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Aflatoxins and Ochratoxinin A in Bean from Iran

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Abstract A study was undertaken to determine levels of aflatoxins and ochratoxin A in bean, using a technique preceded by an immunoaffinity clean-up step. For this purpose, a total of 30 bean samples were analyzed. 16.67% and 10% of examined bean samples contained Aflatoxin B_1 and ochratoxin A more than 0.2 ng g^{-1}. Recoveries were found to be 91.1% and 98.5% for Aflatoxin B_1 and ochratoxin A, respectively, while the detection limit was 0.2 ng g^{-1} for both mycotoxins.

Keywords HPLC · Aflatoxin · Ochratoxin A · Bean

Bean (*Phaseolus vulgaris* L.) is one of the most important grains consumed in the world. Food contamination with toxigenic moulds has attracted increasing attention over the last three decades. Most grains, such as wheat, maize, bean and rice, can be infested by filamentous and microscopic fungi. Some genera can produce toxic secondary metabolites, namely mycotoxins, which impact on food safety (Kuiper-Goodman 1995).

Aflatoxins (B_1, B_2, G_1 and G_2) are mycotoxins produced by the fungi *Aspergillus flavus* and *A. parasiticus*. These mycotoxins are hepatotoxic and carcinogenic in humans. *A. flavus* produces aflatoxins B_1 and B_2, while *A. parasiticus* gives rise to aflatoxins B_1, B_2, G_1 and G_2 (IARC 1993). Aflatoxins are also produced by some other *Aspergillus* species (*A. nomius*, *A. pseudotamarii*, *A. bombycis*), although they are not as important in economical terms as *A. flavus* and *A. parasiticus* (Varga et al. 2004). Aflatoxins occur all over the world in foods and in a wide variety of food raw materials, most commonly in peanuts, pistachio, nuts, cereals, maize, rice and figs.

Ochratoxin A (OTA) is a secondary metabolite produced by mould fungi belonging to several *Aspergillus* and *Penicillium* species during the storage of cereals and other plant-derived products under non-optimal conditions. Among ochratoxin-producing organisms, aspergilli have mostly been suggested to be responsible for OTA contamination of spices by several authors (Varga et al. 2004). OTA has nephrotoxic and hepatotoxic effects and it is likely to be potentially carcinogenic in humans (IARC 1993). It is widespread in products of plant origin and is a common contaminant of cereals, coffee beans and dried fruits. It has also been detected in cereal products, wine and beer (Trucksess et al. 1999; Visconti et al. 2000).

Because of potential health hazards to humans, regulatory levels have recently been documented. The range of worldwide regulations for AFB_1 was from 0 to 30 ng g^{-1} with a total from 0 to 50 ng g^{-1} (Creppy 2002; FAO 1997). Recently, the Codex Alimentarius Commission, Joint FAO/WHO Food Standards Program adopted a limit of 15 ng g^{-1} for total aflatoxins (Codex Alimentarius Commission 2001). Because of high toxicity there is a demand for the determination of minute amount of aflatoxins. A number of sensitive methods for the analysis of aflatoxins in foods and feed have been developed since the discovery of aflatoxins (Gilbert 2000; Horwitz 2000; Feizy et al. 2010) and several of them have been adapted as the AOAC methods (Horwitz 2000). Chemical methods such as thin layer chromatography (TLC) and High-performance liquid chromatography (HPLC) are most commonly used (Gilbert 2000; Horwitz 2000; Feizy et al. 2010).

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The European commission has enforced the limits of OTA in cereals and cereal products with the following levels: 5 ng g^{-1} for raw cereal grains, 3 ng g^{-1} for cereals and cereal products intended for human consumption, 0.5 ng g^{-1} for baby food and cereal-based food intended for young children (European Commission 2006b). There are currently no legal limits for OTA in spices; however, the European Commission has been discussing a limit of 10 ng g^{-1} for OTA in spices (Goryacheva et al. 2006). For the dried vine fruits, soluble coffee and some dried fruits, the European commission has set a maximal permissible limit for OTA at 10 ng g^{-1}. Numerous methods for OTA determination in food have been described, including Enzyme-linked immunosorbent assay (ELISA) and thin layer chromatography (TLC) (El-Kady et al. 1995). Liquid chromatography linked to fluorescence detection (HPLC-FLD) was extensively used for OTA confirmatory analysis (Gonzalez et al. 2006).

This study was designed to determine the presence and levels of AFs and OTA in beans, that especially sold and consumed in Khorasan province of Iran and to compare the results with maximum AFs and OTA tolerance limits that accepted by Iranian national standard.

Materials and Methods

All samples were obtained locally from Mashhad (Khorasan, Iran). Samples were taken according to the alternative sampling plan for official control of mycotoxins in food (European Commission 2006a). A minimum sample size of 1,000 g was applied and samples were mixed for 10 min, and 500 g of samples were ground to a fine powder and stored at -20°C while awaiting analysis. The particle sizes after grinding were below 0.3 mm.

Methanol, acetonitrile, acetic acid and other chemical reagents were HPLC grade and supplied by Merck (Darmstadt, Germany). Aflatoxins and OTA powder was obtained from Sigma (St. Louis, USA). Immunoaffinity columns (Neogen Europe, Ltd., Scotland, UK) for purification and preconcentration of aflatoxins and OTA prior to the quantitative analysis were used. Double distilled water was used in all the experiments. Stock standard solution of aflatoxins and OTA with concentrations of 100 μg mL^{-1} was prepared in acetonitrile and methanol, respectively. Working standard solutions for aflatoxins and OTA were prepared daily by diluting the stock solution with water: methanol (6:4) and methanol: acetic acid (98:2), respectively. All of the working standard solutions were stored in darkness and refrigerator at 4°C.

HPLC analyses were performed on a Sykam (Eresing, Germany) HPLC system equipped with an S2100 pump, an S7131 reagent organizer, an S4011 column thermo controller, an S1122 secondary pump, a RF-10Axl fluorescence detector and a Genesis RP C_{18} analytical column (250 × 4.6 mm, 4 μm) and a chromolith® performance RP18 analytical column (100 × 4.6 mm), respectively for aflatoxins and OTA. The fluorescence detector for aflatoxin was operated at 365 and 445 nm and for OTA was operated at 333 and 443 nm for excitation and emission, respectively. Clarity software was used for data management. For determination of aflatoxins and OTA concentration a UV–Visible spectrum of aflatoxins and OTA stock solution against solvent used for solution in reference cell (Feizy et al. 2010) was obtained using a Shimadzu UV-1700 Pharma spec. (Tokyo, Japan). Spectrophotometer equipped with a standard 10 mm path length spectrophotometer cell.

For aflatoxins determination, 50 g ground bean samples, either non-spiked or spiked with a known volume of an aflatoxins stock solution, was mixed with 40 mL of pure water, 160 mL methanol and 5 g of sodium chloride and blended (Waring 8011S, Torrington, CT) at high speed for 5 min to obtain a homogeneous sample mix. After mixing, the slurry was filtered through filter paper (Whatman No. 4) and 20 mL of this, diluted with 130 mL phosphate buffered saline (PBS) solution. This diluted solution was filtered through glass microfiber filter (Whatman, Inc., Clifton, NJ) and 100 mL was passed through an immunoaffinity column. Aflatoxins were eluted from the column by passing 2 mL of HPLC grade methanol and then 2 mL of HPLC grade water and using gravity to collect the eluate into a glass vial at a flow rate of around 5 mL min^{-1}.

For OTA determination, 25 g ground bean samples, either non-spiked or spiked with a known volume of an OTA stock solution, was mixed with 40 mL of pure water, 60 mL acetonitril and 0.3 g $NaHCO_3$ and blended (Waring 8011S, Torrington, CT) at high speed for 5 min to obtain a homogeneous sample mix. After mixing, the slurry was filtered though filter paper (Whatman No. 4) and 10 mL of this diluted was mixed with 40 mL phosphate buffered saline (PBS) solution. This diluted solution was filtered through glass microfiber filter (Whatman, Inc., Clifton, NJ) and 55 mL was passed through an immunoaffinity column. OTA were eluted from the column by passing 1.5 mL of HPLC grade methanol: acetic acid (98:2 v/v) and then 1.5 mL of HPLC grade water and using gravity to collect the eluate into a glass vial at a flow rate of around 5 mL min^{-1}. For aflatoxins and OTA, 20 μl of eluate were injected into the HPLC.

Linear isocratic elution chromatography for aflatoxins and OTA have done using solvent system water: methanol: acetonitrile (6:2:2 v/v/v) at 40°C and water: acetonitrile: acetic acid (99:99:1 v/v/v) at 30°C, respectively. For aflatoxins, the flow rate was kept constant at 1 mL min^{-1} (mobile phase) and 0.3 mL min^{-1} (reagent). Aflatoxins were quantified using post column derivatization

performed with iodine and aflatoxins were detected by fluorescence detector (excitation 365 nm, emission 445 nm). The iodine and mobile phase was mixed in T-mixer. The reaction taking place at 75°C (Van Egmond et al. 1988). For OTA the flow rate was kept constant at 0.7 mL min^{-1} and 20 μL was injected. OTA was detected by fluorescence detector (excitation 333 nm, emission 443 nm).

An external standard curve was constructed using reference standard aflatoxins and OTA to quantify the OTA content in all samples. Aflatoxins and OTA stock working solution containing 100 ng mL^{-1} was prepared and then diluted to appropriate concentration ranges with methanol: water (4:6 v/v) and methanol: acetic acid (98:2 v/v), respectively for construction of calibration curve. Calibration curve was performed with seven different concentrations with square of correlation coefficient (r^2) $B_1 = 0.998$, $B_2 = 0.998$, $G_1 = 0.998$, $G_2 = 0.999$ and five different concentrations with square of correlation coefficient (r^2) OTA = 0.999. Calibration curves were derived by plotting concentrations as a function of peak area of each aflatoxin and OTA. Calibration curves exhibited good linear regression.

Results and Discussion

Mycotoxin contamination is less commonly reported in bean than many other cereals. In this study 30 bean samples were analyzed to evaluate the concentration of aflatoxins B_1, B_2, G_1, G_2 and OTA with HPLC. As it is shown in Table 1, aflatoxins B_1 and OTA were detected in 16.67% and 10% of bean samples with mean value 0.24 and 0.29 ng g^{-1}, respectively. Aflatoxins B_2, G_1 and G_2 were not detected in any of the bean samples. All contaminated samples had a level of aflatoxin B_1 and total aflatoxins were below the Iranian National Standard No. 5925. Based on Iranian National Standard No. 5925 the total aflatoxins, AFB_1 and OTA levels in bean should be 15, 5 and 20 ng g^{-1}, respectively (ISIRI 2002). Relevant data concerning the analytical system are summarized in Table 2. For aflatoxins B_1, B_2, G_1, G_2 and OTA, LOD were 0.2, 0.1, 0.2, 0.1 and 0.2 ng g^{-1} and LOQ were 0.6, 0.3, 0.9, 0.3 and 0.5 ng g^{-1}, respectively. For the repeatability measurement a standard solution containing 10 ng mL^{-1} of OTA was used.

Accuracy was examined by the determination of the recoveries of the aflatoxins and OTA. The recovery study was performed by comparing the concentration in the bean spiked samples to the respective non-extract standards (aflatoxins and OTA in solution). The area under the peak of each sample was divided by the area under the curve of the quality control sample and multiplied by 100. The recoveries of aflatoxins B_1, B_2, G_1, G_2 and OTA from samples spiked at 5 ng g^{-1} for AFB_1, AFG_1 and OTA, and 1 ng g^{-1} for AFB_2 and AFG_2 were quite good (Table 2). Relative standard deviations within laboratory repeatability (RSDr, n = 6) range from 1.3 to 2.89.

This recovery range is within the guideline of acceptable recovery limits of AOAC and the Codex alimentarius. The AOAC guideline for the acceptable recovery at the 10 μg kg^{-1} level is 70%–125% and Codex acceptable recovery range is 70%–110% for a level of 10–100 μg kg^{-1} for a level of 10–100 μg kg^{-1}, and 60%–120% for a level of 1–10 μg kg^{-1} (Feizy et al. 2010).

Our studies showed that from 30 bean samples, five samples contained AFB_1 and three samples contained OTA with mean value 0.24 and 0.29 ng g^{-1}, respectively. According to results obtained, the mean concentration of AFB_1 and OTA contamination of bean in Mashhad, Iran, had a significant difference with the accepted limits by Iran regulations (5, 20 ng g^{-1} for AFB_1 and OTA, respectively).

Table 1 Mean value and range of bean samples aflatoxins and OTA concentration (ng g^{-1})

Compound	Average (±SD)	Range
AFB_1	0.24 (±0.03)	0.21–0.29
AFB_2	ND	ND
AFG_1	ND	ND
AFG_2	ND	ND
OTA	0.29 (± 0.09)	0.23–0.39

ND not detected

Table 2 Analytical data for the aflatoxins and OTA HPLC system

Compound	t_R (min)	Repeatability (%RSD, n = 6) Peak area	Limit of detection (ng g^{-1})	Limit of quantification (ng g^{-1})	Recovery (%)
AFB_1	14.75	1.3	0.2	0.6	91.1
AFB_2	12.04	2.4	0.1	0.3	97.1
AFG_1	10.72	1.5	0.2	0.9	85.2
AFG_2	8.89	3.2	0.1	0.3	83.4
OTA	5.49	1.4	0.2	0.5	98.5

So it seems that the present status of this mycotoxin is not at risk and won't be a serious problem for the public health. Therefore, it is needed to routinely monitor this as a food quality control measure. The initial approach to control the occurrence of aflatoxins and OTA in bean had to be control the contamination with aflatoxins and OTA in the field, although, is very difficult to control because it is influenced primarily by climatic conditions such as relative humidity and temperature (Applebaum et al. 1982). However, it is reported that the highest concentrations of aflatoxins are associated with the post harvest growth of Aspergillus moulds on poorly stored stuffs (Jay 1992).

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