Aflatoxin contamination level in Iran’s pistachio nut during years 2009–2011

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\textbf{A B S T R A C T}

An efficient monitoring system for sampling, analyzing and issuing the export certificates for pistachio consignments has been established in Iran in recent years. Accordingly, 3181 commercial raw pistachio nut lots were supplied for testing for European export certification since January 2009 till December 2011. Aflatoxin analysis was carried out by high-performance liquid chromatography with fluorescence detection after immunoaffinity column clean up with recoveries ranging from 77 to 99%. Amongst 8203 sub-samples analyzed, aflatoxin B\textsubscript{1} (AFB\textsubscript{1}) was detected in 1921 cases (23.4%) with the mean and median values of 2.18 $\pm$ 13.1 ng/g and < LOD, respectively. Total aflatoxin (AFT) was detected in 1927 sub-samples (23.5%) with the mean and median values of 2.42 $\pm$ 14.7 ng/g and < LOD, respectively. AFB\textsubscript{1} level in 556 (6.78%) and 428 (5.22%) sub-samples was above the maximum tolerable levels set for AFB\textsubscript{1} in Iran (5 ng/g) and European Union (EU) (8 ng/g). The mean contamination levels of AFB\textsubscript{1} (2.18 ng/g) and AFT (2.42 ng/g) were lower than the maximum tolerable levels set in Iran and EU. The contamination levels of pistachio nut for export to EU were ~50% of those found in 2002–2003 indicating a satisfying improvement in hygienic conditions of pistachio cultivation, harvesting and post-harvesting practices in Iran.

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1. Introduction

Pistachio nut is being produced in different regions of the world by various countries, among which Iran is the largest producer (Farzaneh et al., 2012; Zheng, 2011) most of which is exported to other countries. Traditionally, the European Union (EU) is one of the major destinations for Iran pistachio (Cheraghali & Yazdanpanah, 2010). During years 2009–2011, around 20,000 tons pistachio nut were exported to EU from Iran most majorly from Kerman and Rafsanjan located in the center of country (Iranian Pistachio Association website).

Contamination of agricultural products with mycotoxins including aflatoxins is one of the major challenges encountered by producers. Aflatoxins are polyketide secondary metabolites produced by some species of Aspergillus genus, particularly Aspergillus parasiticus and Aspergillus flavus (Cheraghali et al., 2007). Pistachio nuts are amongst the commodities with the highest risk of aflatoxin contamination (Pittet, 1998). The contamination is influenced by environmental factors such as temperature, humidity, and the extent of rainfall during cultivation, harvesting, and post-harvesting stages (Campbell, Molyneux, & Schatzki, 2003; Doster & Michailides, 1994; Emami, Suzanag, & Barnett, 1977). It is important to protect both consumers health and producers benefit through implementing a series of interventions in order to reduce the aflatoxin contamination to as low as practically feasible. Failure to do this, may create devastating economic consequences to producers, and also deprive consumers from a very valuable source of nutrient and pleasure. Many countries have established maximum tolerable levels on mycotoxins occurrence in foods due to the increasing awareness of their harmful carcinogenic, mutagenic and teratogenic effects on human and animals (Set & Erkmen, 2010). In Iran, the maximum acceptable levels for aflatoxin B\textsubscript{1} (AFB\textsubscript{1}) and total aflatoxin (AFT) in pistachio nuts are 5 and 15 ng/g, respectively (ISIRI, 2002). In 1998, the Commission of the
European Communities set the maximum level for AFB1 in a range of commodities for human consumption at 2 ng/g (Moss, 2002). Recently, EU commission increased the maximum level in almonds, pistachios and apricot kernels intended for direct human consumption or use as an ingredient in foodstuffs to 8 ng/g and that for AFT from 4 to 10 ng/g (EC [European Commission], 2010a).

In 1997, based on the unacceptable levels of aflatoxin detected in pistachio consignments arriving in EU ports from Iran, special conditions were imposed on Iran pistachio entering the EU. Thereafter, extensive efforts were made to overcome the problem by Iranian authorities in collaboration with EU authorities based on a multi-approach intervention (Cheragahi & Yazdanpanah, 2010). A comprehensive survey on 3356 pistachio nut samples produced mostly in Kerman and Rafsanjan during March 2002–February 2003 indicated that 11.8% and 7.5% of samples had contamination levels higher than maximum tolerable limits set in Iran for AFB1 and AFT (Cheragahi et al., 2007). Later, a brief study on 100 samples purchased from retail shops and local markets in Esfahan province of Iran from September to November 2007 revealed that 36% and 29% of pistachio nuts exceeded the maximum tolerable limit set for AFB1 and AFT, respectively (Sarhang Pour, Rasti, Zighamian, & Daraei Garmakhani, 2010). These high contamination levels were most probably due to the extensive manipulation and exposure of pistachio nut in retail shops. Efforts were continued due to the enforcement and stricter control measures arising from Commission Decision 2006/504/EC (EC [European Commission], 2006a) on special conditions governing certain foodstuffs imported from certain countries (Ariño et al., 2009), Iranian health and agricultural authorities implemented strict regulations to manage the contamination by promoting good agricultural practices in the orchards and hazard analysis and critical control point principles in storage and processing plants (Farzan et al., 2012). Recently, Ariño et al. (2009) reported the occurrence of aflatoxins in pistachios available in northeast Spain from January to April 2007. They found that although all positive samples originated from Iran, no sample exceeded the maximum permitted level for aflatoxins in pistachio nuts set by EU.

Foodstuffs import/export checking and their monitoring on the borders and domestic markets are commonly carried out by governmental bodies. This is performed by the Ministry of Health in Iran as the official responsible for testing the pistachio nut exporting to EU. A network of Food Control Laboratories supervised by Ministry of Health accomplishes the task. The present paper reports the results of an extensive survey for aflatoxins occurrence carried out by Food Controls Labs to monitor the contamination of raw pistachio intended to export to EU in a period of three years from January 2009–December 2011.

2. Materials and methods

2.1. Sampling

A total of 3181 pistachio nut samples were collected by inspectors of Food Control Offices in Kerman and Rafsanjan since January 2009 till December 2011. The sampling was carried out exactly according to the sampling procedures established by EU. During January 2009–September 2010, samples were taken following the procedure described in Commission regulation (EC) No. 401/2006 (EC, 2006b) and then during October 2010–December 2011 according to the Commission regulation No. 178/2010 (EC, 2010b). The pistachio nut consignments, intended for export to EU, are usually about 25 tons. Accordingly, 100 incremental samples, each weighing 300 or 200 g were mixed together and divided into three or two 10 kg sub-samples. Preparation of sub-samples for analysis and further analytical experiments were then carried out in Toxicology Labs of Food Control Laboratories located in Rafsanjan and Kerman. A total of 1094, 1074 and 1013 samples divided in 3282, 2895 and 2026 sub-samples were analyzed in 2009, 2010 and 2011, respectively.

2.2. Sample preparation

A water slurry of pistachio nut samples was prepared to minimize the sub-sampling errors in aflatoxin analysis (Cheragahi et al., 2007). Therefore, 15 L water was added to each 10 kg sub-sample of pistachio nut, followed by mixing and grinding the mixture by using a slurry machine for 15 min. When ready, 125 g of slurry was taken as the test portion for analysis.

2.3. Chemicals and reagents

Aflatoxin B1, B2, G1 and G2 standards were procured from Sigma (MO, U.S.A). Methanol, acetonitrile (Caledon laboratories Ltd, Canada) and water were high-performance liquid chromatography (HPLC) grade. Sodium chloride, potassium bromide, nitric acid (Merck, Darmstadt, Germany) and phosphate-buffered saline [pH 7.4; 0.20 g KCl, 0.20 g KH2PO4, 1.16 g anhydrous Na2HPO4 (or 2.92 g Na2HPO4.12H2O) and 8.0 g NaCl dissolved in 900 ml water and pH adjusted to 7.4 with 0.1 M HCl or 0.1 M NaOH and diluted to 1 L with water] were used in present research.

2.4. Standard solutions

After preparation of standard solutions of each aflatoxin, their concentration was determined by using an UV–Visible Spectrophotometer (Varian, CARY 100, USA) through AOAC Official method No. 971.22 (AOAC, 2006; chap. 49.2.03). These standards were used to prepare mixed standards for HPLC analysis. The working standard solution was prepared by diluting mixed standards, tertiary stock standard 40 ng/ml (AFB1, AFG1 = 16 ng/g; AFB2, AFG2 = 4 ng/g), with methanol and water.

2.5. Apparatus

Liquid chromatography (LC) analysis was performed using a reverse-phase HPLC system (Dionex, Sunnyvale, California, LP, USA) equipped with a Gilson-Workstation (GX-271 Aspec Gilson, USA), vacuum degasser (Ultimate-3000, Dionex, Sunnyvale, California, LP, USA), temperature-controlled oven (Ultimate-3000, Dionex, Sunnyvale, California LP, USA), and fluorescence detector (RF 2000; Dionex, Sunnyvale, California LP, USA). The Dionex LC column was C18, 250 mm × 4.6 mm, 5 μm. Aflatoxin immunoaaffinity columns (IAC) were purchased from Vicam (MA, USA).

2.6. Chromatographic conditions

Reversed-phase LC determination of aflatoxins was performed using the post-column bromination with KobraCell (Coring System, Gernsheim, Germany) with a flow rate of 0.8 ml/min and fluorescence detection at excitation wavelength 365 nm and emission wavelength 435 nm. The column temperature was adjusted to 36 °C. Retention times for AFG2, AFG1, AFB2 and AFB1 were 10, 11.63, 13.6, and 15.06 min, respectively. The isocratic mobile phase was water–acetonitrile–methanol solution and a ratio of 60:20:30 (v/v/v), containing 120 mg/L KBr and 350 μl HNO3 4 M.

2.7. Extraction and clean up

Samples were analyzed for aflatoxins content based on the AOAC Official Method No. 999.07:2000 (AOAC, 2006; chap. 49.2.29)
with minor modifications (Stroka, Ankle, Jorissen, & Gilbert, 2000). Pistachio nut slurries were extracted with methanol/water/hexane (300 ml/75 ml/100 ml) after which the extract was filtered through MN 619 de filter paper. After filtration, 3.1 ml of extract was diluted with 9.9 ml deionized water and mixed with 8 ml air in workstation. For clean up the diluted extract, aflatoxin immunoaffinity columns were used. First, 5 ml phosphate buffered saline was passed through the column by workstation cleaned needle. Then, 12.6 ml of the diluted filtrate was passed through the aflatoxin column at a flow rate of ca. 1 drop/s. The column was washed with 15 ml water and dried by applying little vacuum. Finally, aflatoxin was eluted with methanol through the following procedure. At first, 0.5 ml methanol was applied on the column which passed by gravity. After 1 min, the second portion of 0.75 ml methanol was applied and collected. The eluate was diluted with 1.75 ml deionized water, after which 100 μl was injected into HPLC.

2.8. Calibration curve

A calibration curve was prepared by using the working standard solutions. The tertiary stock standard was used to prepare the working standard solutions by pipetting appropriate volumes into a set of 20 ml calibrated volumetric flasks and diluting to volume. Concentrations of AFB₁ and AFG₁ were 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 2.4 ng/ml and AFB₂ and AFG₂ were 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6 ng/ml, respectively. An eight point calibration curve was built for each type of aflatoxin. The calibration curve was constructed before the analysis to check the pilot for linearity \((r^2 = 0.998)\) and was used for quantification of aflatoxin. If the content of toxins in the sample was outside the calibration range, a more appropriate calibration curve was prepared, or the injection solution for LC analysis was diluted to an aflatoxin concentration appropriate for the established calibration curve.

2.9. Quality assurance

For evaluation of the reliability of results, in addition to using validated methods, internal and external quality control experiments were performed. Regarding internal quality control, the accuracy and precision of the methods were verified. For this purpose, recoveries of AFB₁, B₂, G₁ and G₂ were recorded by analyzing a blank pistachio nut sample spiked at 4 ng/g for AFB₁ and AFG₁ and 1 ng/g for AFB₂ and AFG₂. According to the recovery values, aflatoxin levels were corrected for recoveries. Regarding external quality control, Food Control Labs participated regularly in proficiency testing of Food Analysis Performance Assessment Scheme (FAPAS) in UK obtaining consistently satisfactory Z-score \((-2 \leq Z\text{-score} \leq +2)\).

3. Results

Method’s performance determined through the internal quality validation showed that the limit of detection (LOD) averaged 0.066 ng/g for AFB₁ and AFG₁ and 0.033 ng/g for AFB₂ and AFG₂. Therefore, the method demonstrated a good performance at low statutory limits. As well, the limit of quantification (LOQ) was acceptable (Table 1) implying the reliability of generated data. The average recoveries and relative standard deviation for reproducibility \((RSD_{d})\) of the analytical method applied for measurement of various aflatoxins in pistachio nut are given in Table 1. Recoveries ranged from the minimum value of 77% for AFG₂ to a maximum value of 99% for AFB₂ and RSD_{d} values were within the acceptable ranges (EC No. 401/2006, Annex II) indicating the good accuracy and precision of the used analytical method.

Results of occurrence of various aflatoxin types in pistachio for export to EU in each year are detailed in Table 2. The mean occurrence levels of AFB₁, AFB₂ and AFT in pistachios for export to EU in year 2011 were lower than those in 2009 and 2010. Sum of occurrence of AFT and AFB₁ during years 2009–2011 indicates that 6282 samples (76.6%) were not contaminated with AFB₁ (<LOD); however, its level in 428 (5.2%) and 556 (6.78%) sub-samples exceeded the maximum tolerable levels set for AFB₁ in pistachio nut by EU (8 ng/g) and Iran (5 ng/g), respectively. Seventy-six and half percent (6276 cases) of all analyzed samples were not contaminated with AFT (<LOD) and its mean occurrence level was 2.42 ± 14.7 ng/g. Samples had a median contamination level < LOD. The mean contamination levels with AFT (2.42 ± 14.7 ng/g) and AFB₁ (2.18 ± 13.1 ng/g) were lower than the maximum tolerable limits set for both Iran (5–15 ng/g) (ISIRI, 2002) and EU (8–10 ng/g). AFT contamination level in 402 (4.9%) sub-samples exceeded one of the maximum tolerable limits set for AFT by EU and codex committee on food additive and contamination. AFB₂ was not detected in solely 6 of pistachio nut samples contaminated with AFT (1927 sub-samples), and the mean value for AFB₂/AFT was 0.94 indicating the major contribution of AFB₂ in aflatoxin contamination of pistachio nut.

4. Discussion

After the impose of ban on pistachio importation to EU from Iran in 1997, various strategies were implemented by Iranian authorities to minimize the aflatoxin incidence in pistachio. Analysis of pistachio for export to EU by Food Control Labs of Ministry of Health as the responsible for sampling, analyzing and issuing the export certificates for pistachio consignments indicate significant improvements. It is observed from Table 2 that more than 95% of pistachios during three successive years since 2009 till near end 2011 had acceptable contamination levels. The mean contamination level in 402 (4.9%) sub-samples exceeded one of the maximum tolerable limits set for AFT by EU and codex committee on food additive and contamination. AFB₂ was not detected in solely 6 of pistachio nut samples contaminated with AFT (1927 sub-samples), and the mean value for AFB₂/AFT was 0.94 indicating the major contribution of AFB₂ in aflatoxin contamination of pistachio nut.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Year & Type of aflatoxin & LOD \(^a\) (ng/g) & LOQ \(^b\) (ng/g) & No. & Spike level (ng/g) & Average recovery (%) & RSD\(^d\) (%) \\
\hline
2009 & B₁ & 0.066 & 0.2 & 86 & 4 & 93 & 9.5 \\
& B₂ & 0.033 & 0.1 & 86 & 4 & 90 & 11.2 \\
& G₁ & 0.066 & 0.2 & 86 & 4 & 98 & 10.6 \\
& G₂ & 0.033 & 0.1 & 86 & 4 & 77 & 14.3 \\
2010 & B₁ & 0.066 & 0.2 & 130 & 4 & 86 & 10.4 \\
& B₂ & 0.033 & 0.1 & 130 & 1 & 99 & 14.4 \\
& G₁ & 0.066 & 0.2 & 130 & 4 & 98 & 11.05 \\
& G₂ & 0.033 & 0.1 & 130 & 1 & 96 & 17.9 \\
2011 & B₁ & 0.066 & 0.2 & 97 & 4 & 88 & 8.7 \\
& B₂ & 0.033 & 0.1 & 97 & 1 & 90 & 10 \\
& G₁ & 0.066 & 0.2 & 97 & 4 & 88 & 10 \\
& G₂ & 0.033 & 0.1 & 97 & 1 & 78 & 9.3 \\
\hline
\end{tabular}
\caption{Performance characteristics of the analytical method.}
\end{table}

\(^a\) Limit of detection. \(^b\) Limit of quantification. \(^c\) Number of spiked pistachio nut samples analyzed using HPLC. \(^d\) Relative standard deviation for reproducibility.
It is obvious from results that coordinated activities by Iranian authorities and EU experts and research projects funded by FAO and Iran national organizations (Cheraghali & Yazdanpanah, 2010; FAO TCP Project in Iran, Pineiro, 2003) have drastically improved the situation. In recent years, the European Commission (EC) has established Rapid Alert System for Food and Feed (RASFF) in order to notify the consumers and member states of any possible contamination found in food consignments entering the community. A substantial number of these notifications are concerned with aflatoxins in foods. The recent statistics published by EC regarding RASFF for aflatoxin contamination confirm a significant reduction in Iran pistachio nut contamination. According to annual reports of RASFF, while in 2005 there were 457 (48% of total) notifications about pistachio nuts from Iran, the figure has reduced to 234 (29% of total) in 2006, and 126 (18% of total) notifications in 2007; whilst, the quantity of imported pistachio nuts in the period 2005–2007 remained constant, approximately 33,000 tons/year (Cheraghali & Yazdanpanah, 2010; RASFF, 2008). This decreasing trend has continued and the percentage of notifications for Iran’s pistachio has continually decreased from 17.5% in 2008 to 9% in 2009 and then to 8.6% and 6.5% in 2010 and 2011, respectively (Fig. 1) (RASFF, 2010, 2012). This means that the incidence of aflatoxin contamination in pistachio nuts imported from Iran to EU is improving which is also reflected in the rejection rates observed. While in 2005, approximately 25% of all consignments offered for import in EU were found to be noncompliant with EU’s aflatoxin contamination regulations, this rate decreased to approximately 10% in 2007 (RASFF, 2008) and approximately 4% in 2011. Results obtained in the present communication are in close agreement with the trend observed.

5. Conclusion

Strategies implemented by Iranian authorities, including Ministry of Health and Ministry of Jihad-e-Agriculture in cooperation with EU experts have been effective and promising in control of pistachio nut contamination with aflatoxins. It is however required to continue and develop the currently established procedures to minimize the number of aflatoxin-positive cases. The procedures are also most probably applicable to dried fruits and other nuts suspected with high mycotoxin contaminations. The current ratio of maximum tolerable level set for AFT (15 ng/g) to that of AFB1 (5 ng/g) in Iran is 3:1 while; the major contribution of AFB1 in total aflatoxin contamination of pistachio nuts in Iran and probably some other countries, suggests reconsidering the maximum tolerable levels and set a more appropriate ratio.

Acknowledgments

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References


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**Table 2**

Occurrence of aflatoxins in pistachio nut for export to EU during 2009–2011.

<table>
<thead>
<tr>
<th>Type of aflatoxin</th>
<th>Year</th>
<th>Percentage of samples in the range (ng/g)</th>
<th>Mean</th>
<th>STDb</th>
<th>Median</th>
<th>Highest level of aflatoxin found (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt; LODa</td>
<td>5–8</td>
<td>8–10</td>
<td>10–20</td>
<td>20–50</td>
</tr>
<tr>
<td>B1</td>
<td>2009</td>
<td>72.37</td>
<td>1.62</td>
<td>1.01</td>
<td>1.77</td>
<td>2.04</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>76.78</td>
<td>1.59</td>
<td>0.48</td>
<td>1.73</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>80.15</td>
<td>1.33</td>
<td>0.35</td>
<td>2.02</td>
<td>1.33</td>
</tr>
<tr>
<td>B2</td>
<td>2009</td>
<td>86.69</td>
<td>1.23</td>
<td>0.36</td>
<td>2.02</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>89.19</td>
<td>0.57</td>
<td>0.14</td>
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<td>0.14</td>
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<tr>
<td></td>
<td>2011</td>
<td>92.21</td>
<td>0.20</td>
<td>0.05</td>
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</tr>
<tr>
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<td>2009</td>
<td>99.60</td>
<td>0.31</td>
<td>0.03</td>
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<td></td>
<td>2011</td>
<td>99.55</td>
<td>0.46</td>
<td>0.03</td>
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<tr>
<td>G2</td>
<td>2009</td>
<td>99.82</td>
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<td>2010</td>
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<tr>
<td></td>
<td>2011</td>
<td>99.65</td>
<td>0.34</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Total</td>
<td>2009</td>
<td>72.31</td>
<td>20.47</td>
<td>1.37</td>
<td>0.98</td>
<td>2.05</td>
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<tr>
<td></td>
<td>2010</td>
<td>78.70</td>
<td>14.33</td>
<td>1.16</td>
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<td></td>
<td>2011</td>
<td>80.10</td>
<td>13.27</td>
<td>1.18</td>
<td>0.52</td>
<td>1.92</td>
</tr>
</tbody>
</table>

a LOD – limit of detection.

b STD – standard deviation.

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**Fig. 1.** Number of notifications on aflatoxin contamination in Iranian pistachio for 2005–2011 [data from: http://ec.europa.eu/food/food/rapidalert/- European commission rapid alert system for food and feed notification on aflatoxins (RASFF, 2008, 2010, 2012) and (Iranian pistachio association website)].


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