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Determination of Aflatoxins in Iranian nuts using HPLC Method.

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ABSTRACT

Aflatoxins, a group of closely related hepatocarcenogenic metabolites produced by certain species of Aspergillus may contaminate plant and plant products. The objective of this study was to detect the level of aflatoxins in Iranian nuts. A total of 80 samples including pistachio (20), almond (20), hazelnut (20) and walnut (20) were randomly collected from the retail markets in Esfahan, Iran and analyzed for aflatoxins B1, B2, G1, G2 and total using HPLC method. Aflatoxins B1, B2, G1, G2 and Total were found in 7.5%, 2.5%, 7.5%, 2.5% and 10% of the analyzed samples by an average concentration of 8.32, 5.635, 3, 067, 1.705 and 10.375 µg/kg, respectively. The concentration of AFB1 in 66.67% of AFB1-positive nut sample were higher than 5 µg/kg and the concentration of AFs total in 37.5% of AFs total-positive nut samples were higher than 15 µg/kg which is the maximum tolerated level by research of Iran. The percentage of pistachio AFs-positive nut samples was significantly ($P<0.05$) higher than almond, hazelnut and walnut samples. The results of the present study showed the importance of periodically monitoring the level of aflatoxins in nuts in Iran.

Keywords: Aflatoxins, nuts, Iran

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INTRODUCTION

Incidences of food contamination have become increas-ingly frequent in recent years raising question about their human health and economic consequences. Aflatoxins (AFs) are highly toxic secondary mould metabolites. Mycotoxi-coses, which can occur in both industrialized and develop-ing countries, arise when environmental, social and eco-nomic conditions combine with meteorological conditions (humidity, temperature) which favor the growth of moulds. Aflatoxins (B1, B2, G1 and G2) are a group of naturally occurring compounds called mycotoxins, produced by certain moulds growing on some food crops during production and storage [1]. Mycotoxins are found in a wide range of foods around the world. Aflatoxins are produced by *Aspergillus flavus, A. parasiticus* and *A. nominus*, mould species that grow in warm humid conditions. They occur mainly in commodities imported from the tropics and sub-tropics, in particular groundnuts, edible nuts, dried figs, spices , maize, rice, wheat, barley, cereal and some other crop [2]. Aflatoxins are considered to be genotoxic carcinogens and have been shown to cause cancer in laboratory animals by reacting with DNA. In humans, aflatoxins have been linked to liver cancer in a number of developing countries, where it is common for some foods that are an important part of the diet to contain high levels of aflatoxins [3, 4]. Aflatoxin B1 is the most toxic aflatoxin. Nuts and their products are consumed as well as part of the ingredient of our daily diet. So it is important to control their occurrence and level in food. Research on Food products has demonstrated that aflatoxins are still being found frequently in food product at level that are of significant concern for consumers. A number of survey and monitoring programs have been carried out in several countries attempting to obtain general pattern of extent of food contamination. Various approaches exist for the determination of aflatoxin in food and feed commodities. Generally, all analytical methods follow the basic protocol of extraction, clean-up, separation, detection, identification and quantification. However, current aflatoxin analysis is done by various methods including thin layer chromatography (TLC), liquid chromatography (LC), high-performance liquid chromatography (HPLC) and ELISA, Although immunoassay-based quantitative methods are fast and promising, for mycotoxin research they have the possibility of producing misleading results because of cross-reaction and interference in the complex matrixes [4-6].

Due to the significant health risks associated with the presence of aflatoxins in foods, it is important to establish a data collection on the occurrence of these toxins in nuts as valuable foods. Iran is one of the largest and production consumers of nuts and dry fruits in the Middle East. On the other hand, various nuts have been produced and consumed widely in Islamic Republic of Iran. The aim of this study was to screen the content of aflatoxins in nut used in Iranian nuts in Isfahan market, Iran.

MATERIALS AND METHODS

Sampling

A total of 80 different types of nuts included 20 samples of pistachio, 20 samples of almond, 20 samples of walnut, 20 samples of hazelnut, were randomly collected from the markets in Esfahan, Iran and stored frozen at – 20 ^{o}C until started analysis. Samples transferred to room temperature and then approximate value homogenized completely.

Chemicals

Aflatoxins B1, B2, G1 and C2 standard solution was prepared from Supellco (USA). aflatoxins concentrations were aflatoxin B1 1.00 µg/mL, aflatoxin B2 0.200 µg/mL, aflatoxin G1 1.00 µg/mL, aflatoxin G2 0.200 µg/mL. The standard solutions were in 100% methanol. Solvents and chemicals used were of highest purity and analytical grade. For clean-up step immunoaffinity column (IAC) AflaCLEANTM (LC Tech, Germany) were used.

HPLC determination of aflatoxins

Aflatoxins were analyzed in nuts according to the method reported by Stroka et al [5]. Five gram of homogenized samples was extracted with 0.5 g NaCl and 30 ml methanol: water: (2:8) by high – speed blender. (In fatty samples n- Hexan was added in order to remove fat). The mobile phase consisted of acetonitrile, methanol: water (17:29:54, v/v/v). The mobile phase was degassed by sonication. The Hichrom

ODS (250× 4.6 mm I.D., 5 mm, Hichrom Ltd., Reading, Berkshire, UK) column was connected as LC column. The column was eluted with a flow rate of 1 ml/min. The aflatoxins were detected at the excitation and emission wavelengths of 365 and 435 nm, respectively. The injection volume was 20 ml. For the quantization of aflatoxins in samples, a separate calibration curve was established for each aflatoxin. Triplicate samples were used for setting calibration curve, determining LODs and extraction recovery. The limit of detection (LOD) and limit of quantitation (LOQ) were defined. Based on the equations, the LOD and LOQ values for aflatoxins are shown in Table 1. The efficacy of method was examined by the determination of the recoveries of the aflatoxins. The recoveries were ascertained by spiking 10, 20, 30 µg/ml of aflatoxin B1 and aflatoxin G1 and 0.2, 0.4, 0.6 µg/ml of aflatoxin B2 and aflatoxin G2 and the extraction result were expressed as percentage that as shown in Table 1.

Statistical Analysis

All statistical analyses were performed using SPSS software, version 16 (SPSS Chicago, IL, USA) and the data were expressed as mean ± standard deviation (SD). A repeated measures analysis of variance (ANOVA) were used to compare the mean of aflatoxins concentration in nut samples in the differences were considered significant at values of $P < 0.05$.

RESULTS AND DISCUSSION

The contamination of food with aflatoxins is a very important case of focus of health scientists. Nuts are food samples which are susceptible for this contamination because of their composition and storage conditions. In according to this results study, overall, 10% of the Iranian nuts samples were found to be contaminated with aflatoxins. The incidence of aflatoxins in Iranian nuts samples as shown in Tables 2 - 6. The data showed that 66.67% of the contaminated samples contained aflatoxin B1 higher than the maximum tolerated level (5 µg/kg) by research of Iran [4]. The aflatoxin B1 and aflatoxin total level in Iranian nuts samples ranged 0.29 - 19.35 µg/kg and 0.15 - 19.35 µg/kg, respectively. Individually 20% of pistachio, 10% of walnuts, 5% of almonds and 5% of hazelnuts samples (as shown in Table 2) were found positive for aflatoxins contamination. The percentage of pistachio aflatoxin positive nut samples was significantly ($P < 0.05$) higher than almond, hazelnut and walnut samples. The incidence of aflatoxins in different types of nuts and nutty products has been studied by several authors from different countries. Khamiri et al. (2008) reported that among 156 nut samples analyzed for aflatoxin B_1, one sample (0.64%) was contaminated at the level of 491 ng/g [7]. Magrine et al. (2011) determined the concentrations of aflatoxins in 100 samples of nut products in Brazil. There was a 50% occurrence of aflatoxins (B_1, B_2, G_1 and G_2) in concentrations ranging from 0.5 to 113 ng/g [8], Chun et al (2007) found that nut samples in South Korea were contaminated with AFs (10.6% of incidence) in the range of 0.20 - 28.2 µg/kg [6]. Mahmoud et al. (2013) found that in the holy city of Mekkah, the incidence of aflatoxins contamination in different nuts, those 70 samples (26.5%) were contaminated with aflatoxin total in levels ranged between 1.0 and 110 µg/kg [9]. Wang and Liu (2006) in Chinese peanut were reported contaminated with aflatoxins being than average level of 80.3 µg/kg and the highest level being 437 µg/kg [10]. In Turkey peanut butter was contaminated with aflatoxins in the range of 8.16 - 75.7 ng/g which aflatoxin B1 was in the range of 2.06 - 63.7 µg/kg [11]. Cheraghali et al (2007) reported that 11.8% and 7.5% of 10068 Iran pistachio nut samples were above the maximum tolerated level (MTL) of aflatoxin B1 and aflatoxins, respectively [12]. Yin - Hui and et al (2008) reported that nut and nutty products were contaminated with aflatoxins ranging in level from 16.6 µg/kg up to 711 µg/kg in Malaysia [13]. Luttfullah and Hussain (2011) reported that the percentage of contamination for total aflatoxins in the samples such as in almonds without shell (30%), walnuts with shell (40%), walnuts without shell (70%), pistachios with shell (20%) and pistachios without shell (50%), that highest contamination levels of aflatoxins in one pistachio sample (14 µg/kg) [14].

In the present study some samples specially pistachio and walnuts were collected in Isfahan market were higher contamination compared to another nuts. Probably that is due to their higher surface area in this nuts, storage conditions in markets, climatic differences such as high temperature and dry conditions, agricultural practices during harvest and storage, moisture content of the samples studied here favored aflatoxin production and also which make samples more susceptible for the fungal growth and contamination. The higher level of aflatoxins in pistachio and walnuts, suggests that a good supervision is necessary about the production and storage of nuts which are used in markets.

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Table 1: Validation of aflatoxins determination by HPLC analysis.

Aflatoxin	LOD (µg/kg)	LOQ (µg/kg)	Calibration Curve[a]	R^2	Recovery (%)
AF B1	0.020	0.360	y = 3E+07x -18092	0.999	112.5
AF B2	0.004	0.072	y = 3E+07x -18487	0.998	106.9
AFG1	0.020	0.360	y = 1E+07x -49473	0.998	114.8
AF G2	0.004	0.072	y = 2E+07x -9853	0.996	109.3

[a]x = concentration of aflatoxins (µg/kg) and y = intensity

Table 2: The results of Incidence of aflatoxin B1 in Iranian nuts samples by HPLC analysis

Sample	Number of Samples	AFB1	Samples Having AFB1>5ppb	AFB1(ppb) (mean ± **S**D)	Contamination Range (ppb)
Pistachio	20	3(15%)	2	7.34 ± 6.774	0.29 - 13.80
Almonds	20	1(5%)	ND	3.43	3.43
Hazelnuts	20	ND[a]	ND	ND	ND
Walnuts	20	2(10%)	2	12.23 ± 10.062	5.12 - 19.35
Total	80	6(7.5%)	4(50%)	8.32 ± 7.077	0.29 - 19.35

[a]Not detected

Table 3: The results of Incidence of aflatoxin B2 in Iranian nuts samples by HPLC analysis

Sample	Number of Samples	AFB2	AFB**2**(ppb) (mean ± SD)	Contamination Range (ppb)
Pistachio	20	1(5%)	4.19	4.19
Almonds	20	ND	ND	ND
Hazelnuts	20	ND	ND	ND
Walnuts	20	1(5%)	7.08	7.08
Total	80	2(2.5%)	5.635 ± 1.084	4.19 - 7.08

Table 4: The results of Incidence of aflatoxin G1 in Iranian nuts samples by HPLC analysis

Sample	Number of Samples	AFG1	AFG1 (ppb) (mean ± **S**D)	Contamination Range (ppb)
Pistachio	20	3(15%)	3.997 ± 3.943	0.15 - 8.03
Almonds	20	1(5%)	2.36	2.36
Hazelnuts	20	1(5%)	0.17	0.17
Walnuts	20	1(5%)	3.88	3.88
Total	80	6(7.5%)	3.067 ± 2.941	0.15 - 8.03

Table 5: The results of Incidence of aflatoxin G2 in Iranian nuts samples by HPLC analysis

Sample	Number of Samples	AFG2	AFG2 (ppb) (mean ± SD)	Median	Contamination Range (ppb)
Pistachio	20	1(5%)	0.83	0.83	0.83
Almonds	20	ND	ND	ND	ND
Hazelnuts	20	ND	ND	ND	ND
Walnuts	20	1(5%)	2.58	2.58	2.58
Total	80	2(2.5%)	1.705 ± 1.807	1.705	0.83 - 2.58

Table 6: The results of Incidence of aflatoxin total in Iranian nuts samples by HPLC analysis

Sample	Number of Samples	Aflatoxin Total	Samples Having AFT >15ppb	AFT (ppb) (mean ± SD)	Contamination Range (ppb)
Pistachio	20	4(20%)	1	9.757 ± 7.297	0.15 - 16.76
Almonds	20	1(5%)	ND	5.79	5.79
Hazelnuts	20	1(5%)	ND	0.17	0.17
Walnuts	20	2(10%)	2	19.005 ± 0.488	18.66 - 19.35
Total	80	8(10%)	3(37.5%)	10.375 ± 7.889	0.15 - 19.35

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CONCLUSIONS

This study presents useful information about the risk of aflatoxins hazard in different types of Iranian nuts. The high occurrence of aflatoxins emphasizes the need for regular monitoring and a more stringent good management practices to prevent the occurrence of aflatoxins in Iranian nuts. This results indicate that aflatoxins contamination of some of nuts may be a public health concern in Iran, since it contributes to the general human exposure to these toxins, especially among children. Therefore is a great need to develop practical and cost-effective methods of preventing mold growth and detoxifying foods containing aflatoxin.

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