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Automated Extraction of Aflatoxin B1 by Disposable Pipette Extraction from Corn followed by LC/MS/MS

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Abstract
The extraction of Aflatoxin B1 residues from contaminated corn samples using disposable pipette extraction (DPX) is described. DPX is a solid-phase extraction (SPE) technique that is based on loosely contained sorbent inside a pipette tip fitted with a screen. This device provides faster extraction because only minimal conditioning steps are needed. A weak anion exchange sorbent (DPX-WAX) was found to provide selective extraction of Aflatoxin B1 from a crop sample. Recovery of the analyte of interest was 81 % with a relative standard deviation of 8 %.

Introduction
One of the major food safety challenges is the abundance of *Aspergillus flavus* and *Aspergillus parasiticus* molds, which are among the fungi that produce toxic secondary metabolites known as aflatoxins. Several aflatoxins are known human carcinogens; of these Aflatoxin B1 is one of the most potent registered carcinogens. Aflatoxin levels in food and animal feed are regulated in most countries and there is great interest in a fast, sensitive, and selective analysis method.

Determining aflatoxin concentrations at trace levels in the presence of large amounts of sample matrix is a challenging task. Whether accurate and precise results are obtained when analyzing matrices as complex as food and animal

feed depends largely on the extraction and cleanup methods used.

In this work, automated extraction and determination of Aflatoxin B1 is described. A solid phase extraction (SPE) technique referred to as disposable pipette extraction (DPX) is used. DPX is based on sorbent loosely contained inside pipette tips. DPX differs from other SPE approaches in that sample solutions are dynamically mixed with the sorbent. The extraction efficiency is dependent on the equilibration time for the analyte between solution and sorbent, rather than flow rates. The mixing step often eliminates the need for sorbent conditioning and allows the use of smaller volumes of, elution solvent, which means that less solvent is typically needed for DPX (< 1mL) than for traditional SPE (~ 2 mL). A schematic of a DPX extraction is shown in Figure 1.

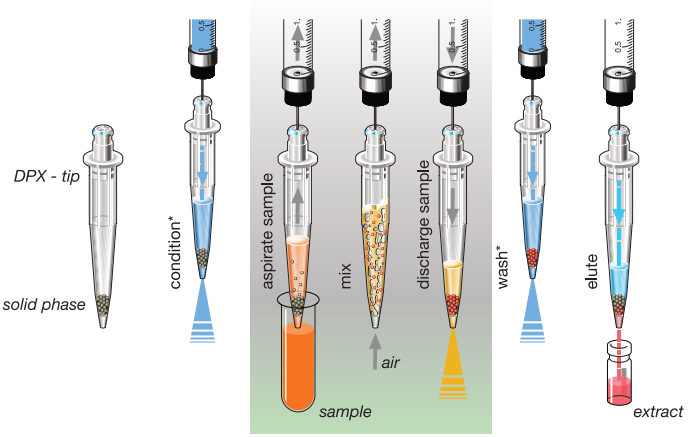

Figure 1. Graphical representation of the DPX extraction process.

EXPERIMENTAL

Materials. DPX-WAX-1mL tips were obtained from DPX Labs, LLC (Columbia, SC). LC vials, inserts and caps were purchased from Agilent (Palo Alto, CA). Methanol and acetonitrile were purchased from Fisher Scientific (Pittsburg, PA) as analytical grade reagents.

A reference standard solution of Aflatoxin B1 was provided by the South Carolina Department of Agriculture (SCDA, Columbia, SC). A working standard solution of the analyte was prepared by diluting the standard solution to 10 µg/mL using acetonitrile.

Instrumentation. All analyses were performed using an Agilent 1100 HPLC instrument with a Restek Ultra Aqueous C18 column (50 × 2.1 mm, 5 µm) coupled to a 3200 Q-Trap mass spectrometer with electrospray ionization source (AB Sciex, Foster City, CA). The GERSTEL MultiPurpose Sampler (MPS) XL was configured with an active wash station. Sample injections were made using a Valco (Houston, TX) six port (0.25 mm) Cheminert C2V injection valve fitted with a 20 µL stainless steel sample loop (Figure 2). Sample preparation and introduction was performed by the MPS under GERSTEL MAESTRO software control using one integrated sequence table for sample preparation and LC-MS/MS analysis.

Figure 2. MPS 2XL MultiPurpose Sampler (MPS) with GERSTEL DPX option.

Sample preparation. Corn-based food samples were processed by blending 20 g with 100 mL of 80 % methanol in water. Several incurred samples were processed and screened at the SC Dept. of Agriculture for aflatoxins, and these solutions were subsequently analyzed using LC/MS/MS. Samples that were screened negative for aflatoxins were used to make matrix-matched standards and calibrators.

Standards were added to blank corn matrix samples (250 µL), diluted with 250 µL of water, and mixed for validation studies. After mixing, the solutions were transferred to clean labeled test tubes (17 x 100 mm) and placed on a GERSTEL MPS XL sample tray for automated DPX extraction. The extractions were performed using DPX-WAX 1 mL TA tips. DPX tips incorporate transport adaptors that enable automated transport of the tips and also serve as connectors for liquid transfer into the tips.

Automated DPX Prep Sequence. The DPX tip was conditioned with 200 µL of methanol-water (20:80) for 10 s. The corn samples containing Aflatoxin B1 were aspirated into the DPX tip and mixed with the sorbent by aspirating 275 µL of air. The solution was equilibrated for 10 seconds before dispensing the entire content into the original culture tube. This procedure was repeated 2 times followed by a wash step with water (200 µL) and final elution (dispensing of clean solution) with 400 µL of acetonitrile into a 2 mL autosampler vial. Finally, the sample was diluted with 850 µL of water prior to analysis. It should be noted that this solution can be solvent evaporated using the GERSTEL evaporation station to improve sensitivity; however, in this case, a solvent evaporation step was not performed because the required detection limit was achieved without the evaporation step.

LC/MS/MS Method Parameters. The mobile phase gradient was generated using acetonitrile containing 0.05 % formic acid and 5 mM ammonium formate buffer containing 0.05 % formic acid as follows: acetonitrile was held at 10 % for 1 minute; increased to 30 % over 1 minute; further increased to 90 % over 6 minutes; and finally held for 1 minute to clean the column. Total flow rate through the column was 0.75 mL/min and the run time was 9 minutes. The analytical column was equilibrated at room temperature (25°C). During the LC-MS/MS run, the eluent from the LC was diverted to waste for 2 minutes, connected to the turbo ion spray source for 7 minutes, and finally diverted to waste for 1 minute. Mass spectrometric analysis was performed in positive electrospray ionization mode using multiple reaction monitoring (MRM) and the following method settings:

Needle voltage:	5 kV
Turbo ion spray, heater temperature	500°C
Nebulizer gas, (nitrogen) pressure	30 psi
Turbo heater gas, (nitrogen) pressure	50 psi
Curtain gas, (nitrogen) pressure	30 psi
Collision gas (CAD, nitrogen) pressure	50 psi.

Results and Discussion

Aflatoxin B1 was successfully extracted from corn samples using the MultiPurpose Sampler with DPX option described in this work. DPX extraction was combined with automated direct introduction of the extract to the integrated LC-MS/MS system and Aflatoxin B1 concentrations were determined successfully. Figure 3 shows an LC-MS/MS chromatogram of a low QC sample.

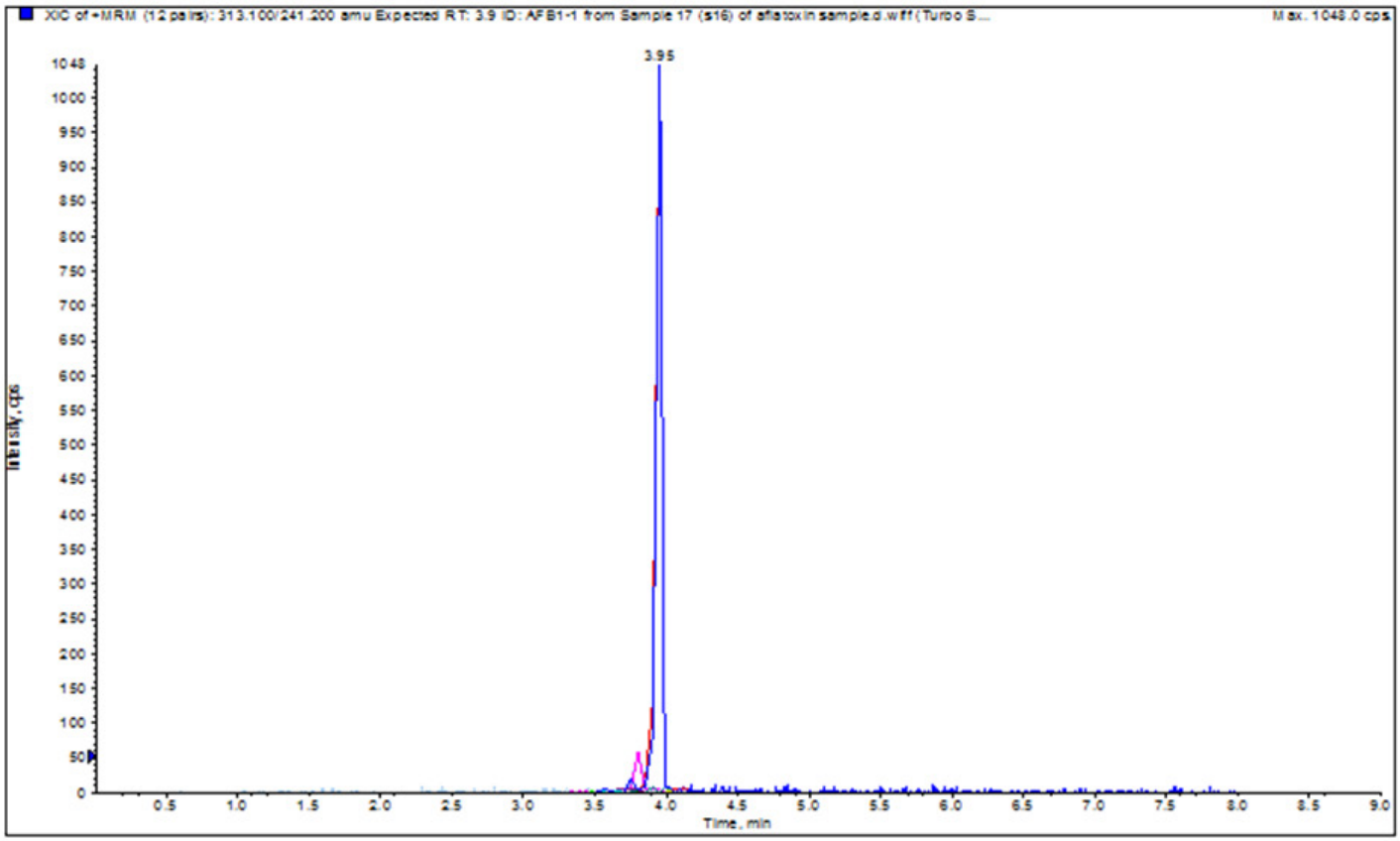

Figure 3. LC/MS/MS chromatogram of Aflatoxin B1 from a low QC sample.

Relative recovery was determined by comparing results from spiked samples (at least 5 replicates) with "matrix-matched" samples prepared by adding Aflatoxin B1 directly to the eluent of the extracted blank matrix. Percent recovery and relative standard deviations were 81.7 % and 8 % respectively. It should be noted that using an internal standard would significantly improve the reproducibility. In this case no internal standard was used.

A representative calibration curve for Aflatoxin B1 is shown in Figure 4. Regression analysis for Aflatoxin B1 using this method resulted in an R^2 value of > 0.999. Limits of Detection and Limits of Quantitation for Aflatoxin B1 were 1.7 and 5.5 ng/mL, respectively. Table 1 summarizes all results obtained for Aflatoxin B1 extracted from a corn matrix using automated DPX.

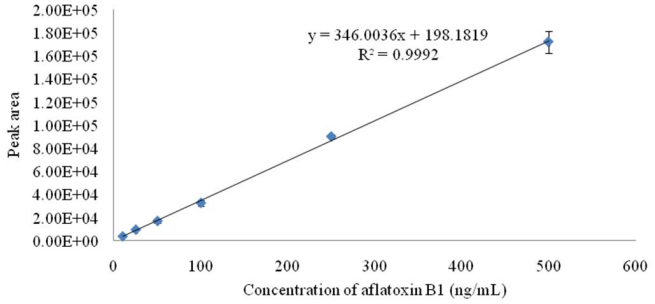

Figure 4. Calibration Curve for Aflatoxin B1.

Table 1. Aflatoxin B1 LC/MS/MS parameters, % Recovery, % RSD , LOD and LOQ.

MRM Parameters	
Q1	313.1
Q3	241.2
DP	69
EP	4
CE	42.8
CEP	16.1
CXP	3.1
Validation Results	
% Recovery	81.70 %
% RSD	8.01 %
LOD	1.66
LOQ	5.54

LOD & LOQs expressed in ng/mL

The DPX method was based on WAX sorbent for this analysis. The WAX sorbent has reversed phase characteristics, which were used to extract and concentrate aflatoxin B1. In addition, this sorbent also binds fatty acids and helps to remove potential matrix interferences. This same method should work equally well with the other aflatoxins (not performed in this study).

The total cycle time per sample for the DPX extraction, sample dilution and injection was 10 minutes, enabling "just in time" sample preparation using the MAESTRO software PrepAhead function. Using this automated procedure for extraction and analysis over 100 samples can be processed per day.

Results obtained using the DPX-based analysis method were compared with the results obtained from immunoassay screening (Table 2). The results from this study correlate well with the immunoassay results, with the main difference being that the immunoassay determines „total aflatoxins". Some of the samples were analyzed using the DPX_based method several days after the immunoassay screening had been performed by the SCDA.

Table 2. Comparison of results for aflatoxin B1 levels obtained using DPX –LC/MS/MS (column one) and immunoassay screening for total aflatoxins (column two).

Sample No.	DPX-WAX (AFB1) (ng/g)	SCDA Total (ng/g)	Sample
S-90016	531.3	724	Corn
S-90031	50.7	54	Farm produced feed
S-90035	0	Negative	Dry dog food
S-90039	12.5	27	Corn
S-90040	44.0	39	Corn
S-90042	15.8	27	Corn
S-90043	0	1	Corn
S-90044	21.0	35	Corn
S-90429	18.4	26	Corn
S-90430	15.2	17	Corn

Conclusions

A fast, reproducible automated extraction method for the determination of Aflatoxin B1 in corn has been developed using disposable pipette extraction and LC/MS/MS. The described DPX extraction requires only small volumes of sample and solvent (approximately 0.5 mL). In addition, high recovery (> 70 %) and good reproducibility were achieved for Aflatoxin B1 with RSDs below 10 %. LOD and LOQ for Aflatoxin B1 were below 10 ng/mL.

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