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Validation of High Performance Liquid Chromatography with Fluorescence Detector Methods for Determination of Aflatoxins in Different Food and Animal Feed Samples

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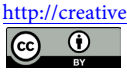

Abstract

Method validation for quantitative analysis of aflatoxins, AFB1, AFB2, AFG1 and AFG2 in sorghum, peanut butter, groundnut and animals feed is presented. Aflatoxins were extracted with a mixture of methanol: acetonitrile: water (60:30:10) and cleaned with Alfa test IAC chromatography before analysis by High Performance Liquid Chromatography coupled with fluorescence detection (HPLC-FLD) by adopting an isocratic chromatographic system using a mobile phase consisting acetic acid: acetonitrile: methanol (59:14:27), the separation of the four aflatoxins was achieved in less than 15 minutes. Calibration curves were linear over the concentration range from 2 - 18 ng/mL for AFB1 and AFG1, 0.4 - 3.6 ng/mL for AFB2 and AFG2, respectively. The LOD and LOQ in spiked samples were found to be 0.02 and 0.05 µg/kg for both AFB1 and AFG1, 0.01 and 0.03 µg/kg for both AFB2 and AFG2. The mean recovery values were in range from 84.2% to 96.9% was obtained. Five samples were found to be contaminated with aflatoxins and the total aflatoxins ranged from 0.02 to 3.26 µg/kg were obtained. Nineteen different samples were found to be contaminated with aflatoxins; the total aflatoxins ranged from 0.27 to 10.48 µg/kg were obtained. The highest total aflatoxins value was obtained in animal feeds.

Keywords

Aflatoxins, HPLC-FLD, Peanut, Peanut Butter, Sorghum and Animal Feeds

1. Introduction

Aflatoxins (AFs) are highly toxic and carcinogenic compounds, being structurally related toxic metabolites produced by Aspergillus flavus, *Aspergillus parasiticus* and *Aspergillus nomius*.

Among which AFB1, AFB2, AFG1, AFG2 are frequently found in foodstuffs, animal feeds and medicinal plants. In general, these mycotoxins are carcinogenic, mutagenic, teratogenic, and genotoxic posing a significant threat to human and animal health through ingestion [1] [2] [3].

Aflatoxins contaminations of peanuts are one of the most important factors determining the quality of peanuts and peanuts products and have caused significant financial losses for producing and exporting countries [4] [5]. The toxicities of mycotoxins have led many countries to set up legislations for their limits in foods that are intended for human or animal consumption [6] [7].

The development of multi-mycotoxin methods allows the determination of several co-occurring mycotoxins in a single run, which is more cost-effective and faster compared to conventional single determination methods. Nowadays, many methods for simultaneous analysis of mycotoxins in foodstuffs, spices and medicinal plants are reported in the literature. These methods include thin-layer chromatography (TLC) [8], enzyme linked immunosorbent assay (ELISA) [9], gas chromatography (GC) and liquid chromatography (LC) with various detectors, for the detection of multiple mycotoxins [10]-[17]. Among these methods available, GC-MS or LC-MS or LC-MS/MS methods are becoming increasingly widespread in recent years. Nevertheless, their application is limited and not available in many quality laboratories worldwide due, not only to their complexity and cost, but also due to the complexity of the sample matrices.

HPLC is considered as the most popular technique in mycotoxins laboratories, the literature concerning simultaneous mycotoxin determination using HPLC is scattered. An HPLC method has been reported for simultaneous determination of AFs and OTA [14] [15] [16] and AFs, OTA and ZEA [17] [18] multi-mycotoxin method for AFs, OTA, ZEA, DON, FB1 and FB2 in corn using a HPLC-FLD and UV detection was developed [19], HPLC-FLD for simultaneous determination of AFTs and OTA in ginger was developed [20]. A sensitive HPLC-FLD method for analysis of AFs (AFB1, AFB2, AFG1 and AFG2) and ZON, α-ZOL, and β-ZOL in Coix seed samples was recently reported [21].

HPLC methods for analysis of AFs in peanut [22], cereal products [23] and animal feeds [24] were developed, however the analysis time for these methods is too long, moreover these methods were applied for only one type of samples. Therefore fast and sensitive HPLC method for analysis of aflatoxins in different food samples is important. This study is aimed to validate HPLC method with FLD for simultaneous analysis of AFB1, AFB2, AFG1, and AFG2 in various samples such as animal feeds, sorghum, peanut and peanut butter. To the best of our knowledge no HPLC method for simultaneous analysis of mycotoxins in various food samples has been published in the literature.

2. Materials and Methods

2.1. Chemicals and Reagents

Methanol (CL chem. Lab) HPLC grade, n-hexane (CARLO ERBA) RPE, hydrochloric acid, 37% dichloromethane were obtained from Scharlau, (Barcelona, Spain), sodium chloride (CARLO ERBA, MARSEILLE, France), acetone, benzene (AnalaR), acetonitrile HPLC grade, trifluroacetic acid (TFA) and chloroform were purchased from (ROMIL, UK), phosphoric acid (AnalaR) and acetic acid were obtained from Riedel-de Haen (Hannover, Germany), sodium sulphate anhydrous was purchased from Prabhat Chemicals (Mumbai, India), citric acid, diethyl ether (Labtech Chemicals) were all profiled by El Walidien Trade and Investment Co. (Khartoum, Sudan). AFB1, AFB2, AFG1 and AFG2 were purchased from Immunolab GmbH (Kassel, Germany).

2.2. Apparatus and HPLC Conditions

The HPLC conditions were Supelcosil LC18 column, 150 × 4.6 mm internal diameter (I.D.), 5 micron particle size; oven temperature 30˚C, fluorescence detection at excitation 360 nm and emission 476 nm, mobile phase consisted of acetic acid (0.1%): acetonitrile: methanol (59:14:27) was used. The flow rate of the mobile phase was maintained at 1.0 mL·min^{-1} and the injection volume of sample solution was 20 µL.

2.3. Method Development

2.3.1. Samples

In order to find blank samples (groundnut, peanut butter, sorghum and animals feed), five samples from each kind were determined using approved single aflatoxins determination method and the sample with the lowest contamination level (not contaminated) of each was used as a blank sample for future spiking during the study (in-house reference materials). These selected samples were used for spiking and validation method during the study.

2.3.2. Sample Preparation for Analysis

Applicability and verification of the method was performed by analysis of replicate spiked samples. Thus, blank samples (groundnut, peanut butter, sorghum, and animals feed) were spiked with an appropriate amount of mixture of aflatoxins to achieve 2.5, 5.0, 1.25, and 2.5 µg/kg for AFB1, AFG1, AFB2 and AFG2, respectively. The spiked samples were mixed well and then kept for 2 h at room temperature.

2.3.3. Extraction of the Samples

Ten gram of a representative powdered samples and spiked samples were accurately weighed and transferred to a glass-stoppered flask, 50 mL of a mixture of methanol, acetonitrile and water (60:30:10) was added and mixed well for 10 minutes on vortex. The mixture was centrifuged for 10 minutes at 5000 r/min, the clean solution was collected. The extraction was repeated with other 50 mL

of mixture and the extract was combined.

2.3.4. Clean-Up and Detection of the Different Mycotoxins

Twenty mL of the extracted solution was taken into a 50 mL centrifuge tube; 30 mL of 0.1 M Phosphate Buffer Solution was added, then mixed well, and filtered through glass microfiber paper. 25 mL of filtrate (equivalent to 1 g of test sample) was collected into a 25 mL graduated cylinder, and proceed immediately with Alfa test IAC chromatography.

The top caps of the column were removed and connected to the reservoir; the end cap of the column was removed. The liquid in the column was passed through until the liquid is about 2 to 3 mm above the column was remained. 25 mL of filtrate was passed through the column by gravity force. The column was dried and washed with 5 mL of Phosphate Buffered Saline Solution and then with 5 mL of water. The column was left to dry and 3 mL of air was forced through the column with a syringe. The aflatoxins were eluted with 2 mL methanol from the column and evaporated and derivatized with TFA solution.

2.4. Analytical Method Performance

The analytical methods were validated with respect to specificity, selectivity, linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, accuracy, and stability [25]. The validation procedure was performed according to Decision 2002/657/EC [26].

2.4.1. Specificity

Specificity is the ability of the method to measure the analyte in the presence of its potential impurities. It was demonstrated by showing that samples were free of interference and was assessed by the absence of interference in the same retention times as examined for mycotoxins.

2.4.2. Selectivity

The selectivity of the method was assured by the use of immunoaffinity purification techniques, (SPE) cartridges column, and a selective fluorescence detector. To test the selectivity of the procedure, a sorghum sample was spiked with AFB1 and AFG1 at contamination level of 2.5 μg/kg and at contamination level of 1.25 μg/kg for AFB2 and AFG2, respectively. The spiked sample was then analyzed according to the procedure described above.

2.4.3. Linearity

Linearity was determined by constructing the calibration curves. Working standard solutions of mycotoxins were prepared in three replicates at concentration levels of 2 - 18 ng/mL for AFB1 and AFG1, 0.4 - 3.6 ng/mL for AFB2 and AFG2, and HPLC measurements were performed. The calibration standard of each concentration was constructed using the peak-area of each mycotoxin versus the nominal concentration of the analytes. The linear relationship was evaluated by the correlation coefficient, y-intercept and slope of the regression line.

2.4.4. LODs and LOQs

Sensitivity of the method was tested by examining the limit of detection (LOD) and limit of quantification (LOQ). The LOD was the lowest absolute concentration of analyte in a sample that could be detected, but not necessarily quantified; it was calculated based on the concentration of the analyte that produced a peak, whose height was thrice the height of the noise from a blank sample (ratio of signal to noise (S/N) = 3). LOQ was the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy; it was calculated by taking three replications of the lowest calibration standard (S/N = 10).

2.4.5. Accuracy

For calculation the percent recovery, the concentrations of the spiked samples (peanut, peanut butter, sorghum, sorghum product, and animals feed) were determined against calibration curves and the equation below was used:

Percent recovery = (obtained concentration/spike concentration in the sample) × 100

2.4.6. Precision

Precision was demonstrated as repeatability (within-day precision) and reproducibility (between-day precision) and expressed as RSD. Each of these precision assays was determined by analyzing three replicates of sorghum samples, spiked with mycotoxins at the levels of 2.5, 5.0, 1.25 and 2.5 μg/kg for AFB1, AFG1, AFB2 and AFG2, respectively. Within-day repeatability was performed by triplicate determination on the same day by the same operator. Between-day repeatability was performed by repeating the same procedure on two different days.

3. Results

3.1. Selectivity and Specificity

The chromatograms obtained in the developed method for the standards and the sample spiked with four aflatoxins are shown in **Figure 1**.

3.2. Linearity and Sensitivity

Calibration curves were linear in the range of levels used; the correlation coefficients of determination (R2) were greater than 0.990 for all analytes (**Table 1**). The LOD and LOQ in spiked samples were 0.02 and 0.05 μg/kg for both AFB1 and AFG1, but for both AFB2 and AFG2 were 0.01 and 0.03 μg/kg, respectively.

3.3. Accuracy and Precision

The recovery (R %) for the developed methods in the spiked samples (peanut, peanut butter, sorghum, and animals feed) were in the range of 84.2% to 96.9% (**Table 2**). The values obtained were in the range of satisfactory levels, as suggested by the Commission Regulations [27].

The precision of the method was evaluated by analyzing three replicate samples

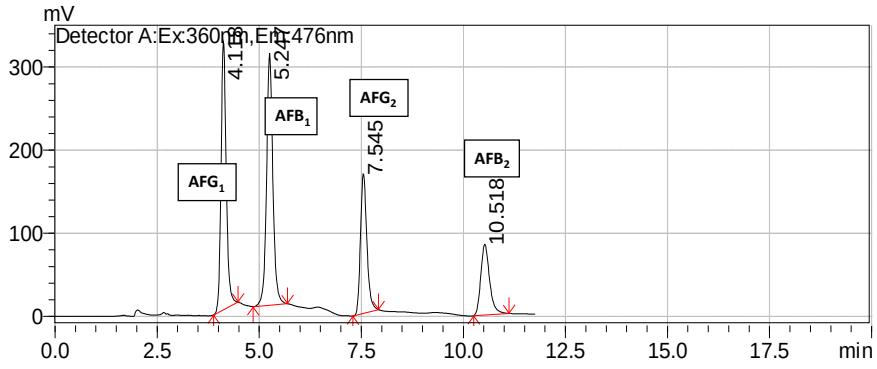

Figure 1. Chromatogram of different aflatoxins in the validated method.

Table 1. Linearity range, limit of detection (LOD) and limit of quantification (LOQ) for different aflatoxins.

Analyte	Linear range (ng/mL)	Slope	Intercept	R^2	LOD (µg/kg)	LOQ (µg/kg)
AFB_1	2 - 18	3434627.02	+52628.24	0.992	0.02	0.05
AFB_2	0.4 - 3.6	5911589.33	+12887.47	0.994	0.01	0.03
AFG_1	2 - 18	200,000	+12733.01	0.993	0.02	0.05
AFG_2	0.4 - 3.6	2695521.41	+2974.84	0.996	0.01	0.03

LOD: Limit of Detection, LOQ: Limit of Quantification, R^2: Correlation coefficients.

Table 2. Recovery and relative standard deviation (RSD) of mycotoxins in different spiked samples (n = 3).

Analyte	Sp.C	Sorghum		Animal feed		Groundnut		Peanut butter	
		R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %
AFB_1	2.5	94.2	1.06	93.3	3.98	90.4	3.32	88.8	6.16
	5	93.3	2.25	91.7	3.11	89.6	5.9	89.6	4.68
AFB_2	1.25	95.4	0.84	90.1	4.55	85.6	8.88	88.8	3.11
	2.5	94.8	0.83	94.5	1.91	90.4	5.91	87.6	3.65
AFG_1	2.5	94.4	1.04	93.4	4.88	88	5.82	90.8	5.94
	5	96.9	0.66	94.9	1.5	84.2	7.72	84.2	11.4
AFG_2	1.25	94.6	0.85	95.2	1.68	87.2	7.79	88.8	3.13
	2.5	95.6	1.26	94.3	2.14	93.2	4.71	90.8	1.52

Sp.C: Spiked Concentration. R %: Accuracy Percent, RSD %: Relative Standard Deviation Percent.

of standard solutions and spike sorghum samples. The within-day and between-day precision of the method for aflatoxins standards are shown in Table 3. The precisions (RSD %) were less than 0.5% which indicate that the precision of the method is acceptable.

3.4. Application to Real Samples

The developed method was applied for the determination of AFB1, AFB2, AFG1,

and AFG2 in 40 samples, namely peanut butter, sorghum, groundnut, and animals feed. In the analysis of these real samples peak identification was based on the comparison between the retention times of aflatoxins standards and was confirmed by spiking standards to the samples.

Nineteen samples were found to be contaminated with aflatoxins (Table 4). The total aflatoxins ranged from 0.27 to 10.48 μg/kg were obtained. The highest total aflatoxins value was obtained in animal feeds.

4. Discussion

The chromatograms obtained at the developed method for blank sample and spiked sample with the four aflatoxins, was an evident that the peaks of different aflatoxins were well separated from each other and no foreign peaks interfered with analytes at the retention times of each aflatoxin. Hence, the selectivity and

Table 3. Accuracy and precision for aflatoxins standard and spiked sorghum.

Analyte	Sp.C	Mycotoxin solution				Spiked sorghum			
		Within-days (n = 3)		Between-days (n = 6)		Within-days (n = 3)		Between-days (n = 6)	
		R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %
AFB_1	2.5	99.2	0.56	98.4	1.21	94.2	1.06	93.4	1.51
AFB_2	1.25	99.4	0.64	97.9	1.02	95.4	0.84	95.7	1.08
AFG_1	2.5	98.6	1.04	97.3	1.84	94.4	1.04	95.1	1.94
AFG_2	1.25	99.6	0.45	98.8	0.72	94.6	0.85	94.8	1.42

Sp.C: Spiked Concentration, R %: Accuracy Percent, RSD %: Relative Standard Deviation Percent.

Table 4. Incidents of different aflatoxins in different food samples and animal feed.

Sample	Groundnut	Sorghum	Peanut butter	Animal feed	Total
Total samples	10	10	10	10	40
Present samples	5	4	2	8	19
Present samples %	50	40	20	80	47.5
Number of present samples of AFB1 (%)	5 (50)	4 (40)	2 (20)	8 (80)	19 (47.4)
Range of AFB1 in present samples	0.32 - 0.91	0.51 - 3.62	0.44 - 0.53	0.68 - 10.48	0.32 - 10.48
Mean of AFB1 in present samples	0.62	1.25	0.485	5.47	2.78
Number of present samples of AFB2 (%)	1 (10)	1 (10)	0	3 (30)	5 (12.5)
Range of AFB2 in present samples	0.27	0.45	0	0.48 - 2.58	0.27 - 2.58
Mean of AFB2 in present samples	0. 27	0.45	0	1.33	0.95
Number of present samples of AFG1 (%)	0	0	0	1 (10)	1 (2.5)
Range of AFG1 in present samples	0	0	0	1.52	0 - 1.52
Mean of AFG1 in present samples	0	0	0	1.52	1.52

specificity of the procedure were considered satisfactory. The four aflatoxins were appeared within 15 minutes; this was faster than most of HPLC-FLD methods reported in the literature [22] [23] [24].

The LOD and LOQ in spiked samples were 0.017 and 0.05 ng/g for both AFB_1 and AFG_1, 0.01 and 0.03 ng/g for both AFB2 and AFG2 and showed lower quantification levels for analytes compared with the previous HPLC-FLD assay described by Gobel and Lusky [27] and little high quantification levels for analytes compared to method reported by Rahmani [28] and agreement with results reported by Aycicek [29].

The results indicate that the accuracy and precision of the method were acceptable, according to the European Commission Regulation 401/2006 [30]. The results obtained from the within-day and between days accuracy study at three concentrations indicated high recoveries of mycotoxins by the proposed method and the RSD values were in the range of 1.07% - 22.05%, indicating high precision. They were all in the range of 70% - 110%, required by European Commission regulation 401/2006 hence they were considered satisfactory [6].

Recovery values of the spiked samples (groundnut, peanut butter, sorghum, and animals feed) and the RSD values were in the range of satisfactory levels, as suggested by the European Commission.

The contamination percent of animal feed by aflatoxins were 80%, 30% and 10% for AFB1, AFB2 and AFG1; respectively which was above than the contamination percent found by Salah [31] the two studies were found the three kinds of aflatoxins in the same order but in other study by Salah Eldeen [32] AFG2 was found in some samples of animal feed.

In this groundnut contamination percent with 50% for AFB1 and 10% for AFB2 in previous study by Esameldin [30] and Salah [33] contamination percent was above 50% for AFB1 in all groundnut samples, but in other study by Esameldeen Bashir [34] only 8% of groundnut sample collected from storage were contaminated with AFB1.

Peanut butter was the lowest kind of sample that has been found to contain aflatoxins and AFB1 was only detected in 20% of the samples this result was different of the study by Salah [33] which detected AFB2 beside AFB1.

In this study aflatoxins found in sorghum samples were AFB1 and AFB2 with percentage of 40% to 10%; respectively, this contamination was similar to that found by Salah Eldeen [31] and different from that found by Abdalla [35] which other kinds of mycotoxins detected in sorghum like OTA with percentage of 3.3% from different state of Sudan.

5. Conclusion

This paper describes fast and sensitive HPLC-FLD method for analysis of aflatoxins AFB1, AFB2, AFG1 and AFG2 in various food samples namely peanut, peanut butter, sorghum, and animals feed. The LOD and LOQ in spiked samples were found to be 0.02 and 0.05 ng/g for both AFB1 and AFG1, 0.01 and 0.03

ng/g for both AFB2 and AFG2. Recoveries ranged from 84.2% to 96.9% with RSD % values in the range of 0.6% - 11.4% for spike sorghum samples. The method was successfully applied to various real food samples and is suitable for use in aflatoxins determination in quality control laboratories.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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