Fast and Effective Determination of Aflatoxins in Grains or Food Using Accelerated Solvent Extraction followed by HPLC

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INTRODUCTION

The fungus *Aspergillus* grows in soil and decaying vegetation, and can colonize and contaminate crops with aflatoxins before harvest or during storage. Aflatoxins are toxic and highly carcinogenic substances, and the presence of aflatoxins B1, B2, G1, and G2 (structures shown in Figure 1) in a variety of processed and unprocessed foods is regulated in countries around the world. The European Commission has set maximum levels for aflatoxin B1 between 2.0 and 8.0 µg/kg and for the sum total of all four of these toxins between 4.0 and 15.0 µg/kg in crops such as nuts, groundnuts, grains, and dried fruits.[1] The U.S. Food and Drug Administration has set action levels (levels where the FDA will take legal action to remove products from the market) of 20 ppb (µg/kg) for the sum total of the four aflotoxins in foods such as corn, peanuts, brazil nuts, and pistachios as well as other foods.[2]

The traditional method for aflatoxins analysis in grains includes soxhlet extraction, sample clean-up using solid-phase extraction (SPE), and separation, identification, and quantification using high-performance liquid chromatography (HPLC). Because of the time-consuming extraction and clean-up steps, sample throughput is limited using this technique.

In this poster we describe the use of an accelerated solvent extraction (ASE®) system followed by on-line SPE-LC for the analysis of aflatoxins in corn and almonds. ASE uses high temperatures during extraction to speed-up the extraction process, while incorporating high pressure to maintain the solvents in their liquid state. The on-line SPE-LC approach automates sample clean-up and aflatoxin analysis, increasing throughput while decreasing labor. The method is validated for linearity, accuracy, method precision, recovery, and system precision.

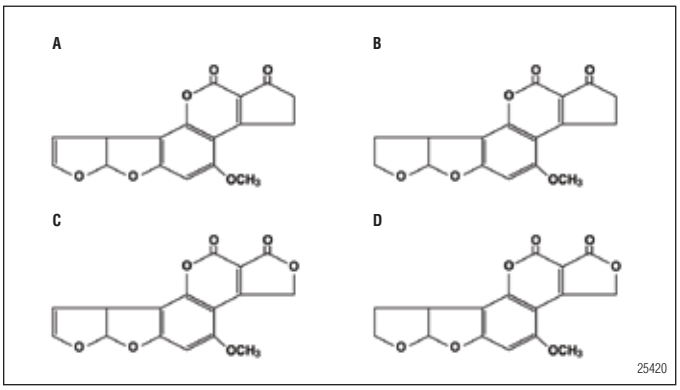


Figure 1. Molecular structures of aflatoxins. (A) Aflatoxin B1, (B) Aflatoxin B2, (C) Aflatoxin G1, and (D) Aflatoxin G2.

INSTRUMENT CONFIGURATIONS

Accelerated solvent extraction was performed on a Dionex ASE 200 system (see Figure 2 for schematic layout of instrument). A 5 g sample (almond or corn) was ground with a mortar and pestle with 3 g of ASE Prep DE, a diatomaceous earth used to remove moisture from the sample and to fill up any empty space in the cell to reduce solvent usage. The ground sample was added to a 22 mL extraction cell and was extracted using 50/50 v/v% methanol/acetonitrile with two 5 min extraction cycles at high pressure and 80 °C. A total solvent volume of 16.5 mL was used in the two cycles to extract the sample. After the complete extraction procedure, the cell was flushed with nitrogen for 120 s to remove any solvent retained in the sample.

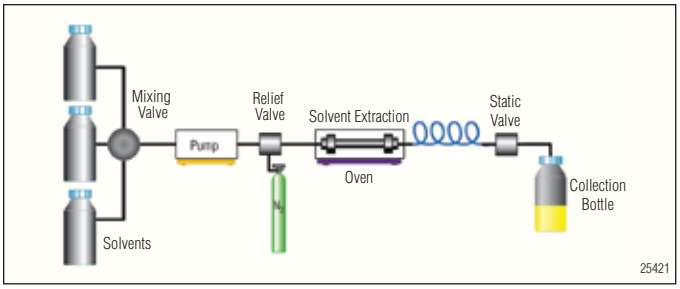


Figure 2. Schematic layout of accelerated solvent extraction.

The extract was analyzed by on-line solid-phase extraction coupled to a high-performance liquid chromatographic system (online SPE-LC). The Dionex UltiMate® ×2 Dual-Gradient HPLC system was used, comprising a six channel on-line degasser; two integrated gradient pumps used for sample loading, sample cleanup, and separation on the analytical column; a cooled well-plate autosampler with split loop injection; a thermostatted column compartment equipped with a 6-port switching valve; a photochemical derivatizer; and a fluorescence detector.

The stationary phase of the Venture™ AF SPE immunoaffinity 15–20 µm 50 × 2.1 mm column selectively retained the target analytes (aflatoxins) from the sample matrix. The enriched analytes were transferred in a back-flush mode to an Acclaim® 120 C18 3 µm, 4.6 × 150 mm column for the reversed-phase separation of the B1, B2, G1, and G2 aflatoxins.

After separation, the aflatoxins B1 and G1 are photochemically derivatized by irradiation with UV light at 254 nm as shown in Figure 3, allowing detection of these aflatoxins with a fluorescence detector. The photochemical derivatization has no influence on the chemical or measurement related properties of aflatoxins B2 and G2.

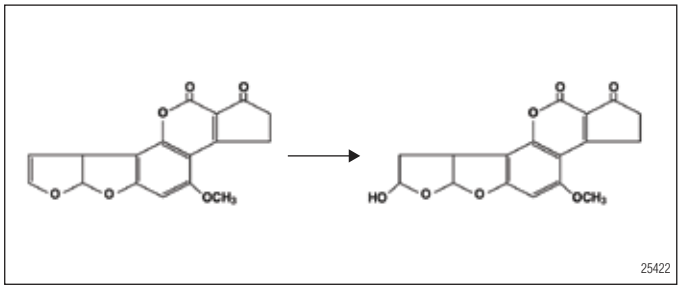
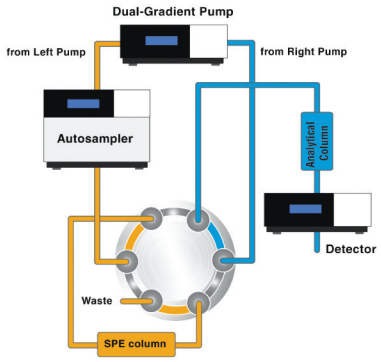
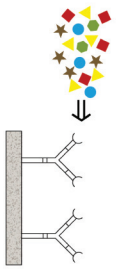
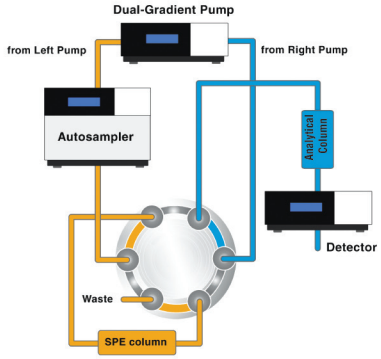
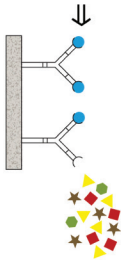
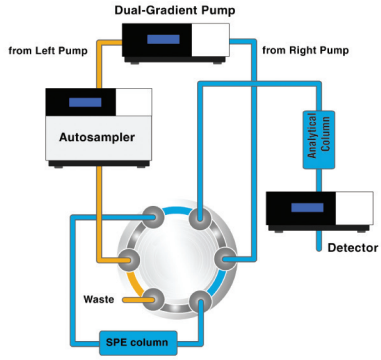
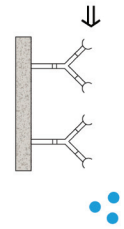
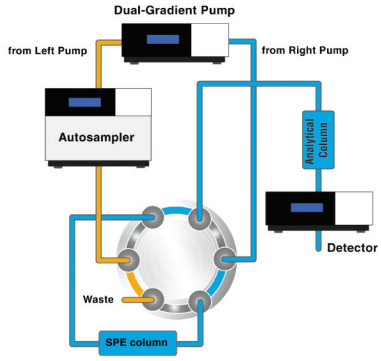
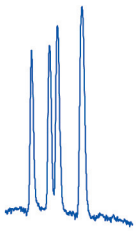


Figure 3. Hydroxylation of aflatoxin B1 by photochemical derivatization with UV light.

Table 1. Program Events During On-Line SPE-LC Analysis

Time (min)	Valve Position	Loading Solvent for SPE Column, 250 µL/min A) Binding buffer B) Water	Transfer / Elution for Analytical Column, 1.00 mL/min A) 20/80 CH_3CN/H_2O B) 22.5/22.5/55 $CH_3OH/ CH_3CN/H_2O$
0.0	1_2	100% A	100% A
5.0		100% A	100% A
5.1		100% B	100% A
10.0	6_1	100% B	100% A
10.1		100%A	100% A
14.5		100%A	100% A
14.6	1_2	100%A	100% B
27.6		100%A	100% B
27.7		100%A	100% A
40.0		100%A	100% A

1. The ASE extract is loaded on the SPE immunoaffinity column with binding buffer (10 mM phosphate, 0.15 M NaCl buffer, pH 7). 2. The analytical column is equilibrated during SPE.		
3. The SPE immunoaffinity column allows for the isolation of the aflatoxins from the extract matrix by selective binding with immobilized antibodies. 4. After the aflatoxins are bound, the binding buffer is flushed from the SPE immunoaffinity column with water.		
5. Analytes are transferred from the SPE immunoaffinity column to the analytical column with 20/80 v/v% acetonitrile/water.		
6. Aflatoxins are separated on an HPLC column with mobile phase 22.5/22.5/55 v/v/v% acetonitrile/ methanol/water. 7. The SPE column is equilibrated with binding buffer for the next injection.		

RESULTS AND DISCUSSION

An SPE-LC method was developed for automated sample clean up and analysis. A typical chromatogram of a standard aflatoxin solution is shown in Figure 4.

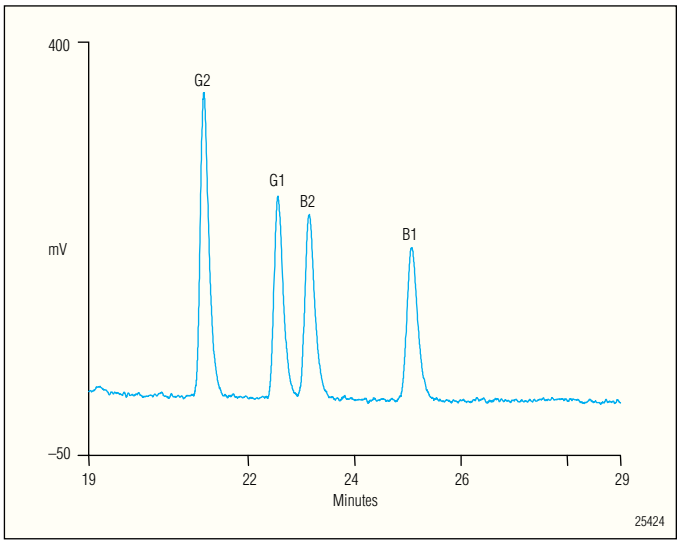


Figure 4. Emission signal for 20 ppb G1 and B1 and 6 ppb G2 and B2. Injection volume was 5 µL.

The recovery of aflatoxins on the immunoaffinity SPE column was determined by comparing the signals from injections of a standard on the analytical and SPE/analytical column configurations. The recoveries for the aflatoxins are listed in Table 2

Table 2. Recovery Results for Four Aflatoxins (n =2)

Aflatoxin	Area mAU*min Direct injection	Area mAU*min With SPE	% Recovery
G2	35.58	38.71	108.8%
G1	46.67	44.00	94.3%
B2	52.54	54.03	102.8%
B1	72.80	72.80	100.0%

Calibration curves were recorded from 0 to 20 ppb for the aflatoxins. Linearity, precision, and limit of detection are listed in Table 3.

Table 3. Linearity, Precision, and Limit of Detection (n=4)

Compound	Regression Coefficient (R^2)	Retention Time[a] Retention (%RSD)	Peak Area[a] (%RSD)	LOD[b] (ppb)
G2	0.997	0.11	1.28	0.6
G1	0.996	0.15	1.34	2.0
B2	0.996	0.14	1.31	0.6
B1	0.996	0.22	1.52	2.0

The method showed acceptable linearity and precision. The limit of detection allows the determination of the toxins in food with maximum acceptable levels of 2 µg/kg for aflatoxin B1 and 4 µg/kg for the sum total of the four toxins set in the European legislation.

SAMPLE ANALYSIS

Almond and corn samples were treated with the ASE system to extract the aflatoxins. The resulting extracts were analyzed with the SPE-HPLC system. Example chromatograms for almond and corn are shown in Figure 5.

The aflatoxin amounts found in the samples are listed in table 4.

The retention time and peak area precision for the samples were similar to those obtained with the standards.

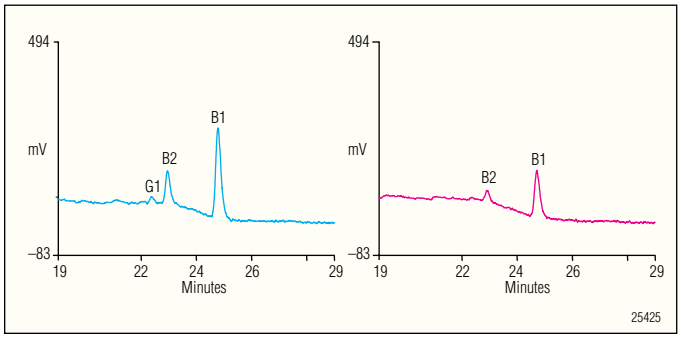


Figure 5. Chromatogram of aflatoxin determination by SPE-LC in almond (left) and corn (right).

Table 4. Amount of Aflatoxins Found in Almond and Corn Samples

Compound	Almond Amount (µg/kg)	Corn Amount (µg/kg)
G2	<LOD	<LOD
G1	3	<LOD
B2	6	1
B1	53	27

CONCLUSION

The combination of accelerated solvent extraction and SPE-LC offers an attractive method for the quantification of aflatoxins in food. The sample extraction time is significantly reduced as a result of the applied temperature and pressure. The labor involved in aflatoxin isolation is reduced by automation of the on-line SPE. The SPE-LC method shows acceptable linearity, precision, selectivity, and recovery for reliable quantification at meaningful concentration limits.

REFERENCES

1) Commission Regulation (EC) No 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs. *Official Journal of the European Union* **2006,** *49,* L364, 5–24.

2) *Action Levels for Poisonous or Deleterious Substances in Human Food and Animal Feed.* Industry Activities Staff Booklet. U.S. Food and Drug Administration, Washington, DC, 2000. http://www.cfsan.fda.gov/~lrd/fdaact.html (accessed Apr 30, 2008)

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