Single-Laboratory Validation for the Determination of Aflatoxin B$_1$, B$_2$, G$_1$, and G$_2$ in Foods Based on Immunoaffinity Column and Liquid Chromatography with Postcolumn Derivatization and Fluorescence Detection

Cemalettin Baltacı · Huri İlyasoğlu · Ferhat Yüksel

Abstract This study presents a method validation procedure for the determination of aflatoxin B$_1$, B$_2$, G$_1$, and G$_2$ in hazelnut, hazelnut paste, walnut, peanut, pistachio, corn, and wheat. The method consisting of clean-up with immunoaffinity column, high performance liquid chromatography with postcolumn derivatization and fluorescence detection was validated in accordance with Commission Regulation 2004/882/EC. The selectivity, linearity, decision limit, detection capability, detection and quantification limits, precision, recovery, ruggedness, and measurement uncertainty of the method were determined. The limit of detection and limit of quantification values (μg/kg) were: aflatoxin B$_1$, 0.02, 0.07; aflatoxin B$_2$, 0.01, 0.02; aflatoxin G$_1$, 0.02, 0.07; and aflatoxin G$_2$, 0.01, 0.03. The relative standard deviation values for the repeatability and within-laboratory reproducibility were below 4 and 5 %, respectively. The recovery values of the spiked samples ranged from 80 to 105 %. These results complied with minimum performance criteria established by regulation 2006/401/EC. Therefore, the procedure can be implemented for the routine analysis of aflatoxins in the studied matrices.

Keywords Aflatoxin · HPLC · Immunoaffinity column · Validation

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Introduction

Aflatoxins (AFs), difuranocoumarin derivatives, are produced by fungi, *Aspergillus flavus*, *Aspergillus parasiticus*, and the rare *Aspergillus nomius* (Juan et al. 2008). Twenty aflatoxins have been identified, and the most of them are metabolites formed endogenously in animals. Aflatoxin B$_1$, B$_2$, G$_1$, and G$_2$ (AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$) are the main AFs occurring naturally in foods and feeds, and AFB$_1$ is the most common and most toxic form. Aflatoxin B$_1$, B$_2$, G$_1$, and G$_2$ are named for their fluorescence characteristics. Aflatoxin B$_1$ and B$_2$ show strong blue fluorescence under UV light, whereas aflatoxin G$_1$ and G$_2$ exhibit greenish yellow fluorescence (Wogan and Busby 1980). AFB$_2$ and AFG$_2$, the dihydroxy derivatives of AFB$_1$ and AFG$_1$, respectively, are biologically inactive until they are oxidized to AFB$_1$ and AFG$_1$ in vivo.

Aflatoxins have been classified as carcinogenic compounds to human. AFB$_1$ has been classified as group 1 of human carcinogens (IARC 1993). Highly toxic, immunosuppressive, mutagenic, and teratogenic effects of aflatoxins have been identified as well (Castells et al. 2008; Hussein and Jeffrey 2001). Moreover, malabsorption syndrome and decrease in bone strength may occur owing to AFs consumption (Nelson et al. 1982). Aflatoxins not only have adverse effects on human health but also cause serious economic losses.

The level of aflatoxins in corn, cereals, sorghum, peanut, hazelnut, walnut, pistachio, corn, and other oil seed crops contaminated by aflatoxins varies with respect to region. Tropical and subtropical region are more convenient for mold growth and toxin production. From previous surveys, about 19 % of peanut samples ($n=1416$) were found to be contaminated with an average value of 1 μg/kg AFB$_1$ (Stoloff

1977) in North America, whereas in Thailand, about 49 % of peanut samples ($n=216$) were contaminated with an average value of 424 μg/kg AFB_1 (Shank et al. 1972).

To ensure food safety, the maximum level of aflatoxin in foods has been set by international organizations. European Commission Regulation 2010/165/EC established limits for AFB_1 and total AFs of 8 and 15 μg/kg, respectively, in hazelnuts, Brazil nuts, groundnuts and other oilseeds, to be subjected to sorting or other physical treatment before human consumption or use as ingredient in foodstuffs. The legal limit of AFB_1 and total AFs in groundnuts and other oilseeds intended for human consumption are 2 and 4 μg/kg, while in hazelnut and Brazil nuts are 5 and 10 μg/kg, respectively (Codex 2006 & EC 165/2010). In Turkey, the maximum level of AFB_1 and total AFs in dried fruit, hazelnut, pistachio, peanut, oil seeds and processed foods were 5 and 10 μg/kg, respectively (TFC 2002). Each country has established its legal limits.

Several methods have been developed to determine AFs in foods; immunoassays techniques (Lee et al. 2004; Li et al. 2001), capillary electrophoresis (Pena et al. 2002), thin layer chromatography (Braicu et al. 2008; Abbas 2005), and high performance liquid chromatography (HPLC) with fluorescence detection (Bacaloni, et al. 2008). Recently, analytical methods based on clean-up with immunoaffinity column and HPLC with postcolumn derivatization and fluorescence detection have gained much attention for the determination of AFs in food matrices. Although several studies have been reported to determine AFs in foods by using these methods (Asis et al. 2002; Daradimos et al. 2000; Stroka et al. 2000), only few validation studies are available which comply with regulation 2004/882/EC and 2006/401/EC (Muscarella et al. 2007; Tavcar-Kalcher et al. 2007).

The aim of this study was to develop a fast, economical, precise, and reliable method for aflatoxin analysis in hazelnut, hazelnut paste, walnut, peanut, pistachio, corn, and wheat. The proposed method consisting of clean-up with immunoaffinity column and HPLC with postcolumn derivatization and fluorescence detection was validated according to the Commission Regulation 2004/882/EC in terms of selectivity, linearity, decision limit, detection capability, detection and quantification limits, precision, recovery, ruggedness, and measurement uncertainty.

Experimental

Samples

Representative samples of hazelnut, hazelnut paste, walnut, peanut, pistachio, corn, and wheat were collected from importer and exporter companies in Trabzon, and sampling was performed according to the sampling method in Turkish food codex (TFC 2007). Each sample was from 10 to 30 kg transferred into polyethylene bags and stored at 20 ± 5 °C. The samples were grounded by using a laboratory grinder (Robout Coupe, R23 grinder) and subsamples were transferred in plastic bags and stored at -5 °C until analysis. To prove homogeneous samples, a homogeneity test was applied (Baltacı et al. 2009).

Chemicals and Reagents

All reagents used were analytical or HPLC grade. Supelco aflatoxin standard solution consisting of 1.0 μg/mL AFB_1, 0.3 μg/mL AFB_2, 1.0 μg/mL AFG_1, and 0.3 μg/mL AFG_2 were obtained from Sigma–Aldrich (St. Louis, MO, USA, catalog number : 46304-U). Stock standard solutions (1 μg/mL AFB_1, 0.3 μg/mL AFB_2, 1 μg/mL AFG_1, and 0.3 μg/mL AFG_2) and working standard solutions (0.726, 1.450, 2.180, 2.910, 3.630 μg/L for AFB_1, 0.216, 0.432, 0.648, 0.864, and 1.080 μg/L, for AFB_2; 0,720, 1.440, 2.160, 2.880, and 3.600 μg/L for AFG_1; 0.223, 0.446, 0.670, 0.893, and 1.112 μg/L for AFG_2) of AFs were prepared in methanol and stored at -20 °C in amber glass vials. HPLC-grade methanol and acetonitrile were supplied from JT Baker (JT. Baker BV, Netherlands). Analytical grade potassium bromide, nitric acid, and sodium chloride were supplied from Sigma–Aldrich (St. Louis, MO, USA). Immunoaffinity columns were obtained from Romer Labs (Romer Labs Diagnostic, Tulln, Austria). Calibrated equipments were used for analysis.

Apparatus

Robot Coupe laboratory grinder (Robot Coupe, R23, USA) and Waring laboratory blender (Waring, Torrington, USA) were used for the extraction. Quantitative analysis was performed on a HPLC system (Shimadzu, Japan) equipped with a system controller CBM-20A, a solvent delivery unit LC-20 AD, a gradient system LC-20AB, an on-line degasser DGU-20A3, an autosampler SIL-20A, a column oven SPD-20A, a scanning fluorescence detector RF-10 AXL, a symmetry ACE C18 column (150×4 mm, 5 μm, Advanced Chromatography Technologies, Aberdeen, Scotland), and a Kobra cell (R-Biopharm, Glasgow, Scotland) for postcolumn derivatization. OQ-PV test of the HPLC system was performed by Shimadzu technical service.

Sample Extraction and Cleanup

First, 25 g representative subsamples were weighed into a blender chamber and 5 g NaCl and 125 ml of 70 % methanol (AOAC 991.31) were added to the chamber. After the

mixture was blended for 2 min at high speed, the extract was filtered through a filter paper (Schleicher&Schwel, ∅240 mm, Dassel, Germany). An aliquot of 5 ml of the filtrate was applied onto immunoaffinity column by using a syringe containing 10 ml of deionized water, and the filtrate was passed through column at a flow rate of 3 ml/min. The column was washed with 20 ml of deionized water at a flow rate of 5 ml/min, and then air was passed through a syringe at least three times. The aflatoxin bound to the column was eluted with 1 ml of methanol at a flow rate of 0.5 ml/min. Aflatoxins were collected into a flask (2.5 ml) and transferred in an amber glass vial.

HPLC Conditions

The isocratic mobile phase consisted of water, methanol, and acetonitrile (6:3:2) and 350 µl of 4 M HNO_3 and 0.120 g of KBr were added to 1 L of the mobile phase. Flow rate was set at 1 ml/min and column temperature was 22 °C. Sample volume of 100 µl was injected in triplicate. The fluorescence detection was set to an excitation wavelength of 360 nm and an emission wavelength of 430 nm. Postcolumn derivatization with electrochemically generated bromine method was used to enhance fluorescence intensity of aflatoxin derivatives. Electrochemical cell supplying 100 µA electrical current was positioned between the column and fluorescence detector by using PTFE tube (30 cm).

Validation Procedure

Method validation procedure was carried out according to Commission Regulation 2004/882/EC. Twenty-one independent blank samples were analyzed to check the sensitivity of the method. Linearity was checked by analyzing standard solutions of aflatoxin B_1, B_2, G_1, and G_2 at five different concentrations. Calibration curves were constructed by plotting the peak area of each standard solution versus the concentration of standard solution. For the determination of LOD and LOQ, ten independent blank samples spiked with standard solutions of aflatoxins were analyzed. To determine detection limit (CC_α) and detection capability (CC_β), twenty-one independent blank samples fortified with aflatoxins were prepared. Recovery and precision were determined by analyzing spiked samples at three different concentrations (2.5, 5, and 7.5 µg/kg) and 20.8, 41.6, and 62.4 µl of aflatoxin B_1, B_2, G_1, and G_2 (3 µg/ml) dissolved in benzene:acetonitrile (98:2) were added to 25 g of the samples, respectively. The homogenized samples were stored in the dark for 2 h, and the solvent was evaporated via nitrogen. Finally, the ruggedness of the method was tested. The spiked samples consisted of hazelnut (*n*:3),

hazelnut paste (*n*:3), walnut (*n*:3), peanut (*n*:3), pistachio (*n*:3), corn (*n*:3) and wheat (*n*:3).

Statistical Analysis

Microsoft Excel statistical software (Microsoft Office Excel 2003, Microsoft Corp., Redmond, WA USA) was used for data processing. The Cochran test and the Grubbs test were performed to eliminate results with too high difference between triplicate measurements and outliers. The equation of calibration curve was determined by using least square regression analysis.

Results and Discussion

### Method Performance Characteristics

Method validation was performed by conducting a single-laboratory study in accordance with the European Union Commission Regulation 2004/882/EC. Method performance characteristics determined were selectivity, linearity, detection and quantification limits, decision limit, detection capability, precision, recovery, ruggedness, and measurement uncertainty.

Selectivity

Selectivity is the degree to which a method can determine particular analyte without interferences from other components. Selectivity of an analytical method towards naturally present substances (metabolites, endogenous substances and matrix constituents, etc) has to be checked before proceeding with the validation procedure. To check the selectivity of the proposed method, the chromatograms of independent blank samples (*n*=21) were compared with the chromatograms of blank samples fortified with aflatoxin B_1, B_2, G_1, and G_2 and naturally contaminated samples. The chromatograms obtained for spiked samples, blank samples, and standard mixture were presented in Fig. 1. No interferences at the retention time of each aflatoxin were observed in the studied matrices. From the chromatograms of a blank sample and a naturally contaminated sample, it is evident that the proposed method has the power of discrimination between the analyte and other matrix components.

Linearity

Calibration curves were constructed by plotting the peak areas of three series of standard solutions at five different concentrations (0.726, 1.450, 2.180, 2.910, and 3.630 µg/L for AFB_1; 0.216, 0.432, 0.648, 0.864, and 1.080 µg/L, for
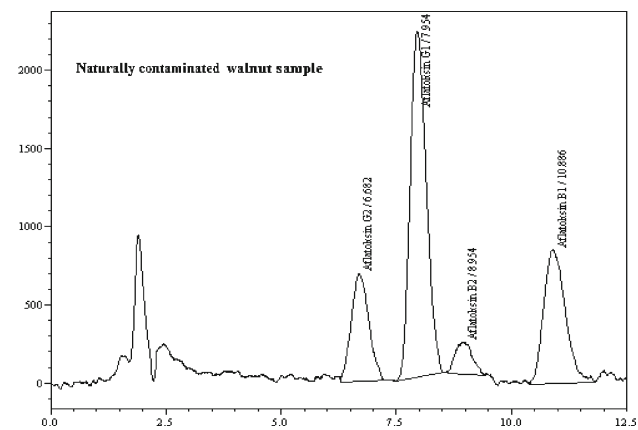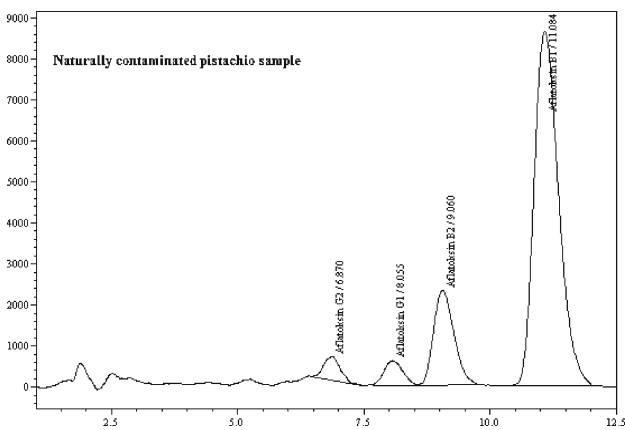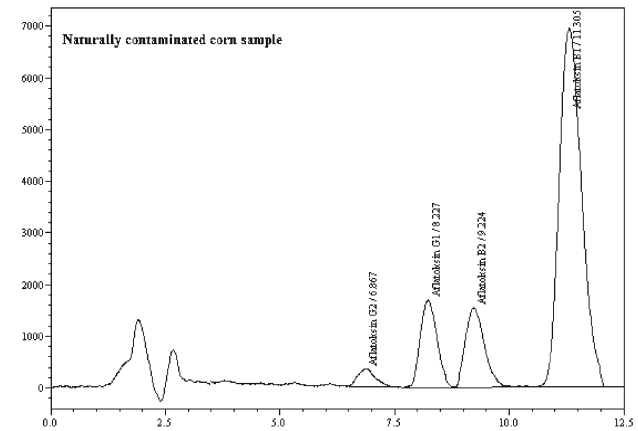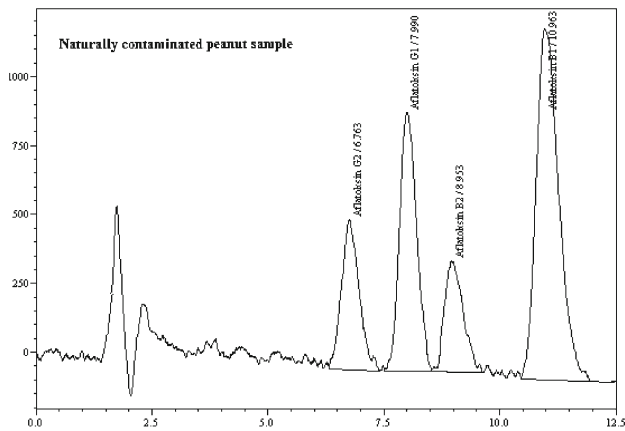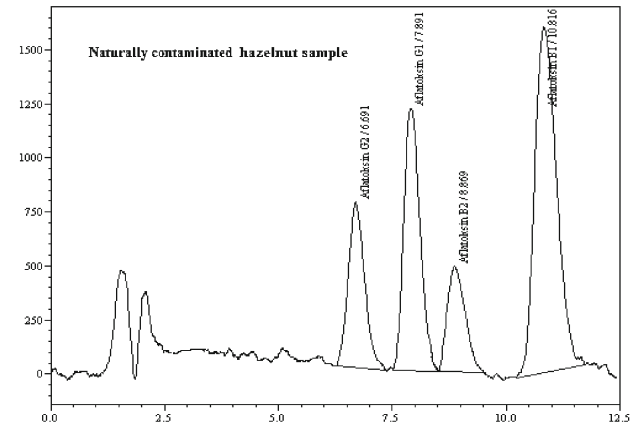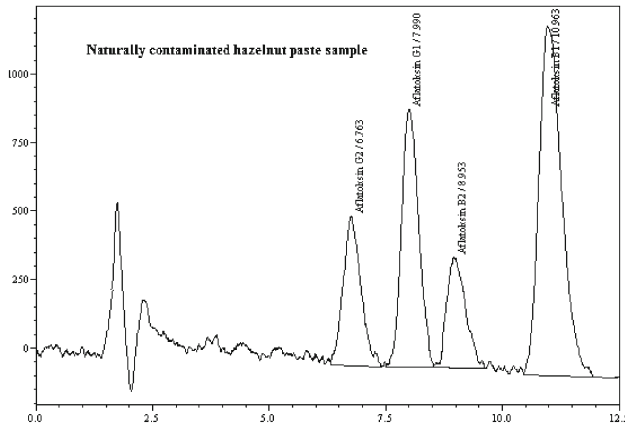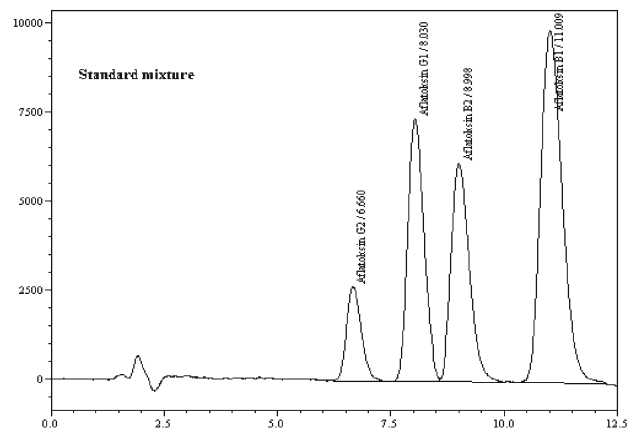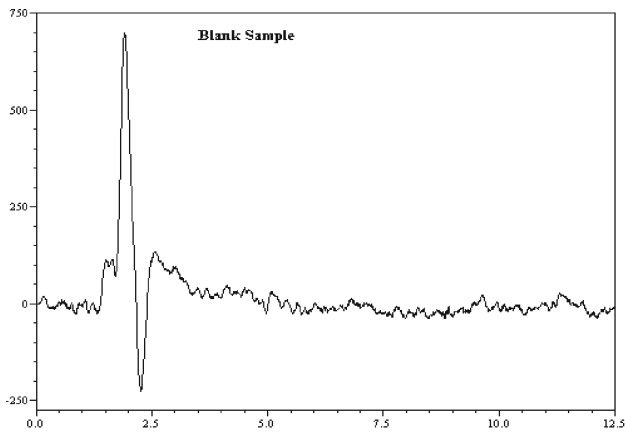
◀ **Fig. 1** The chromatograms obtained for naturally contaminated samples, blank sample, and standard mixture of AFs

AFB$_2$; 0,720, 1.440, 2.160, 2.880, and 3.600 µg/L for AFG$_1$; 0.223, 0.446, 0.670, 0.893, and 1.112 µg/L for AFG$_2$). Least square regression analysis was applied to determine equation of each calibration graph. The equation describing the calibration curve was $y = A(x) + B$, where y is the peak area of standard solution expressed in fluorescence unit and x is the concentration of standard solution expressed in µg/L. The regression coefficient of each calibration graph was found to be higher than 0.999. The regression was tested by performing the F test at 95 % confidence level, and the linearity was obtained in the following ranges: 0.07–9.08 µg/kg for AFB$_1$ in the samples, 0.02–2.70 µg/kg for AFB$_2$ in the samples, 0.07–9.0 µg/kg for AFG$_1$ in the samples, and 0.03–2.79 µg/kg for AFG$_2$ in the samples, respectively. The retention time values (min) were 6.87, 8.35, 9.32, and 11.47 for AFG$_2$, AFG$_1$, AFB$_2$, and AFB$_1$, respectively, with relative standard deviation values of 0.5 % (repeatability) and 3.5 % (within-laboratory reproducibility).

Limit of Detection and Limit of Quantification

Ten blank samples fortified with AFB$_1$ (0.074 µg/kg), AFB$_2$ (0.023 µg/kg), AFG$_1$ (0.070 µg/kg), and AFG$_2$ (0.032 µg/kg) were analyzed to calculate the LOD and LOQ values of the method. The LOD and LOQ values were determined according to Analytical Detection Limit Guidance (ADLG 1996). For the estimation of LOD, the standard deviation of the response (s) was multiplied by the Student's t test value for ten replicates and nine degrees of freedom. The estimated LOD values were verified in accordance with the guidance (ADLG 1996) as well. The LOQ values were calculated by ten times the standard deviation of the response.

$$\text{LOD} = (t \text{ value}) \times (s) \qquad (1)$$

$$\text{LOQ} = 10 \times (s) \qquad (2)$$

The LOD values obtained for AFB$_1$, AFB$_2$, AFG$_{12}$, and AFG$_2$ were 0.02, 0.01, 0.02, and 0.01 µg/kg, respectively. The LOQ values were 0.07, 0.02, 0.07, and 0.03 µg/kg for AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$, respectively.

Detection Limit and Detection Capability

Two new performance characteristics, CC$_\alpha$ and CC$_\beta$, are introduced by Regulation 2002/657/EC (2002). While CC$_\alpha$ refers to the limit above which samples are concluded to be

non-compliant ($\alpha = 5$ %), CC$_\beta$ refers to the smallest content of the substances that may detected, identified and/or quantified in a sample ($\beta = 5$ %). The CC$_\alpha$ and CC$_\beta$ values were determined to analyze 21 blank samples fortified with aflatoxins at the maximum permitted limit (MRL) regulated by Turkish food codex. The CC$_\alpha$ and CC$_\beta$ values of the method estimated according to the following equations are presented in Table 1.

$$\text{CC}_\alpha = \text{the concentration at MRL} + 1.64$$
$$\times \text{ the standard deviation of the fortified samples} \qquad (3)$$

$$\text{CC}_\beta = \text{CC}_\alpha + 1.64$$
$$\times \text{ the standard deviation of the fortified samples} \qquad (4)$$

Precision and Accuracy

Precision was tested by analyzing ten blank samples fortified with aflatoxin B$_1$, B$_2$, G$_1$, and G$_2$ at the concentrations of 0.5, 1, and 1.5 times MRL. For the repeatability test, samples were prepared in six replicates and analyses were performed by the same operator in 1 day. To determine within-laboratory reproducibility, samples (six replicates) were analyzed by three different operators in 3 days over a month. The results for the repeatability expressed with the standard deviation (S$_r$) and the relative standard deviation (RSD$_r$) and the results for the within-laboratory reproducibility expressed with the standard deviation (S$_R$) and the relative standard deviation (RSD$_R$) are presented in Table 2. Regulation 2006/401/EC declared that RSD values shall not exceed the values derived from the Horwitz equation. Both RSD values at the three concentration levels were found to be lower than the reference values (Table 2) calculated from the Horwitz equation. These results revealed that the developed method meets minimum performance criteria established by Regulation 2006/401/EC. Therefore, the precision of the method is acceptable.

Recovery

Samples spiked with aflatoxins at three different concentrations (0.5, 1, and 1.5 times MRL) were prepared to perform the recovery study. The spiked samples (six replicates) were analyzed with the developed method. The recovery values obtained were in the range of 80–105 % (Table 3), complying with the values (70–110 %) recommended by

Table 1 The decision limit (CC_α) and detection capability (CC_β) values of the method calculated at the MRL ($n=21$) for the AFs

Analyte	Added (µg/kg)	Measured±S.D. (µg/kg)	Error α (1.64×S.D.)	CC_α (µg/kg)	Added (µg/kg)	Measured±S.D. (µg/kg)	Error β (1.64×S.D.)	CC_β (µg/kg)
Aflatoxin B$_1$	0.07[a]	0.09±0.02	0.03	0.10	0.12	0.11±0.03	0.05	0.15
	5.0[b]	4.95±0.08	0.13	5.13	5.13	5.12±0.15	0.25	5.38
Aflatoxin B$_2$	0.02[a]	0.03±0.01	0.02	0.04	0.04	0.05±0.01	0.02	0.05
	5.0[b]	4.79±0.08	0.16	5.16	5.16	5.15±0.17	0.28	5.44
Aflatoxin G$_1$	0.07[a]	0.09±0.02	0.03	0.10	0.10	0.11±0.02	0.03	0.14
	5.0[b]	4.99±0.06	0.10	5.10	5.10	5.12±0.13	0.21	5.31
Aflatoxin G$_2$	0.03[a]	0.04±0.01	0.02	0.05	0.05	0.06±0.01	0.10	0.31
	5.0[b]	4.72±0.16	0.26	5.26	5.26	5.24±0.13	0.21	5.48
Aflatoxins (total)	0.19[a]	0.21±0.06	0.10	0.29	0.29	0.26±0.06	0.10	0.39
	10.0[b]	9.85±0.14	0.23	10.23	10.23	10.17±0.21	0.34	10.57
	15.0[b]	14.85±0.11	0.18	15.18	15.18	15.12±0.23	0.38	15.56

[a] limit of quantification

[b] maximum permitted limit

Regulation 2006/401/EC. The recovery of the method was determined by analyzing FAPAS test material (T0474) as well. Recovery was estimated by dividing the detected mean value by the certified value. The estimated recovery values varied from 81.4 to 95.7 %, confirming acceptable values for the accuracy of the method. Therefore, it can be interpreted that satisfactory results are achieved by using the proposed method for the determination of aflatoxin in hazelnut, hazelnut paste, walnut, peanut, pistachio, corn, and wheat.

Table 2 The repeatability and within-laboratory reproducibility of the method, expressed with the standard deviation and the relative standard deviation

	Intra-day ($n=6$)					Inter-day ($n=6$)			
Analyte	Fortification level (µg/kg)	Determination level (µg/kg)	S_r (µg/kg)	Precision RSD$_r$ (%)	0.5×Horwitz value (%)	Determination level (µg/kg)	S_R (µg/kg)	Precision RSD$_R$ (%)	Horwitz value (%)
Aflatoxin B$_1$	2.5[a]	2.43	0.05	2.05	6.99	2.49	0.02	0.80	13.98
	5.0[b]	4.89	0.09	1.84	6.27	4.78	0.07	1.40	12.55
	7.5[c]	7.03	0.12	1.71	5.90	7.12	0.11	1.54	11.81
Aflatoxin B$_2$	2.5[a]	2.39	0.09	3.76	6.99	2.41	0.12	4.98	13.98
	5.0[b]	4.76	0.13	1.26	6.27	4.82	0.13	2.69	12.55
	7.5[c]	7.15	0.08	1.11	5.90	7.25	0.09	1.24	11.81
Aflatoxin G$_1$	2.5[a]	2.52	0.09	3.57	6.99	2.48	0.07	2.82	13.98
	5.0[b]	4.99	0.06	1.20	6.27	4.89	0.08	1.64	12.55
	7.5[c]	7.32	0.07	0.95	5.90	7.27	0.17	2.34	11.81
Aflatoxin G$_2$	2.5[a]	2.38	0.09	3.78	6.99	2.43	0.07	2.88	13.98
	5.0[b]	4.73	0.16	3.38	6.27	4.80	0.06	1.25	12.55
	7.5[c]	7.09	0.04	0.56	5.90	7.11	0.14	1.96	11.81
Aflatoxins (total)	5 [a]	4.69	0.16	3.41	6.27	4.89	0.26	5,31	12.55
	10 [b]	9.86	0.13	1.32	5.66	9.76	0.23	2.36	11.31
	15 [c]	14.95	0.28	1.87	5.32	14.89	0.18	1.21	10.64
Aflatoxins (total)	7.5 [a]	7.13	0.25	3.50	5.91	7.27	0.56	7.7	11.81
	15 [b]	14.81	0.33	2.23	5.32	14.67	0.45	3.06	10.64
	22.5 [c]	21.65	0.48	2.21	5.01	20.98	0.58	2.76	10.01

[a] 0.5×MRL

[b] MRL

[c] 1.5×MRL

Table 3 The recovery values of spiked samples at three different concentrations ($n=6$)

Recovery (%)

Analyte	Added (μg/kg)	Hazelnut	Hazelnut Paste	Peanut	Walnut	Pistachio	Corn	Wheat
Aflatoxin B_1	2.5[a]	102.8±1.9	92.3±1.2	92.8±1.1	95.3±1.9	92.8±1.9	93.8±1.9	96.9±1.7
	5.0[b]	92.3±0.9	93.4±1.9	91.3±0.6	94.3±1.3	92.3±0.9	93.3±0.9	95.2±1.8
	7.5[c]	94.4±1.7	92.4±1.1	104.1±1.8	93.2±1.5	94.4±1.7	95.4±1.1	97.4±1.4
Aflatoxin B_2	2.5[a]	92.5±0.6	93.5±0.9	91.5±0.6	96.5±0.4	92.5±0.6	101.8±0.6	96.6±0.4
	5.0[b]	94.3±0.9	92.3±1.3	93.3±0.9	105.4±0.4	94.3±0.9	91.7±0.9	104.4±0.4
	7.5[c]	98.6±1.6	93.4±1.7	96.6±0.6	96.6±1.6	94.6±1.6	93.6±1.6	97.4±1.6
Aflatoxin G_1	2.5[a]	93.3±1.0	94.5±0.7	92.5±1.0	96.7±1.0	94.5±0.8	93.5±1.1	96.8±1.0
	5.0[b]	94.5±0.8	94.9±0.8	103.5±0.9	97.5±2.0	94.2±0.8	96.5±0.3	96.4±2.0
	7.5[c]	97.8±1.3	94.8±1.2	93.8±1.3	94.8±1.2	95.8±1.3	97.8±1.3	94.8±1.2
Aflatoxin G_2	2.5[a]	80.0±1.3	84.1±0.4	83.6±1.4	88.5±1.6	80.6±1.3	83.6±1.2	89.1±1.3
	5.0[b]	86.8±1.5	83.5±1.4	86.8±1.5	89.6±1.1	82.8±1.5	83.0±1.5	87.4±1.5
	7.5[c]	88.3±0.4	85.4±0.6	88.3±0.4	88.9±0.2	83.3±0.5	82.7±0.4	85.7±1.2
Aflatoxins (total)	5 [a]	97.6±1.8	91.3±1.8	91.6±1.4	96.6±1.8	95.6±1.3	92.6±1.6	98.9±1.6
	10 [b]	91.0±1.7	92.0±1.7	93.1±1.1	99.0±1.7	92.0±1.1	91.0±1.2	99.8±1.5
	15 [c]	90.9±0.5	91.9±0.9	92.3±1.5	94.9±0.5	94.9±0.4	90.9±0.6	97.9±0.5
Aflatoxins (total)	7.5 [a]	98.9±2.1	94.4±2.0	94.2±1.2	104.5±2.1	98.1±1.3	96.5±2.6	95.9±2.2
	15 [b]	95.5±1.8	95.3±1.5	94.2±1.5	97.0±1.7	95.7±1.8	96.6±1.2	96.6±1.6
	22.5 [c]	93.8±0.5	93.5±1.9	94.4±1.5	95.6±1.5	99.1±1.4	93.5±1.6	97.3±1.6

[a] $0.5 \times MRL$

[b] MRL

[c] $1.5 \times MRL$

Ruggedness

Ruggedness was investigated by means of the Youden ruggedness trial, in which seven factors were explored in an experiment requiring eight determinations (Table 4). The factors evaluated for samples were extraction solvent, sample matrix, column temperature, analyst, type of extract for cleanup, reversed phase HPLC column, and mobile phase. The standard deviation for the effects can then be calculated:

$$SD = 2 \sum \left(\frac{\sum_i^2}{n} \right) \qquad (5)$$

where E_i is each of the calculated effects and n is the total number of parameters. To assess if the method is robust compared to each variation, the influence of each variable on the method performance has to be assessed by applying the t test (Bratinova et al. 2009). The experimental t values for each of the effects were calculated using the following formula:

$$t = \frac{E_i \cdot \sqrt{n}}{SD \cdot \sqrt{2}} \qquad (6)$$

The calculated t values presented in Table 4 are lower than the t critical value for $\nu = n-1$ degrees of freedom

($t_{crit}=2.45$, $\nu=6$, 97.5 % confidence level), that is why the method is robust with respect to the changes in the procedure. The results obtained in the ruggedness study reveal that analytical results are not affected by the slight variations in the selected factors. Consequently, the proposed method is applicable for the determination of AFs in hazelnut, hazelnut paste, walnut, peanut, pistachio, corn, and wheat.

Proficiency Test

The technical competence of the laboratory was tested by participating in the FAPAS (Food and Environment Research Agency) proficiency test 04145 in October 2009. The FAPAS test material (hazelnut) was analyzed in triplicate. The Z score calculated by the FAPAS was used to evaluate the results. The Z scores obtained in the FAPAS ring test were 0.9, −1.1, −0.8, −0.8, and −0.9 for AFB_1, AFB_2, AFG_1, AFG_2, and total AFs, respectively. These values were below the reference value of 2 (absolute), indicating the technical competence of the laboratory.

Measurement Uncertainty

Evaluation of uncertainty of the analytical results is compulsory for laboratories accredited according to ISO 17025/

Table 4 Experimental design for the ruggedness study

	+	−	1	2	3	4	5	6	7	8	E_i	$E_i{*}E_i$	t values	SD
Aflatoxin B$_1$	A	a	4.93	4.86	4.92	4.79	−4.81	−4.82	−4.89	−5.12	−0.035	0.00122	0.21	
	B	b	4.93	4.86	−4.92	−4.79	4.81	4.82	−4.89	−5.12	−0.075	0.00563	0.98	
	C	c	4.93	−4.86	4.92	−4.79	4.81	−4.82	4.89	−5.12	−0.010	0.00010	0.02	
	D	d	4.93	4.86	−4.92	−4.79	−4.81	−4.82	4.89	5.12	0.115	0.01322	2.32	0.01069
	E	e	4.93	−4.86	4.92	−4.79	−4.81	4.82	−4.89	5.12	0.110	0.01210	2.12	
	F	f	4.93	−4.86	−4.92	4.79	4.81	−4.82	−4.89	5.12	0.040	0.00023	0.04	
	G	g	4.93	−4.86	−4.92	4.79	−4.81	4.82	4.89	−5.12	−0.070	0.00490	0.86	
Aflatoxin B$_2$	A	a	2.52	2.56	2.47	2.56	−2.51	−2.52	−2.49	−2.45	0.035	0.00123	1.49	
	B	b	2.52	2.56	−2.47	−2.56	2.51	2.52	−2.49	−2.45	0.035	0.00122	1.49	
	C	c	2.52	−2.56	2.47	−2.56	2.51	−2.52	2.49	−2.45	−0.025	0.00063	0.76	
	D	d	2.52	2.56	−2.47	−2.56	−2.51	−2.52	2.49	2.45	−0.010	0.00010	0.12	0.00154
	E	e	2.52	−2.56	2.47	−2.56	−2.51	2.52	−2.49	2.45	−0.040	0.00160	1.94	
	F	f	2.52	−2.56	−2.47	2.56	2.51	−2.52	−2.49	2.45	0.000	0.00000	0.00	
	G	g	2.52	−2.56	−2.47	2.56	−2.51	2.52	2.49	−2.45	0.025	0.00063	0.76	
Aflatoxin G$_1$	A	a	5.02	4.89	4.82	4.97	−4.85	−4.92	−4.89	−5.06	−0.005	0.00002	0.01	
	B	b	5.02	4.89	−4.82	−4.97	4.85	4.92	−4.89	−5.06	−0.015	0.00022	0.09	
	C	c	5.02	−4.89	4.82	−4.97	4.85	−4.92	4.89	−5.06	−0.065	0.00422	1.61	
	D	d	5.02	4.89	−4.82	−4.97	−4.85	−4.92	4.89	5.06	0.075	0.00563	2.14	0.0492
	E	e	5.02	−4.89	4.82	−4.97	−4.85	4.92	−4.89	5.06	0.055	0.00303	1.15	
	F	f	5.02	−4.89	−4.82	4.97	4.85	−4.82	−4.89	5.06	0.120	0.00206	0.78	
	G	g	5.02	−4.89	−4.82	4.97	−4.85	4.92	4.89	−5.06	0.045	0.00202	0.77	
Aflatoxin G$_2$	A	a	2.48	2.47	2.46	2.51	−2.51	−2.52	−2.49	−2.48	−0.020	0.00040	1.73	
	B	b	2.48	2.47	−2.46	−2.51	2.51	2.52	−2.49	−2.48	0.010	0.00010	0.43	
	C	c	2.48	−2.47	2.46	−2.51	2.51	−2.52	2.49	−2.48	−0.010	0.00010	0.43	
	D	d	2.48	2.47	−2.46	−2.51	−2.51	−2.52	2.49	2.48	−0.020	0.00040	1.73	0.00043
	E	e	2.48	−2.47	2.46	−2.51	−2.51	2.52	−2.49	2.48	−0.010	0.00010	0.43	
	F	f	2.48	−2.47	−2.46	2.51	2.51	−2.52	−2.49	2.48	0.010	0.00001	0.06	
	G	g	2.48	−2.47	−2.46	2.51	−2.51	2.52	2.49	−2.48	0.020	0.00040	1.73	

Original parameters: (A) extraction solvent, methanol/water (70:30v/v); (B) sample matrix, hazelnut, hazelnut paste, walnut, peanut, pistachio, corn, wheat; (C) column temperature, 22 °C; (D) analysts, 1; (E) type of extract for cleanup, 10 ml water/5 ml extract; (F) LC column, 150×4 mm, 5 μm, LC-18; (G) mobile phase, water/methanol/acetonitrile (60:30:20$v/v/v$). Changed parameters: (a) extraction solvent, methanol/water (80/20v/v); (b) sample matrix, blank sample; (c) column temperature, 27 °C; (d) analysts, 2; (e) type of extract for cleanup, 10 ml PBS/5 ml extract; (f) LC column, 250×4 mm, 5 μm, ODS 2; (g) mobile phase, water/methanol/acetonitrile (54:17:29, $v/v/v$)

2005, and there are several proposed methods of uncertainty calculations (EURACHEM Guide 1995; EURACHEM/CITAC GUIDE CG 4 2000; and Nordic Committee on Food Analysis, NMKL 2003). In this study, we used the analytical validation parameters obtained from each step of the process. The determination of relative uncertainty (u) has been performed by considering eight sources of uncertainty: (a) volume; (b) mass; (c) calibration curve; (d) method reproducibility and repeatability; (e) preparation of standard; (f) accuracy; and (g) reproducibility of device.

A relative expanded measurement uncertainty was calculated using a coverage factor k of 2, corresponding approximately to a 95 % confidence level (EURACHEM/CITAC GUIDE CG 4 2000) giving values of 9.5, 11.0, 10.5, and 13.5 % for aflatoxins B$_1$, B$_2$, G$_1$, and G$_2$ in foodstuffs.

Conclusions

An analytical method based on clean-up with immunoaffinity column and HPLC with fluorescence detector was validated according to Regulation 2004/882/EC for the determination AFs in food matrices. The method performance characteristics were found to be conformed to minimum performance criteria established by Regulation 2006/401/EC. The validated method provides accurate and precise results and presents rapid and economic procedure. The excellent results achieved in a proficiency test round confirmed laboratory technical competence. The proposed method is well suited to satisfy the demands for accurate and sensitive detection of aflatoxins with sample preparation and cleanup steps. The developed procedure is rapid and

allows the simultaneous determination of aflatoxins in different food matrices with high sensitivity. [2, 3, 4, 9, 12, 13, 14, 15, 16, 18, 19, 20, 21, 25, 26, 27, 28, 31, 34, 36, 37, 40, 42, 44, 48]

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