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Full Length Research Paper

Detection of *Aspergillus* spp. and determination of the levels of aflatoxin B1 in rice imported to Bushehr, Iran

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Aflatoxins (B_1, B_2, G_1 and G_2) are hepatotoxic metabolites produced by *Aspergillus flavus* on a number of agricultural commodities. Their levels were studied in rice samples imported to Iran through a southern port in Bushehr. Aflatoxins analysis was performed by solvent extraction, immunoaffinity clean-up and determination using high performance liquid chromatography (HPLC) equipped with post-column photochemical reactor and fluorescence detector. Among 152 samples analyzed, 75% showed levels of aflatoxin B1 (AFB1) contamination. However, there was no sample with AFB_1 above maximum tolerated level (MTL) of 5 ng/g assigned by Institute of Standard and Industrial Research of Iran (ISIRI). AFB1 concentrations in the samples was 0.09 to 3.3 ng/g. Out of the 152 samples analyzed, about 76.97% were contaminated with total aflatoxin (AFT) with the mean of 0.671 ng/g which is lower than MTL of AFT in rice (30 ng/g) assigned by (ISIRI). AFT concentration ranged from 0.15 to 4.27 ng/g. Contamination of aflatoxin in imported rice was dissimilar between different months. The highest levels of AFB_1 and AFT were detected in rice samples imported in September, while the lowest levels were in rice imported during November.

Key words: Iran, aflatoxins, food safety, rice.

INTRODUCTION

In the production of food crops, losses occur during the growth cycle in the field, harvest and storage where up to 5% of grain weight can reduce the agricultural output (Compton et al., 1993). The risk of contamination by mycotoxins is an important food safety concern for grains and other field crops (Bhat and Vasanthi, 2003; CAST, 2003; Bryden, 2007). The genus *Aspergillus* is distributed worldwide and contains over 180 species. It is one of the most ubiquitous and abundant of all groups of fungi and one of the most studied fungal groups (Dyer, 2007). *Aspergillus flavus* Link:Fr. and *Aspergillus parasiticus* Speare, the two *Aspergillus* sp. of most concern in agriculture, are predominant saprotrophs with limited parasitic ability (Payne, 1998). They are distributed worldwide and infect a number of crops (Jackson and Bell, 1969; ; Speijers and Speijers, 2004). Toxins produced by some *Aspergillus* spp. are called aflatoxins.

Aflatoxins (AFs) were first discovered in Europe in animal feed. AFs are found as contaminants in various agricultural commodities such as maize, rice, sorghum, wheat, oats, spices (black pepper, ginger) and chilli which are considered to be of greater significance world over for human beings (Goyal, 1989). The four major AFs that occur in crops are B1, B2, G1, and G2. *A. flavus* produces aflatoxins B1 and B2, while *A. parasiticus* produces all four aflatoxins; Aflatoxin B1 is the most toxic and best studied of the aflatoxins (Payne, 1998; Zheng

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Abbreviations: **HPLC,** High performance liquid chromatography; **AFB1,** aflatoxin B1; **MLT,** maximum tolerated level; **ISIRI,** Institute of Standard and Industrial Research of Iran; **AFT,** aflatoxin Total.

et al., 2006). AFs are the most potent carcinogens in animal and human populations produced by some strains of *Aspergillus* (Ito et al., 2001; Kurtzman et al., 1987; Payne, 1998). The International Agency for Research on Cancer has designated AFs as a human liver carcinogen (IARC, 1993). They also interfere with the function of the immune system (Hsieh, 1988). A wide variety of animals, including fish, rodents, waterfowl, poultry, swine and cattle can be affected by aflatoxins (Robinson et al., 1982; Smith and Moss, 1985; Higgins et al., 1992). The knowledge that AFs effect on humans and animals has led many countries to establish maximum tolerated level (MTL) on aflatoxin levels allowed in food and feed in the last decades to safeguard the health of humans, as well as the economical interests of producers and traders. Currently, worldwide range of limits for AFB1 and total AF (AFT) are 1 to 20 ng/g and 0 to 35 ng/g, respectively (FAO, 2004; Hussein and Brasel., 2001; IARC, 1993; INSPQ, 2002; Massey et al., 1995).

Rice (*Oryzae sativa L*.) is the most important staple food crop in Iran and cultivated in different areas which have sultry and rainy climate. Since the amount of rice cultivation is not enough for domestic consumption, some country import rice from other regions such as India, Pakistan, Bangladesh and Thailand which are the largest producers of rice in the world. However, some of those countries have frequent and heavy rainfall and floods in coastal areas particularly near harvest, under this climate the development of fungi, especially species of the *Aspergillu*s and *Penicillium* is a common and unresolved problem (Reddy et al., 2004). A number of surveys and monitoring programmes have been carried out in several countries attempting to obtain a general pattern of aflatoxin contamination in rice (Reddy et al., 2008). Regulations for major mycotoxins in commodities and food exist in at least 100 countries, most of which are for aflatoxins, MTL differ greatly among countries (van Egmond and Jonker, 2004). Thus, the rice imported to Iran port is usually examined for aflatoxins contamination. Institute of standards and industrial research of Iran (ISIRI) has set minimum levels of 5 and 30 ng/g for aflatoxin B1 and the total aflatoxins, respectively. Bushehr is the major ports for importing rice (*Oryza sativa*) in south west of Iran. Therefore, the aim of this investigation was to identify the levels of aflatoxin B1 and the total aflatoxins in imported rice to Bushehr port.

MATERIALS AND METHODS

One hundred and fifty two samples were collected randomly by inspectors of Food Control Offices in Bushehr port from May 2010 to February 2011.The rice consignments, intended for import to Iran, are in different sizes but all of the packages are about 50 kg. Samples were taken according to the method of the Iranian National Standard of Sampling for aflatoxin analysis in agricultural products (ISIRI, 1998). Then, all the samples were transferred to Toxicology Labs in Food and Drug Control Laboratory. A minimum size of 2 Kg from each sample was used for analysis. Samples were kept at 20°C in PE bags until analysis.

Reagents and apparatus

All reagents (potassium chloride, phosphoric acid, hydrochloric acid) and solvents methanol, acetonitrile, propanol-2-ol, n-hexane, chloroform) used were of high performance liquid chromatography (HPLC) grade. AF standards were purchased from Sigma Chemical Company, USA. Aflatest immunoaffinity columns (IAC) were purchased from VICAM Company, Watertown, MA, USA. Apparatus characteristics were WATERS 1525 binary HPLC pump, and 2475 Multy λ fluorescence detector. HPLC column (C_{18}, 250 x 4.6 mm: 4 µm) was purchased from Waters, USA.

Sample preparation

To avoid the sub-sampling error due to highly heterogeneous nature of fungal distribution in AF analysis, every sample was grinded with the miller and collected in plastic bag and finally 50 g of test portion from the ground samples were taken for analysis.

Extraction and clean up

Samples were analyzed using a HPLC following AOAC and method (ISIRI, 2004). Samples were extracted with methanol: water:n-hexane (240:60:100, v/v/v). The mixture was shaken for 30 min on a mechanical shaker. The solution was left to sediment and filtered through a WHATMAN Filter No.1. After filtration, the extract was diluted with water and filtered through glass micro fiber filter. Aflatest was used for samples clean up. First, 10 ml phosphate buffer saline (PBS) was passed through the IAC. Then, 75 ml of the filtrate was passed through the IAC at a flow rate of 1 ml/min. The column was washed with water and dried using vacuum. Finally, AF was eluted with methanol using the following procedure. First, 0.5 ml methanol was applied on the column which passed through by gravity.

After 1 min, the second portion of 0.75 ml methanol was applied and collected. The Aflatest was diluted with water and analyzed using HPLC.

AF standards

After preparation of standard solutions of AFs, the concentration of each one was determined using UV spectrophotometer. These standards were used to prepare mixed working standards for HPLC analysis.

Recovery and limit of detection (LOD)

The effectiveness of the extraction procedure was confirmed by sample fortification. The recovery of extraction method was determined by fifty grams of milled rice fortified with a solution of AF in methanol at 5 µg/ml (for B1 and G1) and 1 µg/ml (for B2 and G2) 1 h before extraction . The AF fortification solution was prepared in methanol and used for quantification of analyte recovered after extraction. Sample were fortified with 0.25 ml of this solution in order to have 5 ng /g of AFB1 in rice, which is maximum permitted limit in cereals by National standard of Iran (ISIRI 5925). LOD were 0.07, 0.08, 0.1, 0.07 and 0.32 ng/g for AFB1, AFB2, AFG1, AFG2 and Total AF, respectively.

Table 1. Mean and standard deviation of aflatoxin B_1 (AFB_1) and total aflatoxins (AFT) (ng/g) in the examined rice samples.

Month	N	ND	AFB1 (ng/g)	AFT (ng/g)
May	4	1	0.633 ± 0.404	1.057 ± 1.27
June	8	0	0.875 ± 0.851	0.806 ± 0.03
July	10	1	0.155 ± 0.052	0.935 ± 1.087
August	20	1	0.426 ± 0.424	0.626 ± 0.509
September	23	4	0.579 ± 1.010	0.882 ± 1.294
October	13	4	0.144 ± 0.133	0.155 ± 0.133
November	19	6	0.584 ± 0.288	0.646 ± 0.371
December	2	1	0.99	0.15
January	30	11	0.426 ± 0.202	0.526 ± 0.202
February	23	9	0.685 ± 0.377	0.927 ± 0.581

N: Number of samples; ND: not detected.

Analysis of AF using HPLC

AF was quantified by reverse-phase HPLC and 2475 Multy λ fluorescence detector with post column derivatization (PCD) involving bromination (Stroka et al., 2000). The waters HPLC system was applied with a Kobra cell and addition of bromide to the mobile phase. After dilution of AF eluate with water, 100 µl was injected into HPLC. Mobile phase was water:methanol:acetonitrile (600:300: 200, v/v/v) and 350 µl of nitric acid 4 M and 120 mg of potassium bromide with a flow rate of 1 ml/min. The fluorescence detector was operated at an excitation wavelength of 365 nm and emission wavelength of 435 nm. The calibration curve for each individual AF including AFB1, AFB2, AFG1 and AFG2 was used to check for the linearity and quantification of AF in rice samples.

RESULTS AND DISCUSSION

Among 152 samples analyzed, 38 samples (25%) were not contaminated with AFB1 (<LOD). A high proportion of samples (75%) showed positive to AFB1 contamination (Table 1). Mean of AFB1 in the samples was 0.46 ng/g. However, there was no sample with AFB1 above MTL of 5 ng/g assigned by Institute of Standard and Industrial Research of Iran (ISIRI, 2002) (Figure 1). Maximum level of AFB1 in rice samples was 3.3 ng/g. Among 152 samples analyzed, 35 samples (23.03%) did not show any AFT contamination. However, 76.97% of samples were contaminated with AFT with the mean of 0.671 ng/g which is lower than MTL of AFT in rice in Iran (30 ng/g) (ISIRI, 2002) (Table 1). Maximum level of AFT in rice samples was 4.27ng/g (Figure 1). Levels of aflatoxins in rice samples were different between different months. The highest levels of AFB1 and AFT detected were in rice samples imported in September. The lowest levels were detected in rice imported during November. In a similar study from Iran by Mazaheri (2009), among 71 rice samples analyzed, AFB1 was detected in 59 samples (83% of the total). The mean of AFB1 was 1.89 ng/g for all samples. Total AF was detected in 83% of samples. Mean of AFT was 2.09 ng/g. AFB1 levels in two samples (2.8%) were above the MTL of AFB1 in Iran (5 ng/g).

Another survey conducted by Food Standards Agency, UK in 2002 to determine the levels of mycotoxins in rice showed that levels ranged from 0.2 to 1.8 mg/kg. All levels found were below the EC legislative limits of 2 mg/kg aflatoxin B1 and 4 mg/kg total aflatoxin in cereal products for direct human consumption. Sales and Yoshizawa (2005) reported that the incidence of AFB1 in rice from the Philippines ranged from 0.025 to 11.0 mg/kg. In another study, the AFB1 contamination was detected in 37 samples of rice grains from China. They found that 92% of the samples were positive to AFB1 (Liu et al., 2006). Toteja et al. (2006) reported the presence of AFB1 in parboiled rice collected from 11 states in India; 38.5% of the samples were positive to AFB1. Reddy and Reddy (2009) reported that from 1200 samples from India, 67.8% were positive to AFB1. The highest AFB1 found in the samples was 38.5 mg/kg. Prasad et al. (1987) tested 56 samples of stored rice among which 12 were positive for aflatoxin. Levels of aflatoxins ranged from 184 to 2830 mg/kg.

Jayaraman and Kalyansundaram (1990) reported that 35% of the samples of raw rice bran and parboiled rice bran showed the presence of aflatoxin B1. It was shown that bran of parboiled rice supports higher aflatoxin production than bran of raw rice. Study from Sri Lanka (Bandara) et al. (1991) reported that almost all the samples of parboiled rice, AFB1 and AFG1 contents were significantly higher than the raw milled rice. Cultivar differences in the amount of aflatoxin B1 and G1 was showed by Sinha and Dubey (1991). A survey on the prevalence of aflatoxin B1 (AFB1) in rice bran in coastal and interior districts of Tamil Nadu and Andhra Pradesh, India revealed that 62% of the samples contained AFB1 and the levels far exceeded the permissible limit of 50 mg/kg (Elangovan et al., 1999).

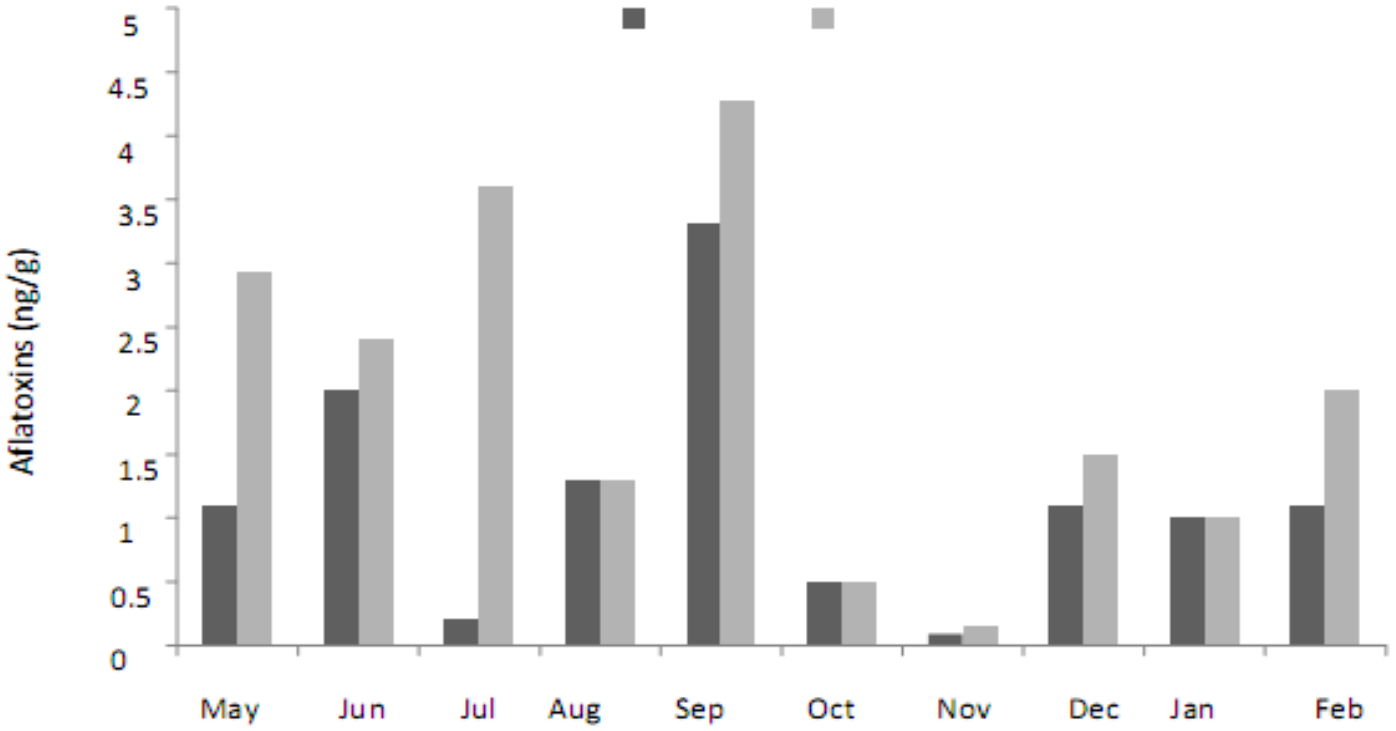

Figure 1. Maximum levels (ng/g) of aflatoxin B_1 (AFB_1) and total aflatoxins AFT detected in the examined rice samples in different months (2010 May-2011 Feb).

Conclusion

This study shows that rice samples collected from imported Iran via Bushehr port varied in AFB1 and total AF contamination. In the rice samples, AFB1 levels were below the ISIRI and internationally acceptable limits for human consumption. But, detection of small quantities of aflatoxins in most of the samples warrants further investigations, since intake of rice is very high in Iran. The average per capita consumption of rice in Iran is 45.5 kg, which makes Iranians the 13th biggest rice consumers. Our investigation demonstrates that rice market represents a significant source of exposure to aflatoxin. These data suggest that public health efforts to interrupt aflatoxin exposure during an aflatoxicosis event must include both an assessment of aflatoxin contamination and the replacement of contaminated rice in accordance with the strict laws on permissible limits of aflatoxin levels in food and feed products.

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