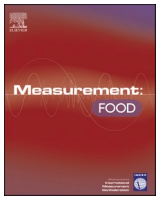
Measurement: Food 13 (2024) 100124



Contents lists available at ScienceDirect

Measurement: Food

journal homepage: www.elsevier.com/locate/meafoo





Comprehensive and sensitive validation of the method and determination of measurement uncertainty for simultaneous specification of aflatoxin B1, B2, G1 and G2 in nuts

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ARTICLE INFO

Keywords:
Aflatoxin
Nuts
Validation
Uncertain measurement
Proficiency test performance
High pressure liquid chromatography

ABSTRACT

In this study, a comprehensive and sensitive validation study of the legally used method was conducted to simultaneously determine the levels of aflatoxin B1, B2, G1, and G2 in nuts. The applied method has been extensively evaluated in terms of in-laboratory detection limit and quantification limit, linearity, selectivity, precision, recovery, accuracy, adequacy, and measurement uncertainty. Levels of aflatoxins were examined by a validated immunoaffinity column-high performance liquid chromatography-post column derivatization-fluorescence detection (IAC-HPLC-PCA-FLD) analytical method. Detection limits (LOD) for aflatoxin G2, G1, B2, and B1 were found to be 0.05 μg/kg, 0.13 μg/kg, 0.06 μg/kg, and 0.15 μg/kg, respectively. The regression coefficient (R^2) was greater than 0.999 according to constructed calibration curves for all aflatoxin components. Thus, the curves exhibited satisfactory linearity within the analyzed range. The combined standard uncertainty values for precision (RSD%) studies were below 10%. It showed a robust and reliable sensitivity with <10% RSD with the test procedure. Average recovery values were detected as 67-84% for hazelnut and 60-89% pistachio. All aflatoxin components in hazelnut paste, which is the proficiency test material, were determined within an appropriate |Z|-score range (≤ 2). The relative expanded uncertainty of measurement was 0.80 μg/kg for AFB1 and AFG1, 0.83 μg/kg for AFB2 and AFG2, and 0.78 μg/kg for total AFs. The results showed that the approved method for the investigation and monitoring of aflatoxins in hazelnuts and pistachios is highly sensitive, robust, reliable, selective, and reproducible. Validation parameters can be successfully used in routine analysis to monitor aflatoxins in nuts for compliance with current regulations.

1. Introduction

Food safety is an important priority for human and animal health. Mycotoxins, which pose a major threat to food safety, are toxic secondary metabolites commonly produced by molds in various food products including nuts, dried fruit, grains, spices, and feed [1]. Aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin B1 (AFB1), and aflatoxin B2 (AFB2) are mycotoxins, which are toxic and even carcinogenic metabolites produced by *Aspergillus flavus, parasiticus and nomius* [2–4]. *A. nomius and parasiticus* produce both G and B aflatoxin, while *A. flavus* produces only B aflatoxin. Aflatoxins can cause grave chronic diseases resulting from their acute toxic, immunotoxic, estrogenic, teratogenic, and nephrotoxic influences and are among the most potent carcinogens known [5,6]. Among aflatoxins, AFB1 has been classified as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC) [7,8]. Mycotoxins can be ingested directly from contaminated agricultural goods or indirectly through the ingestion of products of animal origin. Control of mycotoxins in foods is very important for the protection of consumer health [9]. Although hazelnuts, walnuts, almonds, pistachios, dried fruits and spices contain a low amount of water, and their richness in unsaturated fat and protein limits spoilage by microorganisms, environmental conditions in cultivation, processing or storage allow the formation of aflatoxins [10,11]. Exposure to these contaminants may pose a health risk to humans because of the high toxicity of AFs. To lower the possible risk, the Joint FAO/WHO Committee on Food Additives (JECFA) advises minimizing the amount of

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https://doi.org/10.1016/j.meafoo.2023.100124
Received 20 July 2023; Received in revised form 30 November 2023; Accepted 4 December 2023
Available online 7 December 2023
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mycotoxins in foods. Therefore, many nations have implemented legislation to restrict AFs in food items; measurement and monitoring of AFs in food products are essential [3,5].

Nuts and pistachios are often raised for global trade. Hazelnut is mostly manufactured in Turkey, Italy, and Azerbaijan (550.000 tons, 80.000 tons, and 45.000 tons per year, respectively), while pistachios are mostly produced in Iran (14.7%), Turkey (13%), and USA (57.2%) [12]. The maximum legal limits (MLLs) allowed for hazelnuts and pistachios in Turkey and the European Union (EU) are 10 μg/kg for total aflatoxins (total AFs) and 5.0 μg/kg for AFB1. Additionally, the ML for processed foods and dried fruit is 2.0 g/kg for AFB1 and 4.0 g/kg for total AFs [13–15].

Aflatoxins can also occur in a variety of raw products, including nuts, grains, coffee, cocoa, spices, and dried fruits, and in products derived from industrial processes due to their stability. One of the important problems in the production of nuts like pistachio and hazelnut is the formation of aflatoxins. Tree nuts such as almonds, hazelnuts, walnuts, and pistachios are rich in unsaturated fat and protein. Since these foods contain low amounts of water, they limit the spoilage of microorganisms. In addition, contamination of nuts by fungi may occur if environmental conditions during cultivation, storage, or processing allow fungal growth. Therefore, consumption of nuts is one of the major contributors to human exposure to mycotoxins, especially aflatoxins. Studies on the detection and advancement of analytical test processes are ongoing because the production of aflatoxins in food products is a critical global issue [16–18]. In order to ensure food safety, many analyses are required for official control purposes for the legislative provisions regulating mycotoxin levels in foods. Due to the possibility of coexistence of contamination-susceptible matrices, the wide variety, and the wide range of mycotoxin concentrations, the development and validation of validated versatile multi-mycotoxin methods are essential. For the simultaneous quantification of aflatoxins in food items, it is therefore vital to have established analytical methods for use in control, monitoring, and risk assessment studies. A limited number of studies conducted to develop these methods in recent years contain performance characteristics data made by method validation [19,20]. Delivering a quantitative and/or qualitative answer with a manageable level of uncertainty is the goal of an analytical approach. Therefore, "validation" actually refers to "measuring uncertainty" theoretically. In actual practice, method validation entails assessing a number of method-performance attributes, including accuracy, selectivity/specificity, trueness, linearity, recovery, operating range, limit of detection, limit of quantification (LOQ), applicability robustness, and sensitivity [11].

Enzyme-linked immunosorbent assay (ELISA), gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography, and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) methods are widely used for the quantification of aflatoxins [21–23]. A suitable cleaning and extraction technique is required to increase sensitivity, eliminate interference and detect trace levels prior to chromatographic analysis. Liquid-liquid extraction (LLE), solid phase extraction (SPE), and immunoaffinity columns are commonly used for cleaning methods for mycotoxins [24–26]. Fluorescence-detection, for the simultaneous specification of aflatoxins in quantification tests of mycotoxins, HPLC separation in combination with post-column derivatization is frequently utilized.

In a study conducted with the HPLC-FLD method for the detection of AF in hazelnut samples in Turkey, the detection limit ranged from 0.21 to 0.30 μg kg^{-1}. While AF was not detected in hazelnut shells, 12% of six raw hazelnut kernel samples and 8.3% of five roasted hazelnut kernel samples contained AF [15]. The LOD and LOQ values were 1.07 g/kg and 0.32 g/kg for AFG1 and AFB1, respectively, and 0.19 g/kg and 0.63 g/kg for AFB2 and AFG2, respectively, in research by Fu et al. using the 991.31 method for aflatoxin measurement in maize and peanut samples [26].

Compared to other aflatoxin detection methods, the IAC-PCD-HPLC-FLD method has the advantages of being fast, selective, robust, sensitive and inexpensive when adapted to a routine analysis [25,27,28]. A qualitative or quantitative result should be presented using an analytical approach, and the level of uncertainty should be acceptable. Therefore, each of the parameters used in "verification" contributes to "measuring the total uncertainty". Validation of the method is accomplished by evaluating several method-performance characteristics such as LOQ, LOD, working range, accuracy, linearity, sensitivity, selectivity/specificity, precision, recovery, robustness, and applicability [11,29–32].

Since even the presence of very low amounts of aflatoxins in food products is very dangerous for human health, analyzes for these toxins must be accurate and robust. In addition, to accurately determine aflatoxin levels, the determination method needs to be verified to obtain reliable and accurate results with high sensitivity. In this study, a laboratory validation was aimed at the sensitive and rapid application of the method in routine analyses by using 9 different validation parameters for the simultaneous detection of AFB1, AFB2, AFG1, and AFG2 in hazelnuts and pistachios with IAC-HPLC-PCD-FLD. In addition, as far as we know, the suitability of the technique for the intended use according to the parameters of the legally applied analytical performance has been presented for the first time in such a comprehensive way.

2. Materials and methods

2.1. Reagents, chemical and apparatus

Acetonitrile (chromatography grade-CAS: 75-05-8), methanol (chromatography grade- CAS: 67-56-1), HPLC grade water (CAS: 7732-18-5), and nitric acid (HNO_3, >65%- CAS: 7697-37-2) were purchased from Sigma-Aldrich (MO, St. Louis). Sodium chloride (CAS: 7647-14-5) and potassium bromide (CAS: 7758-02-3) (analytical grade) were purchased from Merck (Germany, Darmstadt). Afla Test immunoaffinity columns (VICAM, Watertown, MA - Cat. No: G1010) were used. Before use, all glassware was thoroughly rinsed with water after being cleansed with 0.5 mol/l HNO_3. Reference materials were purchased from UME-9909 TUBITAK National Metrology Institute (Gebze-Kocaeli, Turkey) for hazelnut and FAPAS-T04377QC Food Analysis Performance Evaluation Program (FERA, Sand Hutton, UK- Product Code: FCMA2-NUT14QC) for peanuts. Waring laboratory blender (Waring-8011Eb, USA) and Robot Coupe laboratory grinder (Robot Coupe, R2, France) were used for extraction. Aflatoxin analyses were performed on the Agilent 1260 Infinity HPLC (Santa Carla, ABD) system. Agilent technical service completed the Operational Qualification- Performance Verification (OQ-PV) test on the HPLC system.

2.2. Sample collections

For validation studies, 2 kilograms of hazelnut (*Corylus avellana*, Giresun-Turkey) and pistachio (*Pistacia vera L.*, Gaziantep-Turkey) samples, and 100 grams of walnut (*Juglans regia L.,* Eskisehir-Turkey), corn (*Zea mays,* Karaman-Turkey), peanut (*Arachis hypogea,* Osmaniye-Turkey), and almond (*Prunus spp.,* Karaman-Turkey) samples were purchased from supermarkets in closed packages to appraise the applicability of the method. The samples were ground using a laboratory grinder and stored in sealed containers at +4°C until analysis.

2.3. Standard solutions preparation

Mixed Aflatoxin standard stock solution for HPLC was purchased from Supelco (Bellefonte. PA. ABD) ready-to-use in methanol in a vial containing 1000 ng/ml AFG1 (Lot No: LB83051) and AFB1 (Lot No: LC06494), 300 ng/ml AFG2 (Lot No: LC09578) and AFB2 (Lot No: LC20105). A second step mixed standard solution was prepared from mixed AF standard solution (1 ml) in methanol (10 ml) (100 ng/ml AFG1 and AFB1, 30 ng/ml AFG2 and AFB2). In order to create a six-point calibration from this solution, working solutions were prepared

freshly on the day of use in 10 ml calibrated measuring balloons (Table 1). Second stage stock standard solutions were used within 3 months after preparation and stored at -18°C. Working standard solutions were stored at +4°C using stock standard solutions for 1 week.

2.4. Extraction and clean-up

Extraction and clean-up of aflatoxin from hazelnut, pistachio, peanut, walnut, almond, and corn were performed as described in AOAC official method 991.31 [17]. In this method, the aflatoxin from the liquid phase obtained as a result of methanol-water extraction of hazelnut and pistachio was purified by passing through IACs. According to the method, at first, 25 g of hazelnut and pistachio samples were weighed and separately transferred to the blender jars together with 5 g of sodium chloride. 125 ml extraction solvent (70% methanol: 30% HPLC-grade water, v/v) was added into the jars. High-speed mixing was provided for exactly 2 minutes to allow the samples to interact with the solvent and extracts were filtered with fluted filter paper (VICAM, 24 cm). Into clean beakers, 30 ml of HPLC grade water was added along with 15 ml of filtrates and shaken vigorously. After the mixtures were filtered through glass microfiber filters (VICAM, 11 cm-1.5 μm), they were ready to be passed through the IACs. All 15 ml of the mixtures were pumped through the IACs for the clean-up procedure at a constant flow rate of 2 drops per second. IACs were washed with using 20 ml of HPLC-grade water at the same constant flow rate. Aflatoxin components were eluted into vials by passing 1 ml of ethanol through the IACs by gravity instead of pumping. Then, they were ready for the HPLC analysis by diluting the elutes with 1 ml of HPLC-grade water. The measurement tools (such as analytical balance, micropipette and graduated cylinder) used in the whole study were calibration certificated.

2.5. HPLC-FLD conditions

Measurements were carried out using an Agilent 1260 Infinity HPLC system equipped with an Agilent 1260 Infinity Quaternary Pump, a fluorescence detector (Agilent 1260) and an auto-sampler. The data was processed with Agilent Openlab Software. C18–ODS2 (250 mm-5μm-4.6 mm, Waters, Milford-MA, ABD) column was used for the separation and quantitative analysis of aflatoxin components during an analysis period of 25 minutes for each sample with an injection volume of 100 μL. The FLD detector was fixed at 360 nm excitation and 440 nm emission to obtain better spectrums. The mobile phase was prepared as 120 mg/L KBr and 100 μL 65% HNO_3 in methanol: water: acetonitrile (20:60:20, v/v/v). The flow rate was set 1 ml/min at room temperature (24 °C). The post-column aflatoxin derivatization as AFB2, AFB1, AFG2, and AFG1 was performed using Cobra-Cell inserted into the HPLC system.

2.6. Validation procedure (measuring uncertainty)

Validation studies were carried out using hazelnut and/or pistachio samples in terms of limit of detection and limit of quantification, selectivity, linearity, precision, recovery, accuracy, proficiency and uncertain measurement.

Table 1
Preparation of working standard solutions for the calibration curve.

Working standard	Aliquot of second-stage standard solution (μl)	The final concentration of working standards for calibration (ng/ml)			
		AFB1	AFB2	AFG1	AFG2
1	30	0.3	0.09	0.3	0.09
2	50	0.5	0.15	0.5	0.15
3	120	1.2	0.36	1.2	0.36
4	250	2.5	0.75	2.5	0.75
5	400	4.0	1.2	4.0	1.2
6	600	6.0	1.8	6.0	1.8

2.6.1. LOD-LOQ

Limit of detection and limit of quantification were assigned to check the validity of the HPLC procedure used for aflatoxins. Blank hazelnut samples were spiked with 0.5 ng/ml for AFG1, AFB1, and 0.15 ng/ml for AFG2, AFB2 from the aflatoxin mix standard. Ten independent runs for AFG1, AFB1 and AFG2, AFB2 were operated on a total of 10 spike samples. The acquired data were statistically evaluated, and if there was no significant difference between the injections (independent sample t-tests), the standard deviation (SD) of all injections was calculated. LOD and LOQ values were determined using the following formulas Eqs. (1) and ((2)).

$$LOD = SD \times 3 \quad (1)$$

$$LOQ = SD \times 10 \quad (2)$$

2.6.2. Linearity

Standards at six different concentrations (ranging from 0.09 to 6.0 ng/ml) were prepared to establish the linear measuring range. Three injections of each standard concentration were operated to measure the aflatoxins. Six-point calibration curves were plotted and the presences of aflatoxins were determined by calculating the peak area (2D Chemstation Software). The correlation coefficient, slope, and y-intercept of the regression line were used to assess the linear relationship.

2.6.3. Specificity and selectivity

The capacity to quantify the analyte in the presence of any potential contaminants is known as specificity. The absence of interference at the same retention times used to test for aflatoxins was used to establish that the samples were free of interference. The selectivity of the method was achieved through immunoaffinity purification techniques, a derivatization unit, and a selective fluorescence detector. To test the selectivity of the procedure, AFB2, AFB1, AFG2, and AFG1 were added to a hazelnut and pistachio samples within contamination ranges of 0.6 to 0.36 ng/ml. The added samples were then examined using the methods outlined above to see if the peaks had separated.

2.6.4. Precision

Precision was evaluated by repeatability and reproducibility studies. In these precision studies, HPLC equipment, the number of analysts, and reagents remained constant. For the repeatability studies, AF-free hazelnut and pistachio samples were spiked from the standard solution with AFG1 and AFB1 2.0 ng/ml, AFG2 and AFB2 0.6 ng/ml. Repeatability 10 independent assays with added AF standards as recommended by the Eurachem validation protocol (≥10) were tested on the same day as intermediate precision [30].

For the reproducibility study, the AF-free hazelnut sample was 1.2 ng/ml for AFB1-AFG1, 0.36 ng/ml for AFB2-AFG2, and 3.1 ng/ml for AFG1-AFB1, 0.95 ng/ml for AFG2-AFB2 for AF-free pistachio sample was added from the standard solution. The samples prepared in this way were completed in 6 days by two different analysts performing one independent study each day.

2.6.5. Recovery

The recovery studies were performed with hazelnut and pistachio butter by two analysts for six days. Average % recovery values were calculated according to the formula below for each by the addition of a known amount of mycotoxins standards separately into blank hazelnut (1.2 ng/ml for AFB1, AFG; 0.36 ng/ml for AFB2, AFG2) and pistachio butter (3.1 ng/ml for AFG1, AFB1; 0.95 ng/ml for AFG2, AFB2) samples.

The formula was "%R = C_1 x 100 / C_2" which was the recovery, the value read in the spiked sample (expected concentration) and spike concentration (calculated concentration) were abbreviated as R, C_1 and C_2, respectively. The values read in the spiked samples were determined by extrapolating the absolute responses in the calibration curve. Expected concentrations were compared with calculated ones for the 100%

recovery.

2.6.6. Accuracy

The accuracy of the method was based on the analysis of six independent studies conducted on each of the hazelnut UME RM-9909 and the pistachio FAPAS-T04377QC quality control materials separately by the same operator in one day. The quality control materials were certificated by TUBITAK and FAPAS (Food Analysis Performance Assessment Scheme) according to the z-score of the results obtained with using different methods and devices by six different laboratories participating in the proficiency test for this material. Absolute and relative errors were calculated for both hazelnuts and pistachios by comparing quality control materials containing known amounts of natural aflatoxins with the average of six independent measurements separately using them.

2.6.7. Proficiency

The proficiency test performance was carried out following the "ISO/IEC 17043: 2010 Conformity Assessment - General Requirements for Proficiency Testing" standard, through the TUBITAK UME Reference Materials Laboratory in December 2020. Evaluations of proficiency test results were made by TUBITAK. Assigned values were determined based on the median value of participant (9 different laboratories) results for aflatoxins. Horwitz Equations modified version was used to determine the standard deviation value of the reference material's proficiency test. Hazelnut paste containing mixed aflatoxins (AFG1, AFB1, AFG2 and AFB2) was used as the proficiency test material provided by TUBITAK UME. After the extraction and clean-up of the test material, triplicate aflatoxin analyzes were performed by the same operator within one day by HPLC accordingly to the AOAC official method 991.31. The average of the results was then submitted to TUBITAK to determine the Z-score.

2.6.8. Uncertain measurement

For laboratories accredited according to ISO 17025/2017, it is mandatory to evaluate the uncertainty of analytical results after validation studies. There are several suggested uncertainty calculation methods such as EURACHEM Guide 2014, ISO Guide 2008, Nordic Committee on Food Analysis and NMKL 2003. In this study, analytical validation parameters were used at each step in the validation of the method. The relative uncertainty (u) was determined by taking into account various sources of uncertainty, including standard preparation (volume and stock standard), sample preparation (mass and volume), calibration curve, repeatability of the method device reproducibility.

The relative expanded uncertainty (U) of measurement was calculated using the coverage factor k=2, which corresponds to approximately a 95% confidence level.

2.6.9. Statistical analysis

Microsoft Excel software (Microsoft Office Excel 2003, Redmond, WA USA) was used for data processing. The equation of the calibration curve was determined using least squares regression analysis. Data were analyzed using descriptive statistics [including average means values and standard deviations (SDs) via SPSS software. version 20 (IBM SPSS Statistics)]. Independent sample t-tests and analysis of variance (ANOVA) were used to analyze the data. A significant level was accepted as $p < 0.05$.

3. Result and discussion

3.1. Optimization of HPLC-FLD

The HPLC method was initially optimized to enable the identification and quantification of total aflatoxins. Column temperature and mobile phase were optimized as key HPLC parameters. Appropriate column temperature and mobile phase selection are important as it allows for satisfactory elution of analytes, ensure maximum retention, and prevent carryover between samples [33]. The addition of 100 μl of 65% nitric acid to the water may contribute to the ionization since the higher peak heights were obtained with the addition of nitric acid to the mobile phase. Methanol-acetonitrile-water (containing nitric acid) was used as the mobile phase. Analyzes were performed under different column temperature conditions, including 22, 24, and 30°C. It was determined that the peak shapes showing the best separation of the analytes were at 24°C.

3.2. Analytical performance

Performance characteristics of the method are a way to check whether an analytical method is suitable to meet legal requirements. The efficiency of the method (extraction, clean-up, IACs, post-column derivatization, and the condition of the HPLC-FLD) was appraised by standard addition quantification procedure. Quality parameters like LOD-LOQ, linearity, selectivity, precision, recovery, accuracy, and proficiency were examined for the development of AF components determination before the sample analysis. Validation data obtained from studies with quality parameters are given in the tables.

The linear range in the calibration curves was chosen as 0.3-6.0 ng/ml for AF B1 (Fig. S1) and G1 (Fig. S2), and 0.09-1.82 ng/ml for AF B2 (Fig. S3) and G2 (Fig. S4). The regression coefficient (R^2) was greater than 0.999 according to constructed calibration curves for a total of four aflatoxin components. Thus, the curves exhibited satisfactory linearity within the analyzed range. The calculated LOD and LOQ values of mixed aflatoxin spiked hazelnut samples were given in Table 2. The LOD and LOQ values obtained in this study were found to be within the limits of EU legal requirements (maximum limits of 5.0 μg/kg for AFB1 and 10.0 μg/kg for total aflatoxin) [34].

In the Leong et al study in nuts, the levels of four individual aflatoxin LOD and LOQ values were reported as 0.3 – 0.6 μg/kg for both AFG1 and AFB1 and 0.1 – 0.4 μg/kg for both AFG2 and AFB2, respectively [35]. In another validation study were conducted for aflatoxins in Brazil nuts by Campos et al. While AF B2, B1, G1 and G2 values for LOD were 0.03, 0.05, 0.05 and 0.05 μg/kg, respectively, LOQ values were 0.10, 0.18, 0.18 and 0.18 μg/kg, respectively [11]. The LOD and LOQ values were 0.3 g/kg and 0.6 g/kg for AFG1 and AFB1, respectively, and 0.1 g/kg and 0.4 g/kg for AFG2 and AFB2, respectively, in a research by Leong et al. [26] utilizing for aflatoxin analysis in hazelnuts using HPLC. The LOD and LOQ values were 0.32 g/kg and 1.07 g/kg for AFB1 and AFG1, respectively, and 0.19 g/kg and 0.63 g/kg for AFB2 and AFG2, respectively, in a research by Fu et al. [29] using the S/N method for aflatoxin measurement in maize and peanut samples.

Solubility in each aflatoxin compound exhibited good chromatography with an acceptable baseline. Fig. 1 indicates chromatograms illustrative of the procedure's selectivity. From the chromatograms of the working standards, samples with added aflatoxins, and blank samples, it can be seen that the aflatoxin peaks are well distincted from each other. Also, there were no foreign peaks interfering with the analytes at the retention times of each aflatoxin, which were 22.687, 17.930, 16.503, and 13.204 min for AF B1, B2, G1, and G2, respectively. As a result, the procedure selectivity and specificity were deemed adequate.

The precision of the whole method was evaluated for both reproducibility (intra-day precision) and intermediate precision (inter-day precision). Repeatability was evaluated by applying the entire

Table 2
LOD and LOQ values obtained from mixed aflatoxin spiked hazelnut samples (μg/kg).

Hazelnut	G_2	G_1	B_2	B_1	TOTAL
AVERAGE	0.123	0.401	0.135	0.427	1.085
SD	0.016	0.043	0.021	0.052	0.030
LOD	0.05	0.13	0.06	0.15	0.02
LOQ	0.16	0.43	0.21	0.52	0.07

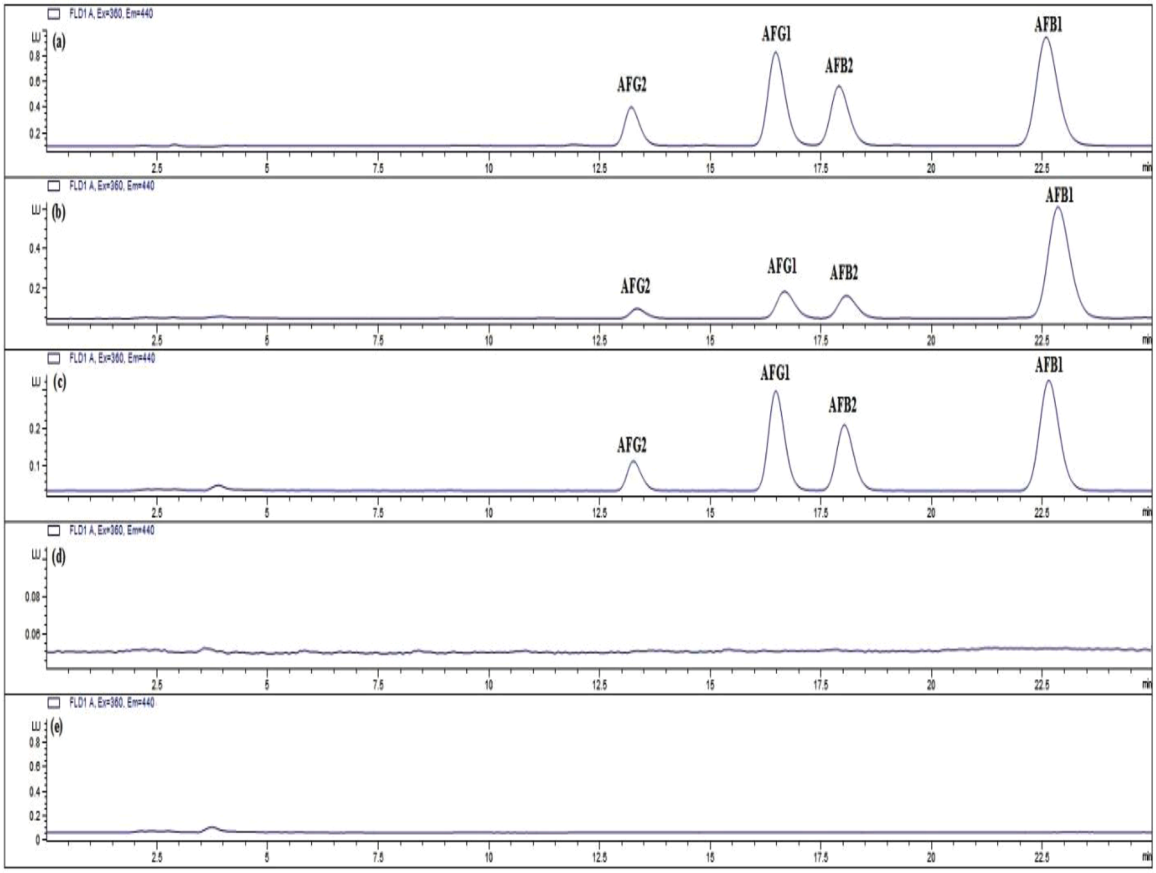


Fig. 1. Chromatograms of (a) 4 ng ml^{-1} for AFG1-AFB1 and 1.2 ng ml^{-1} for AFG2-AFB2 standards, (b) 1.2 ng ml^{-1} for AFG1-AFB1 and 0.36 ng ml^{-1} for AFG2-AFB2 extracted spiked hazelnut sample, (c) 0.6 ng ml^{-1} for AFG1-AFB1 and 0.18 ng ml^{-1} for AFG2-AFB2 extracted spiked pistachio sample, (d) blank hazelnut sample (e) blank pistachio sample.

procedure to hazelnut and pistachio samples on the same day with all aflatoxins added at specific concentrations (Table 3). For the reproducibility study, aflatoxin standards were added at certain concentrations to the hazelnut and pistachio samples that do not contain aflatoxin, and an independent study was conducted by two different analysts each day and completed in 6 days (Table 4). Every sample was injected three times (instrumental replicates). All of the precisions (RSD, %) were below 10%. A solid and reliable precision with an RSD of <10% was indicated by the test procedure.

In the study of Arroyo-Manzanares et al. in peanuts, the RSD% levels of four different aflatoxin intra-day precision and inter-day precision values were found to be 5.1 - 9.6 for AFB1, 4.6 - 9.5 for AFB2, 3.0 - 9.1 for AFG1, 6.3 - 10.4 for AFG2 [36]. In the repeatability study conducted on peanuts, the RSD% levels of intraday precision values for AF B2, B1, G2, and G1 were determined in the range of 5.5 - 9.6 [22]. Precision studies have RSD values consistent with our previous studies and in all cases, sensitivities of less than 10% were achieved compared to current legislation [30,37,38].

In addition, for the repeatability and reproducibility studies, combined standard uncertainty calculations were made according to the Eq.

Table 3
Repeatability for determination of AF B2, B1, G2 and G1 in hazelnut and pistachio samples.

	Hazelnut (μg/kg)					Pistachio (μg/kg)				
Compound Analysis	B1	B2	G1	G2	Total	B1	B2	G1	G2	Total
1	2.023	0.618	2.033	0.539	5.213	4.596	0.675	1.867	0.446	7.584
2	2.039	0.633	2.034	0.516	5.222	4.596	0.682	1.861	0.461	7.600
3	2.034	0.623	2.026	0.543	5.226	4.605	0.675	1.856	0.466	7.602
4	2.024	0.631	2.041	0.532	5.228	4.596	0.676	1.846	0.459	7.577
5	2.032	0.630	2.030	0.509	5.201	4.589	0.675	1.858	0.461	7.583
6	2.025	0.619	2.022	0.517	5.183	4.601	0.675	1.849	0.479	7.604
7	2.035	0.633	2.030	0.500	5.198	4.600	0.677	1.845	0.461	7.583
8	2.026	0.634	2.039	0.520	5.219	4.607	0.676	1.856	0.479	7.618
9	2.025	0.626	2.019	0.518	5.188	4.607	0.681	1.859	0.466	7.613
10	2.037	0.625	2.025	0.531	5.218	4.592	0.670	1.848	0.479	7.589
Average	2.030	0.627	2.030	0.523	5.210	4.599	0.676	1.855	0.466	7.595
Absolute Error	0.006	0.006	0.007	0.014	0.016	0.006	0.003	0.007	0.011	0.014
Relative Error	0.003	0.009	0.003	0.026	0.003	0.001	0.005	0.004	0.023	0.002
% Relative Error	0.296	0.932	0.349	2.586	0.307	0.135	0.499	0.390	2.297	0.185

Table 4
Reproducibility for determination of AF B1, G1, B2 and G2 in hazelnut and pistachio samples (μg/kg).

Reproducibility for the Hazelnut								
	1st analyst				2nd analyst			
Compound Day	B1	B2	G1	G2	B1	B2	G1	G2
1	0.909	0.281	0.908	0.255	1.076	0.338	1.062	0.296
2	0.913	0.277	0.901	0.226	1.020	0.332	1.031	0.280
3	1.002	0.316	1.011	0.295	0.913	0.278	0.892	0.246
4	0.917	0.277	0.881	0.217	1.009	0.321	1.010	0.293
5	0.953	0.289	0.911	0.254	1.032	0.337	1.046	0.274
6	0.981	0.285	0.875	0.212	1.046	0.331	1.030	0.290
Average	0.946	0.288	0.915	0.243	1.016	0.323	1.012	0.280
Absolute Error	0.039	0.015	0.049	0.031	0.056	0.023	0.061	0.019
Relative Error	0.042	0.051	0.054	0.029	0.055	0.071	0.061	0.066
% Relative Error	4.151	5.120	5.408	2.871	5.468	7.057	6.051	6.622
Reproducibility for the Pistachio								
	1st analyst				2nd analyst			
Compound Day	B1	B2	G1	G2	B1	B2	G1	G2
1	2.537	0.798	2.488	0.549	2.552	0.813	2.501	0.538
2	2.582	0.806	2.512	0.557	2.591	0.808	2.518	0.545
3	2.641	0.824	2.516	0.532	2.600	0.813	2.507	0.578
4	2.778	0.837	2.559	0.536	2.684	0.825	2.520	0.543
5	2.652	0.927	2.507	0.623	2.630	0.912	2.493	0.620
6	2.667	0.922	2.484	0.593	2.758	0.950	2.633	0.597
Average	2.643	0.852	2.511	0.565	2.636	0.854	2.529	0.570
Absolute Error	0.082	0.058	0.027	0.036	0.074	0.061	0.052	0.034
Relative Error	0.031	0.068	0.011	0.063	0.028	0.072	0.021	0.059
% Relative Error	3.113	6.753	1.069	6.331	2.819	7.203	2.061	5.907

(3) (Table 5).

$$RSD_m = \sqrt{\frac{RSD_1^2(n-1) + RSD_2^2(m-1)}{n+m-2}} \quad (3)$$

Here, RSD_1; Relative standard deviation of the analysis results of the first matrix, RSD_2; Relative standard deviation of the analysis results of the second matrix, n; Number of repetitions of the first matrix, m; It is the number of analysis repetitions of the second matrix.

Table 6 shows the results for recoveries over 6 days for aflatoxin components spiked at blank hazelnut and pistachio samples by two analysts. Average recoveries were obtained from spiked samples for AFB1, AFB2, AFG1, and AFG2 respectively; first analyst hazelnut samples of 78, 79, 76, 67%, pistachio samples of 85, 89, 81, 59% and second analyst hazelnut samples of 84, 84, 84, 77%, pistachio samples of 85, 89, 81, 60%. The recovery rates are within the values recommended by the European Committee for Standardization (CEN) criteria, and the Commission Regulation (EC) No. 401/2006 for regulatory purposes; for AFB1 and AFG1 (hazelnut: 1.2 mg/l, pistachio: 3.1 mg/l spiked, identical at 1-10 ng/ml) need to be within the range of 70 to 110 % and for AFB2 and AFG2 (hazelnut: 0.36 mg/l, pistachio: 0.95 mg/l spiked, identical at < 1.0 ng/ml) need to be within the range of 50 to 120% [39–41].

The study tests the detection accuracy of hazelnut (Fig. 2a) and pistachio (Fig. 2b) quality control materials (UME RM-9909 and FAPAS-T04377QC, respectively) containing known concentrations of aflatoxin and the results for the analysis of test materials are shown in Table 7. Based on the certification values (standard deviation and range for |z|) of the quality control material, the measured values of the total and four aflatoxin components can be seen to be in a satisfactory range with respect to the assigned values.

Table 5
Combined standard uncertainty values for precision studies.

Compound	B1	B2	G1	G2	Total
RSD_m (Repeatability)	0.002	0.007	0.004	0.024	0.003
RSD_m (Reproducibility)	0.039	0.066	0.036	0.082	0.040

The appropriate performance for the calculated Z-scores and the acceptable value of the results was $|Z| \leq 2$. According to the data obtained as a result of the analysis with the proficiency test material provided by TUBITAK UME (Fig. 2c), total aflatoxin components in hazelnut paste are in the Z-score range given in Table 8.

The measurement uncertainty calculates the test method's overall accuracy. Total uncertainty was calculated according to Eq. (7) by combining the errors due to standard preparation (u_{St}, rectangular distribution), sample preparation (u_{Smp}, rectangular distribution), calibration curve (u_{Co}), repeatability (u_R), and method reproducibility (u_{Rp}) according to the Eurachem guideline, and expressed as expanded uncertainty (U), taking into account the coverage factor (k) 2.0 (95% confidence interval). The relative expanded uncertainty of measurement was 0.80 μg/kg for AFB1 and AFG1, 0.83 μg/kg for AFB2 and AFG2, and 0.78 μg/kg for total AFs (Table 9). Uncertainty coming from volume in standard preparation (Eq. (4)), total uncertainty from sample preparation (Eq. (5)), uncertainty from the calibration curve (Eq. (6)), uncertainty calculations arising from repeatability (Eq. (3)) and reproducibility (Eq. (3)) were made according to the following equations.

$$U_{vtotal} = \sqrt{\left(U_{v100\mu l}\right)^2 + \left(U_{v1000\mu l}\right)^2 + \left(U_{v10ml}\right)^2} \quad (4)$$

$$U_{sample} = \sqrt{\left(U_{v1ml}\right)^2 + \left(U_{v10ml}\right)^2 + \left(U_{v50ml}\right)^2 + \left(U_{v100ml}\right)^2 + \left(U_{weighing}\right)^2} \quad (5)$$

$$U(c_0) = \frac{S}{B_1}\sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(C_0 - \overline{C})^2}{S_{xx}}} \quad (6)$$

S: Residual Standard Deviation, B_1: Slope, p: Number of readings for sample measurement, n: Number of measurements made for calibration, C_o: Determined solution concentration,

C: Average of different calibration standards, S_{xx}: $\Sigma(C_i\text{-}C_{ort})^2$.

Table 6
The calculated recovery values of the hazelnut and pistachio aflatoxin studies by two analysts.

Recovery for the hazelnut (%)								
	1st analyst				2nd analyst			
Compound Day	B1	B2	G1	G2	B1	B2	G1	G2
1	75.75	78.06	75.67	70.83	89.67	88.95	88.50	82.22
2	76.08	76.94	75.08	62.78	85.00	87.37	85.92	77.78
3	83.50	87.78	84.25	81.94	76.08	73.16	74.33	68.33
4	76.42	76.94	73.42	60.28	84.08	84.48	84.17	81.39
5	79.42	80.28	75.92	70.56	86.00	88.68	87.17	76.11
6	81.75	79.17	72.92	58.89	87.17	87.11	85.83	80.56
Average	78.82	79.86	76.21	67.55	84.67	84.96	84.32	77.73
Recovery for the Pistachio (%)								
	1st analyst				2nd analyst			
Compound Day	B1	B2	G1	G2	B1	B2	G1	G2
1	81.84	84.00	80.26	57.79	82.32	85.58	80.68	56.63
2	83.29	84.84	81.03	58.63	83.58	85.05	81.23	57.37
3	85.20	86.74	81.16	56.00	83.87	85.58	80.87	60.84
4	89.61	88.11	82.55	56.42	86.58	86.84	81.29	57.16
5	85.55	97.58	80.87	65.58	84.84	96.00	80.42	65.26
6	86.03	97.05	80.13	62.42	88.97	100.0	84.94	62.84
Average	85.25	89.72	81.00	59.47	85.03	89.84	81.57	60.02

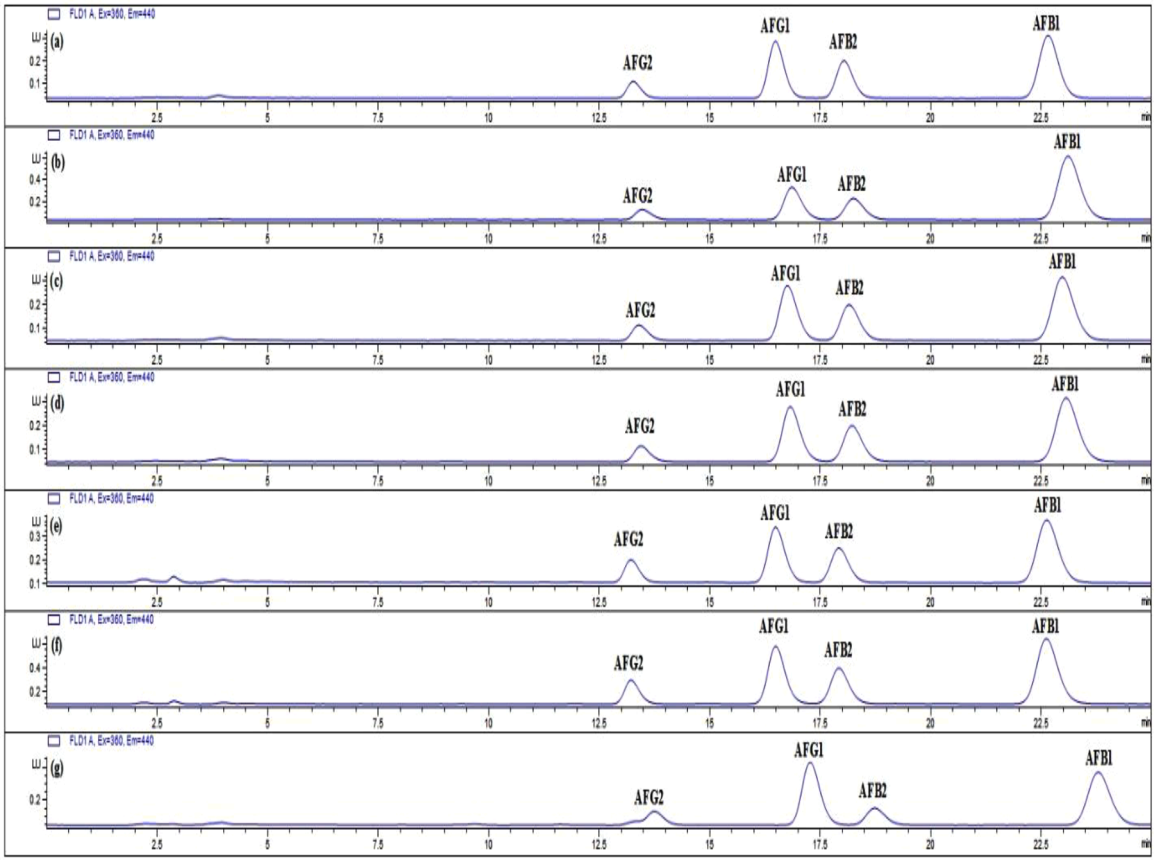


Fig. 2. Chromatograms of (a) CRM hazelnut sample, (b) CRM pistachio sample, (c) the proficiency test material, (d) walnut sample (e) corn sample, (f) peanut sample, (g) almond sample.

$$U_{Afl_m} = \sqrt{\left(\frac{u_{St}}{St}\right)^2 + \left(\frac{u_{Smp}}{Smp}\right)^2 + \left(\frac{u_{Co}}{Co}\right)^2 + \left(\frac{u_R}{R}\right)^2 + \left(\frac{u_{Rp}}{Rp}\right)^2} \quad (7)$$

3.3. Method application

This method, whose robustness was determined by validation studies, was successfully applied to AF B1, B2, G1, G2, and total AFs measurements in walnut (Fig. 2d), corn (Fig. 2e), peanut (Fig. 2f), and

Table 7
The accuracy analysis of six independent studies with hazelnut and pistachio quality control materials.

	Hazelnut (μg/kg)					Pistachio (μg/kg)				
Compound Analysis	B1	B2	G1	G2	Total	B1	B2	G1	G2	Total
1	3.040	0.677	4.788	0.878	9.383	6.365	0.877	1.245	1.105	9.592
2	3.023	0.661	4.774	0.877	9.335	6.153	0.828	1.210	1.040	9.231
3	3.006	0.663	4.756	0.853	9.278	6.351	0.875	1.246	1.088	9.560
4	3.005	0.658	4.748	0.908	9.319	6.312	0.866	1.231	1.070	9.479
5	2.998	0.668	4.754	0.932	9.352	6.323	0.863	1.239	1.074	9.499
6	2.987	0.656	4.732	0.877	9.252	6.323	0.869	1.241	1.066	9.499
Average	3.010	0.664	4.759	0.888	9.320	6.305	0.863	1.235	1.074	9.477
Assigned Value	3.170	0.66	4.290	1.070	9.110	7.84	0.939	1.420	1.480	11.70
Absolute Error	0.160	0.004	0.469	0.183	0.210	1.536	0.076	0.185	0.406	2.223
Relative Error	0.051	0.006	0.109	0.171	0.023	0.196	0.081	0.130	0.274	0.190
% Relative Error	5.053	0.581	10.93	17.06	2.303	19.59	8.094	13.01	27.44	19.00

Table 8
AFB1, AFB2, AFG1, AFG2, and total aflatoxin determination results and z scores in hazelnut paste.

Compound	AFB1	AFB2	AFG1	AFG2	Total
Assigned Value (μg/kg)	3.39	0.51	4.40	0.88	9.01
Standard deviation of the test (μg/kg)	0.75	0.11	0.97	0.19	1.98
Result	2.9	0.53	3.91	0.68	8.02
Standard deviation of the lab (μg/kg)	0.12	0.0	0.2	0.05	0.36
|Z score|	|-0.7|	|0.2|	|-0.5|	|-1.1|	|-0.5|

Table 9
Components of measurement uncertainty of aflatoxins in nuts (μg/kg).

Uncertainty components	Uncertainty values				
	AFB1	AFB2	AFG1	AFG2	Total AF
Standard preparation	0.130	0.162	0.130	0.148	0.077
Sample preparation	0.376	0.376	0.376	0.376	0.376
Calibration curve	0.018	0.021	0.017	0.024	0.066
Repeatability	0.002	0.008	0.004	0.025	0.003
Reproducibility	0.039	0.066	0.036	0.082	0.040
Combined Uncertainty	0.400	0.415	0.400	0.414	0.392
Expanded Uncertainty	0.80	0.83	0.80	0.83	0.78

almond (Fig. 2g) samples collected from supermarkets. Each sample was taken from 4 different markets and a total of 16 samples were analyzed in 3 replicates. AF B1, B2, G1, and G2 were present in all samples, and levels above the LOQs were not detected in 62.5% of the samples, AFB1 levels above LOD were measured in all analyzed samples, and measurable levels (>LOQ) of AFB1 were detected in 6 samples. The highest AFB1 in samples above measurable levels was 0.092 and the average AFB1 in samples was 0.114 μg/kg. These maximum and average values are far below the maximum allowable level (5.0 μg/kg) for AFB1 in nuts such as hazelnuts and peanuts, which are offered for direct human consumption by the EU and Turkey. AFB2 was detected at very low levels in 5 of the samples. In only 2 samples, AFB2 was determined to be higher than the LOQ (0.035-0.048 μg/kg, respectively). The mean AFG1 in the samples was 0.036 μg/kg and the mean AFG2 was 0.013 μg/kg. The order of total AF amount in the samples was determined as peanut (0.196 μg/kg) > corn (0.178 μg/kg) > walnut (0.155 μg/kg) > almond (0.151 μg/kg). These average values are well below the maximum allowable level (10 μg/kg) for total AF in nuts and oilseeds offered by the EU and Turkey for direct human consumption. The results found are consistent with our previous study and with the determined values of AFB1 in food samples from other studies [22,42–45].

There was no significant difference between AFB1, AFG1, AFB2, and AFG2 determined in food samples (hazelnuts, pistachios, peanuts, walnuts, almonds, and corns) in statistical studies (p >0.05). These results showed that the method is reliable and easily applicable in nut samples.

In a study by Hu et al., high sensitivity (LOQs: 0.20–0.40 μg/kg), good recoveries (80.5%–106%) for the simultaneous analysis of four aflatoxins under optimum sample pretreatment conditions, and acceptable precision were achieved [46]. In another study, in the method validation analysis of simultaneous extraction of AFB1, AFB2, AFG1, and AFG2 from rice using methanol-water (80:20, v/v) extraction and IAC, standard deviations were between 5 and 11%, recoveries were 86–92%, the LOQ was determined from 0.31 to 1.06 μg/kg and the LOD from 0.09 to 0.32 μg/kg [47]. In other study, measurement uncertainty for the detection of aflatoxin B1 in corn and hazelnuts using an HPLC method with pre-derivatization with trifluoroacetic acid and fluorescence detection was calculated using samples whose concentration ranged from 2 to 20 mg. The calculated relative standard uncertainty for aflatoxin B1 was determined as 16.3% [48]. The method we used is compatible with previous studies.

4. Conclusion

In this study, a single laboratory validation is aimed at the simultaneous detection of AFB1, AFB2, AFG1, and AFG2 in hazelnuts and pistachios with IAC- HPLC-PCD-FLD, using 9 different validation parameters to apply the method in routine analysis in a comprehensive, sensitive and fast manner. The RSD% levels of intra-day and inter-day precision values in hazelnut were 0.30 - 4.15 for AFB1, 0.93 - 5.12 for AFB2, 0.35 - 5.41 for AFG1, 2.59 - 2.87 for AFG2. The RSD% levels of intra-day and inter-day precision values in pistachios were determined as 0.14 - 3.11 for AFB1, 0.50 - 6.75 for AFB2, 0.39 - 1.07 for AFG1, 2.30 - 6.33 for AFG2. The combined standard uncertainty values (RSD_m) for aflatoxins in precision studies ranged from 0.002 to 0.024 for repeatability and 0.036 to 0.082 for reproducibility. Average recoveries were obtained from spiked samples for AFB1, AFB2, AFG1, and AFG2 respectively; first analyst hazelnut samples of 78, 79, 76, 67%, pistachio samples of 85, 89, 81, 59% and second analyst hazelnut samples of 84, 84, 84, 77%, pistachio samples of 85, 89, 81, 60%. Based on the certification values (standard deviation and range for |z|) of the quality control and qualification material, the measured values of the total and four aflatoxin components were determined to be in a satisfactory range with respect to the assigned values. The relative expanded uncertainty of measurement was 0.80 μg/kg for AFB1 and AFG1, 0.83 μg/kg for AFB2 and AFG2, and 0.78 μg/kg for total AFs. The approved method for the investigation and monitoring of aflatoxins in hazelnuts and pistachios can be successfully used in routine analyzes in terms of compliance with current regulations. Future research is needed to develop lower-cost, more accurate, faster, non-toxic, and non-polluting technologies to detect aflatoxins.

Funding source

This research was supported without a project by the Technological Research & Application Center of Karamanoğlu Mehmetbey University.

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Hacer Sibel Karapinar: Conceptualization, Formal analysis, Investigation, Project administration, Software, Supervision, Writing – original draft, Writing – review & editing. **Numan Eczacioglu:** Conceptualization, Formal analysis, Investigation, Project administration, Software, Supervision, Writing – original draft, Writing – review & editing. **Faruk Dogan:** Conceptualization, Formal analysis, Investigation, Project administration, Software, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank the Karamanoglu Mehmetbey University Scientific and Technological Research & Application Center for the financial support in the publication of this article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.meafoo.2023.100124.

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