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Original Article

Detection of Aflatoxins in Peanut Samples Using HPLC in Isfahan, Iran

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Abstract

Background and Aim: Aspergillus, Fusarium, and Penicillium are fungi commonly found in farm produce, including peanuts, that can produce mycotoxins such as Aflatoxin. Aflatoxins are toxic byproducts of fungal metabolism that can cause cancer. They are found in various food items. This study aimed to identify Aflatoxins in peanut specimens using HPLC in Isfahan, Iran.

Materials and Methods: One hundred fifty 300-gram peanut samples were collected from markets in Isfahan province, Iran, and cultivated on Sabouraud glucose agar (SDA). The fungi were classified using the standard slide culture technique, and aflatoxin analysis was performed using the HPLC approach.

Results: The most prevalent isolated fungi among the 150 peanut specimens were Aspergillus, Penicillium, and Rhodotorula. The occurrence of total aflatoxin, AFB1, AFB2, AFG1, and AFG2 were 85%, 85%, 74%, 35%, and 45%, respectively. 5% of peanut samples exceeded the maximum permissible limits (5 and 15 µg/kg) for AFB1 and total aflatoxins, respectively, as established by European Union guidelines.

Conclusion: The results suggest that peanuts are an appropriate medium for various fungal growth and mycotoxin formation, emphasizing the significance of testing peanuts for aflatoxin and fungal contamination before distributing them to the general public.

Keywords: Peanut, High-performance liquid chromatography (HPLC), Fungi, Aflatoxins (AFTs)

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Introduction

Aflatoxins (AFs) has exhibited irreversible detrimental effects such as carcinogenic properties, compromised immune systems, and developmental irregularities on humans, presenting a significant health hazard (1, 2). Around 20 forms of AFs exist, with four primary categories, i.e. B1 and B2 (Blue) and G1 and G2 (Green-Blue), classified based on their ultraviolet light fluorescence (3). All AFTs can exhibit heat resistance (4), with AFB1 being the most potent and carcinogenic. Peanut aflatoxin Aspergillus contamination cases have been recorded globally (5-7). For instance, contamination levels of 80% in India (8), 23.5% in the United States (9), and 82% in Brazil (10) have been reported. Occurrence of aflatoxins in various

food grains such as peanuts underscores food safety concerns, necessitating the improvement of storage and transportation methods to prevent illnesses such as cancer (1, 11). Techniques such as thin layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA), and high-performance liquid chromatography (HPLC) are beneficial for detecting AFs in different food items (12). The present study aimed to determine AFs in peanut samples using HPLC from Isfahan, Iran.

Materials and Methods

Sample Collection

During this study, 150 peanut specimens weighing 300g each were collected from retail shops and community marketplaces in the central region of Isfahan. These specimens were submerged in a solution containing 0.4% sodium hypochlorite for 2 minutes, then washed twice with uncontaminated distilled water and placed on filtering paper to dry. Decontaminating surfaces is an established method for removing fungal spores from food surfaces (13). Subsequently, the peanuts were cultivated on Sabouraud Dextrose Agar (SDA) and incubated at a temperature of 25°C for a four-day period. Various fungal species were identified based on the macroscopic features of their colonies, such as coloration on both sides, presence of radial striations or concentric rings, and texture of the colony's exterior, which could be smooth or uneven. Moreover, characteristics such as a powdery, downy, cottony, woolen, or velvety appearance were inspected under a microscope. The standard slide culture method was utilized to ensure precise fungal identification (14).

Sample Preparation and Clean Up

The AOAC official method 999.07 was slightly modified (20) to obtain AFs from the samples and adjust chromatographic conditions. To extract the AFs, 50 grams of peanuts were mixed with 5 grams of sodium chloride and 300 milliliters of methanol: H2O (80:20 v/v) for 30 minutes. An additional amount of 100 milliliters of n-hexane was used for peanuts. The filtrate was diluted with 130 milliliters of deionized water, and 20 milliliters of the filtrate was passed through a glass microfiber filter. The remaining 75 milliliters of the filtrate were purified using an Afla test IAC column. First, the Afla test column was prepared with 10 milliliters of phosphate-buffered saline (2-3 mL/min). Subsequently, 75 milliliters of the diluted sample extract were passed through the column (2-3 mL/min), followed by a rinse with 15 milliliters of water. To elute AFB1, 0.5 milliliters of HPLC grade methanol was introduced, followed by another 0.75 milliliters of the same solvent one minute later. The eluent was combined with HPLC-grade water to obtain a volume of 3 milliliters. Finally, 100 microliters of the resulting mixture were injected into the HPLC system (20) for analysis.

AF Standards

The concentrations of individual AF standard solutions were measured using a UV spectrophotometer and then combined to form mixed working standards for HPLC analysis. The guidelines for assessing the concentration and purity of the aflatoxin standards have been provided in the AOAC Official Methods of Analysis (15), and the criteria for these standards have also been also defined (Rodricks 1973).

Analysis of AF Using HPLC

Reverse-phase HPLC and a fluorescence detector were utilized in conjunction with a postcolumn derivatization chamber for bromination to quantify AFs (Stroka et al. 2000). The PCDC technique was executed using a Kobra cell, and potassium bromide was incorporated into the mobile phase. After diluting the AF eluate using water, 100 milliliters were introduced into the HPLC apparatus. The employed analytical column was a C18, featuring a 4.6 mm diameter, 250 mm length, and 5 µm particle dimensions. The mobile phase consisted of water, methanol, and acetonitrile at a ratio of 54:29:17 (v/v/v), maintaining a flow rate of 1 milliliter per minute. The fluorescence detector operated at excitation and emission wavelengths of 365 nm and 435 nm, respectively. A 5-point calibration curve was generated daily for each AF, including AFB1, AFB2, AFG1, and AFG2, to evaluate linearity and calculate AF concentrations in peanut specimens. The elution order was AFG2, AFG1, AFB2, and AFB1. The limit of detection (LOD) for AFB1, AFB2, AFG1, and AFG2 was established at 0.1 µg/kg, while for the combined AFs, it was 0.4 µg/kg. The limit of quantification for all AFs was determined to be 0.8 µg/kg (15).

Quality Assurance

In order to ensure the credibility of the AF analysis

findings, verified methods were employed, and both internal and external quality assurance experiments were conducted. The procedures' accuracy and consistency were verified through internal quality control procedures. Recovery rates for AFB1 and AFG1 at 10 µg/kg and AFB2 and AFG2 at 2 µg/kg were established by analyzing a blank sample of pistachio nuts spiked with these toxins. AF concentrations were adjusted according to the values obtained. As part of their external quality assurance efforts, the Iran National Food Control Laboratories participated in proficiency testing through the Food Analysis Performance Assessment Scheme in the United Kingdom.

Statistical Analysis

The data were analyzed using SPSS software version 18, and the Kruskal-Wallis test was used to compare the levels of the four aflatoxins ($p<0.001$).

Results and Discussion

Fungal Isolation

Of the 150 peanut samples, Penicillium and Rhodotorula were the most commonly isolated fungi, accounting for 30% of all isolates, followed by Aspergillus niger (24%), Rhizopus (14.66%), Aspergillus flavus (13.33%), and Cladosporium (12%). The least frequently isolated fungi were Scopulariopsis and curvularia, both at 1.33%.

AF Analysis Using the HPLC Method

The extraction process was improved in order to facilitate the total removal of the aflatoxins. The performances of the used analytical procedure were acceptable for the intended aim. Recovery studies were conducted by spiked uncontaminated samples with AFs standard solution.

We investigated average recoveries and relative standard deviation for repeatability (RSDr) of the analytical methods used for AFB1, AFB2, AFG1, and AFG2 in peanuts. The results have been indicated in Table 1. Both recoveries and RSDr of AF were in the acceptable range. The retention times for B1, B2, G1, and G2 were: 8.2, 7.2, 6.4, and 5.8, respectively. Figure 1 shows the sample obtained peaks for AFB1, AFB2, AFG1, and AFG2 by IAC HPLC.

Out of the 100 specimens that were examined, 85 (85%) were found to have AFB1 present. Within this group of specimens, 92% had AFB1 levels lower than

Table 1: Average recoveries and coefficient of variations for aflatoxins spiked into blank peanut samples analyzed using HPLC and fluorescence detector.

Aflatoxins	No.	Spike Level (µg/kg)	Average recovery (%)	RSDr (%)
B1	10	10.0	97.3	8.0
B2	10	2.0	95.6	6.8
G1	10	10.0	97.8	5.8
G2	10	2.0	84.1	5.1

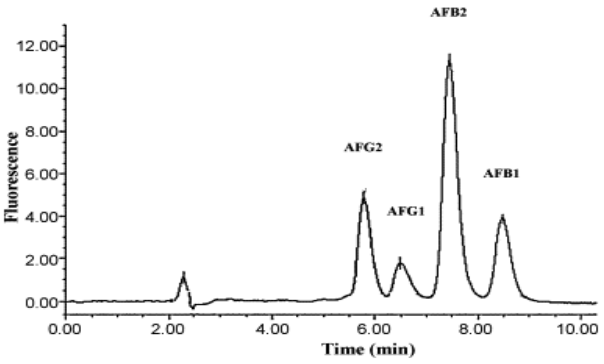

Figure 1. Chromatogram aflatoxin B1, B2, G1 and G2.

the maximum acceptable level (MAL) established by the Industrial Research and Standards Institute of I.R. Iran for peanuts, which is 5 µg/kg. However, 8% of the specimens exceeded this threshold. On the other hand, 15 specimens (15%) did not show any evidence of AF presence. The average presence of AFB1 in the remaining 85 specimens was 8.79 µg/kg (Table 2). This value was lower than both the MAL for AFs in Iranian peanuts (15 µg/kg) and the provisional maximum limit suggested by the Codex Committee on Food Additives and Contaminants for AFs (15 µg/kg). Out of all the specimens analyzed, 91% had AF concentrations below the MAL for AFs in peanuts established by I.R. Iran (15 µg/kg), with only 9% exceeding it. The mean presence levels for AFB1, AFB2, AFG1, AFG2, and AFT in the

Table 2: Aflatoxins in total samples.

Aflatoxin	No. of samples analyzed	Samples without AF (<LOD)a	Mean
B1	100	15	6.2
B2	100	15	1.91
G1	100	65	0.56
G2	100	55	0.12
Total aflatoxin	100	15	8.79

peanut specimens were 6.2, 1.91, 0.56, 0.12, and 8.79 µg/kg, respectively (Table 2).

Contamination of food products by fungi such as oilseeds can result in spoilage and render them unsuitable for consumption. Certain fungi can produce mycotoxins that pose significant risks to human and animal health under specific conditions (16, 17). In this investigation, we analyzed 150 peanut samples from various retail and wholesale nut stores in Isfahan and found that Aspergillus was the most prevalent fungal contamination (41.33%), followed by Penicillium (30%) and Rhodotorula (30%).

Consistent with our findings, a study conducted by Gürses *et al*. in Turkey revealed that Aspergillus and Penicillium were the primary isolated fungi in peanuts (18). Aspergillus and Penicillium have also been identified as common fungi found in edible oilseeds, including pistachios, almonds, and hazelnuts, in other research studies such as the study conducted by Pirzmani *et al*. in Iran (19). The high prevalence of Aspergillus in these oilseeds, including their seeds and shells, can be attributed to factors such as storage conditions, low humidity, and mycotoxin production on the substrate (11, 20-22).

In Saudi Arabia, 34.3% of A. flavus and 38.8% of A. niger were isolated from peanut samples. It is partly related to the existence of a suitable substrate for the growth of this fungus or due to the low abundance of A. flavus in the soil of the area (23). In our study, out of 20 samples infected with A. flavus, 17 (85%) were infected with total Aflatoxin with a maximum value of 6.20 µg / kg and none of them exceeded the Iranian standard (15 µg / kg). A comparison of the present results with the European standard indicated that the amounts of total aflatoxin and aflatoxin B1 in 5 samples were higher than the allowable standard level of Aflatoxin in peanut seeds.

In a study conducted by Ding *et al.*, high-performance liquid chromatography was used to determine the levels of aflatoxin B1 in peanuts. The results indicated that 25% of the samples had levels of aflatoxin B1 ranging from 0.7-0.720 µg/kg. More than 95% of the peanut samples had low levels of aflatoxin B1 and total aflatoxins with concentrations below 1 µg/kg. However, one sample had an exceptionally high level of 720 µg/kg, and approximately 1% of the samples exceeded China's permissible limit of 20 µg/kg (24).

In Sao Paulo, Brazil, Atayde *et al*. reported aflatoxin contamination in 5% of peanut seeds (concentrations between 1-1.7 µg/kg) and 13.8% of shells (concentrations between 1.117-1.8 µg/kg) (25). Using HPLC, Imani Nejad *et al*. discovered aflatoxin contamination in almost 74.3% of 35 walnut samples in Tehran, with 20% exceeding the standard of 15 µg/kg (26).

In our study, total aflatoxin contamination levels in Iranian peanut seeds ranged from undetectable to 6.20 ppb, which is below the limit for aflatoxin B1 (5 µg/kg) and total aflatoxins (15 µg/kg). The detection level reached up to 4 ppb, which is significantly different from other investigations. Disparities in the results could be due to different determinants such as sampling methods, storage locations, climatic conditions, and pre-or post-harvest circumstances. Taking proper measures to ensure food safety and prevent aflatoxin contamination is crucial.

The results of the present study are consistent with Akbas *et al*.'s findings, which suggest that aflatoxin production can be significantly affected by factors such as geographic location, agricultural practices, and crop susceptibility to fungal presence during harvesting and storage (27). Peanut seeds are particularly conducive to aflatoxin production, and storing moldy seeds under normal conditions can pose a high risk of elevated aflatoxin levels.

According to Gonçalez et al.'s research, low levels of aflatoxin concentration in peanuts may be due to water activity (aw) levels being lower than the optimal amount required for A. flavus growth and aflatoxin production. Our study's prevalent drought conditions might have also contributed to this situation (28).

In a study conducted by Hussain et al., the incidence rates of AFB1, AFB2, AFG1, and AFG2 in peanut oils were 70%, 51.7%, 3.3%, and 0%, respectively. The average total AFs was 8.59 µg/kg, with a range of 0.12 to 55 µg/kg. Out of 60 samples, 5% (three samples) exceeded the permissible limit for AFB1 contamination (20 µg/kg) based on national regulations. The AF levels in the samples from three distinct areas of Peshawar showed a remarkable distinction ($P<0.05$) (6).

Conclusion

The link between liver cancer and aflatoxins is well-established, and peanuts are highly susceptible to

fungal growth and mycotoxin formation. Therefore, it is crucial for producers to follow strict sanitation measures during harvesting and food preservation to avoid contamination. While a clean harvest does not guarantee the complete prevention of fungal contamination in peanuts, it is still a crucial step to take in preventing aflatoxin contamination.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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