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Aflatoxin in pistachio nuts used as ingredients in Gaz sweets produced in Isfahan, Iran

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In Isfahan province of Iran, pistachio and almond are used specifically as ingredients to produce Gaz sweets, which are a typical product of Isfahan and exported to many countries. In this survey, 112 samples were gathered from different corresponding Gaz producers from 2007 to 2012 to evaluate the occurrence of aflatoxins (AF) in these products. Samples were analysed for AFB1, AFB2, AFG1 and AFG2 using immunofinity column clean-up (IC) and quantified by high-performance liquid chromatography (HPLC) with fluorescence detection. Among the samples analysed, AFB1 was detected in 13.4% of the samples above the maximum limit (ML) as set in Iran (5 µg/kg). Total AF (AFT) was above the ML in 9.8% of the samples. This study showed that full consideration is equally essential for nuts used as ingredients in special food products such as the Gaz sweets as for nuts for direct human consumption. It suggests a need for modifying the current trend of supervision.

Keywords: aflatoxin; pistachio nut; Iran; Isfahan; Gaz sweet; ingredient

Introduction

Aflatoxins (AF) are toxic and carcinogenic metabolites developed by certain fungi or mould that grow in nuts, seeds and legumes. They are the most intensively researched mycotoxins in the world because of their demonstrated potent carcinogenic effects. However, they are allowed at low levels because it is practically impossible to remove them completely from food products. Moreover, it is believed that eating small amounts of AF poses only little risk over a lifetime. As it is realised that absolute safety is never fulfilled, many countries have attempted to limit exposure to AF by establishing maximum limits (ML). The highest risk of contamination with AF has been observed in corn, peanut, pistachio and fig (Pittet 1998). Pistachio is cultivated in Iran in large volumes. In addition to the local consumption, there is an extensive demand for it in the international markets. Moreover, pistachio and almond are provided as main ingredients of a famous sweet in Isfahan, Iran, called Gaz. While many AF measurements have been done on the contamination of these nuts, there has been almost no attempt to survey safe use of the same products when used as ingredients in Gaz sweets.

As pistachio is an important export item in Iran, several studies on the possibility of its contamination with AF have been performed. Cheraghi et al. (2007) assessed the incidence of AF in Iran’s pistachio nuts during 2002–2003. They analysed 3356 samples of domestic pistachio nuts for AFB1, AFB2, AFG1 and AFG2, using immunofinity column clean-up (IC) and high-performance liquid chromatography (HPLC) and TLC scanners. The samples were divided into sub-samples and about 10,000 AF analyses were performed. AFB1 was detected in 36.7% of the samples, but only 11.8% of the samples contained AFB1 above the ML. AFT were observed in 28.3% of the samples and 7.5% had a total AFT content above the ML. This study was repeated in 2009–2011 by Dini et al. (2013), to evaluate the effect of a more control and closer monitoring during the last decade on pistachio production in Iran. This time 3181 commercial raw pistachio nut samples divided into 8203 sub-samples were tested. AF were analysed by HPLC with fluorescence detection after IC clean up, with recoveries in the range of 77 to 99%. AFB1 was detected in 23.4% of the subsamples, where only 5.2% of the subsamples were above the permitted level. AF were revealed in 23.5% of the subsamples with a mean value lower than the ML. Less than 5% of the samples exceeded the ML. In total, the contamination level was reduced by 50% when compared to the previous study. AF incidence in pistachio nuts in Isfahan province, Iran, was studied by Sarhangpour et al. (2010). They tested 100 pistachio nut samples on AF purchased in 2007 from local markets in Isfahan. AFT, AFB1, AFB2, AFG1 and AFG2 in the pistachio samples were 95, 95, 42, 64 and 28%, respectively. Out of the total number of samples, 36 and 29% exceeded the ML for AFB1 and AFT, respectively.

Reviewing this literature showed room for more investigations on AF contamination in nuts and especially in nut products. To be specific, there is no study on pistachio used as an ingredient in Gaz, a typical sweet of Isfahan, Iran, that is exported to many countries, hence having

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consumers worldwide. Also, there was an expectation that the quality of the pistachio picked up for use in products like Gaz is inferior to what is presented in the market for direct consumption. This was the incentive for the current study on AFB1 and AFT in pistachio used as ingredients for Gaz sweets.

Materials and methods

Sampling
Pistachio samples (n = 112) of 3 kg were taken from local factories producing Gaz sweets by expert food inspectors of the food and drug administration of Esfahan, according to the EU sampling procedure for nuts (EC2006). The samples were tested in the Toxicology Department of the Food Control Laboratory in Isfahan during 2007–2012.

Sample preparation
In order to minimise sub-sampling errors, water slurry was prepared by adding 1.5 l of water to each 1.0 kg pistachio. The mixture was ground and mixed with a laboratory blender, and 125 g slurry of each sample was taken for analysis.

Chemicals and reagents
For AFB1, B2, G1 and G2 standards were purchased from Sigma Chemical Company (St. Louis, MO, USA). All solvents were of HPLC or analytical grade. Sodium chloride, potassium bromide and nitric acid were purchased from Merck (Darmstadt, Germany). Aflatoxin immunoaffinity columns were purchased from Vicam Company (Watertown, MA, USA). For phosphate buffer saline (PBS) solution, a mixture of 0.2 g KCl, 0.2 g KH₂PO₄, 1.16 g anhydrous Na₂HPO₄ and 8 g NaCl were dissolved in 900 ml water, adjusted to pH 7.4 and diluted to 1 l with water.

AF standard solutions
First, a standard solution of each individual AF was prepared and its concentration was determined by UV spectrophotometry (Shimadzu, Tokyo, Japan; AOAC 2006). These standard solutions were used to prepare mixed standard solutions for HPLC analysis. Then working standards were provided by diluting the mixed standard solutions with methanol and water.

Apparatus
Liquid chromatography was performed using an HPLC system (Dionex, Sunnyvale, USA) with vacuum degasser, temperature-controlled oven, C18, 250 mm × 4.6 mm, 5 μm column and fluorescence detector (AOAC method 999.07), with minor modification (Stroka, Ankle, et al. 2000; Stroka, van Otterdijk, et al. 2000).

Extraction and clean-up
Pistachio slurries were extracted using methanol/water/hexane (300 ml/75 ml/100 ml). After filtration, the extract was diluted with water and filtered through a glass microfibre filter. For clean-up ICs were used. First, 10 ml PBS solution was passed through the IC. Then, 75 ml of the filtrate was passed through the column at a flow rate of 1 drop/s. The column was washed with 15 ml of water and dried by applying little vacuum. AF was eluted with methanol as follows: at first, 0.5 ml methanol was applied on the column and passed through by gravity. After 1 min, 0.75 ml of methanol was applied, the eluate collected, diluted with water and 100 μl was injected in the HPLC.

AF analysis
Samples were quantified by HPLC with fluorescence detection using post-column bromination with a Kobra cell. The mobile phase was a water/methanol/acetonitrile solution containing 120 mg KBr and 350 ml 4M HNO₃. The flow rate was 0.8 ml/min. The fluorescence detector was operated at an excitation wavelength of 365 nm and emission wavelength of 435 nm.

Calibration curve
Each working day, a 6-point calibration curve was constructed for each individual AF, checked for linearity and used for quantification. If AF content in the sample was outside the calibration curve, a dilution of the sample was prepared and re-analysed.

Quality assurance
The accuracy and precision of the validated method was verified by recording the recoveries of AFB1, AFB2, AFG1 and AFG2 through analysing a blank sample spiked at 5 μg/kg. AF levels were corrected for recoveries.

Results
Average recoveries and relative standard deviations for repeatability (RSDr) applied for AFB1, AFB2, AFG1 and AFG2 are shown in Table 1. Both recoveries and RSDr of AF were in the acceptable ranges (EC 2006), indicating good precision of the analytical method. Averaged limit of detection (LOD) was determined using the signal-noise ratio to be 0.06 μg/kg for AFB1 and AFG1 and 0.03 μg/kg for AFB2 and AFG2. MLs of AFB1 and AFT are different according to various standards. In this section those of Iran are used as a criterion. In the “Discussion” section, other standards will be mentioned too.

Among the 112 samples analysed, 77 (68.7%) samples were not contaminated with AFB1 (<0.06 in Figure 1). AFB1 was detected in 35 samples (31.2%) and only the level in 15 samples (13.4%) was above the ML of AFB1 in pistachio
nuts in Iran (5 µg/kg). Mean and median of the samples are shown in Table 2. Among samples analysed, 75 samples (67%) did not show any AFT contamination (Figure 2). The remaining 37 samples (33%) contained AFT, and the level of 11 samples (9.8%) was above the ML (15 µg/kg).

Discussion

The results of this study can be compared with several others. In a recent survey in South Korea (Chun et al. 2012), 85 samples were tested, out of which 10.6% were contaminated with AF up to 28.2 µg/kg. Also in Malaysia (Leong et al. 2010), 32 out of 196 samples (16%), were contaminated with AFTs above the ML. In Turkey, during 2008–2009, Set and Erkmen (2010) tested total AFT and AFB1 contamination, pH, water activity, mould and yeast counts in 82 unpacked and packed products. For unpacked products, 17% and 23% of the samples contained AFT and AFB1 above the legal limits, respectively, while only one packed sample exceeded the AFB1 limit. In Greece, the production chain of pistachio nuts from farm to storage was studied by Georgiadou et al. (2012) and the conditions and practices that resulted in AF contamination were investigated. A total of 20 samples, 5 from each of the 4 pistachio orchards monitored, were taken for AF analysis. The first sample was taken at early maturity, the second at maturity, the third at harvest, the fourth at the end of drying and the fifth at storage (45 days after drying). The maturity stage was observed to be the first stage of AF contamination above the ML. AF level was higher at harvest. A higher level of contamination was measured in orchards with heavy insect infestation. The level of contamination with AF was still high but a little bit lower at drying and in storage. Quantification of AFB1 in post-harvest raw peanuts and its dietary risk in China was the subject of a study by Ding et al. (2012). They examined a total of 1040 samples and detected AFB1 in 25% of the samples. Monte Carlo and bootstrap methods were employed to estimate AFB1 intake in children and adults, and their potential risk of liver cancer was evaluated. The results suggested that AFB1 contamination in raw peanuts and its corresponding dietary risk were low. All together, non-permissible detected AF contaminations varied from 5 to 36% for AFB1 and 5 to 29% for AFT.

On the other hand, this study focused on AF contamination of pistachio as an ingredient, not as a product for direct human consumption. It shows non-permitted contamination levels of AFB1 and AFT for 13.4% and 9.8% of the samples, respectively. The interesting point is that these figures are almost corresponding to the median of the range of variation of results in the above cited studies. At the same time, these figures are considerably larger than the corresponding figures of reference (Dini et al. 2013) that were released recently. It confirms the need to pay more attention to the local markets in relevant countries, as nowadays it is obligatory in Iran to check samples of pistachio ingredients of Gaz for AF contamination.

Table 1. Average recoveries and relative standard deviations for repeatability (RSDr).

<table>
<thead>
<tr>
<th>AF</th>
<th>No.</th>
<th>Spike level (µg/kg)</th>
<th>Average recovery (%)</th>
<th>RSDr (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>39</td>
<td>5.0</td>
<td>80.1</td>
<td>10.3</td>
</tr>
<tr>
<td>AFB2</td>
<td>39</td>
<td>1.0</td>
<td>93.4</td>
<td>10.7</td>
</tr>
<tr>
<td>AFG1</td>
<td>39</td>
<td>5.0</td>
<td>92.2</td>
<td>9.6</td>
</tr>
<tr>
<td>AFG2</td>
<td>39</td>
<td>1.0</td>
<td>80.8</td>
<td>14.9</td>
</tr>
</tbody>
</table>

Figure 1. AFB1 in pistachio nuts used as ingredients in Gaz sweets, Isfahan, Iran, 2007–2012.

Table 2. Mean, median and standard deviation of AFB1 and AFT (µg/kg).

<table>
<thead>
<tr>
<th>AF</th>
<th>N</th>
<th>Samples&lt;0.06</th>
<th>Samples with&lt;0.06 &lt; AF &lt;ML</th>
<th>Samples with AF &gt; ML</th>
<th>Mean</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>112</td>
<td>77</td>
<td>20</td>
<td>15</td>
<td>6.2</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>AFT</td>
<td>112</td>
<td>75</td>
<td>26</td>
<td>11</td>
<td>11.5</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Another point of interest is the difference between the ML levels for AF contamination worldwide. While MLs for AFB1 and AFT for pistachio in Iran are 5 µg/kg and 15 µg/kg, respectively, these are 8 µg/kg and 10 µg/kg in the European Union (EC 2010a, 2010b). In the United States, ML for AFT is 20 µg/kg, so it is considerably higher. The results of this study, as presented above and other Iranian studies of Cheraghali et al. (2007), Sarhangpour et al. (2010) and Dini et al. (2013), were based on the domestic standard (ISIRI 2002). When using EU limits, the AF contamination determined in this study rises to 17.0%, both for AFB1 and AFT.

Conclusions
In this study, AF levels in pistachio nuts that are used as ingredients in Gaz, a typical sweet of Isfahan province of Iran, were investigated. It was observed that 13.4% of the samples were contaminated above the AFB1 limit and 9.8% was above the AFT limit as set in Iran. These percentages were considerably higher than the corresponding figures released recently out of an extensive study on pistachio for export in Iran. This suggests a need to pay more attention to pistachios provided at local markets and for use as an ingredient in products like Gaz sweets.

References