

## Application Note

# Determination of Aflatoxins in a Wide Range of Food and Agricultural Commodities Using Immunoaffinity Chromatography Column Clean-up Coupled 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 (nutmeg, red chili, black pepper, cocoa, roasted coffee, dog food, 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 satisfactory, between 82% and 119%, 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.

## Benefits

- High performance - the method meets AOAC method performance requirements
- Comprehensive - VICAM's AflaTest WB SR+ column binds aflatoxin B1, B2, G1, and G2
- Flexible - one standardized method is suitable for analysis of a range of different commodities

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## Introduction

Mycotoxins are toxic, secondary metabolites of molds that can occur in food and agricultural products via many contamination pathways, including production, harvest, storage, processing, and transport. 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, but are also responsible for significant losses in revenue and the potential erosion of brand and reputation.

Mycotoxins 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/port of entry. 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 in 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.<sup>1</sup> This emphasizes the need for increased analytical testing, 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, which are more time-consuming, but can be used to provide a more comprehensive view of the level of contamination.<sup>2</sup> Determination of aflatoxins is a challenging task. Sample matrices may be complex (often with high lipid content), placing additional pressure on laboratories to be able to determine the very low concentrations needed to verify compliance with regulatory limits. The combination of clean-up using immunoaffinity chromatography (IAC) columns, 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.<sup>3</sup> Typically, organic extracts require dilution with aqueous solvent prior to application on the IAC column, reducing the sensitivity of the method. Recently, VICAM launched a new IAC product, the AflaTest WB SR+, which can endure a high concentration of organic solvent, so no dilution is required. The AflaTest WB SR+ IAC column binds aflatoxin B1, B2, G1, and G2, in addition to M1, M2, and sterigmatocystin. The objective of this study was to evaluate the performance of the VICAM's AflaTest WB SR+ column, using two separate HPLC/UPLC platforms, for the determination of aflatoxin B1, B2, G1, and G2 in difficult food matrices; three spices, cocoa, roasted coffee, dog food, and a Traditional Chinese Medicine (TCM), using a standardized testing procedure.

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## Experimental

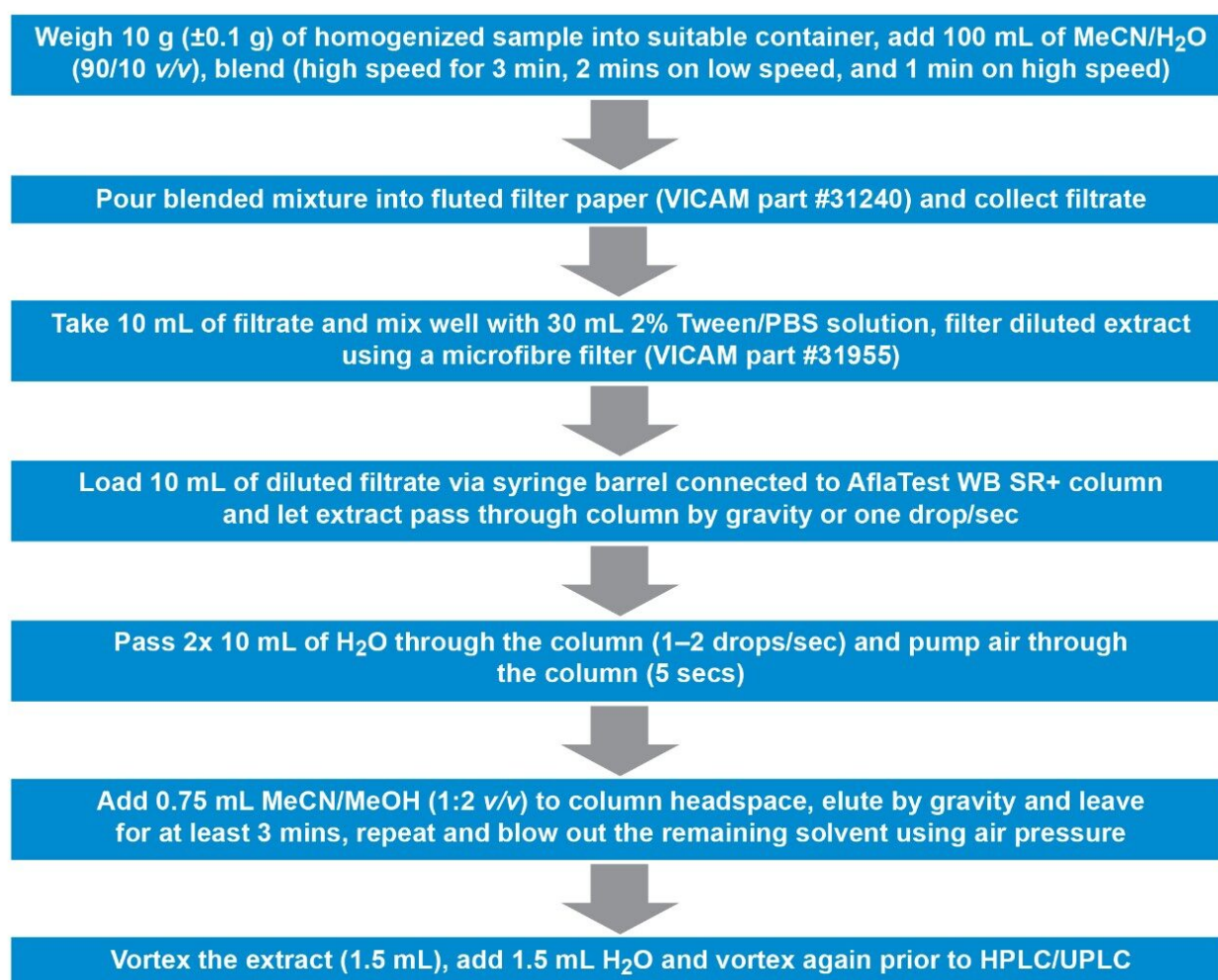
### Sample Description

Samples of the TCM *Fallopia multiflora*, spices (nutmeg, red chili, black pepper), cocoa, roasted coffee, and dog food, were obtained commercially and, apart from dog food, were ground into homogenous powders.

### Sample Extraction and Clean-up

An overview of the details of sample extraction and clean-up for aflatoxins is given in Figure 1. Test portions from

each sample were extracted using liquid extraction with acetonitrile and water (90/10, v/v) and homogenized in a blender. The filtered extract is then diluted with a buffer solution and applied to the AflaTest WB SR+ IAC column containing specific antibodies to aflatoxin. The aflatoxins bind to the antibody on the column. The column is then washed with water to remove co-extractives from the column, prior to elution of the aflatoxins with a mixture of methanol and acetonitrile. Subsequent determination of aflatoxins in the extract was by using HPLC/UPLC with fluorescence detection (see Figure 1 for more detail). More details on the use of the AflaTest WB SR+ is can be found [here <https://www.vicam.com/products/aflatest-wb-sr-plus>](https://www.vicam.com/products/aflatest-wb-sr-plus) .



*Figure 1. Overview of the details of sample extraction and clean-up for aflatoxins in a wide range of foodstuffs.*

Calibration standards were prepared in a mixture of acetonitrile, methanol, and water (1:1:2 v/v) from two different working standards containing combinations of aflatoxins with ratios of AFB1:AFB2:AFG1:AFG2 equal to 5:1:3:1 (Sigma 48487-U) and 0.3:1:0.3:1 (Supelco #CRM46304).

## Method Conditions

### HPLC Conditions

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:	Nova-Pak C <sub>18</sub> , 4 $\mu$ m, 3.9 mm x 150 mm (P/N WAT086344)
Column temp.:	25 °C
Sample temp.:	25 °C
Injection volume:	50 $\mu$ L
Flow rate:	0.8 mL/min
Mobile phase A:	Water (55%)
Mobile phase B:	Methanol (45%)
Run time:	12 min

### UPLC Conditions

LC system:	ACQUITY UPLC H-Class PLUS with FTN Sample Manager
Detection:	ACQUITY UPLC FLR 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:	ACQUITY UPLC HSS T3 1.8 $\mu\text{m}$ , 2.1 x 100 mm (P/N 186009468)
Column temp.:	25 °C
Sample temp.:	25 °C
Injection volume:	3 $\mu\text{L}$
Flow rate:	0.3 mL/min
Mobile phase A:	Water (55%)
Mobile phase B:	Methanol (45%)
Run time:	8 min

## Data Management

Chromatography software: Empower 3

## Method Validation

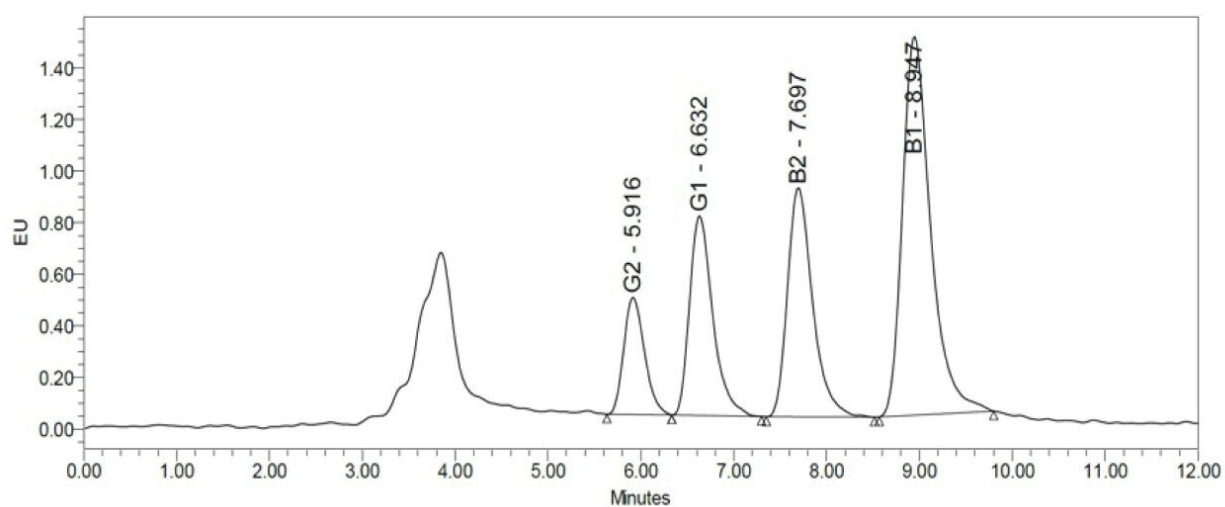
Validation was performed by replicate analysis of spiked test portions of samples thought to be blank. For the food ingredients, five replicates were prepared at 3 spiking levels, at 4, 20, and 100  $\mu\text{g}/\text{kg}$  total aflatoxins, whereas three replicates of the TCM samples were spiked at 5 and 20  $\mu\text{g}/\text{kg}$  total aflatoxins. The standard solutions used for spiking were the same as those described above. The following parameters were assessed: sensitivity, selectivity, trueness, and within-laboratory repeatability (RSD<sub>r</sub>).

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## Results and Discussion

### Chromatography

A typical HPLC chromatogram, using isocratic conditions, is shown in Figure 2. The improvements in chromatographic efficiency offered by using ACQUITY UPLC Columns with sub-2- $\mu$ m porous particles has been used to improve sensitivity, resolution, and speed. Figure 3 shows the efficient separation for aflatoxins within 7 minutes using UPLC. Both columns provided excellent retention and peak shape for all the analytes and resulted in complete separation of all the aflatoxins of interest.



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Figure 2. HPLC chromatogram from analysis of black pepper spiked with 4.0  $\mu$ /kg total aflatoxin.

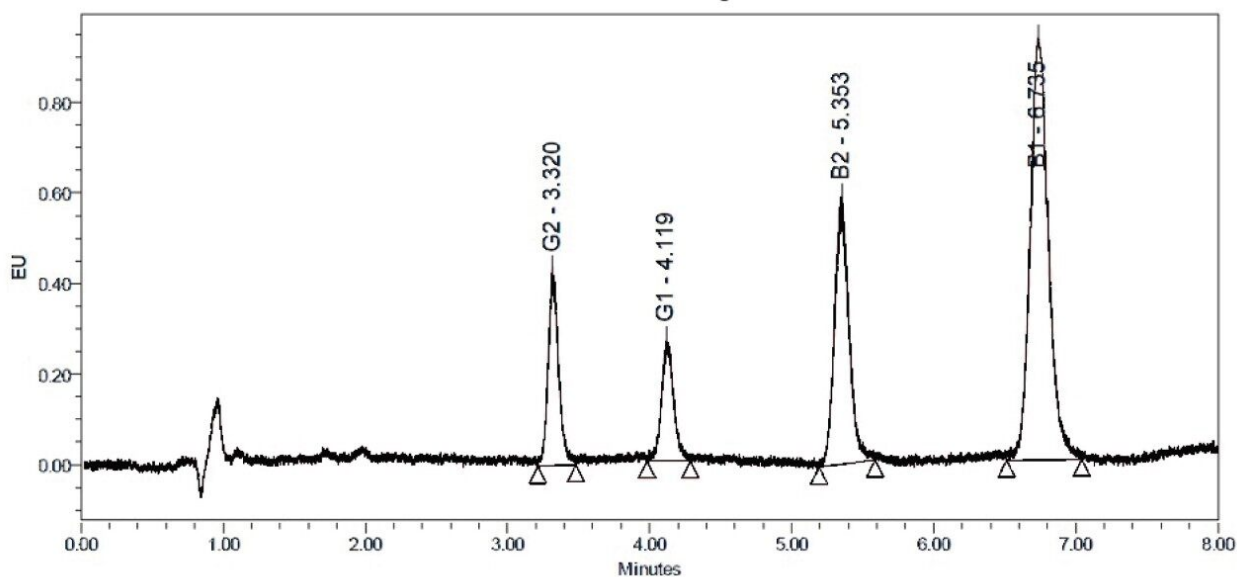


Figure 3. UPLC chromatogram from analysis of TCM spiked with 5.0 µg/kg total aflatoxin.

## 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.<sup>4</sup> 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.<sup>5</sup> The use of a large volume flow cell within the Waters ACQUITY UPLC FLR Detector has nullified the need for any post-column derivatization and provided very low limits of quantification for aflatoxins.<sup>6</sup> The sensitivity of both methods, as shown by the S/N for the peaks in the chromatograms above, shows that they are suitable for checking compliance with regulatory maximum limits worldwide.

No signals were detected in the extracts from most of the blank samples that could lead to detection of false reporting of non-compliant samples. For example, one can compare chromatograms from the analysis of black pepper in Figure 4. The exception was the detection of aflatoxins in the nutmeg sample. The data from the spike at 4 µg/kg was significantly impacted by the presence of aflatoxins in the nutmeg and so a full set of results were not reported at the lowest level. An indication of performance was provided by spiking at 10 µg/kg (n=2) to add to the complete data set at the higher levels. Blank correction was used to calculate the concentration in the spiked test portions.



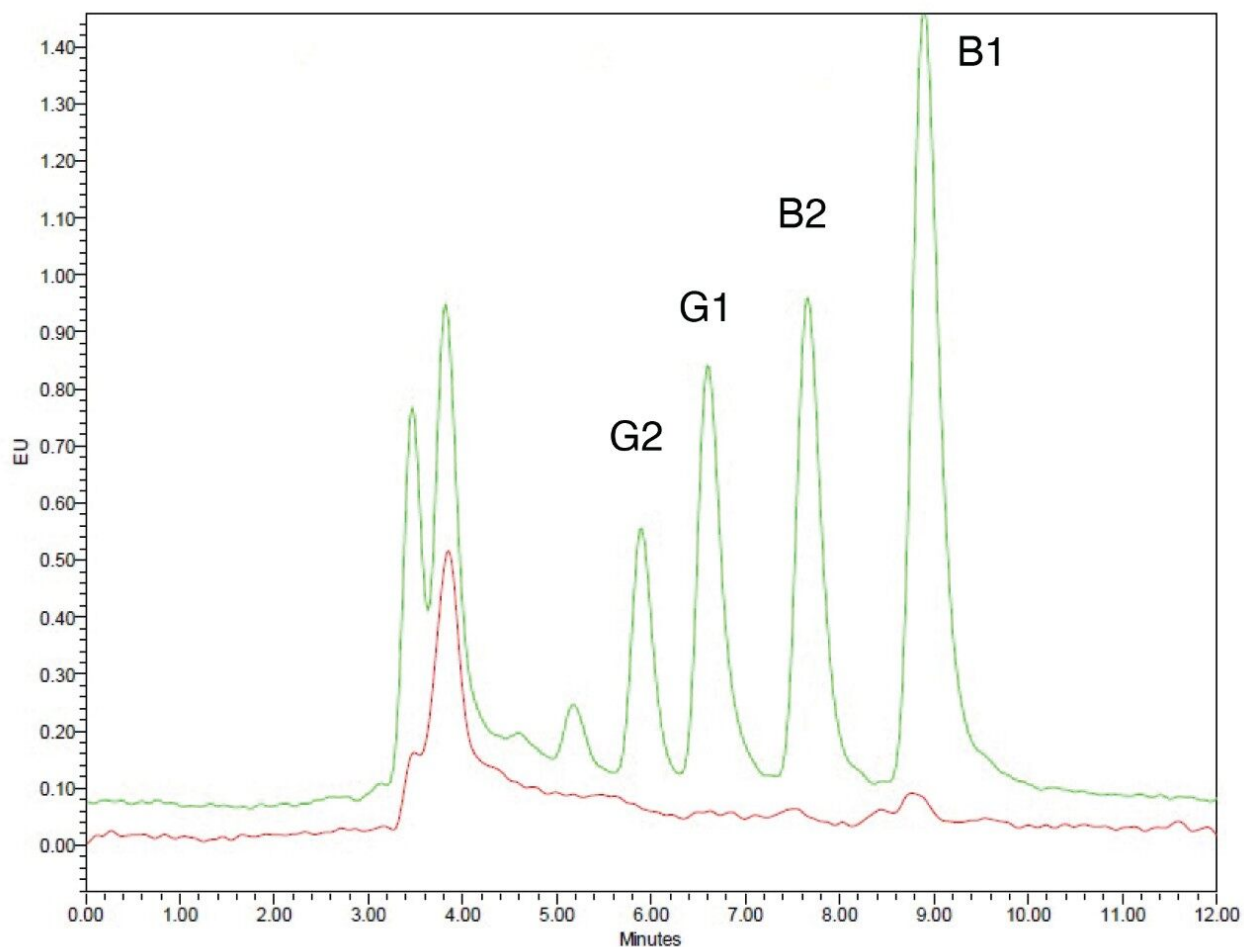


Figure 4. HPLC chromatograms from analysis of black pepper before (red) and after spiking (green) with 4.0 µg/kg total aflatoxin.

## Trueness and Repeatability

The trueness, expressed by recovery, was evaluated using the HPLC data from the analysis of the spiked sample. Mean values for recovery ranged from 82% to 119%, with three values slightly above the requirements defined by CODEX<sup>7</sup> and the European Commission<sup>8</sup> but within the range provided by AOAC<sup>9</sup> (see Table 1 and Figure 5). Repeatability ( $RSD_r$ ) was excellent with mean values between 0.8% and 9.5% (see Table 2 and Figure 6). As recommended values are often derived from the Horwitz Equation, at these very low concentrations, the recommended value for  $RSD_r$  is 14.5%. Table 3 shows the results from the analysis of the TCM using the AOAC criteria for dietary supplements and botanicals.<sup>10</sup>

Aflatoxins	Spike (µg/kg)	Recommended recovery (%)		Mean recovery (%)					
		Commission regulation (EC) No 401/2006	AOAC	Chili powder	Black pepper	Cocoa	Roasted coffee	Nutmeg	Dog food
G2	0.46	50-120	40-120	107	102	100	96	104#	87
	2.3	70-110	40-120	116	107	107	101	102	99
	11.5	80-110	60-115	108	104	103	98	101	86
G1	1.5	70-110	40-120	109	104	102	96	102#	84
	7.7	70-110	40-120	114	107	107	95	99	94
	38.5	80-110	60-115	107	101	108	95	99	84
B2	0.46	50-120	40-120	103	100	95	86	98#	82
	2.3	70-110	40-120	110	103	98	93	84	94
	11.5	80-110	60-115	102	100	96	92	94	82
B1	1.5	70-110	40-120	102	96	99	92	97#	88
	7.7	70-110	40-120	110	101	101	89	119	87
	38.5	80-110	60-115	101	100	99	91	91	82

Table 1. Summary of mean values for recovery at each spiking concentration (#10 g/kg n=2).

Aflatoxins	Spike (µg/kg)	Recommended RSD <sub>r</sub> (%)		Mean repeatability (% RSD <sub>r</sub> )					
		Commission regulation (EC) No 401/2006	AOAC	Chili powder	Black pepper	Cocoa	Roasted coffee	Nutmeg	Dog food
G2	0.46	14.5	16	5.5	2.1	6.9	4.4	0.4#	5.0
	2.3		16	2.7	2.3	1.4	1.5	3.1	5.9
	11.5		22	3.9	1.8	4.1	1.9	4.7	4.3
G1	1.5		16	6.8	2.4	6.3	7.8	0.6#	4.5
	7.7		16	3.9	5.4	2.2	1.6	5.0	5.6
	38.5		22	2.9	1.8	6.8	1.8	4.9	4.5
B2	0.46		16	6.5	2.4	6.2	3.7	0.6#	4.1
	2.3		16	4.2	3.5	1.8	2.4	5.5	6.4
	11.5		22	3.3	1.7	5.0	1.7	4.9	4.0
B1	1.5		16	7.9	4.5	6.3	7.4	1.3#	6.1
	7.7		16	4.9	3.9	2.4	2.5	9.5	7.2
	38.5		22	3.6	2.0	7.1	1.6	5.0	4.2

Table 2. Summary of mean values for repeatability at each spiking concentration (#10 g/kg n=2).

Aflatoxins	Spike (µg/kg)	AOAC recommendations		Mean observed values	
		Recovery (%)	RSD <sub>r</sub> (%)	Recovery (%)	RSD <sub>r</sub> (%)
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

Table 3. Summary of mean values for recovery and repeatability for TCM.

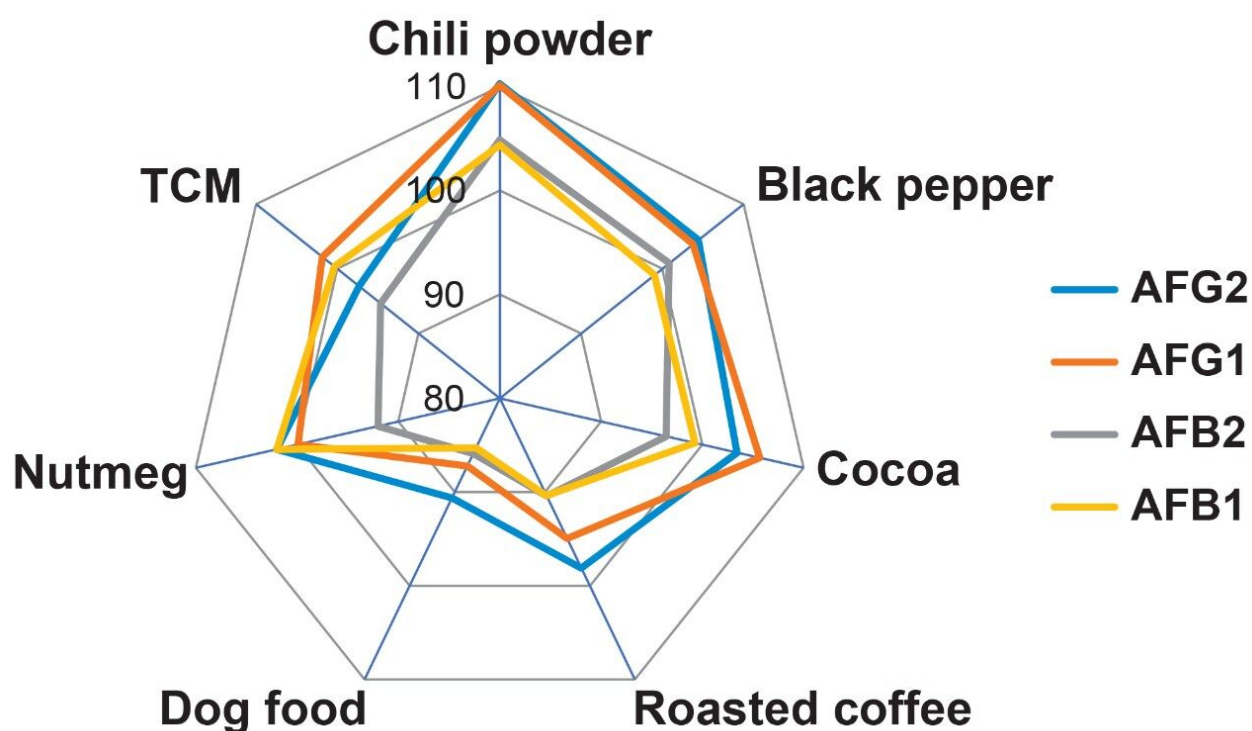


Figure 5. Radar plot of the overall mean values for recovery for the food commodities (%).

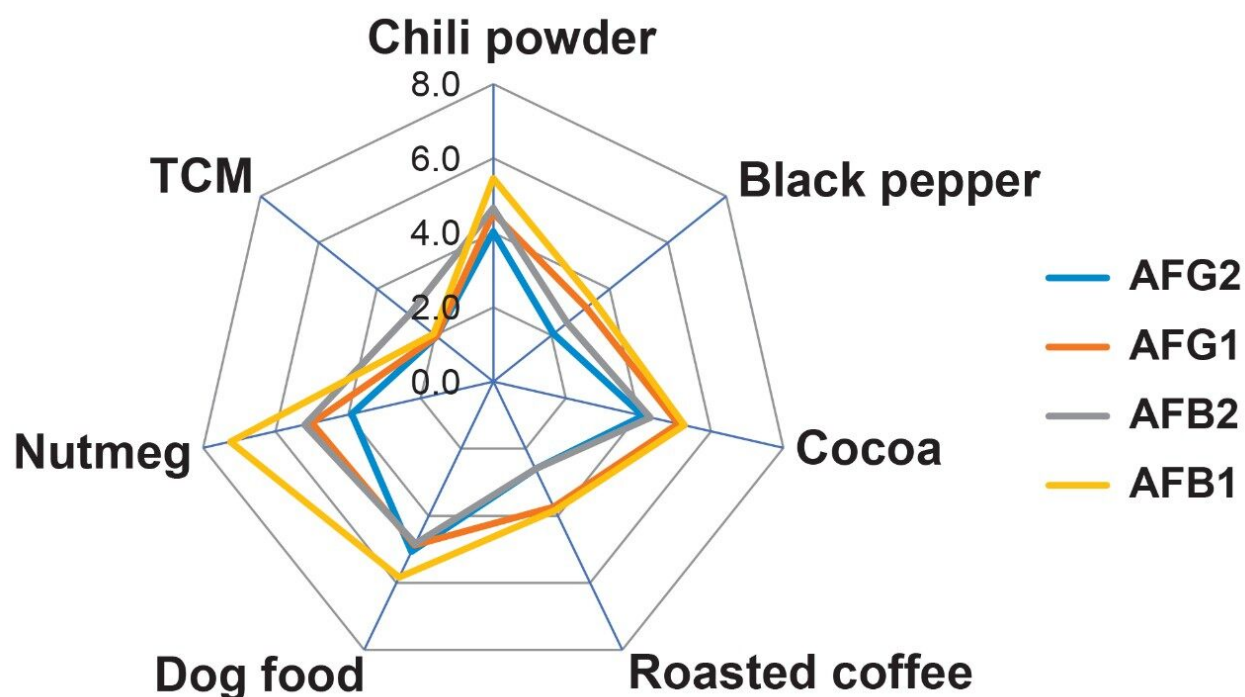


Figure 6. Radar plot of the overall mean values for repeatability for the food commodities (% RSD<sub>r</sub>).

## Conclusion

Using a 90% acetonitrile extraction with AflaTest WB SR+ IAC clean-up column can eliminate matrix co-extractives, even from analysis of complex commodities, with good recovery and precision. 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. This method has the required sensitivity, selectivity, and overall performance to be used to check compliance with regulatory limits for aflatoxins in a wide range of crops and foodstuffs.

## References

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