DETERMINATION OF AFLATOXINS IN A WIDE RANGE OF FOOD AND AGRICULTURAL COMMODITIES USING IMMUNOAFFINITY CHROMATOGRAPHY COLUMN CLEAN-UP WITH UPLC OR HPLC WITH FLUORESCENCE DETECTION

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ABSTRACT

Aflatoxins are carcinogenic mycotoxins that have adverse health effects on both humans and animals consuming contaminated food and feed, respectively. A method has been developed for the highly sensitive and selective determination of regulated aflatoxins in a wide range of commodities. The extraction of aflatoxins from representative commodities of interest (corn, wheat, oats, red chili, black pepper, cocoa and a traditional Chinese medicine) was performed using liquid-liquid extraction and then immunoaffinity column clean-up on the new AflaTest WB SR+ column. Chromatographic separation was demonstrated using both HPLC (Alliance) and UPLC (ACQUITY UPLC H-Class PLUS) platforms, using fluorescence detection, supported with post-column derivatization and large flow cell, respectively. The performance of the method was evaluated through replicate analysis of spiked test portions of seven different matrices. Overall recovery was shown to be excellent, between 92% and 116%, with relative standard deviations lower than 8%. The method was found to be specific as no interference peaks were observed for blank samples. The method has been demonstrated as suitable for monitoring compliance with regulatory limits set for aflatoxins in food commodities globally.

INTRODUCTION

Mycotoxins are toxic, secondary metabolites of molds that can occur in food and agricultural products via many contamination pathways, including production, processing, transport, and storage. Fungal growth and mycotoxin production depend on biological (susceptible crop) and environmental factors, with the emphasis on regional climatic conditions during plant development and crop harvest. Mycotoxins are well established to have health impacts both in humans and animals (e.g. recent pet deaths in the USA) but are also responsible for significant losses in revenue and the potential erosion of brand and reputation.

Mycotoxin consistently constitute the highest risk category for notifications in the European Rapid Alert System for Food and Feed (RASFF) and often result in consignments being rejected at border control. The most toxic, and carcinogenic group of mycotoxins commonly found in food and animal feed is the aflatoxins, typically found in nuts, nut products, corn, and grains but have also been reported a wider variety of crops, including coffee, cocoa, and spices, and in foods of animal origin such as milk. Aflatoxins are regulated in many countries of the world. A recent review of contemporary published papers in the field showed a high number of aflatoxin contaminations in food at levels that exceed a regulatory limit of 20 µg/kg and 4 µg/kg set for foods for human consumption in the USA and European Union, respectively.[1] European regulatory levels of 0.1 ug/kg aflatoxin B1 in cereal based foods for infants and young children provide the additional challenge of being able to quantitate very low levels of aflatoxins. This emphasizes the need for increased analytical testing and robust methods as an effective strategy for prevention, control, and periodic monitoring of mycotoxin in all stages from field to the consumer.

A variety of testing solutions exist for determination of aflatoxins, ranging from easy to use, rapid tests, which can be used at the point of production, to lab-based reference methods that are more time-consuming, but can be used to provide a more comprehensive view of the level of contamination.[2] Determination of aflatoxins is a challenging task, due to the complexity of the matrix (often with high lipid content), the low concentrations in which these compounds are usually present, and the strict low regulatory limits. The combination of clean-up using immunoaffinity columns (IAC), based on antibodies, and analysis by HPLC with fluorescence detection has been a pre-requisite step to achieve the desired sensitivity and selectivity and has been successfully used as a cost-effective way to check compliance with the regulatory limits for aflatoxins for many years.[3] Recently, VICAM launched a new IAC product, the AflaTest WB SR+, which can endure a high concentration of organic solvent whilst maintaining excellent recoveries. The objective of this study was to examine the performance of using VICAM's AflaTest WB SR+ column, with two separate HPLC/UPLC platforms, in both grains (corn, wheat, and oats) and difficult food matrices such as spices (chili powder and black pepper), cocoa, and a traditional Chinese medicine (TCM), using a standardized testing procedure.

In addition, the AflaTest WB SR+ column was evaluated for its ability to bind sterigmatocystin, another potent liver carcinogen and precursor to aflatoxin.

METHODS

For Chili Powder, Black Pepper, Cocoa, and Medicinal Herbs

Weigh 10 g of homogenized sample into suitable container, add 100 mL of MeCN/H_2O (90/10 v/v) and blend for 3 mins

Pour blended mixture into fluted filter paper and collect filtrate

Take 10 mL of filtrate and mix well with 30 mL 2% Tween/PBS solution, filter diluted extract using a microfiber filter

Load 10 mL of diluted filtrate via syringe barrel connected to AflaTest WB SR+ column and let extract pass through column by gravity or one drop/sec

Pass 2x 10 mL of water through the column (1-2 drops/sec) and pump air through the column

Add 0.75 mL MeCN/MeOH (1:2 v/v) to column headspace, elute by gravity and leave for at least 3 mins, repeat

Vortex the eluate (1.5 mL), add 1.5 mL water and vortex again prior to HPLC/UPLC

For Corn, Wheat, and Oats

Weigh 25 g of homogenized sample, add 100 mL of MeCN/H_2O (80/20 v/v), and blend on high speed for 2 mins

Pour blended mixture into fluted filter paper and collect filtrate

Take 10 mL of filtrate and mix well with 40 mL PBS solution, filter diluted extract using a microfiber filter

Load 10 mL of diluted filtrate via syringe barrel connected to AflaTest WB SR+ column and let extract pass through column by gravity or one drop/sec

Pass 10 mL of purified water through the column (1-2 drops/sec)

Add 0.75 mL MeCN/MeOH (1:2 v/v) to column headspace, elute by gravity and leave for at least 3 mins, repeat

Vortex the eluate (1.5 mL), add 1.5 mL water and vortex again prior to HPLC/UPLC

HPLC conditions for aflatoxins

LC System:	Alliance e2695
Detection:	Multiwavelength Fluorescence Detector 2475 with PhCR Photochemical Reactor (P/N 600001222) Excitation 360 nm Emission 440 nm
Vials:	Deactivated Amber Glass 12 x 32 mm Screw Neck Vial (P/N 186000846DV)
Column(s):	Nova-Pak C18, 4 µm, 3.9 mm X 150 mm (P/N WAT086344)
Column Temp.:	25 °C
Sample Temp.:	25 °C
Injection Volume:	50 µL
Flow Rate:	0.8 mL/min
Mobile Phase	Water:methanol (55:45 v/v) Isocratic
Software	Waters Empower™

UPLC Conditions for aflatoxins

LC System:	ACQUITY UPLC H-Class with FTN SM
Detection:	ACQUITY Fluorescence Detector with large volume cell (P/N 205000609) Excitation 360 nm Emission 440 nm
Vials:	LCGC Certified Clear Glass Screw Neck Vial, 12 x 32 mm, 2 mL (P/N 186000307C)
Column(s):	ACQUITY UPLC HSS T3 1.8 µm, 2.1 x 100 mm (P/N 186009468)
Column Temp.:	25 °C
Sample Temp.:	25 °C
Injection Volume:	3-6 µL
Flow Rate:	0.3 mL/min
Mobile Phase	Water:methanol (55:45 v/v) Isocratic
Software	Waters Empower™

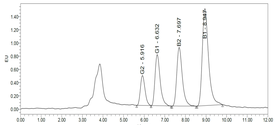


Figure 1: HPLC chromatogram from analysis of a black pepper sample spiked with 4.0 µg/kg total aflatoxin

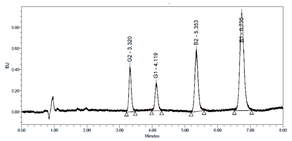


Figure 2: UPLC chromatogram from analysis of an extract of Fallopia Multiflora spiked with 5.0 µg/kg total aflatoxin

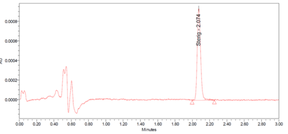
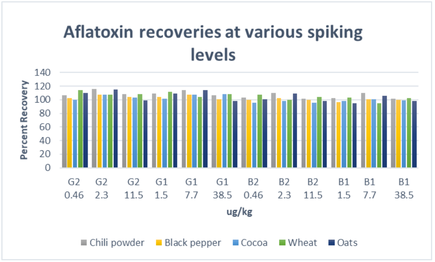


Figure 3: UPLC chromatogram from analysis of corn sample spiked with 25 µg/kg Sterigmatocystin

RESULTS



		Recommended Recovery (%)	Mean Recovery (%) (n=5)*				
Aflatoxins	Spike (µg/kg)	AOAC	Chili powder	Black pepper	Cocoa	Wheat	Oats
G2	0.46	40-120	107	102	100	114	110
	2.3	40-120	116	107	107	107	115
	11.5	60-115	108	104	103	108	99
G1	1.5	40-120	109	104	102	112	109
	7.7	40-120	114	107	107	104	114
	38.5	60-115	107	101	108	108	98
B2	0.46	40-120	103	100	95	107	101
	2.3	40-120	110	103	98	100	109
	11.5	60-115	102	100	96	104	98
B1	1.5	40-120	102	96	99	103	95
	7.7	40-120	110	101	101	95	106
	38.5	60-115	101	100	99	102	98

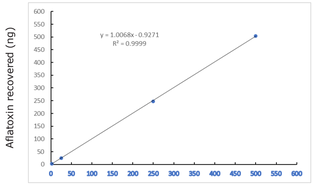
*Percent CV Values below 8% (not shown but available upon request)

Recovery from a traditional Chinese medicine- Fallopia Multiflora

		Recommended AOAC		Mean Observed Values	
Aflatoxins	Spike (µg/kg)	Recovery (%)	RSD, (%)	Recovery (%)	RSD, (%)
G2	5.0	75-120	8.0	99	3.1
	20	70-125	15	96	0.8
G1	5.0	75-120	8.0	105	3.1
	20	70-125	15	99	0.8
B2	5.0	75-120	8.0	96	4.1
	20	70-125	15	93	1.7
B1	5.0	75-120	8.0	103	3.3
	20	70-125	15	98	0.8

Total Aflatoxins Recovery in Spiked Corn Extract

Spike Level (ng)	Mean ng (n=3)	Mean % Recovery (n=3)	SD	CV (%)
2	1.92	96.17	0.02	1.08
25	24.96	99.84	0.54	2.17
250	247.75	99.1	3.79	1.53
500	503.95	100.79	1.92	0.38



Aflatoxins	LOD (ng)	LOQ (ng)
G2	0.008	0.015
G1	0.034	0.083
B2	0.007	0.012
B1	0.037	0.057

Sterigmatocystin Recoveries (%) in Spiked Corn Samples

Spike Level (µg/kg)	Mean % Recovery (n=3)	SD	CV (%)
0	0.0	0.0	-
4	92.4	7.3	7.9
5	97.8	5.6	5.7
25	94.6	1.1	1.2
50	98.4	0.4	0.4

DISCUSSION

Chromatography
A typical HPLC chromatogram, using isocratic conditions, is shown in Figure 1. The improvements in chromatographic efficiency offered by using ACQUITY UPLC columns with sub 2µm porous particles has been used to improve sensitivity, resolution, and speed. Figure 2 shows the efficient separation for aflatoxins within 7 minutes. Both columns provided excellent retention and peak shape for all the analytes and resulted in complete separation of all the aflatoxins of interest.

Sensitivity and selectivity
HPLC-FLD, using isocratic conditions, often after derivatization to boost sensitivity for AFB1 and AFG1, has been employed for the detection of aflatoxins for many years.[4] Unlike the iodine and Kobra cell, on-line post-column continuous photolytic derivatization performs the derivatization photochemically without additional chemicals added to the mobile phase. This approach has been validated as AOAC methods for the determination of aflatoxins in corn and peanuts.[5] The use of a large volume flow cell within the Waters ACQUITY Fluorescence Detector has nullified the need for any post-column derivatization and provided very low limits of quantification for aflatoxins.[6] The sensitivity of both methods, as shown by the signal/noise (S/N) for the peaks in the chromatograms and LOD/LOQ data, shows that they are suitable for checking compliance with regulatory maximum limits worldwide.

Trueness and Recovery
The trueness, expressed by recovery, was evaluated using the UPLC and HPLC data from the analysis of spiked samples. The mean recoveries for each of the commodities, at three or more concentrations, were within the range of 92% to 116 % and hence were within the criteria set out by the AOAC.[7] The results from the analysis of the traditional Chinese medicine meet the AOAC criteria for dietary supplement and botanicals.[8]

Cross-reactivity
The AflaTest WB SR+ immunoaffinity column binds aflatoxins B1, B2, G1, G2, and sterigmatocystin. The antibody also binds aflatoxins M1 and M2 (data not shown but available upon request).

CONCLUSION

- **High performance** - The AflaTest WB SR+ method exceeds AOAC method performance requirements giving > 90% mean recovery of aflatoxins B1, B2, G1, and G2 aflatoxins in both grains (corn, wheat, and oats) and difficult to analyze food matrices such as chili, cocoa, and traditional Chinese medicine.
- **Comprehensive** - VICAM's AflaTest WB SR+ column binds aflatoxin B1, B2, G1, and G2, as well as aflatoxin M1, M2, and sterigmatocystin
- **Flexible** - One standardized method is suitable for a range of different commodities.
- **Wide testing range**– AflaTest WB SR+ can detect total aflatoxin levels from 0.05ng to 500ng with recoveries greater than 90%.
- **Fast and easy**- The UPLC option provides an opportunity to shorten the analytical run time and the use of a large volume flow cell in the fluorescence detector eliminates the need for post-column derivatization.
- **Overall performance**- These methods have the desired sensitivity, selectivity, and overall performance to be used to check compliance with regulatory limits for aflatoxins in a wide range of crops and foodstuffs.

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