Extraction of Aflatoxins and Ochratoxin from Dried Chili Using ISOLUTE® Myco Prior to LC-MS/MS Analysis

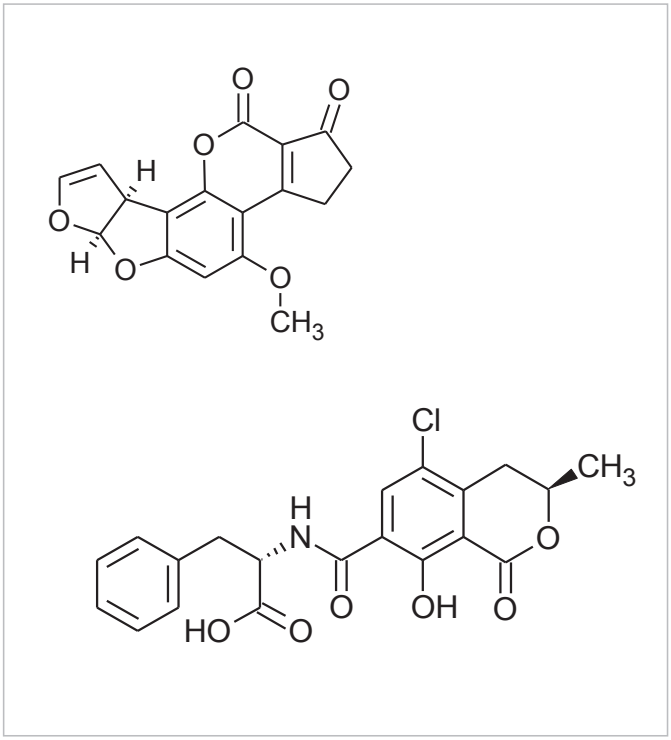

Figure 1. Structures of Aflatoxin B1 and Ochratoxin A

Introduction

This application note describes a solid phase extraction (SPE) protocol for the extraction of a range of mycotoxins from dried chili (pimiento) using ISOLUTE® Myco with LC-MS/MS analysis.

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 chili.

The method described in this application note achieves high recoveries of relevant mycotoxins from dried chili (pimiento) with %RSDs and LOQs that meet the requirements set in European Union regulations for measurement of these analytes. 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, aflatoxin B2, aflatoxin G1, aflatoxin G2, ochratoxin A

Sample Preparation Procedure

Format: **ISOLUTE® Myco 60 mg/3 mL Columns (Tabless), part number 150-0006-BG**

Sample Pre-treatment

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.
Extraction:	Mix the ground sample (5g) with 80% acetonitrile (aq) (20 mL). Place the sample pre-treatment tube on a shaking table for 30 mins. Transfer the extract to a 50 mL centrifuge tube and centrifuge at 4000 g for 10 minutes.
Dilution:	Take the supernatant (2 mL), transfer to a new 50 mL centrifuge tube and dilute with water (32 mL). Centrifuge diluted extract at 4000 g for a further 10 minutes.

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Solid Phase Extraction

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

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 (2 x 2.5 mL)
Interference Wash 2:	Wash the column with 10% acetonitrile (aq) (2 x 2.5 mL)
Drying:	Dry the column for 30 seconds at maximum vacuum, -0.5 bar/7 psi
Elution 1:	Elute with 0.1% formic acid in 40% acetonitrile (aq) (2 mL)
Elution 2:	Elute with 1.0% ammonia (conc.) in methanol (2 mL)
Post Elution:	Dry the eluate in a stream of air or nitrogen using a SPE Dry (35 °C, 20 to 40 L min^{-1}) or TurboVap® LV (15 bar at 35 °C for 40 min). Reconstitute in 0.1 % acetic acid in acetonitrile : methanol : water (1 : 1 : 8, v/v/v, 1mL). Syringe-filter using a 0.2 µm PTFE membrane prior to analysis.

HPLC Conditions

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}
Injeection:	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)
aflatoxin G2	3.3
aflatoxin G1	3.6
aflatoxin B2	3.9
aflatoxin B1	4.1
ochratoxin A	6.1

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MS Conditions

Ions were selected in order to achieve maximum sensitivity, and the MS was operated in positive ion polarity mode, using multiple reaction monitoring.

Instrument: AB Sciex Triple Quad 5500 (Warrington, UK)

Source: Turbo-V ESI

Desolvation temp.: 500 °C

Curtain gas: 30 psi

Spray voltage: +5.0 kV

Gas 1: 60 psi

Gas 2: 60 psi

Collision gas: 7 psi

Table 2. Positive Ion Mode - MRM Parameters

MRM transition	RT	Compound ID	DP, V	EP, V	CE, V	CXP, V
331.1>313.1	3.3	aflatoxin G2 1	100	10	33	12
331.1>245.1	3.3	aflatoxin G2 2	100	10	41	12
331.1>257.1	3.3	aflatoxin G2 3	100	10	41	12
329>243.1	3.6	aflatoxin G1 1	80	10	37	12
329>200	3.6	aflatoxin G1 2	80	10	53	12
315.1>287	3.6	aflatoxin B2 1	100	10	35	12
315.1>259.1	3.9	aflatoxin B2 2	100	10	40	12
315.1>243.1	3.9	aflatoxin B2 3	100	10	51	12
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
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

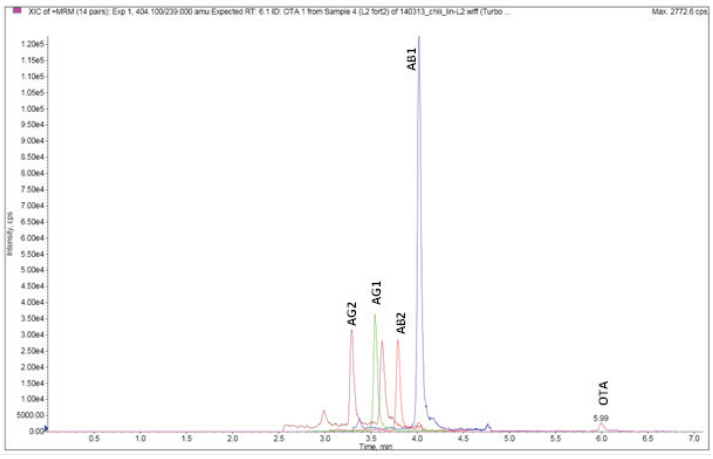

Figure 2. Extracted ion chromatogram (positive ion mode) using ISOLUTE® Myco protocol at 5 µg kg^{-1} (aflatoxins and ochratoxin A) from chili

Validation Criteria

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

Analytes	Working Range, µg kg^{-1} (pg µL^{-1} on column)
aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2,	0.5 to 80 (0.046875 to 7.5)
ochratoxin A	5.0 to 80 (0.46875 to 7.5)

LOQ was determined from the lowest matrix-matched standard meeting EU repeatability and recovery criteria; or estimated from the S/N ratio of the blank where incurred levels of mycotoxins were present in the sample (e.g AB1).

Repeatability (%RSDr) 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.

Recovery was determined as a % of ISOLUTE® Myco extract spike before sample prep to spike after at the EU MRL.

Results

The extracted ion chromatogram in figure 2 demonstrates chromatography at 5 µg kg^{-1} (aflatoxins and ochratoxin A) from a spiked extraction of 2 g ground chili. Good linearity was achieved for all analytes in all both matrices as demonstrated in the example charts shown in **Figures 3 and 4**.

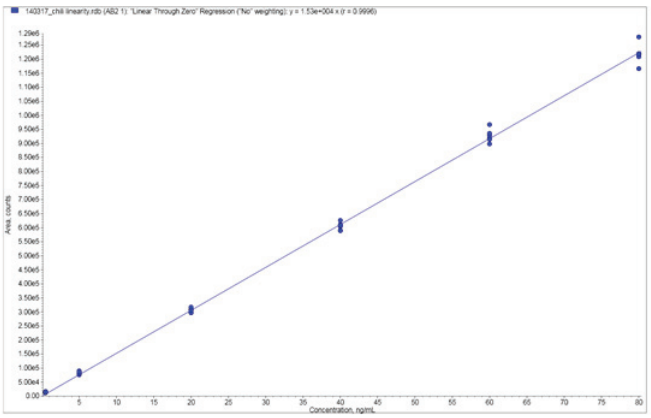

Figure 3. Calibration curve for aflatoxin B2 from ground chili using the ISOLUTE® Myco protocol from 0.5 – 80 µg kg^{-1}

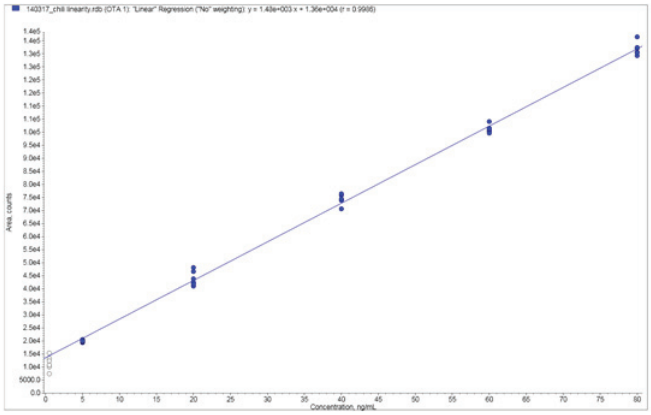

Figure 4. Calibration curve for ochratoxin A from ground chili using the ISOLUTE Myco protocol from 5 – 80 µg kg^{-1}

All analytes extracted using the ISOLUTE Myco protocol achieved the limits of quantities and recovery required by the current European standards for mycotoxin analysis as shown in **Table 3**.

Table 3. Analyte recovery and limit of quantitation data for a range of mycotoxins from chili using the ISOLUTE® Myco protocol

Analyte	r²	LOQ / µg kg^{-1}		%RSD$_r$		Recovery %	
		Target	Actual	Target	Actual	Target	Actual
aflatoxin B1	0.9988	5	2.0	20	3.2	70 to 110	108
aflatoxin B2	0.9992	5	2.0	20	3.2	70 to 110	106
aflatoxin G1	0.9986	5	2.0	20	2.9	70 to 110	106
aflatoxin G2	0.9980	5	4.0	20	2.7	70 to 110	89.1
ochratoxin A	0.9972	15	10	20	2.7	70 to 110	108

Alternative Sample Pre-Treatment Strategy

Weigh the ground sample (2 x 1 g) into separate 7 mL Omni International sample tubes containing ceramic 2.8 mm bead lysing matrix (approximately 2 g / n=23) and add 80% acetonitrile (aq) (4 mL). Homogenize the sample in an Omni Bead Ruptor 24 using the following settings: disruption at 5.5 m s^{-1} for 2 x 45 s with a 10 s pause.

Centrifuge the sample tubes at 4000 x g for 10 minutes. Transfer the supernatant (2 x 2 mL) to a new centrifuge tube and dilute with water (16 mL). Centrifuge the tube at 4000 x g for 10 minutes. Proceed to solid phase extraction (page 2).

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, 100/120V	1
C103199	TurboVap® LV, 220/240V	1

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EUROPE
Main Office: +46 18 565900
Toll Free: +800 18 565710
Fax: +46 18 591922
Order Tel: +46 18 565710
Order Fax: +46 18 565705
order@biotage.com
Support Tel: +46 18 56 59 11
Support Fax: + 46 18 56 57 11
eu-1-pointsupport@biotage.com

NORTH & LATIN AMERICA
Main Office: +1 704 654 4900
Toll Free: +1 800 446 4752
Fax: +1 704 654 4917
Order Tel: +1 704 654 4900
Order Fax: +1 434 296 8217
ordermailbox@biotage.com
Support Tel: +1 800 446 4752
Outside US: +1 704 654 4900
us-1-pointsupport@biotage.com

JAPAN
Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com
jp-1-pointsupport@biotage.com

CHINA
Tel: +86 21 2898 6655
Fax: +86 21 2898 6153
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