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Development of an Automated Sample Preparation and Analysis Workflow for Mycotoxin Residues in Different Food Matrices

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Abstract
In this report, we describe a completely automated sample preparation workflow for the extraction and screening of multi-mycotoxin residues in different food matrices (corn, wheat) by LC/MS/MS. The extraction and cleanup was performed using a GERSTEL MultiPurpose Sampler (MPS XL) followed by LC/MS/MS determination using an AB SCIEX QTRAP® 4500. The automated sample preparation workflow involved centrifugation, dispersive solid phase extraction (dSPE) and evaporative concentration, providing extraction efficiencies greater than 70 % with RSDs less than 15 % for most analytes.

The LC/MS/MS method was developed for screening for a panel of 14 mycotoxins (aflatoxins, trichotecenes and fuminosins) using the Scheduled MRM™ algorithm in combination with fast polarity switching, achieving excellent linearity (R^2 values of 0.98 or greater) , average accuracies greater than 88 % and limits of quantitation lower than the action levels established by the EC and FDA.

Introduction

One of the major challenges in food safety is the abundance of the naturally occurring contaminants known as mycotoxins. Mycotoxins are secondary metabolites (by-products) that are produced by different types of filamentous fungi such as Aspergillus (Aflatoxins), Penicillum (Ochratoxin A) and Fusarium (trichothecenes, fumonisins, deoxynivalenol and zearalenone) [1]. The presence of these compounds in agriculturally grown products is an important concern due to the health risks they pose to humans and livestock [2]. For this reason, it is crucial to have monitoring and surveillance methods that screen for mycotoxin presence in a variety of food and feed.

The available analytical methods typically require many manual steps making it a quite labor-intensive and time consuming process. In this report, we describe a completely automated sample preparation workflow using a GERSTEL MPS XL autosampler configured for the extraction of multi-mycotoxin residues from different food products combined with extract cleanup and LC/MS/MS determination. This workflow features centrifugation, spiking and calibration curve generation - in addition to a dispersive SPE (dSPE) technique referred to as disposable pipette extraction (DPX) combined with evaporative concentration and subsequent LC/MS/MS determination.

DPX is based on sorbent loosely contained inside pipette tips, which is used to remove matrix interferences and provide a clean extract for analysis. Two different automated sample cleanup strategies using DPX were evaluated in order to determine how efficiently they could extract a panel of 14 different mycotoxins for subsequent LC-MS/MS determination. The LC/MS/MS method utilized a Scheduled MRM™ algorithm in combination with fast polarity switching. The LC-MS/MS method enabled successful identification and quantification of multi-mycotoxin residues in a number of DPX extracts of agricultural commodities (corn, wheat). In addition full scan MS/MS spectra were acquired to allow library searching for increased confidence in identification.

Experimental

Materials. 1 mL ampoules of the following mycotoxins were obtained from Romer Labs for this study: Mix 1 (Aflatoxins B1, B2, G1 and G2), Fumonisin B1 (FB1) Zearalenone (ZEN). A trichothecenes A&B dry standard containing 0.2 mg of the following mycotoxins was obtained from Trilogy Analytical Laboratory: Fusarenon-X (FUS-X), Deoxynivalenol (DON), 3-Acetyldeoxynivalenol (3-DON), HT-2 Toxin (HT-2), T-2 Toxin (T-2), Diacetoxyscirpenol (DAS) and Neosolaniol (NEO).

MycoSpin 400 multitoxin columns were purchased from Romer Labs. 100 mg of the cleanup sorbent contained in the MycoSpin columns were packed in empty 1mL DPX tips, and are referred as DPX-MYCO tips. DPX-WAX-1 mL (30 mg, 10-20 µm) tips were obtained from DPX Labs. Mycotoxin free and Quality Control (QC) corn and wheat midds samples with known concentrations of mycotoxins were donated by the Maryland Department of Agriculture. All solvents used were reagent grade.

Automated Sample Preparation. All automated sample preparation steps for the mycotoxin determination were performed using a dual-head MultiPurpose Sampler (MPS XL) equipped with a CF-100 dual position centrifuge, mVAP multi-evaporation station, mVORX vortex and DPX Option (All from GERSTEL) as shown in Figure 1.

Figure 1. GERSTEL MultiPurpose Sampler (MPS XL) configured for automated extraction of mycotoxins from food samples.

The automated sample preparation workflow was set up in the MAESTRO PrepSequence and performed by the MPS. It is detailed in the flowchart shown below. (Figure 2)

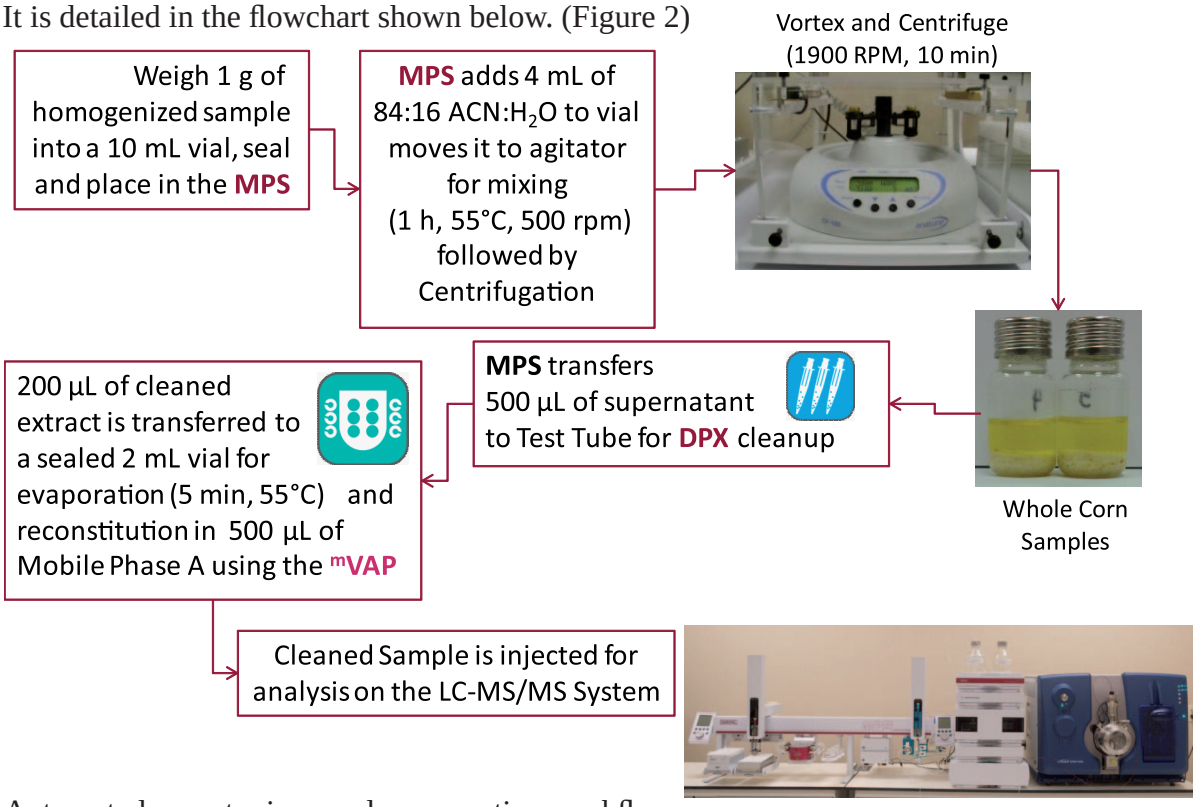


Figure 2. Automated mycotoxin sample preparation workflow.

Using the GERSTEL MAESTRO software, it is possible to enhance the productivity of the sample preparation workflow by using the PrepAhead feature, which allows staggering different stages of the sample preparation workflow. A graphic representation of this feature and its benefits are shown in Figure 3.

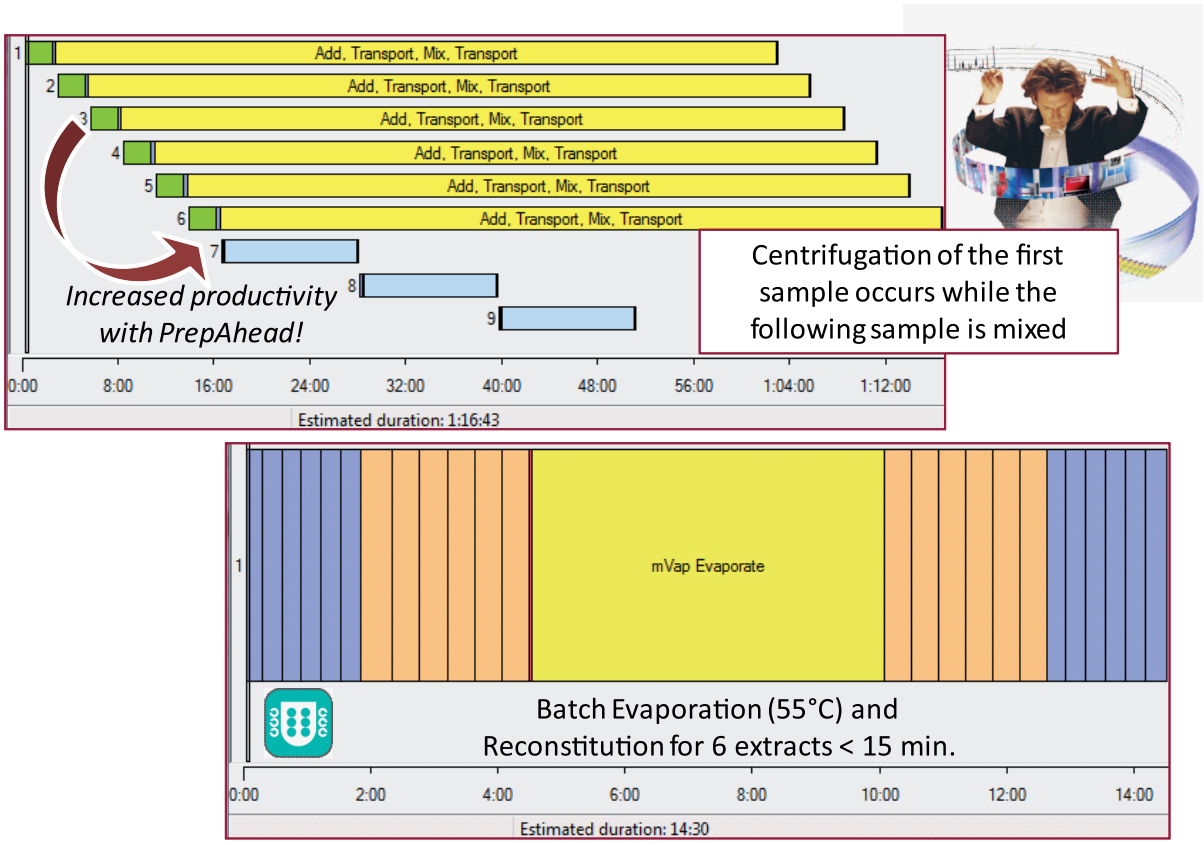


Figure 3. Graphical representations of sample preparation using PrepAhead combined with Batch evaporation all controlled by MAESTRO software.

LC/MS/MS Parameters. The LC/MS/MS analyses were performed using an Agilent 1200 Series LC pump configured with a Phenomenex Gemini 5 µm (110 Å, 150 x 4.6 mm) column1, an AB SCIEX® QTRAP® 4500 LC/MS/MS System and GERSTEL MPS autosampler configured with a Modular Active Washstation. Sample injections were made using a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 50 µL stainless steel sample loop.

Mobile Phase: A – Water/Methanol/Acetic Acid (89:10:1) + 5 mM ammonium acetate
B – Methanol/Water/Acetic Acid (97:2:1) + 5 mM ammonium acetate

LC Gradient:

Time (min)	Flow (µL/min)	% B
0.00	700	10
2.00	700	10
12.00	700	95
16.00	700	95
16.01	700	10
20.00	700	10

Run time: 20 minutes.
Injection volume: 50 µL
Column Temperature: 40°C

Source/Gas Parameters

CUR:	10 psi
IS:	4000 V (+ESI)
IS:	-4000 V (-ESI)
TEM:	550°C
GS1:	50 psi
GS2:	50 psi
CAD:	Medium

MRM Parameters

Dwell time:	Scheduled MRM™
DP:	Optimized
Q1 Resolution:	UNIT
Q3 Resolution:	UNIT
MRM detection window:	30 s
Target scan time:	0.1 s

The MRM transitions used for the compounds are shown in table 1.

Table 1. MRM transitions used for detection.

Compound	Ret. Time [min]	Precursor Ion	Product Ions	ESI Mode
Aflatoxin B1	11.8	313.1	285.2/241.1	ESI+
Aflatoxin B2	11.4	315.1	287.1/259.1	ESI+
Aflatoxin G1	11	329	243/200	ESI+
Aflatoxin G2	10.6	331.1	245.1/313.2	ESI+
Ochratoxin-A	14.1	404	239/102	ESI+
Fumonisin B1	12.5	722.5	704.4/334.4/352.3	ESI+
Neosolaniol	8.8	400.2	215/185	ESI+
Diacetoxyscirpenol	11.9	384.2	307.2/105.2	ESI+
HT-2 Toxin	12.8	447.4	345.1/285.1	ESI+
T-2 Toxin	13.6	484.3	215.2/185.1	ESI+
Zearalenone	14.4	317.1	131.1/175	ESI-
Deoxynivalenol	7.2	355	59/353	ESI-
3-Acetyldeoxynivalenol	10.3	397	59.1/337.1	ESI-
Fusarenon-X	8.7	413	59/353	ESI-

The AB SCIEX® QTRAP® 4500 LC/MS/MS System was operated with Turbo V™ source and Electrospray Ionization (ESI) probe. Approximately 35 MRM transitions were monitored in both positive and negative polarity. The Scheduled MRM™ algorithm was used in combination with fast polarity switching using Analyst® 1.6.1 Software and MultiQuant™ 2.1 Software was used for quantitative data processing. For increased confidence in compound identification EPI spectra at a scan speed of 10000 Da/s were acquired using a dynamic fill time for optimal MS/MS quality. EPI spectra were generated using the standardized Collision Energy (CE) of ±35 V with Collision Energy Spread (CES) of 15 V to ensure a characteristic MS/MS pattern independent of the compound's fragmentation efficiency. MS/MS spectra were searched against the Mycotoxin spectral Library version 1.0

Results and Discussion

Automated dSPE strategies. DPX differs from other SPE approaches in that sample solutions are dynamically mixed with the sorbent within the pipette tip. The extraction efficiency is dependent on the equilibration time between solutions and sorbent, rather than flow rates through a packed bed.

Two strategies were evaluated to ensure maximum recovery of all mycotoxins as detailed in the flowchart in Figure 4. Relative recoveries were determined by comparing results from spiked corn samples (at least 5 replicates) with "matrix-matched" samples prepared by adding the neat mixture of mycotoxin standards directly to the eluent of the extracted blank matrix. The total DPX extraction time ranged between 5-9 minutes per sample allowing high throughput sample preparation.

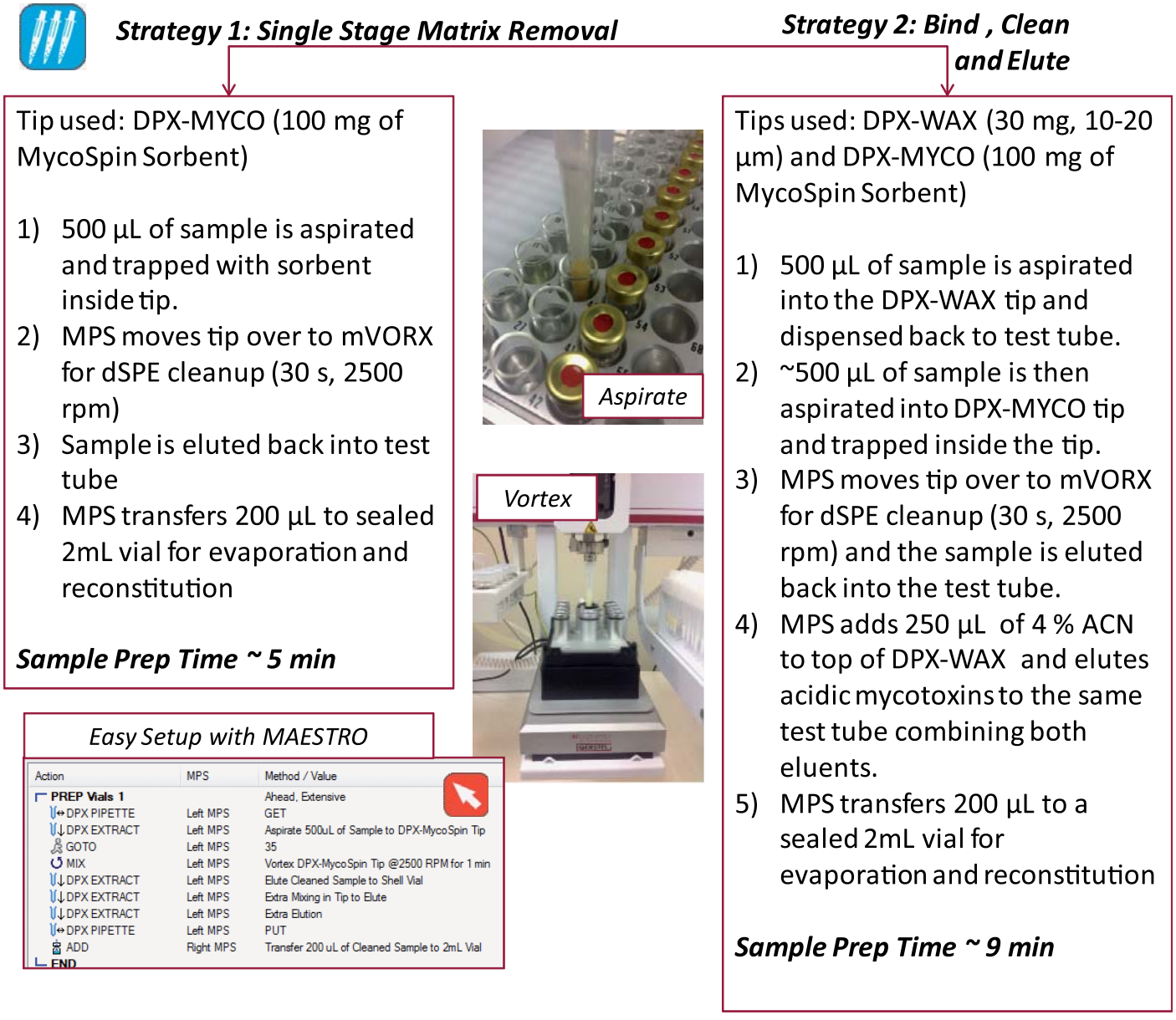


Figure 4. DPX strategies used for the extraction of mycotoxins from corn samples.

Strategy 1 averaged analyte recoveries in the range 15-110 % with relative standard deviations (RSDs) of 4-15 %, whereas Strategy 2 averaged slightly higher recoveries ranging from 45-120 % with RSDs of 2-15 %. (Figure 5)

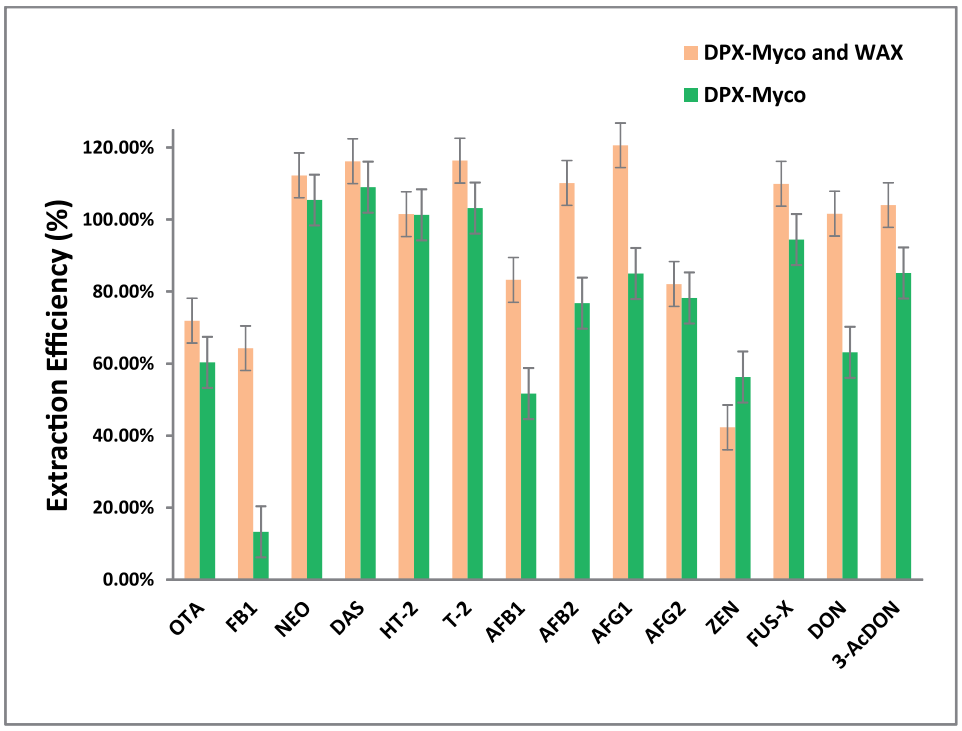


Figure 5. Automated DPX extraction efficiencies for all mycotoxins analyzed.

It was observed that using Strategy 1, the FB1 mycotoxin irreversibly bound to the resin in the DPX-MYCO, however by initially using DPX-WAX in Strategy 2 the FB1 mycotoxin selectively binds to the resin and can be eluted thereby improving its recovery, suggesting the possibility of including the rest of the fumonisin mycotoxin family. This WAX sorbent also has reversed phase characteristics, which can selectively extract some mycotoxins of interest. It should be noted that using an internal standard would significantly improve the reproducibility. In this preliminary study no internal standards were used.

Automated Workflow Method Validation. Figure 6 shows MRM chromatograms from a mycotoxin-fortified corn sample extract at 10 ng/g using fast polarity switching. All 14 mycotoxins were successfully monitored in this sample matrix at low concentrations using the automated DPX-LC/MS/MS sample preparation workflow.

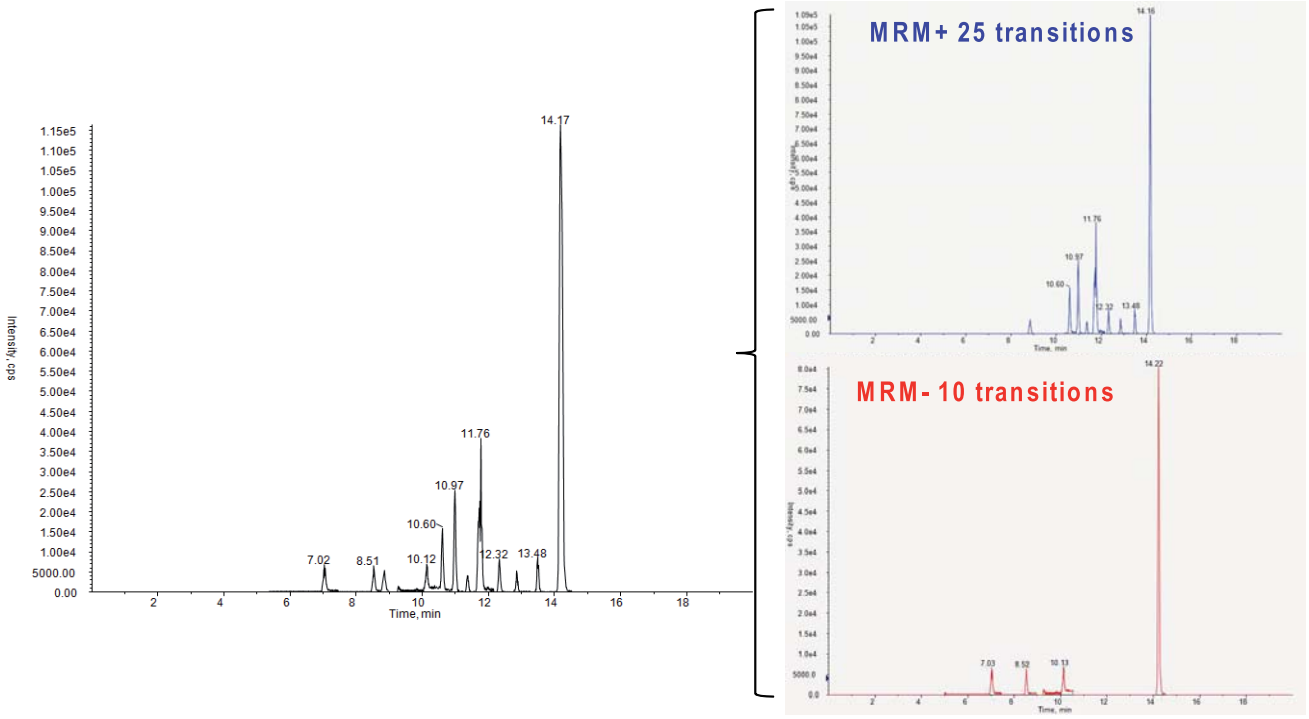


Figure 6. Representative MRM chromatograms from a mycotoxin-fortified corn sample extract at 10 ng/g.

Figure 7 shows representative calibration curves for DON and OTA obtained from neat standards that were prepared automatically. The resulting calibration curves were shown to be linear from at least 2 to 500 ng/mL for the mycotoxins monitored, using a linear 1/x regression method, reaching limits of quantitation lower than the action levels established by the FDA and EC3,4. Sample data were processed using MultiQuant™ software version 2.1. The 'Multicomponent' query automatically calculates and compares MRM ratios for compound identification and highlights concentrations above a user specified maximum residue level.

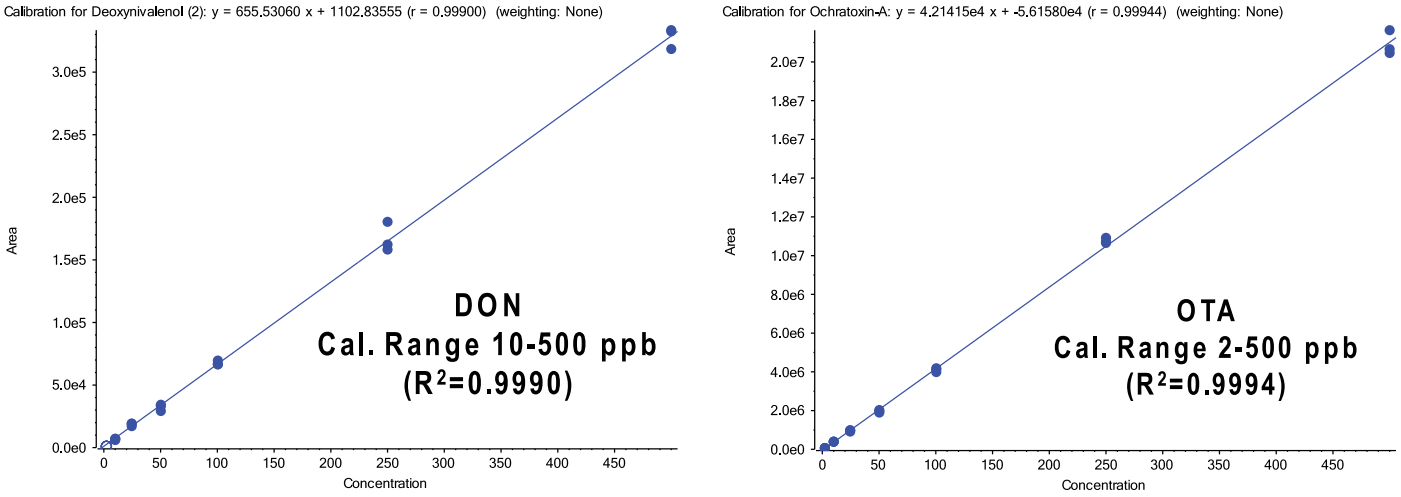
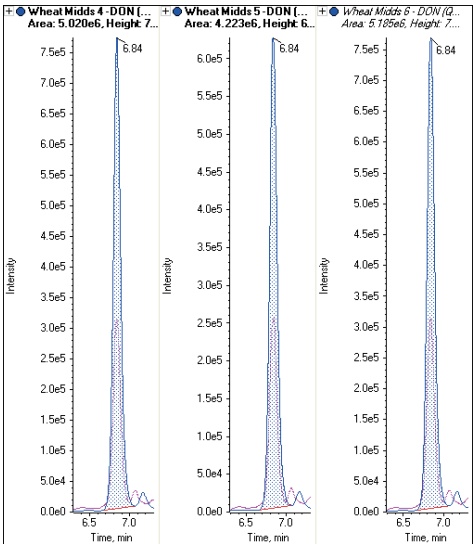


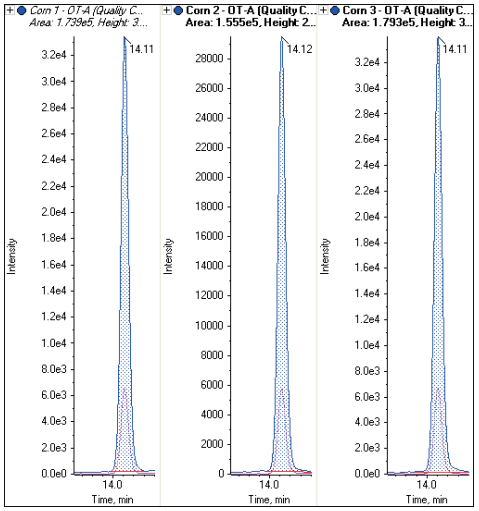
Figure 7. Calibration curves for DON and OTA.

Figure 8 shows overlaid MRM chromatograms from QC samples (wheat midds and corn) containing DON (250 ng/g) and OTA (12-20 ng/g) respectively. Method accuracy for both extracts averaged > 88 % with RSDs less than 10 %.



Wheat Midds

Compound (MRM)	QC sample Concentration (ng/g)	% Average Accuracy (n=6)	% RSD (n=6)
DON (1)	250	94.6 %	10.4 %
DON (2)		95.0 %	10.5 %



Corn

Compound (MRM)	QC sample Concentration (ng/g)	% Average Accuracy (n=6)	% RSD (n=6)
OTA (1)	12-20	82.3 %	8.9 %
OTA (2)		82.8 %	7.9 %

Figure 8. MRM chromatograms from QC samples (wheat midds and corn) with method accuracies and RSDs.

LC/MS/MS analysis of incurred samples. For improved accuracy, compound identification was performed using full scan MS/MS experiments with automated library searching to compare the unknown with a standard spectrum. The dependent MS/MS spectra were acquired using the EPI mode of the QTRAP® system after being triggered from a Scheduled MRM™ IDA survey scan. The rapidly collected high quality MS/MS data were used for mass spectral library searches, using LibraryView™ Software 1.0, to increase the confidence of detection. Extracted spectra and library search Purity Score values using an MS/MS library search algorithm are shown in Figure 9 for a corn sample contaminated with FB1.

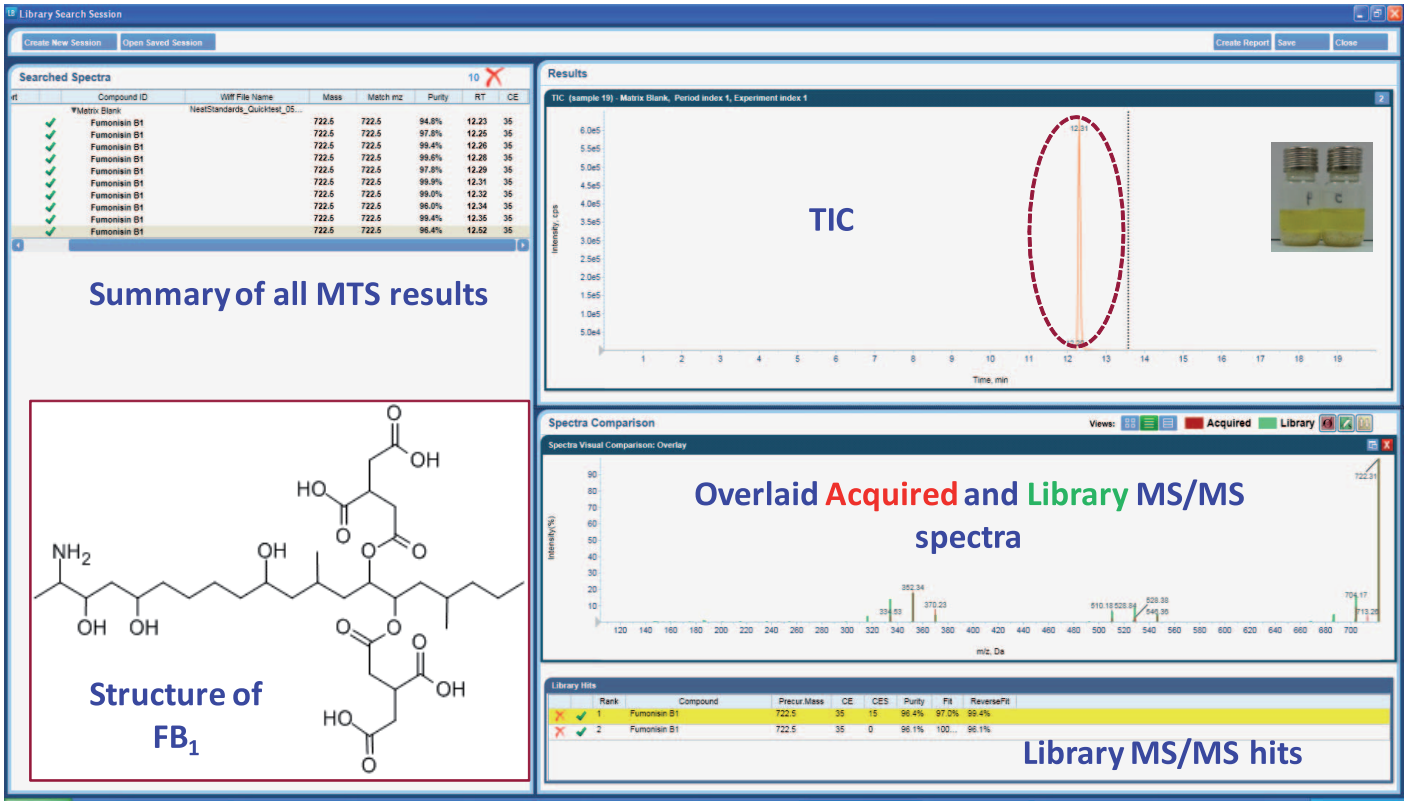


Figure 9. Automated library confirmation for FB1 found in extract of contaminated corn sample.

Conclusions

As a result of this work, we were able to demonstrate:

- A completely automated sample preparation workflow for the efficient extraction and determination of multi-mycotoxin residues in different food matrices using the dual head GERSTEL MPS XL autosampler.
- Automated dSPE using DPX requires only small volumes of sample (~500 µL), enabling fast sample preparation (5-9 min/sample) with average extraction efficiencies greater than 70 % and good reproducibility (% RSD < 15 %) using 2 different cleanup strategies for all mycotoxins.
- Using the AB SCIEX QTRAP® 4500 LC/MS/MS System a method for the screening of a panel of 14 mycotoxins was successfully developed using the Scheduled MRM™ algorithm in combination with fast polarity switching, achieving excellent linearity (R^2 values of 0.98 or greater) , average accuracies greater than 88 % and limits of quantitation lower than the action levels established by the EC and FDA.
- The QTRAP®allowed high accuracy compound identification by performing full scan MS/MS experiments using the Enhanced Product Ion mode after being triggered from a Scheduled MRM™ IDA survey scan with automated library searching capabilities to compare spectra of unknown compounds with standard spectra.

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