Determination of aflatoxins in nuts of Tabriz confectionaries by ELISA and HPLC methods

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ABSTRACT

Purpose: Aflatoxins (AFs) are a group of mycotoxins and secondary metabolites of various species of Aspergillus. There are various forms of aflatoxins including B1, B2, G1, G2, M1 and M2 types. Aflatoxins cause important health problems and have high potential effect on liver cancer. Therefore, numerous investigations have been conducted during last three decades. The aim of this work is to determine the contamination levels of nuts used by the confectionaries in Tabriz. Methods: A total of 142 samples including 35 almond, 26 walnut, 4 seeds of apricot, 6 sunflower seeds kernel, 6 sesame seed, 6 peanuts, 32 pistachio, 13 hazelnuts and 14 cashews samples were collected from Tabriz confectionaries. The ELISA method was employed for the screening of total aflatoxins. Results: In 13 cases (28.1% of pistachios, 5.1% of walnuts and 7.1% of cashews) contamination rate of higher than 15 ppb were observed. The HPLC method was applied for the confirmation of ELISA results. Aflatoxin B1 was the highest detected AFs. Conclusion: The overall results of the tested samples indicated that the rate of contamination of pistachios is higher than the other tested samples.

Introduction

Aflatoxins (AFs) are a group of mycotoxins and secondary metabolites of strains of the fungi Aspergillus spp. There are various forms of aflatoxins including B1, B2, G1, G2, M1 and M2 types. AFs have commonly been found to contaminate a wide variety of food stuffs such as nuts, rice, wheat, barley, cereal and some other crops. AFB1, known as a powerful hepatocarcinogenic and genotoxicogenic substance and has been classified as group 1 carcinogen. The 8, 9-epoxide aflatoxin B1 is the active ingredient to the carcinogenesis effect of AFB1. Generally tropical condition such as high temperature and moisture lead to fungal proliferation and production of AFs. Poor storage condition can also contribute to fungal growth and increase the risk of aflatoxin contamination. Aflatoxin effects are very strong in liver carcinogenesis. Therefore AFs have an important role in public health. 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.

AFs are regulated in more than 76 countries. Currently the worldwide MTL for AFB1 and total AFs are 1 - 20 ng/g and 0 - 35 ng/g respectively. European Commission regulating set limits for AFB1 and total AFs 2 and 4 ng/g respectively in nuts, groundnuts, dried fruit and cereals since 1998. Research on Food products has demonstrated that AFs are still being found frequently in food product at level that are of significant concern for consumers. 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. The aim of this study was to screen the content of AFs in nut used in the confectionary products in Tabriz market as a leader for these products in Iran and also a first report in this regard.

Materials and Methods

Chemicals

AFB1, B2, G1 and C2 standard solution was prepared from Supelco (USA). aflatoxins concentrations...
were AFB1 1.00 µg/mL, AFB2 0.200 µg/mL, AFG1 1.00 µg/mL, AFG2 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) AflaCLEAN™ (LC Tech, Germany) were used.

**Sampling**

A total of 142 different types of nuts included 26 samples of walnut, 35 samples of almond, 32 samples of pistachio, 13 samples of hazelnut, 4 samples of seeds of apricot, 14 samples of cashew, 6 samples of sunflower seeds kernel, 6 samples of sesame seeds and 6 samples of peanut were purchased from Tabriz market and confectionaries and stored frozen at –20°C until started analysis. Samples transferred to room temperature and then approximate value homogenized completely.

**Enzyme – linked immunosorbent assay (ELISA) analysis.**

For analysis of samples by ELISA, Euroclon kit was used with detection limit of 4 ppb. Analysis was performed according to the protocol of the manufacture. ELISA microplate reader (State Fax® 2100, Awareness, USA) was used to read the absorbance of samples and standard solutions at 450 nm. The extraction of aflatoxins from nuts was carried out using a 33% methanol solution. After filtering with filter paper (whatman No. 1, USA) 500 µl of the filtrate and 500 µl of 33% methanol were transferred to microtubes and stored at –20°C until started analysis. Samples transferred to room temperature and then approximate value homogenized completely.

**Extraction procedure**

Aflatoxins were analyzed in nuts according to the method reported by Stroka et al. Briefly, in 10 g of fatty samples (pistachio, peanut, and walnut), 1 g of NaCl added and blended with 40 ml of methanol: water (80:20) and 20 ml of n-hexane for 3 min. After separation of the two phases, n-hexane was eliminated. Nonfat samples were extracted only with 40 ml of methanol: water (80:20). The extract was filtered with filter paper (whatman No. 1 USA) and 10 ml of filtrate was passed by glass microfiber filter.

**Immunoaffinity column clean-up**

An aliquot of 7 ml was diluted with 43 ml of PBS buffer (pH 7.4). An immune affinity column AflaCLEAN™ (LC Tech, Germany) was conditioned with 10 ml of PBS buffer by gentle syringe pressure at a flow rate of 1 ml/min. Then, the mixture of the filtrate diluted extract (50 ml) was applied to the IAC column (1–2 drops per second), followed by a washing with 20 ml of bidistilled water and then dried with air. Aflatoxins were then slowly eluted from the IAC with 2 ml methanol into a glass vial.

**HPLC determination of aflatoxins**

After the ELISA screening, HPLC confirmation proceeded with contaminated samples (≥15 µg/kg total AFs). Briefly 5 g 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) was defined as the analyte concentration that gives a signal equal to: \( y_b + 3.3s_b \)

where \( y_b \) is the signal of the blank and \( s_b \) is its standard deviation. Similarly, the limit of quantitation (LOQ) was defined as: \( y_b + 10s_b \)

In the unweighted least-squares method, it is quite suitable in practice to use sxy instead of sb, and the value of the calculated intercept instead of \( y_b \) Thus: \( \text{LOD} = 3.3 \, s_{xy}/b \) and \( \text{LOQ} = 10 \, s_{xy}/b \) where b is the slope of the regression line (19, 20). Based on the above equations, the LOD and LOQ values for aflatoxins are shown in table 1.
Aflatoxins in nuts…

Table 1. Validation of aflatoxins determination by HPLC analysis.

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>LOD (µg/kg)</th>
<th>LOQ (µg/kg)</th>
<th>Calibration curve</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>0.38</td>
<td>1.26</td>
<td>y = 30729x + 7714</td>
<td>0.999</td>
</tr>
<tr>
<td>AFB2</td>
<td>0.08</td>
<td>0.28</td>
<td>y = 64499x + 12356</td>
<td>0.999</td>
</tr>
<tr>
<td>AFG1</td>
<td>0.42</td>
<td>1.4</td>
<td>y = 11004x + 8230</td>
<td>0.996</td>
</tr>
<tr>
<td>AFG2</td>
<td>0.05</td>
<td>0.19</td>
<td>y = 27323x + 7461</td>
<td>0.997</td>
</tr>
</tbody>
</table>

* Limit of detection (LOD).
* Limit of quantification (LOQ).
* x = concentration of aflatoxins (µg/kg) and y = intensity.

The efficacy of method was examined by the determination of the recoveries of the AFs. The recoveries were ascertained by spiking 10, 20, 30 µg/ml of AFB1 and AFG1 and 0.2, 0.4, 0.6 µg/ml of AFB2 and AFG2 and the extraction result were expressed as percentage that were ranging from 85.6 to 112.3% (table 2).

Table 2. Recoveries of aflatoxins in edible nuts.

<table>
<thead>
<tr>
<th>Aflatoxins</th>
<th>spiked aflatoxin µg/ml</th>
<th>Recovery (%)</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>10</td>
<td>95</td>
<td>2.1</td>
</tr>
<tr>
<td>AFB2</td>
<td>0.2</td>
<td>90.3</td>
<td>3.5</td>
</tr>
<tr>
<td>AFG1</td>
<td>0.4</td>
<td>97</td>
<td>2.9</td>
</tr>
<tr>
<td>AFG2</td>
<td>0.6</td>
<td>112.3</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Precautions in handling and decontamination of aflatoxins

The handling of preparation of standards, working solutions and samples must be done in fume hood with suitable protective clothing (laboratory cover, mask and gloves). All glass wares of laboratory accessories should be immersed in a 10% sodium hypochlorite before cleaning or discarding. Accidental spill and laboratory surface should be cleaned with 1% sodium hypochlorite.

Results and Discussion

The contamination of food with AFs 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. The incidence of AFs in different types of nuts and nutty products has been studied by several authors from different countries.

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. In China peanut was reported contaminated with AFs being than average level of 80.3µg/kg and the highest level being 437µg/kg. In Turkey peanut butter was contaminated with AFs in the range of 8.16 – 75.7 ng/g which AFB1 was in the range of 2.06 – 63.7 µg/kg. Cheraghal and et al (2007) reported that 11.8% and 7.5% of 10068 Iran pistachio nut samples were above the MTL of AFB1 and AFs respectively. Yin – Hui et al (2008) reported that nut and nutty products were contaminated with AFs ranging in level from 16.6 µg/kg up to 711 µg/kg.

In this study, at first ELISA method was used for screening contaminated samples. ELISA analysis is suitable for determination of contaminant in a large number of samples in a shorter time. According to the results, between 142 samples, 13 samples were contaminated above the maximum tolerated level (15 µg/kg). The most contaminated samples were walnuts (76.9%) and pistachio (53.12%), the least amount of contamination found in samples of almonds, hazelnuts and apricots.

Table 3. The results for confirmation of Presence of aflatoxins in different samples by HPLC analysis.

<table>
<thead>
<tr>
<th>Sample category</th>
<th>Incidence</th>
<th>Aflatoxins * (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No b</td>
<td>%</td>
<td>Total c</td>
</tr>
<tr>
<td>Almond</td>
<td>5/35</td>
<td>14</td>
</tr>
<tr>
<td>Walnut</td>
<td>20/26</td>
<td>76</td>
</tr>
<tr>
<td>Apricot</td>
<td>1/4</td>
<td>25</td>
</tr>
<tr>
<td>Sesame</td>
<td>0/6</td>
<td>0</td>
</tr>
<tr>
<td>Sunflower</td>
<td>0/6</td>
<td>0</td>
</tr>
<tr>
<td>peanut</td>
<td>2/6</td>
<td>33</td>
</tr>
<tr>
<td>Hazelnut</td>
<td>1/13</td>
<td>7</td>
</tr>
<tr>
<td>pistachio</td>
<td>17/32</td>
<td>53</td>
</tr>
<tr>
<td>Cashew</td>
<td>2/14</td>
<td>14</td>
</tr>
</tbody>
</table>

* AF concentration was not corrected for the recovery result.
* Incidence no was represented by positive/total sample of the particular category.
* Total was represented by the summation of aflatoxin B1, B2, G1 and G2 levels.
* Not detected. (The samples with total Aflatoxin lower than 15 µg/kg were not tested by HPLC)
The HPLC used for confirmation and quantification of AFs levels in contaminated samples. From 13 samples with contamination upper than 15 µg/kg, 9 samples were pistachio, 3 walnut and 1 cashew samples. The HPLC method used for determination of AFs in the above mentioned samples successfully confirmed the results of ELISA method. Also the results of HPLC showed that contamination with AFB1 were higher than other AFs, which is a potent carcinogen (table 3). In the present study some samples were collected as Powder form , the contamination in these kinds of samples were higher compared to intact ones. Probably that is due to their higher surface area and also storage conditions in confectionaries which make samples more susceptible for the fungal growth and contamination. The higher level of AFs in some nuts, especially about AFB1, suggests that a good supervision is necessary about the production and storage of nuts which are used in confectionaries.

Conclusions
This study presents useful information about the risk of AFs hazard in different types of nuts and their products. The high occurrence of AFs emphasizes the need for regular monitoring and a more stringent food safety system in order to control the AFs at the lowest possible level.

Acknowledgement
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Conflict of interest
The authors report no conflicts of interest.

References