Development of Optimized Multiclass Clean-up Methods for LC-MS/MS Analysis of Mycotoxins in Multiple Food/Feed Matrices Incorporating a Novel SPE Column

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Introduction

Mycotoxins are a group of toxic and often carcinogenic or genotoxic metabolites produced by several strains of fungi found on food crops worldwide. Mycotoxins have great potential to cause harm to humans, crops and farmed animals, example structures shown in ***Figure 1***.

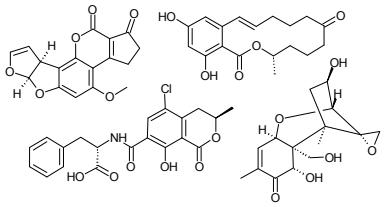

Figure 1. *Typical Mycotoxin Structures – aflatoxin B1, zearalenone, deoxynivalenol, ochratoxin A (clockwise from top left)*

As a result, a wide range of food and feedstuff substrates require testing for mycotoxin contamination. The diversity of analyte structure and food substrate generates a significant analytical challenge. Traditionally, mycotoxins have been analyzed using multiple methods, each optimized for a single mycotoxin or group of closely related toxins. Multi-analyte approaches are being made increasingly possible by the adoption of liquid chromatography-tandem mass spectrometry (LC-MS/MS) based analyses. Due to the selective nature of LC-MS/MS, highly selective sample preparation techniques are often no longer essential to meet the residue limits required. However, appropriate sample preparation remains necessary in order to minimize matrix effects and maximize assay robustness.

Experimental

Reagents

Standards were purchased from LGC (Teddington, UK). Acetic acid (HAC), formic acid, ammonium hydroxide, ammonium acetate and toluene were purchased from Sigma-Aldrich Chemical Co. (Poole, UK). Chromatographic solvents were LC/MS grade and purchased from LGC/Promochem (Teddington, UK). 18.2 MΩ-cm water was drawn fresh daily from a Direct-Q 5 (Merck Millipore, Watford, UK)

Sample Preparation

Sample extraction: Optimized extraction conditions are tabulated below in ***Table 1***. Briefly, a sub-sample of substrate was mixed with a specific volume of extraction solvent. The mixture was then extracted using the method and duration shown in ***Table 1***.
Extraction work-up: The extract was centrifuged at 4000 x g for 10 min. Supernatant was transferred to a 50 mL tube and diluted with water to give a 10% final concentration of organic solvent. The diluted extract was centrifuged at 4000 x g for 10 min. Pre-treatment for small polar mycotoxins such as deoxynivalenol required separate pre-treatment 100% aqueous conditions.

Table 1. *Substrate-Optimized Extraction Conditions*

Substrate	Mass / g	Extraction Solvent	Solvent Vol. / mL	Extraction Method	Duration / min
Grain	5	50% acetonitrile (aq)	20	shake	30
Feed	5	1% HCOOH / 75% acetone (aq)	40	shake	30
Nuts	5	80% acetonitrile (aq)	20	shake	30
Chili (dried)	5	80% acetonitrile (aq)	20	Shake	30
Infant Formula (dried)	as mfr instructions	1% HCOOH (aq)	as mfr instructions	ultra-sonicate	20
Apple (juice)	1:1	10mM ammonium acetate pH 5 + adjusted to pH 5	20 (total)	mix	N/A

Solid Phase Extraction Cleanup: ISOLUTE® Myco SPE Columns, 60 mg / 3 mL (tabless); 150-0006-BG. ***Table 2*** below illustrates substrate-specific optimized SPE procedures.

Table 2. *Substrate-Optimized ISOLUTE Myco SPE Procedures*

Step	Grain & Feed	Nuts	Chili	Infant Cereal	Infant Formula	Juice (apple)
Condition (2 mL)	MeCN	MeCN	MeCN	MeCN	MeCN	MeCN
Equilibrate (2 mL)	H_2O	10mM NH_4OAC	H_2O	H_2O	H_2O	10mM NH_4OAC pH5
Load (3 mL)				(5 mL)	(5 mL)	(1 mL)
Wash 1 (2 mL)	H_2O	10mM NH_4OAC	H_2O	H_2O (5 mL)	H_2O	H_2O (3x 1mL) dry 5 min
Wash 2 (2 mL)	10% MeCN	10mM NH_4OAC / 10% MeCN	10% MeCN	10% MeCN (5 mL)	10% MeCN (5 mL) dry 5 min	Toluene (1 mL) dry 5 min
Elute 1 / Wash 3 (2 mL)	0.1% HCOOH / MeCN	0.1% HCOOH / MeCN	0.1% HCOOH / 40% MeCN	0.1% HCOOH / 60% MeCN	hexane (5 mL) dry 5 min	MeCN (1 mL)
Elute 2 (2 mL)	0.1% HCOOH / MeOH	0.1% HCOOH / MeOH	1% NH_4OH (conc) / MeOH	0.1% HCOOH / MeOH	0.1% HCOOH / MeCN	N/A

Clean-up for small polar mycotoxins such as deoxynivalenol required 100% aqueous load/wash conditions (details supplied on request).
SPE Work-up: The eluates were combined where necessary and dried under a stream of air at 45 °C for a period dependent on eluent composition. The eluates were reconstituted in 1 mL 0.1 % acetic acid in 1:1:8 MeOH:MeCN:H2O (v/v/v) and syringe-filtered using a 0.2 µm PTFE membrane.

HPLC Conditions

Instrument: Waters Alliance 2795 (Waters Assoc., Milford MA, USA) or Shimadzu Nexera (Shimadzu Europa Gmbh, Duisburg, Germany).
Patulin HPLC Column: Kinetex Phenyl-Hexyl 50 x 2.0 mm id, 2.6 µm (Phenomenex, Cheshire UK).
Mycotoxins HPLC Column: Kinetex C18-XB 50 x 2.0 mm id, 2.6 µm (Phenomenex, Cheshire UK).

Mass Spectrometry

Instrument: Ultima Pt triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) with electrospray interface or Triple Quad 5500 mass spectrometer (AB Sciex, Framingham, US) with a Turbo-V electrospray interface.
ESI Analysis: Positive and/or negative pseudomolecular ions were fragmented to their most abundant product ions for MRM (Waters) or sMRM (AB Sciex) experiments.
Chromatography and mass spectrometry experimental details can be supplied on request.

Results

Resin Development

Sample pre-treatment conditions were established during resin development. We found that 50% MeCN gave good recoveries for most classes of mycotoxins. However, for mycotoxins with significant polar character (e.g. deoxynivalenol): extraction, SPE equilibration and load steps required 100% aqueous conditions. SPE conditions were established during resin development. A 60 mg resin bed binds 100% of a suite of mycotoxins when loading up to 10 mL of 200 µg kg^{-1} spiked flour extract (data not shown). The optimum % organic in the wash solvent was determined to be 10% as demonstrated in ***Figure 2***. This can be increased to 20% where recovery of aflatoxins is not required. Equilibration/wash conditions were investigated. Optimum SPE performance was obtained using weak solvents, typically H_2O. Low concentration ammonium acetate was found to improve recoveries where the substrate contained lipids or had increased ionic strength.

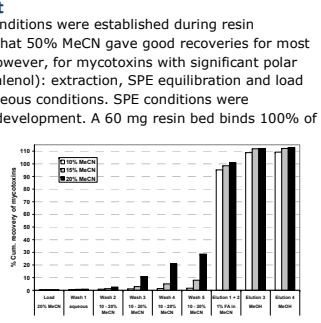

Figure 2. *Cumulative elution profile of mycotoxins varying wash procedure*

Reconstitution

Recoveries were stabilized using 0.5% HAC in preference to 0.1% (and HCOOH) as demonstrated in ***Figure 3***; 1:1:8 MeOH:MeCN:H_2O (v/v/v) gave the best results, 20% MeOH was an acceptable alternative.

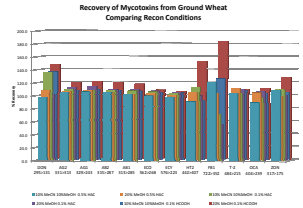

Figure 3. *Comparison of reconstitution conditions on mycotoxin suite recoveries from ground wheat*

Recoveries

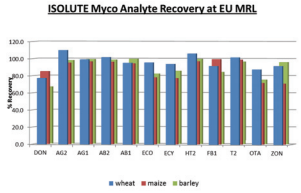

Figure 4. *Recovery of a suite of spiked mycotoxins from grains using ISOLUTE Myco (2 µg kg^{-1} afla/OTA, 20 µg kg^{-1} others)*

Figures 4 through 10 demonstrate SPE recoveries at EU MRLs using the optimized methods shown in ***Table 2***. The recovery of a suite of mycotoxins from differing grain substrates is demonstrated in ***Figure 4***. With the exception of DON, recoveries were between 70% and 110% of spiked.

The recovery of mycotoxins from ground soya or animal feed using the optimized method from ***Table 2*** is demonstrated in ***Figure 5***. 19/20 analyte recoveries were between 85% and 109%.

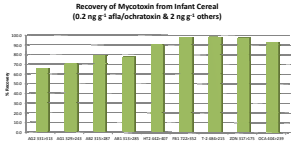

Figure 8. *Recovery of mycotoxins from infant cereal using a SPE method optimized for ISOLUTE Myco (0.2 to 2 µg kg^{-1} spike)*

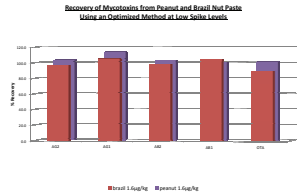

Figure 6. *Recovery of mycotoxins from two nut matrices using a SPE method optimized for ISOLUTE Myco (1.6 µg kg^{-1} spike)*

Where substrates had high lipid content (e.g. nuts), recoveries of ochratoxin A were optimized by increasing MeCN concentration to 80% and changing the equilibration/wash solvent to 10 mM ammonium acetate. The use of hexane and salt as defatting agents; and the use of non-polar organic solvent washes for interference removal gave no observable benefit to the method. The recovery of mycotoxins using the optimized method is demonstrated in ***Figure 6*** and was 89% to 105% (113% for AG1 in peanuts) at a spike of 1.6 µg kg^{-1}.

Pigment and phenolic co-extractives in ground chili gave rise to low S/N and high suppression. The effects were mitigated by reducing the % organic in elute 1 and adding 1% NH_4OH to elute 2. Recoveries are demonstrated in ***Figure 7***.

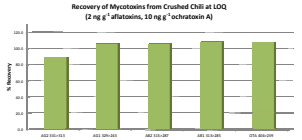

Figure 7. *Recovery of mycotoxins from ground chili using a SPE method optimized for ISOLUTE Myco (2 to 10 µg kg^{-1} spike)*

Infant cereal presented the additional challenge of very low ppb MRLs. We were able to meet these for the most part as demonstrated in ***Figure 8***. However, aflatoxin B1 LOQ was above the EU MRL and aflatoxin G2 recovery was below the EU criterion due to matrix effects observed at the same retention time.

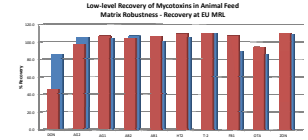

Figure 5. *Low-level recovery of mycotoxins spiked into animal feed or ground soya using the optimized ISOLUTE Myco Method (5 µg kg^{-1} afla/OTA; 100 µg kg^{-1} Z/F/D/T spike)*

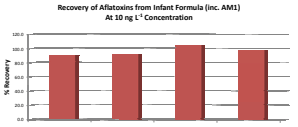

Figure 9. *Recovery of aflatoxins from infant formula using the optimized SPE method optimized for ISOLUTE Myco*

Determination of AM1 at an adequate level as demonstrated in ***Figure 9*** required the use of AB2 as a surrogate internal standard. The use of isotopically labelled AM1 is expected to give a more robust method.

The benefit of alternative wash strategies for widely differing substrates is demonstrated in ***Figure 10***. Patulin recovery is lower using a toluene interference wash. However, signal is increased (not shown) and suppression is reduced to tolerable levels. Reduced signal suppression brings a concomitant improvement to method LOQ. Validation data for the presented recoveries above are available on request.

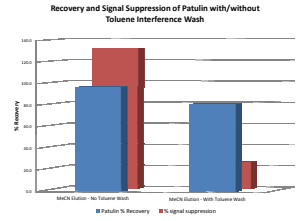

Figure 10. *Recovery and signal suppression of patulin with and without a toluene interference wash (100 ng mL^{-1} patulin spike)*

Conclusions

- The optimized selectivity of the ISOLUTE Myco polymer allows for simultaneous extraction/measurement of multiple mycotoxins.
- The novel polymer used in ISOLUTE Myco is able to recover between 70% and 110% of spike for a majority of mycotoxins from a wide range of substrates using simple catch-and-release methods to below most EU MRLs.
- ISOLUTE Myco demonstrates sufficient sample clean-up to facilitate robust determination of mycotoxins using LC-MS/MS instrumentation.

Part Number: P105