

## Preliminary Survey of Aflatoxins in Mashhad's Roasted Red Skin Peanut Kernels during February to May 2016

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### ABSTRACT

**Introduction:** Aflatoxins (AFs) are a group of mycotoxins created as metabolic items for the most part by three types of *Aspergillus* including *Aspergillus flavus*, *Aspergillus parasiticus* and the uncommon *Aspergillus nomius*. Eighteen aflatoxins have been identified up to now, but only six of them have been found in food and feed.

**Methods:** The occurrence of aflatoxins in 32 samples of roasted red skin peanut was determined using HPLC with a Chromolith column. All samples were purchased from retail shops and local markets in Mashhad city. The method was based on the extraction of samples and aflatoxins determination after post-column derivatization by Kobra Cell and fluorescence detection at excitation and emission wavelengths of 365 and 435 nm, respectively.

**Results:** Mean levels of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub> and total aflatoxins were found to be 57.17, 2.56, 12.51, 1.42 and 85.16 ng g<sup>-1</sup>, respectively. Aflatoxins B<sub>1</sub> (AFB<sub>1</sub>) was detected in 12 samples (37.5%) with a mean value of 57.17 ± 90.32 ng g<sup>-1</sup> and a maximum level of 243.61 ng g<sup>-1</sup>. AFB<sub>1</sub> levels exceeded Iran maximum tolerate limit (5 ng/g) in 7 out of 32 peanut samples. 21.8% of these peanut samples exceeded the maximum tolerate limit set for total aflatoxins by codex and Iran (15 ng g<sup>-1</sup>).

**Conclusion:** According to the obtained results, more effort is needed to control aflatoxin levels in Mashhad's peanut. This survey provides valuable information on aflatoxin contamination in peanut products marketed in Iran as well.

**Keywords:** Chromatography, High Pressure Liquid, Aflatoxin, Arachis, Khorasan Razavi, Mashhad

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## Introduction

Mycotoxins are toxic organic compounds with different chemical structures produced by various molds. They have toxic effects on humans and animals. Aflatoxins (AFs) are a group of mycotoxins which are produced as secondary metabolites *Aspergillus*; a spoilage fungus; particularly *Aspergillus flavus* and *Aspergillus parasiticus* <sup>(1-2)</sup>. About eighteen compounds in the aflatoxin group are identified, but aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub> and M<sub>1</sub> are the most important and monitored ones <sup>(3)</sup>. The International Agency for Research on Cancer (IARC) has classified aflatoxin B<sub>1</sub> as a type I carcinogenic compound that is effective on the r liver <sup>(4)</sup>. Different foods including dried fruit, cereals, nuts, oil seeds, cocoa, spices, pulses and beans can be contaminated with aflatoxin <sup>(5)</sup>.

The world wide range of limits for aflatoxin B<sub>1</sub> and total aflatoxins are 1-20 ng g<sup>-1</sup> and 0-35 ng g<sup>-1</sup>, respectively <sup>(6)</sup>. In the European Union, the maximum residue levels (MRLs) of aflatoxin B<sub>1</sub> and the total aflatoxin level in peanut products cannot be greater than 8 and 15 ng g<sup>-1</sup>, respectively <sup>(7)</sup>. Natural aflatoxins contamination in peanut has been studied in different countries. Analysis of four hundred peanuts samples and peanut products in Sudan determined or bared that the mean percent of aflatoxin contamination was 2%, 64%, and 11% for peanut seeds or nucleus, peanut butter, peanut cake, and roasted peanuts, respectively <sup>(8)</sup>. In another study in Indonesia, 29 of 82 peanut samples (35%), were contaminated with aflatoxin (range 5 to 870 ng g<sup>-1</sup>) <sup>(9)</sup>. Ghosia et al. (2011) <sup>(10)</sup> showed that 40% of peanut with shell and 50% of peanut without shell samples were contaminated with aflatoxins. A survey in the Philippines on peanut-based products showed that 60% of the samples contain AFB<sub>1</sub> and AFG<sub>1</sub> in the range of 1-244 ng g<sup>-1</sup> and 6-68 ng g<sup>-1</sup>, respectively <sup>(11)</sup>. A 0.3 ng g<sup>-1</sup> aflatoxin contamination in peanut in the Rabat-sale area, Morrocco, reported by Juan et al. (2008) <sup>(12)</sup>. In a survey which worked on raw peanut kernels in Malaysia, Arzandeh et al. (2010) <sup>(13)</sup> reported that 78.57% of the samples were contaminated with aflatoxin, that 10.71%

overpassed the maximums acceptable limit of 15 ng g<sup>-1</sup> set by the Codex. In a study, Chun et al. (2007) <sup>(14)</sup> showed AFB<sub>1</sub> contamination with concentration of 0.2 ng g<sup>-1</sup> in 25% of raw peanuts in South Korea and 20-200 ng g<sup>-1</sup> in peanut samples in Argentina and Senegal. On the other hand, there are some studies on roasted peanuts. Younis et al. (2003) <sup>(8)</sup> analyzed four hundred samples of peanut types expected to be naturally contaminated in Kuwait, the percentage of aflatoxins contamination were 14, 11, 26, and 64 for peanut cake, roasted peanuts, peanut kernels, and peanut butter, respectively. The reason for lower aflatoxin content in roasted peanuts may be attributed to the destructive action of heat upon aflatoxins during processing.

There are several analytical methods for aflatoxins determining in food <sup>(15)</sup>. The most common method is HPLC with fluorescence detection (HPLC-FLD) combined with precolumn or postcolumn derivatization; other analytical methods such as thin layer chromatography (TLC) <sup>(16)</sup> and enzyme-linked immunosorbent assay (ELISA) <sup>(17)</sup> were used for aflatoxin determination in foods.

Peanut (*Arachis hypogaea*) is a nutritious grain that is high in protein and fat. About 140,000 tons of peanuts are produced in Iran every year, and because of its low price and extensive nationwide consumption, additional amounts are imported from India and China. However, if peanuts are not properly stored, they are vulnerable to development of a variety of harmful fungi, including mycotoxin. According to the previous results on aflatoxin contamination of peanut, it is clear that more information on aflatoxins in peanut is needed to get a better estimation of the potential problem. The monitoring and elimination of mycotoxin in food and feed products should be implemented by the industry and government in order to bring the consumers and manufacturers to the concerns of quality and public health. Therefore, the purpose of the present study is to investigate the natural occurrence and distribution of aflatoxins in roasted red skin peanut. The

consumption of peanut is widespread in Iran; so, this study is the first report on the natural occurrence of aflatoxins in peanuts from retail shops in Mashhad city of Khorasan Razavi province.

## Methods

### Sample collection and storage

Thirty-two roasted red skin peanut kernel samples (each month 8 samples) collected from retail shops and local markets in Mashhad (Khorasan Razavi, Iran) during February to May 2016. The selected samples have been chosen from imported, processed and locally-made products. The collection of sample was based on the Iranian national standard aflatoxin sampling method no. 12004. Samples were taken exactly according to the sampling procedure of EU for sampling of nuts<sup>(18)</sup>. The peanut packages were about 50 kg following taking one 3 kg sample (10 incremented samples, each 300 g), and mixed (sub-sample). Subsamples were stored under cool conditions until analysis. All samples were transferred to the laboratory at ambient temperature and were kept at +4 °C until initial sample preparation, after which they were stored at -20°C until needed for analysis. Before analysis, the samples were allowed to reach the ambient temperature and returned to -20°C immediately after analysis.

For minimizing the sub-sampling error in aflatoxins analysis, water slurry of peanut samples was prepared. For that matter 1.5L of water was added to one kg of peanut. The resulting mixture was blended for 15 min with slurry machine. Finally, 125g of test portion from the slurry was taken for analysis. Each analysis was repeated three times and the average was reported.

### Chemical and reagents

Methanol, acetonitrile, water, and other chemical solvents and reagents were HPLC grade and supplied by Merck (Darmstadt, Germany). The powders of aflatoxins were obtained from Sigma (St. Louis, USA). For purification and preconcentration of aflatoxins prior to the quantitative HPLC analysis, immunoaffinity columns (Vicam Company, Water town, MA,

USA) were used. Stock standard solution of aflatoxins with concentrations of 10 µg ml<sup>-1</sup> was prepared in methanol. This standard was used to prepare mixed working standards for HPLC analysis. The range concentrations of aflatoxins B<sub>1</sub> and G<sub>1</sub> for construction of external standard calibration curve were 0.4-10 ng ml<sup>-1</sup> and for aflatoxins B<sub>2</sub> and G<sub>2</sub> were 0.08-2 ng ml<sup>-1</sup>. These working standard solutions were prepared daily by diluting the stock solution with water: methanol (6:4) and stored in darkness and refrigerator at 4°C.

### Apparatus

HPLC analysis were performed on a Waters (USA) HPLC system equipped with a Waters 600 pump, a Waters 600 column thermo-controller, a Waters In-line Degasser and Waters 474 Fluorescence Detector and a Chromolith® performance RP18 analytical column (100×4.6mm). The guard column was Chromolith® RP18. The fluorescence detector was operated at 365 and 435 nm for excitation and emission, respectively. UV-visible spectra of aflatoxins stock solution were obtained using a Shimadzu UV-1700 Pharma-Spec spectrometer (Tokyo, Japan). Spectrophotometer equipped with a standard 10 mm path length spectrophotometer cell.

### LC determination of aflatoxins

Linear isocratic elution chromatography has been done using solvent system water: methanol: acetonitrile (55:30:15 v/v/v) with 350 µL of 4 M nitric acid and 119 mg L<sup>-1</sup> potassium bromide at 40 °C. The flow rate was 1 ml min<sup>-1</sup>. Derivatization was performed using post-column bromination with Kobra Cell (R-Biopharm Rhone Ltd, Glasgow)<sup>(19)</sup>.

### Extraction and purification of aflatoxins

Peanut slurries and 5 g NaCl were blended using a blender (Waring 8011S, Torrington, CT) with 400 ml of methanol: hexane (75:25) for 5 min to obtain a homogeneous sample mix<sup>(19)</sup>. The mixture was centrifuged for 5 min at 2000 rpm. The extract was filtered through filter paper (Whatman No.4), then 20 ml aqueous methyl alcohol phase was mixed with 130 ml phosphate buffer saline (PBS)

solution and filtered through glass microfiber filter (Whatman, Inc. Clifton, NJ, USA) and 100 ml passed through an immunoaffinity column. Aflatoxins were eluted from the column by passing 1.5 ml of HPLC grade methanol and then 1.5 ml of HPLC grade water and using gravity to collect the eluate into a glass vial. A 50  $\mu$ l aliquot of eluate was injected into the HPLC.

#### Sample fortification and calibration

Accuracy was examined by the determination of the recoveries of the aflatoxins. Spiking was carried out in three levels, 2.5, 5, and 10  $\text{ng ml}^{-1}$  by adding 125, 250 and 500  $\mu$ L of aflatoxin mixture ( $\text{AFB}_1=\text{AFG}_1=1000 \text{ ng ml}^{-1}$ ,  $\text{AFB}_2=\text{AFG}_2=200 \text{ ng ml}^{-1}$ ) in methanol to 125 g slurry peanut samples, respectively. The relative standard deviations for within laboratory repeatability ( $\text{RSD}_r$ ,  $n=9$ ) was

from 6.45 to 8.58. Statistical analysis was reported as Mean  $\pm$  SD and percentages. All statistical analyses were carried out in Excel 2010.

Linearity was estimated by injecting aflatoxin standard solutions covering the range 0.4-10  $\text{ng g}^{-1}$  for aflatoxins  $\text{B}_1$ ,  $\text{G}_1$  and 0.08-2  $\text{ng g}^{-1}$  for aflatoxins  $\text{B}_2$ ,  $\text{G}_2$ . The square of correlation coefficient ( $r^2$ ) were more than 0.999 ( $\text{B}_1=0.9997$ ,  $\text{G}_1=0.9991$ ,  $\text{B}_2=0.9989$ ,  $\text{G}_2=0.9992$ ). The recoveries of aflatoxins  $\text{B}_1$  and  $\text{B}_2$  and  $\text{G}_1$  and  $\text{G}_2$  from spiked blank samples were quite good (Table 1). The method performance characteristics showed in Table 2. The European Commission<sup>(18)</sup> criteria for methods to be used for control purposes in terms of recovery, repeatability ( $\text{RSD}_r$ ) limit of detection (LOD), and limit of quantification (LOQ).

**Table 1.** Analytical data of aflatoxin determination in peanut by HPLC analysis

Toxin	LOD <sup>a</sup> ( $\text{ng g}^{-1}$ )	LOQ <sup>b</sup> ( $\text{ng g}^{-1}$ )	RSD <sub>r</sub> % (n=9) <sup>c</sup>	Recovery	
				Concentration	Recovery
AFB <sub>1</sub>	0.30	0.90	8.16	2.5	85.26 $\pm$ 4.38.64
				5	93.22 $\pm$ 7.64
				10	81.96 $\pm$ 4.74
AFB <sub>2</sub>	0.04	0.15	8.58	0.5	92.29 $\pm$ 6.07
				1	101.26 $\pm$ 6.55
				2	89.42 $\pm$ 8.23
AFG <sub>1</sub>	0.30	0.90	7.92	2.5	86.13 $\pm$ 3.75
				5	92.5 $\pm$ 7.71
				10	82.67 $\pm$ 6.48
AFG <sub>2</sub>	0.04	0.15	6.45	0.5	87.05 $\pm$ 3.85
				1	80.34 $\pm$ 3.64
				2	79.77 $\pm$ 5.98

**Table 2.** Range and concentrations of aflatoxins in peanut samples (n=32)

Toxin	Number of positive samples <sup>a</sup>	Mean $\pm$ SD <sup>b</sup> ( $\text{ng g}^{-1}$ )	Number of positive samples, $\text{ng g}^{-1}$				Range
			<0.3	0.3-5	5-15	>15	
AFB <sub>1</sub>	12	57.17 $\pm$ 90.32	20	5	2	5	0.90-243.61
AFB <sub>2</sub>	16	2.56 $\pm$ 3.34	24	5	1	2	0.36-6.40
AFG <sub>1</sub>	3	12.51 $\pm$ 20.02	29	2	1	-	0.90-69.25
AFG <sub>2</sub>	3	1.42 $\pm$ 1.85	30	2	-	-	0.17-3.45
AFT	12	95.72 $\pm$ 123.98	18	7	-	7	0.76-308.31

<sup>a</sup> samples above limit of detection

<sup>b</sup> mean  $\pm$  standard deviation of positive sample

## Results

In this study, 32 peanut samples were analyzed to evaluate the concentration of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> by HPLC with fluorescence detection. Table 2 presents the mean and range of total aflatoxin levels in the analyzed samples. Among these products, 16 samples were contaminated with at least one type of aflatoxins. As shown in Table 1, aflatoxin B<sub>1</sub> and total aflatoxins were detected in 12 samples (37.5%). Aflatoxin B<sub>2</sub> was detected in 16 samples (50%), and aflatoxin G<sub>1</sub> and G<sub>2</sub> were detected in 3 samples (9.38%). Our data revealed (Table 2) that, the AFB<sub>1</sub> and total aflatoxin levels in 5 and 7 samples (23.8% and 21.8%) were above the Iranian National Standard<sup>(20)</sup> limit of 5 and 15 ng g<sup>-1</sup> and European Union<sup>(7)</sup> limit of 8 and 15 ng g<sup>-1</sup>, respectively. Besides, the total aflatoxins in 7 samples (21.8%) were above the Codex Committee on Food and Contaminants. The results in Table 2 showed that, AFB<sub>1</sub> was detected in 12 samples (37.5%) at the range of 0.90-243.61 ng g<sup>-1</sup>, and the mean value of AFB<sub>1</sub> was 57.17 ng g<sup>-1</sup>. The incidences of total aflatoxins in peanut samples were 21.8% with the range of 0.76-308.31 ng g<sup>-1</sup> and mean value of 95.72 ng g<sup>-1</sup>. The mean value of AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> was 2.56 ng g<sup>-1</sup>, 12.51 ng g<sup>-1</sup> and 1.42 ng g<sup>-1</sup>, respectively.

## Discussion

In comparison to the other agricultural products, peanut is very susceptible to aflatoxins contamination. It may be due to the kernels develop and nature beneath the surface and domination of *Aspergillus flavus* of the peanut field soil. There are two possible causes of aflatoxin contamination in peanuts, drought stress in winter (pre-harvest) and the existence of undesirable moisture and temperature conditions during storage (post-harvest)<sup>(21)</sup>.

A study in Tabriz (Iran)<sup>(22)</sup> indicated that 15.15% of the roasted peanuts and 17.3% of raw samples were contaminated with aflatoxins. In this study, Ostad-Rahimi et al. explained that it can be the result of susceptible conditions such as high temperature and humidity, low light intensity, and long-term storage. In the another study, Shadbad et

al.<sup>(23)</sup> showed that some nuts were contaminated with aflatoxins in Tabriz. The incidence rates of aflatoxin were 14.29% (almonds), 76.92% (walnuts), 25% (apricots), 33.33% (peanuts), 7.69% (hazelnut), 53.13% (pistachios), and 14.29% (cashews); aflatoxins contamination in sunflower and sesame seeds was not detected, that is similar to the results of our study. Hong et al.<sup>(24)</sup> determined AFB<sub>1</sub> and AFB<sub>2</sub> in peanuts and corn based products. AFB<sub>1</sub> was detected in six peanut samples (20%) of the 20 total samples and in corn based products analyzed with concentrations ranging from 2.5 to 33.4 ng g<sup>-1</sup>. AFB<sub>2</sub> was detected in seven (35%) out of the 20 samples analyzed at concentration of 0.2 to 101.8 ng g<sup>-1</sup>. Bakhiet et al.<sup>(25)</sup> measured aflatoxins level in 60 peanut samples by TLC. These samples were collected from four different Sudan areas. The AFB<sub>1</sub> concentration in these samples was ranged from 17.57-404 ng g<sup>-1</sup> kernels. The aflatoxin B<sub>1</sub> concentration in our study was ranged 0.90-243.61 ng g<sup>-1</sup>, which is lower than the investigated peanuts in this study. In comparison, this study is in agreement with other previous studies on the natural occurrence of aflatoxins in peanut samples from other countries.

## Conclusion

In this study, the incidence aflatoxin level in peanut in Mashhad city was investigated. This high content of aflatoxins could be related to pre-harvest and post-harvest practices, processing storage, transportation, and marketing. Although the present study was limited in both number of samples and locations that samples were collected, aflatoxins contaminated peanuts were consistently found. Since this article reports the data of the first survey on the presence of aflatoxins in peanut samples in Mashhad city of Khorasan Razavi province of Iran, the monitoring and elimination of mycotoxin in peanut samples should be implemented and frequently concerns of quality and public health. Therefore, continuous monitoring of aflatoxins in food and environmental is essential to ensure that consumers are not being exposed to unacceptable levels of these natural toxins. It also enables us to understand the process



at the environmental level and based on the obtained data, a database could be for future use.

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#### Conflict of interest

The authors declare no conflict of interest.

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