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The Determination of Mycotoxins in the Composition of Walnut (*Juglans Regia L.*) Fruits Cultivated in Different Geographical Regions of Uzbekistan by High–Performance Liquid Chromatography Method and Their Comparative Analysis

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ABSTRACT: Mycotoxins are secondary metabolites produced by various fungi and are known to have significant adverse effects on human and animal health. If food products are contaminated with mycotoxins, their toxicity can cause various diseases. In this scientific research work, residual mycotoxins in nutrients were combined with liquid–liquid extraction (LLE) and analyzed through the use of reversed–phase high–performance liquid chromatography (RP–HPLC). These scientific studies were conducted in the experimental biology laboratory of Gulistan State University to determine the amount of aflatoxin (AFB2, AFG1, AFG2) in the samples of walnuts cultivated in six regions of Uzbekistan. The study revealed the detection of aflatoxins AFG_1 type 0.003 in the designated region labeled as S20, AFB_2 and AFG_2 0.001 in J25, and AFG_2 0.002 in S30. However, it is important to note that these levels did not above the maximum residue level standards established by the Republic of Uzbekistan. However, the aflatoxins produced by fungi are released based on the development of fungi at a temperature of 25–300^{0}C, therefore it is recommended to store walnut fruits in dry conditions at a temperature of 150^{0}C. All the methods used during scientific analysis can demonstrate high sensitivity and accuracy. All methods are successfully used to identify mycotoxins in the composition of walnut fruits, separate them from fungi and specify the amount of ingredients.

KEYWORDS: mycotoxin, composition, aflatoxin, walnut, Uzbekistan, juglans regia l., fungi, geographical region, high–performance liquid chromatography (HPLC), reversed–phase (RP), liquid–liquid extraction (LLE), zearalenone, deoxynivalenol, ochratoxin A., fumonisins.

INTRODUCTION

Mycotoxins are a class of secondary metabolites that are present in many food sources, plants, and plant–derived products, and are produced and released by fungus [1–4]. Food, plants, and plant products serve as valuable sources of nutrition for the growth and development of fungi due to their high organic content. Fungal organisms have the ability to release mycotoxins, including deoxynivalenol (DON), aflatoxins (AF), zearalenone (ZEA), and ochratoxin A (OTA), as a defensive mechanism throughout their developmental processes [1–3]. The aforementioned toxins are characterized by their molecular composition, which is both minimal in size and exhibits a stable nature. Enzyme inhibitors exert an influence on the metabolic regulation of both humans and animals.

Over four hundred mycotoxins, possessing distinct chemical structures and characteristics, have been found by the isolation from various fungus species. Included in this group are AF, fumonisins (FBs), A–type trichothecenes (T–2 and HT–2 toxins), B–type trichothecene–DON, and nivalenol, which exhibit elevated levels of mycotoxins. Furthermore, many forms of mycotoxins, including ZEA, OTA, patulin (PAT), and citrine (CIT), have been found [5].

When the concentration of mycotoxins surpasses the permissible limits and enters the human body through food consumption, it can result in toxicity, potentially leading to fatality in certain instances [6]. Consequently, numerous nations, in the process of importing food commodities from foreign sources or managing their inventory of both finished goods and raw materials, conduct regular assessments of these products to ascertain the presence of mycotoxins. The implementation of regulatory limits and monitoring systems on a global scale has been undertaken in order to mitigate the risk of mycotoxin exposure in both human and

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animal populations. Numerous nations have established guidelines pertaining to the maximum permitted or safe levels. Consumer protection legislation in most countries establishes legal thresholds for various types of mycotoxins found in significant food and feed commodities, such as cereals, nuts, fruits and their derivatives, as well as milk and milk products [7, 8].

The presence of mycotoxins in products intended for human consumption is subject to regulatory limits set by the European Commission. These limits include 2 µg/kg for AFB1, 4 µg/kg for the combined sum of AFB1, AFG1, AFB2, and AFG2, up to 3 µg/kg for OTA, 750 µg/kg for DON, and 75 µg/kg for ZEA [9, 10].

Nevertheless, the existing quantitative thresholds fail to consider the collective impact of many mycotoxins and are primarily derived from evaluating the individual hazard level of a single component. The current study focuses on the ongoing development of methodologies for quantifying the levels of mycotoxins and toxins present in various products.

At present, the analysis of mycotoxins and their quantitative indicators in goods is accomplished by the utilization of high–performance liquid chromatography (HPLC) or ultra–performance liquid chromatography (UPLC), as well as mass spectroscopy (MS) or tandem mass spectroscopy (MS/MS) equipment [11–14].

Solid phase extraction (SPE) and liquid–liquid extraction (LLE) are commonly employed techniques for the isolation of mycotoxins from various product compositions, as well as for the determination of their structural characteristics and activity parameters. Currently, the most commonly employed extraction phases among solid–phase extractions (SPEs) include C_{18} and polymeric material immunoaffinity column (IAC) approaches, which rely on extraction connected to a sorbent [15].

Moreover, fluorescence (FLD) or ultraviolet light (DAD) HPLC techniques are extensively employed in the identification and analysis of several subgroups of toxins present in food commodities. HPLC–based approaches have been found to exhibit notable attributes such as high speed, efficiency, and environmental friendliness in the context of separation techniques [16].

This article presents the findings of a research endeavor that aimed to detect and evaluate mycotoxins in walnut fruits (Juglans regia L.) cultivated in several geographical settings. The investigation employed HPLC–based methodologies for this purpose. Walnuts are cultivated across several regions of Uzbekistan, and presently, they are being sold to international markets. Additionally, it is necessary to draw conclusions regarding the populationɘs desire for the exportable nut product, its composition, and the adverse health effects associated with excessive consumption of saturated fatty acids. The objective was established to ascertain the various categories and quantities of aflatoxins present in samples of walnut fruits cultivated across all regions of Uzbekistan.

MATERIALS AND METHODS

The walnut fruits (Juglans regia L.), which are currently experiencing growing global demand, were selected as the subject of this research. These fruits were sourced from several geographical regions in Uzbekistan and thereafter stored in a dry and cool environment at a temperature of 15°C. (Table 1).

The macroscopic and chromatographic (HPLC) identification of all samples was carried out in the chemical analysis department of the Laboratory of Experimental Biology.

Table 1. The samples of walnut (Juglans regia L.) fruit from different geographic regions of Uzbekistan

Code of samples	Geographical area	Details of delivery	Date of delivery
S20	Sirdaryo region	Ismoiljonova O. Mirzaobod district	5.09.2022
N85	Navoi region	Kenjaev A. Kitob district	13.10.2022
J25	Jizzakh region	Ergashev U. Zomin district	15.10.2022
Q75	Kashkadarya region	Jumaev J. Nurota district	22.12.2022
S30	Samarkand region	Bekpulatov Kh. Payarik district	05.01.2023
T10	Tashkent region	Ergasheva N. Okkurgon district	15.11.2022

The research facilities employed suitable criteria to ascertain the presence of aflatoxins. For this study, we employed aflatoxin samples (specifically, 10 µg AFG1, 10 µg AFB2, and 10 µg AFG2) that were dissolved in 1 ml of acetonitrile. These samples were generated by the Sigma firm, a recognized provider of chemical products. The production of acetonitrile was carried out by Avantor® (France) specifically for HPLC.

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REAGENTS

The analysis involved the use of glacial acetic acid (CH_3COOH), ammonium hydroxide ($NH_3{\cdot}H_2O$, NH_4OH), sodium hydroxide ($NaOH$), hydrochloric acid (HCI), various salts (Na_2HPO_4, NaH_2PO_4, $MgSO_4$, $NaCl$), and a selection of organic solvents (C_2H_5OH, hexane–C_6H_{14}, ethylacetate–$C_4H_8O_2$, benzene–C_6 H_6, chloroform–$CHCI_3$).

The Agilent 1260 Infinity II Rapid Resolution Liquid system was utilized for the purpose of conducting chromatographic analysis. The Chem Station application was utilized to do the analysis using the Pump G7111A 1260 Quat Pump VL, autosampler vialsampler G7129A 1260, UV–VIS diode array detector G7115A 1260 DAD, and G7121A 1260 FLD. The utilization of an ultrasonic bath, namely the “Guangdong gt ultrasonic” model, was employed to enhance the dissolution of the solutions. The experiments were conducted using an Infinity Lab Poroshel 20EC–C18 (150 mm x 4.6 mm, 4 micron) analytical column manufactured by Agilent Technologies USA.

The mobile phase employed in this study was a binary gradient composed of acetonitrile (A) and a 0.5% solution of acetic acid (B). The gradient mode encompassed the following indicators: At time 0 minutes, the composition of the mixture was found to be 85% A and 15% B. After 4 minutes, the composition changed to 60% A and 35% B. At 5 minutes, the composition remained at 60% A but increased to 40% B. Finally, after 10 minutes, the composition shifted to 40% A and 60% B. The autofluorescence (AF) was measured using excitation and emission wavelengths of 365 and 460 nm, respectively.

A separate analysis was conducted for each toxin, and the chromatographic conditions are listed in Table 2 below.

Table 2. The chromatographic parameters for determining the flatoxins in the contents products

Standard name	Wavelength, nm	Flow rate, ml/min	Thermostat temperature, 0C
AFB2	DAD 254	1	30
AFG1	FLD 365–455	1	30
AFG2	FLD 365–455	1	30

The determination of toxins in the products was conducted in accordance with the applicable standards. In this study, chromatograms were generated for standard samples of aflatoxins, utilizing solutions that were previously produced (Figure 1 and 2).

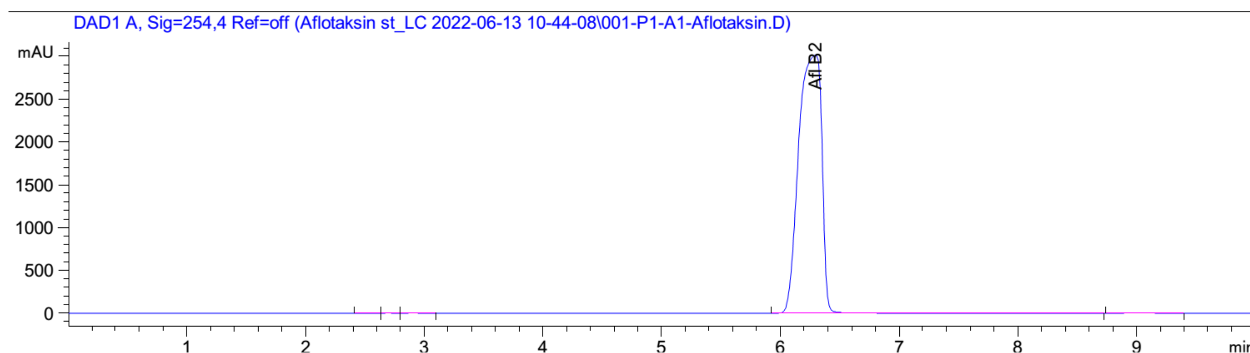

Figure 1. The chromatogram of AFB2 obtained as a standard

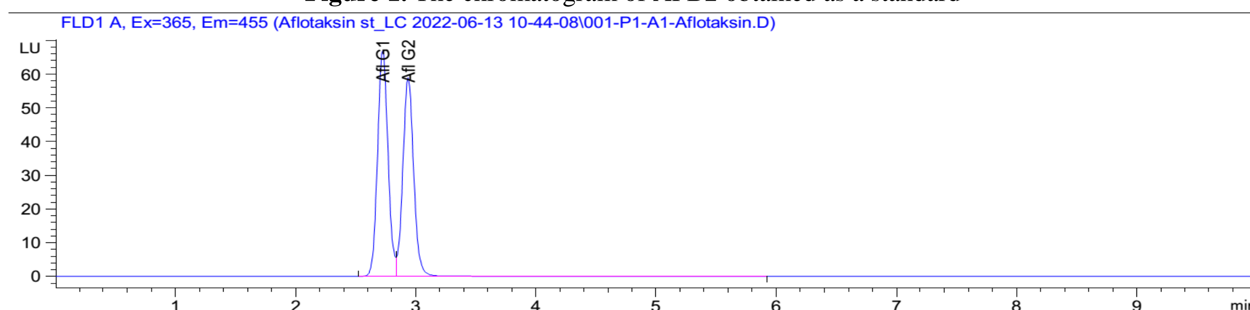

Figure 2. The chromatogram of AFG1 and AFG2 obtained as standard

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THE PREPARATION OF SAMPLES FOR ANALYSIS

Crop samples obtained from various places within our nation are meticulously pulverized to a particle size lower than 0.1 cm utilizing a blender. The process of optimal extraction was conducted in the following manner. In this particular instance, a solution of 125 ml was created by combining water and acetonitrile in equal quantities. Subsequently, 5 g of sodium chloride salt was introduced into the solution and thoroughly mixed. Following the dissolution of the salt, a quantity of 25 g from the specimen is measured, subsequently introduced into the previously prepared solution, and subjected to extraction through agitation using a magnetic stirrer for duration of 5 minutes. The samples were subjected to analysis using the high–performance liquid chromatography method in order to determine the quantity of aflatoxins present.

The samples exhibit negligible differences and undergo filtration using a 0.45 mm filter after a specific extraction period. Subsequently, the filtered samples are transferred into the analytical apparatus for further examination.

RESULTS AND DISCUSSION

The present study focused on investigating the levels of aflatoxin B2, G1, and G2 in walnuts cultivated throughout several regions within the Republic of Uzbekistan. In this study, calibration curves were generated through the preparation of solutions containing AFG1, AFG2, and AFB2 standards at concentrations ranging from 0.5 to 10 µg/ml. The calculations were conducted using the mean peak areas (n = 2.3). Additionally, the relative standard deviations (RSD) and calibration curve equations for each mycotoxin were computed and presented in Table 3.

Table 3. The statistical and productivity indicators for calibration and relative standard deviations based on AFG1, AFG2, and AFB2 standard samples

Standard name	Linear range	Regression equation	R^2
AFG1	0,5–10	Formula: y = mx + b m: 6049.82891 b: 4.52734e–1 x: Amount [mg/ml] y: Area	0,99999
AFG2	0,5–10	Formula: y = mx + b m: 28.52766 b: –6.77128 x: Amount [mkg/ml] y: Area	0,99998
AFB2	0,5–10	Formula: y = mx + b m: 599998.68381 b: 2962.27547 x: Amount [mg/ml] y: Area	0,99823

The walnut fruit samples were subjected to extraction in order to facilitate analysis. The analysis was conducted using HPLC instrument that had been calibrated using AFB2, AFG1, and AFG2 standards. The analysis was performed using an Infinity Lab Poroshell 20EC–C18 (150 mm x 4.6 mm, 4 micron) analytical column manufactured by Agilent Technologies USA. The results obtained are documented in Table 4.

Table 4. The quantitative analysis results of aflatoxins in the content of walnut fruits cultivated in different geographical regions of Uzbekistan

№	Sample code	AFB2	AFG1	AFG2	The total amount of aflatoxins
1	S20	–	0,003		0,003
2	N85	–	–	–	–
3	J25	0,001	–	0,001	0,002

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4	Q75	–	–	–	–
5	S30	0,002	–	–	0,002
6	T10	–	–	–	–

The above quantitative limits hygienic standards for food insecurity of the Republic of Uzbekistan Sanitary rules and norms 0366–19 quantitative limits established in the regulatory document were studied.

The findings of this study indicate that the levels of aflatoxins produced by fungi in walnuts cultivated in various locations of our nation are compliant with the consumption limits outlined in regulatory literature.

The findings of the study indicate the presence of mycotoxin–producing fungus in walnut samples cultivated within the Syrdarya region. Fungal infection can manifest at different stages of plant development in the field or subsequently during the processes of drying and storage, provided that moisture and temperature conditions are conducive. In certain instances, the precise demarcation of the initiation of fungal development can pose challenges. In the context of storage, when the surrounding humidity surpasses the equilibrium relative humidity of the food, the food will undergo a state of wetness, resulting in an increase in its moisture content (w). The investigation of the rise in water content during storage has been examined by previous studies [17, 18, 19] (refer to Table 2) in the context of fungal proliferation and vulnerability to mycotoxin synthesis. Consequently, it is imperative to regulate the storage conditions of the stored commodities.

Table 5. The types of fungi, their growth and optimal conditions for mycotoxin production

Fungi	Growth		Optimal growth		Optimal conditions for toxin production	
	Temperature	a_w	Temperature	a_w	Temperature	a_w
Aspergillus flavus/paraziticus	15–44 °C	0,91–0,99	35 °C	0,95	33 °C	0,99
Aspergillus ochraceous	10–40 °C	0,80–0,98	24–31 °C	0,96–0,98	25–30 °C	0,98
Aspergillus carbonarius	8–40 °C	0,90–0,93	32–35 °C	0,94–0,99	30–35 °C	

In terms of temperature preferences, the majority of fungal species exhibit mesophilic characteristics and thrive within a temperature range of 5 to 35°C. These fungi have optimal growth rates when exposed to temperatures ranging from 25 to 30°C. The occurrence of growth–accelerating temperature circumstances is closely associated with the chemical processes essential for fungal development. These reactions are most efficient within an ideal temperature range that facilitates increased growth rates.

CONCLUSIONS AND RECOMMENDATIONS

The optimization of mycotoxin detection in walnuts cultivated domestically was achieved through the utilization of high–performance liquid chromatography (HPLC) methodology. In the present study, mycotoxins were isolated from the saline environment using liquid–liquid extraction techniques employing organic solvents. Furthermore, additives that may compromise the accuracy of the analysis were effectively separated from the mycotoxins during the analytical process. The levels of mycotoxins present in the selected samples for analysis were assessed and verified to be within acceptable limits for ingestion.

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