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Article

ANIMAL FOOD, PET FOOD, AND PLANT NUTRIENT METHODS

Development and Validation of a Method for Direct Analysis of Aflatoxins in Animal Feeds by Ultra-High-Performance Liquid Chromatography with Fluorescence Detection

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Abstract

Background and Objective: Aflatoxin (AF) contamination is one of the major regulatory concerns for animal feed. As feed is a complex analytical matrix, validated methods on AFs in feed are scanty. The available methods involve a derivatization step before AF analysis by high-performance liquid chromatography (HPLC) with fluorescence detection (FLD). The aim of this study was thus to develop and validate a simple and rapid method for direct analysis of AFs (AFB1, AFB2, AFG1, AFG2) in a range of animal feed matrices.

Methods: Feed samples were extracted with 80% methanol, followed by dilution with water and immmunoaffinity column cleanup. AFs were estimated using an ultra-high performance liquid chromatography (UHPLC) instrument. Use of a large volume flow cell in FLD allowed direct analysis of all AFs with high sensitivity. The method was thoroughly validated in a range of feed matrices.

Results: This sample preparation workflow minimized co-extractives, along with matrix interferences. In pigeon pea husk feed, the method provided a limit of quantification (LOQ) of 0.5 ng/g for each AF with recoveries of AF- B1, B2, G1, and G2 as 71.5, 75.6, 82.4, and 78.2%, respectively. The precision (relative standard deviation, RSD) was below 5%. A similar method performance was also recorded in other matrices, including wheat bran feed and poultry feed.

Conclusions: The optimized method is suitable for regulatory testing because it is simple, robust, cost-effective, and high throughput in nature, with high sensitivity and selectivity.

Highlights: Our workflow has provided a straightforward method for the analysis of AFs in a wide range of animal feed matrices with high sensitivity, selectivity, throughput, and cost-effectiveness. The method allowed a direct analysis of AFs by UHPLC-FLD without a step of derivatization.

Mycotoxins are the secondary metabolites produced by filamentous fungi that can contaminate food as well as animal feed during production, processing, and storage. Among mycotoxins, aflatoxins (AFs) are of major concern for animal feed because of their frequent detection and associated risks to animal health. For instance, while monitoring 830 animal feed consignments (by using immunoassay technique), Kang'ethe et al. (2013) found 80% of samples contaminated with AFB1, with 67% having contaminations above the maximum level (ML) set by the European Commission (1). Similarly, Kocasari et al. (2013)

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reported 55 and 61.7% of feed samples (used for dairy cattle and beef cattle) with contaminations of AFs in the range of 54.3–117 ng/g (2). In another study, Bilal et al. (2014) reported AFB1 contamination in 34.9% of feed samples with residues up to 11.4 ng/g (3).

AFs are difuranocoumarin derivatives produced by various strains of *Aspergillus flavus* and *A. parasiticus* (4) in a wide range of cereals (e.g., rice, wheat, corn), oilseeds (e.g., mustard, peanut), and animal feeds (e.g., oil cakes, maize feeds, wheat feeds). The International Agency for Research on Cancer (IARC) has classified AFB1 as a group 1 human carcinogen (5). Besides, AFs are also responsible for harmful effects on household animals due to both acute and chronic toxicities. They are reported to cause acute liver damage, induce tumors and liver cirrhosis, and also result in teratogenic effects (6). Young animals are most susceptible to AF toxicity, where oral ingestion might cause gastrointestinal dysfunction, reproduction malfunction, hepatotoxicity, and anemia (7, 8).

Cereals- and oilseed-byproducts are often used as components of feed concentrates for domestic animals. Considering the levels of AF detections and their potential toxic effects to animals, the regulatory agencies across the world have recommended ML for AFs with respect to different feeds and age of animals. According to the United States-Food and Drug Administration (US-FDA), the action level for AFB1, AFB2, AFG1, and AFG2 in peanut and corn-based animal feeds for beef cattle and swine is 200 ng/g, whereas, for immature cattle, it is 20 ng/g (9). On the other hand, according to the European Commission Directive 2003/100/EC, the ML of AFB1 for "all feed materials" (with moisture level of 12%) is set at 20 ng/g with exceptions in "complete feeding stuff" (with moisture level of 12%), where the suggested ML is 10 ng/g for calves and lambs, and 5 ng/g for dairy animals (10).

Accurate analysis of AFs is paramount for evaluating the safety of animal feed. As animal feed is at the beginning of the food chain, any contamination of feeds may reach the final consumer through food matrices, such as milk, eggs, and meat products. To verify compliance with regulatory limits, and to obtain an accurate understanding of the prevalence of AFs in animal feed, analytical methods must be developed which are fit-for-application in the above-stated matrices.

Over the past two decades, HPLC-based methods have largely been reported for determination of mycotoxins in food and feed (11–14). Because of its high sensitivity, accuracy, and cost-effectiveness, HPLC coupled with fluorescence detection (FLD) is widely used for determination of AFs (15, 16). To enhance sensitivity, these methods involved a derivatization step for AFs before measurement by FLD. For example, Sahin et al. (2016) reported an HPLC-FLD method in dairy cattle feed, which provided the limits of quantification (LOQ) of 0.181, 0.153, 0.197, and 0.168 ng/g for AFB1, AFB2, AFG1, and AFG2, respectively, after derivatization with potassium bromide (17). Application of this method in monitoring reported 26.3% of the cattle feed samples to be contaminated with AFs at various levels. In another study, Mohammed et al. (2016) also reported an LOQ of 1.10 ng/g for AFB1 in sunflower seed cake using a similar HPLC-FLD method involving derivatization (18).

In recent years, liquid chromatography tandem mass spectrometry (LC-MS/MS) has been gaining significance due to the possibilities of multi-mycotoxin monitoring. For example, Monbaliu et al. (2009) reported an LC-MS/MS method for simultaneous analysis of 23 mycotoxins, which included AFs, in three different feed matrices, namely sow feed, wheat, and maize (19). Elsewhere, Grio et al. (2010) developed a method for simultaneous analysis of AFs, and ochratoxin A in animal feed and pet food by UHPLC-MS/MS (20). In other study, Zhang et al. demonstrated effective applications of stable isotope dilution assay (SIDA) for multi-mycotoxin analysis by LC-MS/MS (21–23).

The AOAC official method for AFB1 in cattle feed involves a derivatization step through KOBRA cell (24). Earlier, we reported a novel method for direct analysis of AFs in a wide range of food matrices, which involved a large volume FLD flow cell (25). However, we have not come across with any FLD-based method where AFs in feed are estimated without a derivatization step. Given this, the current study was undertaken to develop and validate a direct analysis method for AFs with a wide applicability in feed matrices. This method holds promise to evolve as an official method for regulatory analysis of AFs in animal feeds.

Experimental

Apparatus

Equipment used for the sample preparation involved a heavy-duty mixer (Vishvakarma Machine Tools, Rajkot, India), an orbital shaker (Scigenics Instruments, Mumbai, India), a high-speed centrifuge (Kubota Corp., Tokyo, Japan), a micro-centrifuge (Dlab Instruments, Hyderabad, India), and a vacuum manifold (Waters India Pvt. Ltd., Bangalore, India).

Chemicals and Reagents

(a) *Reagents*.—Methanol, acetonitrile (both HPLC gradient grade), acetic acid, sodium chloride, and Tween 20 (polyoxyethylene sorbitan monolaurate) were procured from Merck (Bangalore, India). Water used for UHPLC analysis was obtained through a water purification system (Pall India Pvt. Ltd., Bangalore, India).

(b) *Reference standards*.—Certified reference standards of AFB1, AFB2, AFG1, and AFG2 having >95% purity were purchased from Merck (Bangalore, India).

(c) *Reference materials*.—Two reference materials, *viz*. rice (Lot No. 01-NC-AFBRI-17; AFB1= 15.1±1.1 ng/g, AFB2= 2.1±0.2 ng/g), and corn (Lot No. IAFCOR0118-01; AFB1= 10.72±0.96 ng/g, AFB2= 1.23±0.16 ng/g) were obtained from Trilogy Analytical Pvt. Ltd. (Hyderabad, India), and used to test the method accuracy.

(d) *Columns for cleanup*.—An OASIS® hydrophilic lipophilic balance—solid-phase extraction (HLB SPE) cartridge (3 mL, 30 mg, Waters Corp., Milford, MA, USA) and monoclonal antibody-based AFLATEST® immunoaffinity column (IAC, 3 mL, VICAM, Milford, MA, USA) of capacity 100 ng (AFs) were tried for cleanup. The recoveries of AFs through these columns ranged between 95–100% as claimed in the certificate of analysis provided by the manufacturers.

(e) *Standard solutions*.—Stock solutions (500 μg/mL) were prepared by dissolving each standard (5 mg) in methanol (10 mL) in amber-colored glass vials. Intermediate concentrations were prepared by mixing of the stock solutions, followed by appropriate dilution with methanol. The calibration standards were prepared by serial dilution of intermediate solutions (0.05–10 ng/mL) in 1:1 ratio of methanol–water (+ 0.1% acetic acid, v/v).

Ultra-High-Performance Liquid Chromatography with Fluorescence Detection (UHPLC-FLD)

An ACQUITY UHPLC system fitted with FLD (Waters Corp., Manchester, UK) was used for analysis of AFs. The FLD

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consisted of a large volume (13 μL) flow cell to support high sensitivity direct analysis of AFs. A UPLC® BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters Corp.) was used for chromatographic separation. The column was maintained at 40°C. The UHPLC flow rate was maintained at 0.4 mL/min, with injection volume set at 10 μL. The mobile phase comprised 0.1% acetic acid in water (A), methanol (B), and acetonitrile (C) in the ratio of 64:18:18. The instrument was operated in isocratic mode with a total run time of 5 min. The excitation and emission wavelengths of FLD were set at 365 and 456 nm, respectively.

Test Matrices

Feed matrices, namely, pigeon pea husk (PPH), poultry feed (PF), and wheat bran feed (WF) were procured from a local feed manufacturer (Pune, India). Test portions (1 kg) were finely ground and sieved through a square mesh of 1 mm size. These grounded samples were stored in air tight polytetrafluoroethylene (PTFE) containers at room temperature under darkness, and subsequently used for the method optimization and validation studies. The method accuracy (recovery, %), and repeatability (RSD, %), were initially evaluated in PPH at 0.5, 2, and 4 ng/g levels. The method was further validated in PF and WF matrices at 2 and 4 ng/g levels.

Sample Preparation

(a) *Extraction*.—The samples were extracted by a previously reported method (25), with some modifications. Initially, 12.5 g of sieved sample was drawn in a 250 mL capacity PTFE bottle. To it, 12.5 g of water was added and mixed thoroughly. Furthermore, the extraction solvent (methano–water, 100 mL, 8:2, v/v), and NaCl (5 g) were added and shaken for 30 min. The extract was then centrifuged at 2800 ×*g* for 5 min. An aliquot of 3 mL was drawn, and again centrifuged at 5600 ×*g* for 5 min. The supernatant (3 mL) was diluted with 12 mL of distilled water. Finally, this diluted solution was mixed with 0.1 mL of Tween 20 to reduce turbidity.

(b) *IAC cleanup*.—The diluted sample was loaded on an IAC and was allowed to pass through without application of any vacuum. The column was washed with 10 mL of distilled water and eluted with methanol (2 × 0.5 mL). This extract (1 mL) was evaporated slowly to dryness, then reconstituted in 0.5 mL methanol–water [acidified with 0.5% acetic acid (1:1)], and finally injected into the UHPLC-FLD instrument.

(c) *HLB SPE*.—A separate experiment was conducted to substitute IAC cleanup with solid-phase extraction, as per the protocol reported in grains and grain products (26). At first, the column was conditioned with 2 mL of methanol, and this was followed by equilibration with 2 mL of water. The diluted sample was loaded on the cartridge and allowed to pass through under gravity. Afterward, the cartridge was washed with 2 mL of water and eluted with 2 mL of methanol–water [acidified with 0.5% acetic acid (1:1)]. Finally, the extracted sample was injected into UHPLC-FLD for determination.

Method Validation

A single-laboratory validation experiment was carried out, where the method performance was evaluated in terms of linearity (0.05–10 ng/g for all AFs), sensitivity, LOQ, limit of detection (LOD), accuracy (recovery, %), and precision. In PPH, the recovery and precision studies were conducted at 0.5, 2, and 4 ng/g levels. In PF and WF, the same was conducted at 2 and 4 ng/g levels.

Analysis of Market Samples

Different animal feed samples (10 each), namely maize feed (MzF), peanut cake (PC), pigeon pea husk (PPH), wheat bran feed (WF), poultry feed (PF), mixed animal feed concentrate (MAF), and dog feed (DF) were purchased from local markets of Pune, India. These samples were analyzed by the optimized method. The positive samples were analyzed in six replicates to check method precision.

Results and Discussion

Sample Preparation

Grinding and sieving through a square mesh of 1 mm in size resulted in a fine powder. The recoveries of all four AFs in PPH feed ranged between 70–100%. The feed matrices derived from peanut, maize, and soybean had oily and starchy components. The UHPLC-FLD analysis of feed extracts without a cleanup showed high levels of matrix interferences in the chromatograms, which masked the signals of AFs. Cleanup through an HLB cartridge failed to remove these co-eluting co-extractives, and thus selective detection and quantification of individual AFs was difficult. On the other hand, when the extract was passed through an AFLATEST® IAC, it proved quite effective in removing the co-extractives, and as a result, the chromatogram exhibited a smooth baseline with no interferences (Figure 1).

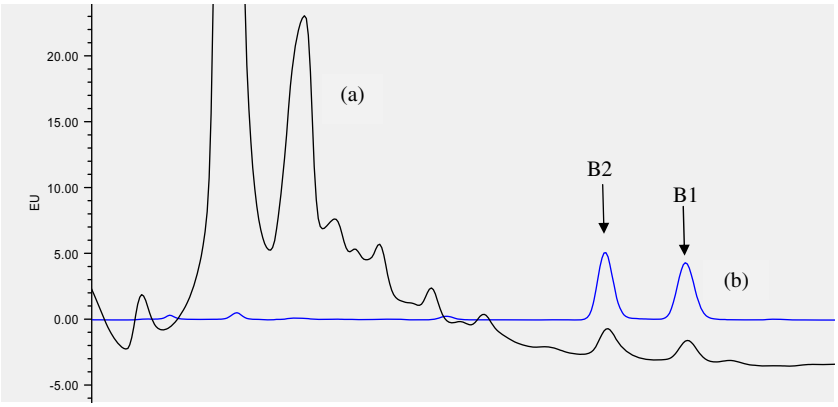

Figure 1. A chromatogram showing peaks of B1 and B2 after cleanup with (a) HLB and (b) IAC in an incurred MzF sample.

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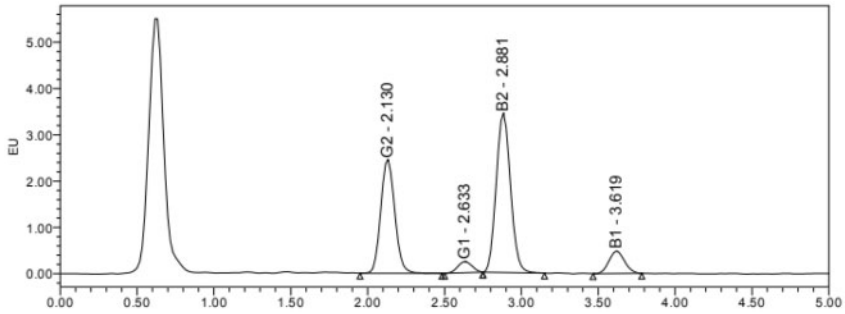

Figure 2. A chromatogram of PPH feed, when spiked at LOQ level (0.5 ng/g of each AF).

So, the IAC cleanup was selected for the final method. The IAC cleanup procedure, however, took a longer time for loading, washing, as well as elution. The addition of Tween 20 to the final extract prior to loading onto the IAC cartridge speeded up the elution process as this strategy cleared turbidity (27) of the extract. Being a surfactant, the addition of Tween 20 improved the solubility of the suspended particles and maintained hydrophilic lipophilic balance, which in turn reduced turbidity of extracts in all feed matrices (28).

The final method, involving sample extraction with methanol–water (80:20), followed by IAC cleanup showed acceptable recovery and repeatability for all AFs. The recoveries of all four compounds were within 70–110%, with high precision at LOQ and higher levels.

Chromatography

The method provided a chromatographic runtime of only 5 min, which is much less than the earlier methods that demonstrated a longer runtime of 20 min (29) and 30 min (30). The unique combination of the optimized emission wavelength (456 nm) and excitation wavelength (365 nm) provided selective detection, and quantification of AF–G2, G1, B2, and B1 with the highest signal intensities [signal-to-noise (S/N) ratio]. The compound-specific retention times were 2.1, 2.6, 2.8, and 3.6 min for AF–G2, G1, B2, and B1, respectively, which were repeatable across the batches of analysis with deviations within ±0.1 min. A chromatogram showing detection of each AF, when spiked at LOQ level (0.5 ng/g of each AF) in PPH feed, is given in Figure 2.

Method Validation

The performance of the method was assessed using the AOAC and the EU criteria (24, 31, 32), which are described below.

(a) *Linearity and sensitivity*.—For each compounds, calibration linearity was established in the range of 0.05 to 10 ng/g, with correlation coefficient of >0.999 (*See* Supplemental Figures_calibrations). The LOQ (S/N > 10) of AFs for PPH feed was 0.5 ng/g, whereas, for PF and WF, the LOQ was 2 ng/g.

(b) *Accuracy, recovery, and repeatability*.—For both (rice and corn) reference materials, the differences in the measured concentrations of AFB1 and AFB2 were within 10% of the reference values. This established confidence on method accuracy. The method, when evaluated at the LOQ level (0.5 ng/g) in PPH feed (Figure 2), provided the recoveries of 71.5, 75.6, 82.4, and 78.2% for AF–B1, B2, G1, and G2, respectively. The recovered concentrations at various spiking levels are presented in Table 1. At 2 ng/g, the recoveries of AF–B1, B2, G1, and G2 in PPH were 91.4, 95.2, 85.4, and 79.3%, respectively. At 4 ng/g, the corresponding recoveries were 82.3, 86.1, 91.4, and 80.3%, respectively. The WF matrix, which predominantly consisted of wheat bran (33), had the recoveries of 104.6 (AFB1), 86.5 (AFB2), 70.1 (AFG1), and 70.3% (AFG2), when spiked at 2 ng/g each. For the same matrix, at 4 ng/g level, the recoveries were 86.3 (AFB1), 78.0 (AFB2), 77.8 (AFG1), and 70.3% (AFG2). Values for recovery from analysis of spiked PPH and WF (33) matrices were within the range 70–105%. Extraction of the PF matrix, which mainly comprised maize, soybean meal, and peanut cake (34), generated an oily extract, and the recoveries were lower, in the range of 70–77% (Tables 1 and 2). In all cases,

Table 1. Recovered concentrations of AFs in feed matrices ($n = 6$)

Analyte	Spike level, ng/g	PPH	PF	WF
G2	0.5	0.36	ND[a]	ND
	2	1.59	1.53	1.41
	4	3.29	2.82	2.85
G1	0.5	0.38	ND	ND
	2	1.71	1.42	1.40
	4	3.44	3.07	3.11
B2	0.5	0.41	ND	ND
	2	1.90	1.51	1.73
	4	3.66	3.06	3.12
B1	0.5	0.39	ND	ND
	2	1.82	1.51	1.73
	4	3.21	3.06	3.12

[a] ND = Not done.

Table 2. Recovery (%) ± RSD of AFs in feed matrices ($n = 6$)

Analyte	Spike level, ng/g	PPH	PF	WF
G2	0.5	71.50 (± 4.9)	ND[a]	ND
	2	79.32 (± 4.8)	76.60 (± 4.7)	70.30 (± 3.2)
	4	82.33 (± 4.9)	70.52 (± 3.6)	71.29 (± 2.3)
G1	0.5	75.60 (± 4.6)	ND	ND
	2	85.41 (± 4.9)	70.95 (± 4.3)	70.10 (± 4.1)
	4	86.12 (± 4.2)	76.80 (± 4.6)	77.84 (± 4.3)
B2	0.5	82.35 (± 4.8)	ND	ND
	2	95.20 (± 4.6)	75.80 (± 4.8)	86.46 (± 4.8)
	4	91.41 (± 4.1)	76.13 (± 4.9)	78.04 (± 4.0)
B1	0.5	78.15 (± 4.0)	ND	ND
	2	91.40 (± 3.3)	71.99 (± 4.6)	104.61 (± 4.3)
	4	80.35 (± 4.2)	76.20 (± 4.2)	86.29 (± 3.9)

[a] ND = Not done.

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Table 3. Estimated levels of AFs in feed samples along with RSDs ($n = 6$)

Analyte	MzF	WF	PC	PF	PPH	AFC	DF
G2	1.49 (±10.42)[a]	ND[b]	ND	ND	ND	ND	ND
G1	1.48 (±10.11)	ND	1.50 (±10.54)	ND	ND	ND	ND
B2	1.92 (±11.48)	0.53 (±4.17)	7.13 (±9.98)	0.66 (±11.52)	0.08 (±5.02)	0.10 (±10.08)	1.53 (±11.22)
B1	4.43 (±10.55)	ND	32.54 (±12.65)	0.66 (±10.21)	0.10 (±4.85)	1.44 (±9.98)	6.87 (±8.89)
Total	9.31	0.53	41.17	1.32	0.18	1.54	8.40

[a] RSD %.
[b] ND = Not Detected.

the repeatability (RSD) was below 5%. The starchy nature of WF matrix and oily nature of PF matrix interfered with the cleanup procedure, and the addition of surfactant (Tween 20) did not improve the method performance. When WF and PF matrices were spiked at 0.5 ng/g, the S/N was less than 10:1 for all AFs. Hence, validation of the method in these matrices was conducted at 2 and 4 ng/g levels.

As noted, the LOQ (0.5 ng/g) established in this study was much lower than the ML of EC (the lowest level of 5 ng/g for AFB1) and US-FDA (20 ng/g for total AFs). The method performance at this LOQ was in compliance with the analytical quality control requirements (32). Recoveries of AFs were repeatable, with precision RSD (%) less than 5% for all AFs.

Market Sample Analysis

The results pertaining to market samples are presented in Table 3. In all the positive samples, except in peanut cake and dog feed, the concentrations were below the lowest ML of EC (5 ng/g for AFB1). Peanut cake and dog feed were found to be contaminated with AFB1 at 32.5 ng/g and 6.9 ng/g, respectively. The precision (RSD) of all feed samples ranged between 4.2–12.7% (Table 3), which established satisfactory repeatability and robustness of the method.

Conclusions

The method illustrated in this research is simple, rapid, sensitive, cost-effective, and reproducible. Considering the method applicability and compliance to MLs, it can be suitably implemented for regulatory testing of AFs in animal feed matrices. In the future, the method can be subjected to multi-laboratory validation, and recommended to regulatory agencies for consideration as an official method for analysis of AFs in feed.

Conflict of Interest

The authors declare no conflict of interest.

Supplemental Information

Supplemental information is available on the *J. AOAC Int.* website.

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