Occurrence of aflatoxin B1 in pistachio nuts during various preparing processes: tracing from Iran

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Abstract:
Aflatoxin B1 (AFB1) is a secondary metabolite produced by some *Aspergillus* species, which have harmful impacts on human health such as carcinogenicity, teratogenicity, acute and chronic toxicity. In this study, the occurrence of AFB1 in pistachio nuts during various preparing processes was investigated in two pistachio farms from Qazvin province, Iran, from September to November 2012. High Performance detected level of AFB1 and Liquid Chromatography (HPLC) contained fluorescence detector using especial immunoaffinity columns. The results showed that AFB1 was present in the samples (100%) in during pre-harvesting, harvesting and post-harvesting stages. The highest mean value of AFB1 (27.58±2.12 µg/kg) was found in pistachio samples after harvesting process. Forty-six of 84 pistachio nuts (54.76%) in various process periods exceeded the maximum tolerable limit (2µg/kg) that set for AFB1 by European Union regulations. In addition, the results indicated that AFB1 levels in the samples had increased over storage time ($P<0.05$). As a result, post-harvest and storage strategies are the best time to prevent mold growth and aflatoxins production.

Keywords: Aflatoxin B1, HPLC, Pistachio.

Introduction

Mycotoxins are the toxic metabolites produced by fungi that may cause a serious hazard to health. Aflatoxin B1 (AFB1) is extremely toxic among other mycotoxins that may be produced by some species of *Aspergillus* such as *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. AFB1 may be caused by teratogenic, carcinogenic and mutagenic effects to human, animals, plants and even microorganisms (Cheraghali et al., 2007). Aflatoxin-producing fungi can contaminate and spoil crops such as tree nuts, peanuts, corn, and cotton (Farzaneh et al., 2002). Among nuts, pistachios has a high nutrient density; and provides an excellent source of copper, manganese and vitamin B6; pistachios offer a high amount of total polyphenols antioxidants; and are the only nut to contain significant amounts of lutein and zeaxanthin. Pistachios also offer a high satiety level and as an in-shell snack, have a slower consumption time.

The Pistachio nut is a commodities with the highest risk for aflatoxin contamination (Dini et al., 2013). The pistachio is a member the family of cashews family, originally from central Asia and the middle east, and is now cultivated in various parts of the world such as Iran, Syria, Lebanon, Turkey, Greece, Xinjiang (China), Tunisia, Kyrgyzstan, Tajikistan, and recently in the United States, on the west coast. Based on the Food and Agriculture Organization (FAO) statistics, Iran is the largest pistachio nut producer in the world and produced approximately 190,000 Mt of pistachio nut in 2004, which is approximately 63.33% of the world's pistachio production (FAO, 2004). Pistachios are the lowest in calories, and fat, they offer a good source of protein and are among the highest in fiber content for a nut (3 grams/ serving). Thirty pistachios make a satisfying healthy snack for just approximately 100 calories per serving- a wise choice when watching your weight (FAO, 2004; Set and Erkmen, 2010).

With factors such as water/ moisture content, substrate composition, storage time, the presence of a shell and the fact that insects may impact fungal growth and the AFs production during cultivation, harvesting, and post-harvesting stages (Campbell et al., 2003; Dostr and Michailides, 1994; Schatzki and Ong, 2001).

The AF content in food and fodder has been determined as a rule by conventional analytical techniques such as: thin layer, gas or liquid chromatography, spectrofluorometry and spectro-photometer (Lebiedzinska and Ganowiak, 1995).

The knowledge that mycotoxins can have serious effects on humans and animals has led many countries to establish a maximum tolerable level (MTL) on mycotoxins in food and feedstuffs in the last decades to safeguard the health of humans, as well as the economical interests of producers and traders. Currently, worldwide range for the limits of AFB1 and Aflatoxin total are 1-20 ng/g and 0-35 ng/g, respectively (FAO, 2004). The purpose of this study study was to exaime and determine the AFB1 levels in the pistachio samples collected from the pistachio farms in the Qazvin province of Iran, and compare the AFB1 levels, in the samples collected during pre-harvesting, harvesting and the post-harvesting stages.

Materials and Methods

Sampling: Totally, eighty-four pistachio nut samples were taken from two pistachio farms from the Qazvin province in Iran during September to November 2012. Various concentrations of AFB1 were determined during pre-harvesting, harvesting and post-harvesting stages. The samplings were readied according to Georgiadou et al. (2012).

Reagents: AFB1 standards for the experiments were purchased from Sigma Chemical Company, St. Louis, MO. All solvents used for the experiments were of either HPLC or analytical grade. Afla test immunoaffinity columns (IAC) were purchased from Vicam Company, Watertown, MA. HPLC column (Partisil 5 ODS3) was purchased from Hichrom Limited, Berkshire, England. HPLC instrument with separation module 2695 (Waters, Milford, MA) and fluorescence detector 474 Waters were used.

Aflatoxin analysis; Extraction and Clean up: Samples were analyzed using an high-performance liquid chromatography [HPLC] system method (the AOAC official method 999.07). Prepration for HPLC analysis, pistachio nut slurries were extracted with methanol/water/ hexane (300 mL/ 75 mL/ 100 ml). After filtration, the extract was diluted with water and filtered through the glass microfiber filter. To remove any remaing residue from the samples, Afla test IACs were used. First, 10 ml of phosphate saline buffer (PBS) was passed through the IAC. Then, 75 ml of the filtrate (10 ml extract + 65 ml PBS) was passed through the IAC at a flow rate of CA. 1 drop/s. The column was washed with 15 ml PBS and dried by applying a slight vacuum. Subsequently, the AF was eluted with methanol by the following procedure. First, 0.5 ml methanol was introduced on-injected into the column, which passed through by gravity. After 1 min, the second portion of 0.75 ml methanol was introduced on-injected into the column and collected. The elute was diluted with water and analyzed by HPLC. After the preparation of the standard solutions of individual AFB1, the concentration of each solution was determined using a UV spectrophotometer. This standard was used to prepare the mixed working standards for HPLC analysis (Stroka et al., 2000).

Aflatoxin analysis; HPLC Analysis: AFB1 was quantified by reverse-phase HPLC and a fluorescence detector with a postcolumn derivation chamber (PCDC) involving brominating. PCDC was achieved with a Kobra cell and the addition of potassium bromide to the mobile phase. After dilution of AF elute with water, 100 mL was injected into the HPLC. The analytical column used was C18, 5 mm, 250 mm and 4.6 mm

Table 1. Occurrence of AFB1 in pistachio nut samples in Qazvin province, Iran (during September to November 2012). Means ± SD within the column with different superscripts are significantly different (P<0.05).

	Sample(N)	Min	Max	Mean	SD	Positive	EU Exceed legal limit n (%)
Pre harvesting	28	1.28	21.96a	4.11	6.56	28(100%)	6 (21.4)
Harvesting	28	1.79	14.75b	5.93	5.46	28(100%)	16 (57.1)
Post harvesting	28	1.53	77.12c	27.85	5.68	28(100%)	24 (85.7)
Total	84	1.28	77.12	12.54	16.82	84(100%)	46 (54.76%)

i.d. Mobile phase was water:methanol:acetonitrile (54:29:17, v/v/v) with a flow rate of 1 mL/min. The fluorescence detector operated at an excitation wavelength of 365 nm and emission wavelength of 435 nm. Each working day, a 5-point calibration curve was built for AFB1, checked for the linearity and used for the quantification of AFB1 in pistachio nut samples. The Limit of detection (LOD) for AFB1 was 0.1 ng/g and limit of quantification (LOQ) for all AFB1 was 0.8ng/g.

Statistical analyses: Data were analyzed by analysis of variance (ANOVA) using SPSS 17. Statistically significant effects were accepted at the P<0.05 level.

Result

The results of the analyses of the AFB1 levels in pistachio nut samples are shown in Table 1. Among the 84 analyzed samples, AFB1 was detected in all pistachio samples (100%). The means of AFB1 in all analyzed samples were not in agreement with both Maximum Tolerance Limits of AFB1 means in the pistachio nut (2ng/g).

The overall mean level of AFB1 in the samples was 12.54±16.82 ppb (Table 1). However, 46 (54.7%) samples (Table 1) were higher than the maximum tolerance level of AFB1 in accordance with the European Union standard (2 ppb) and the majority of these samples, comprised of the harvested pistachio nuts (P<0.05). The mean concentration of AFB1 in various periods of analysisof pistachio nuts had significant differences (P<0.05). The lowest and highest mean concentrations of AFB1 were registered in the pre-harvesting and post-harvesting pistachio nut samples, respectively (4.11 6.56 and 27.58±5.68 ppb). The post harvesting pistachio nut samples had the highest value of AFB1 (77.12 ppb) among all the samples collected.

Discussion

The occurrence of AFB1 in pistachio nuts has been studied by many researchers in the various countries. In Mexico, 2.2% of the analyzed pistachio nuts contained a level of Aflatoxin higher than 20ng/g (JECFA, 1998). In Sweden, 9.5% of pistachio nuts contained AFB1 higher than 2ng/g (Thuvander et al., 2001).

Food Contamination with mycotoxins has been surveyed in the various region of Iran. However, this study did not find any published data regarding the Qazvin pistachio nuts. The MTL of Aflatoxins (15 ng/g) and AFB1 (5ng/g) in the pistachio nuts in Iran is close to USA (20 ng/g), but quite different when compared with gthe statndards proposed by the EU to the Codex Committee (2ng/g for AFB1& 4ng/g AFT) (Dini et al., 2013; ISIRI, 2002).

A comprehensive survey on pistachio nut produced in Kerman and Rafsanjan during March 2002, February 2003 indicated that 11.8% of samples collected had contamination levels higher than the maximum tolerable standard limits set in Iran for AFB1 (Cheraghali et al., 2007). 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% of the pistachio nuts exceeded the maximum tolerable limit set for AFB1(Sarhangpour et al., 2010). According to the additional studies on 3181 commercial raw pistachio nut groveswere sampled for testing for European export certification, for the period of January 2009 until December2011, AFB1was detected in 23.4% with the mean and median values of 2.18 ± 13.1 ng/g and <LOD, respectively, that were lower than the maximum tolerable standard levels of Iran and the EU (Dini et al., 2013). This study found, 54.7% samples were higher than the maximum tolerance level of AFB1 according to EU standard and the majority of the samples were of the harvested pistachio nut group which demonstrated a higher aflatoxin rate than other studies. These high contamination levels were most probably due to the post harvesting conditions, and the extensive manipulation and exposure of pistachio nut in retail shops. It has been documented that tropical conditions such as high temperatures and moisture, monsoons, unseasonal rains during harvest, and flash floods lead to fungal proliferation and production of AFs.

Strategies implemented by the authorities, including the Ministry of Health and Ministry of Agriculture in cooperation, and with the European Union are effective and promising in control experts aflatoxin contamination in pistachio. To be effective in the long term, aflatoxin risk reduction efforts in the developing countries needs to also be directed to:

1. educating families, farmers, stakeholders along the value chain as well as governments about the health risks associated with mycotoxins and the social and economic costs of reducing this risk;
2. reducing the risk of aflatoxins and other harmful mycotoxins by the application of appropriate agricultural practices;
3. investing in local capacity to support further activities both to reduce mycotoxins in agricultural products and to monitor mycotoxin levels in crops and the local population; and
4. providing the tools (data and risk management capacity) for locally driven policy reform that creates an effective regulatory environment to ensure domestic food safety in rural and urban areas and also facilitates trade opportunities in the region.

Conclusion

The results obtained from this study collaborate what previous research has reported, that aflatoxins are common contaminants of the pistachio nut, and are considered to be a main concern for public health.

Therefore, public health authorities enforce more stringent regulations on Aflatoxin prevention, by monitoring the pistachio more closely and the possible added products from harvesting, which is past on to the consumer and by informing producers of the possible hazards of AFS infection.

Analysis of the pistachio nut samples demonstrated a greater risk on the economy as well as the public health issue. This study also provided useful information regarding the risk of aflatoxins hazard and seeks to raise the awareness among consumers, researchers, farmers and traders concerning the importance of improving the processing methods particularly during the harvesting process, drying methods transportation/storage, and to establish a strigenent monitoring program for the pistachio nut. This monitoring process can be used as a basis for risk analysis of aflatoxins, therewith maintaining the aflatoxins comtamination rates at the lowest possible level. As a result, post-harvest and product conservation strategies are necessary to prevent fungal growth and aflatoxins contamination.

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