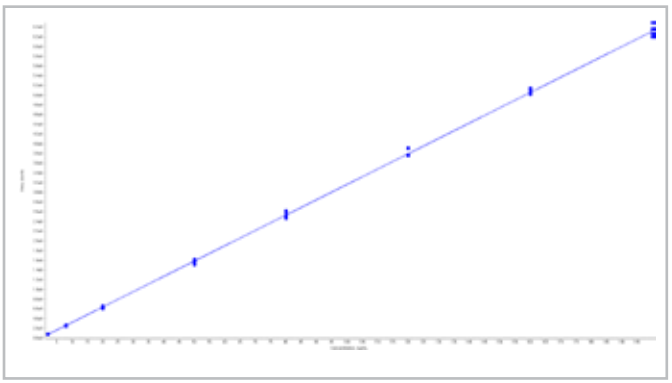


Figure 5. Calibration curve for HT-2 toxin from ground composite horse feed using the ISOLUTE Myco protocol from 2 – 200 ng mL^{-1}

Table 4. Analyte recovery and limit of quantitation data for a range of mycotoxins from ground soya using the ISOLUTE® Myco protocol

Analyte	r²	LOQ / µg kg^{-1}		%RSD$_r$		Recovery %	
Soya		**Target**	**Actual**	**Target**	**Actual**	**Target**	**Actual**
deoxynivalenol	0.9996	900	40	20	2.1	70 to 110	45.9
aflatoxin B1	0.9997	100	1.6	20	1.8	70 to 110	106.8
ochratoxin A	0.9995	50	1.6	20	2.8	70 to 110	94.7
T-2 toxin	0.9988	N/A	1.6	30	1.5	70 to 110	109.8
HT-2 toxin	0.9995	N/A	40	30	1.4	70 to 110	109.4
fumonisin B1	0.9959	5000	320	20	7.1	70 to 110	107.3
zearalenone	0.9997	100	40	25	2.3	70 to 110	109.8

Table 5. Analyte recovery and limit of quantitation data for a range of mycotoxins from composite horse feed using the ISOLUTE® Myco protocol

Analyte	r²	LOQ / µg kg^{-1}		%RSD$_r$		Recovery %	
Horse feed		**Target**	**Actual**	**Target**	**Actual**	**Target**	**Actual**
deoxynivalenol	0.9996	900	40	20	2.2	70 to 110	84.8
aflatoxin B1	0.9990	100	1.6	20	8.1	70 to 110	100.9
ochratoxin A	0.9989	50	1.6	20	5.2	70 to 110	85.9
T-2 toxin	0.9991	N/A	1.6	30	1.7	70 to 110	109.4
HT-2 toxin	0.9998	N/A	40	30	5.0	70 to 110	105.0
fumonisin B1	0.9925	5000	400	20	6.5	70 to 110	89.2
zearalenone	0.9992	100	40	25	4.9	70 to 110	108.8

Ordering Information

Part Number	Description	Quantity
150-0006-BG	ISOLUTE® Myco 60 mg/3 mL column (Tabless)	50
121-1016	Biotage® VacMaster™-10 Sample Processing Manifold complete with 16 mm collection rack	1
121-2016	Biotage® VacMaster™-20 Sample Processing Manifold complete with 16 mm collection rack	1
C103198	TurboVap® LV, 110V	1
C103199	TurboVap® LV, 220V	1

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