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Survey and Determination of Mycotoxins in Wheat from Iran by LC/Fluorescence Using Immunoaffinity Column Cleanup

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Abstract: Mycotoxins are small, toxic chemical products formed as secondary metabolites by a few fungal spices that readily colonise crops and contaminate them with toxins in the field or after harvest. In this study, a Liquid Chromatographic Method was developed for the simultaneous determination of Aflatoxins (AFs) (B_1, B_2, G_1, G_2), Ochratoxin A (OTA) and Zearalenone (ZEA) toxins in wheat. For this purpose, a total of 231 wheat samples were analyzed. Mycotoxins were extracted and purified from the samples using Immunoaffinity (IAC) columns. The 2.67 and 2.21% of examined wheat samples contained AFB_1 and OTA. The method was based on the extraction of AFs, OTA and ZEA finely ground wheat sample with 80% methanol and 60% acetonitrile solution, respectively. The 2.60 and 2.16% of examined wheat samples contained AFB_1 and OTA. LOD was 0.27, 0.04, 0.23, 0.04, 1.18 and 21.56 for AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZEA, respectively. Considering these results these special commodities should not be a health concern for these collectives, however, special attention should be focused on imported foods distributed in special retail shops.

Key words: HPLC, aflatoxin, ochratoxin A, zearalenone, wheat

INTRODUCTION

Wheat is the one of most important grains consumed in the world. Food contamination with toxigenic moulds has attracted increasing attention over the last three decades. Most grain, 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). Mycotoxins are secondary metabolites produced by fungi when they grow on agricultural products before or after harvest or during transportation or storage. Currently, >400 mycotoxins are identified in the world but the most important groups of mycotoxins that are of major health concern for humans and animals and occur quite often in food: AFs, OTA, trichothecenes (deoxynivalenol, nivalenol), ZEA and fumonisins.

The International Agency for Research on Cancer (IARC, 1993) has classified naturally occurring mixtures of AFs as carcinogenic to humans (group 1), OTA and fumonisins as possible carcinogens to humans (group 2B) and ZEA as group 3 carcinogen. Health hazards posed by mycotoxins in human and animals have prompted >100 countries to establish regulatory limits for some of the well-known mycotoxins such as the AFs in the most frequently contaminated foods (Van Egmond *et al*., 1988).

AFs (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 AFB_1 and B_2 while A. parasiticus gives rise to AFB_1, B_2, G_1 and G_2 (IARC, 1993). AFs 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*. AFs 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.

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 nonoptimal conditions. Among OTA producing organisms, Aspergilli have mostly been suggested to be responsible for OTA contamination of spices by several researchers (Varga *et al*., 2004). OTA has nephrotoxic and hepatotoxic effects and it is likely to have carcinogenic potential 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*., 2008).

Because of potential health hazards to humans, regulatory levels have recently been documented. The

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range of worldwide regulations for AFB_1 was from 0-30 ng g^{-1} with a total from 0-50 μg kg^{-1} (Creppy, 2002). Recently, the Codex Alimentarius Commission, Joint FAO/WHO Food Standards Program adopted a limit of 15 μg kg^{-1} for total AFs. Because of high toxicity there is a demand for the determination of minute amount of AFs. There are several types of chromatographic methods available for mycotoxins analysis. Traditionally, the most popular methods used for mycotoxins analysis are Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) and Capillary Electrophoresis (CE). These methods require extensive sample preparation and are expensive to perform. Therefore, a rapid and sensitive technique for routine assay of mycotoxins in foods is necessary. Over the last 20 years, the importance and application of immunoassays, especially Enzyme-Linked Immunosorbent Assay (ELISA) has grown significantly (Lee *et al*., 2004). ELISA test kits became very popular recently due to their relatively low cost and easy application and their results could be comparable with those obtained by other conventional methods such as TLC and HPLC. The European commission has enforced the limits of OTA in cereals and cereal products with the following levels: 5 μg kg^{-1} for raw cereal grains, 3 μg kg^{-1} for cereals and cereal products intended for human consumption, 0.5 μg kg^{-1} for baby food and cereal based food intended for young children (EC, 2006). There are currently no legal limits for OTA in spices, however, the European Commission has been discussing a limit of 10 μg kg^{-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 μg kg^{-1}.

This study was designed to determine the presence and levels of AFLs, OTA and ZEA in wheat that especially sold and consumed in Khorasan Province of Iran and to compare the obtained results with maximum AFLs, OTA and ZEA 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 (EC, 2006). A minimum sample size of 1000 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). AFLs, OTA and ZEA powder was obtained from Sigma (St. Louis, USA). Immunoaffinity columns (Neogen Europe, Ltd. Scotland, UK) for the purification and preconcentration of AFLs, OTA and ZEA prior to the quantitative analysis were used. Double distilled water was used in all the experiments. Stock standard solution of AFLs, OTA and ZEA with concentrations of 100 μg mL^{-1} was prepared in methanol and acetonitrile, respectively. Working standard solutions for AFLs, OTA and ZEA were prepared daily by diluting the stock solution with water:methanol (6:4), methanol:acetic acid (98:2) and water:methanol (10:90), respectively. All of the working standard solutions were stored in darkness and refrigerator at 4°C.

AFs: About 50 g of ground sample was mixed with 200 mL of extractant solution (80% methanol, 20% water) and 5 g of sodium chloride for 5 min and filtered. About 20 mL of filtered solution was diluted with 130 mL of phosphate buffer solution and 100 mL was passed through the immunoaffinity column. After this the columns were washed with 20 mL of PBS were eluted with 2 mL of methanol HPLC grade and 2 mL of milli-Q water.

OTA: About 25 g of ground sample was mixed with 100 mL of extractant solution (75% acetonitrile in water) and 0.3 g $NaHCO_3$ for 5 min and filtered. About 10 mL of filtered solution was diluted with 40 mL of phosphate buffer solution and drained through the immunoaffinity column. After this the columns were washed with 20 mL of PBS were eluted with 1.5 mL of acetic acid:water (98:2) HPLC grade and 1.5 mL of milli-Q water.

ZEA: About 25 g of ground sample was mixed with 100 mL of extractant solution (85% acetonitrile in water) and 1 g of sodium chloride for 5 min and filtered. The 10 mL of filtered solution was diluted with 65 mL of phosphate buffer solution and 65 mL was passed through the immunoaffinity column. After this the columns were washed with 20 mL of PBS were eluted with 2 mL of methanol grade HPLC and 2 mL of milli-Q water.

HPLC analysis: 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 and a RF-10Axl fluorescence detector. For aflatoxin and zearalenone analysis, Genesis RP C_{18} analytical column (150×4.6 mm, 5 μm) and for ochratoxin analysis, Chromolith® performance RP18 analytical column (100×4.6 mm) was used. The excitation wavelength for determination of AFLs, OTA and ZEA was 365, 333 and 275 nm and the emission wavelength was 445, 443 and 450 nm, respectively.

For aflatoxin, ochratoxin and zearalenone, mobile phase consisted of a solution of water:methanol: acetonitrile (60:20:20), water:acetonitrile:acetic acid (99:99:1) and methanol:acetonitrile:water (8:46:46), respectively.

Clarity Software was used for data management. For determination of AFLs, OTA and ZEA concentration a UV-Visible spectrum of AFLs, OTA and ZEA stock solution against solvent used for solution in reference cell was obtained using a Shimadzu UV-1700 Pharma spec. (Tokyo, Japan). Spectrophotometer equipped with a standard 10 mm path length spectrophotometer cell.

An external standard curve was constructed using reference standard AFs and OTA to quantify the OTA content in all samples. AFLs, OTA and ZEA stock working solution containing 100 ng mL^{-1} was prepared and then diluted to appropriate concentration ranges with water:methanol (6:4), methanol:acetic acid (98:2) and water:methanol (10:90), respectively. For construction of calibration curve, calibration curve was performed with seven different concentrations with square of correlation coefficient (r^2) AFB_1 = 0.995 AFB_2 = 0.995, AFG_1 = 0.995, AFG_2 = 0.995 and six different concentrations with square of correlation coefficient (r^2) OTA = 0.986 and six different concentrations with square of correlation coefficient (r^2) ZEA = 0.993 calibration curves were derived by plotting concentrations as a function of peak area of each AFs, OTA and ZEA. Calibration curves exhibited good linear regression.

RESULTS AND DISCUSSION

Mycotoxin contamination is less commonly reported for wheat than for many other cereals. In this study 231 wheat samples were analyzed to evaluate the concentration of AFB_1, B_2, G_1, G_2, OTA and ZEA with HPLC. As it is shown in Table 1, AFB_1 and OTA were detected in 2.60 and 2.16% of wheat samples with mean value 1.01 and 4.194 ng g^{-1}, respectively. AFB_2, G_1 and G_2 were not detected in any of the wheat samples. All contaminated samples had a level of AFB_1 and total AFs were below the Iranian National Standard No. 5925. Based on Iranian National Standard No. 5925 the total AFs, AFB_1 and OTA levels in wheat were 15, 5 and 20 ng g^{-1}, respectively (ISIRI, 2002). Relevant data concerning the analytical system are summarized in Table 2. For AFB_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 AFs and OTA. The recovery study was performed by comparing the concentration in the wheat spiked samples to the respective non-extract standards (AFs 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 AFB_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 for within laboratory repeatability (RSDr, n = 6) range from 1.3-3.2. 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.*, 2011).

The studies showed that from 231 wheat samples, 6 samples contained AFB_1 and 5 samples contained OTA with mean value 1.01 and 4.194 ng g^{-1}, respectively. According to results obtained, the mean concentration of AFB_1 and OTA contamination of wheat in Mashhad, Iran

Table 1: Distribution of mycotoxins in wheat samples

		Frequency distribution			
Toxins	ND*	0.1-0.5	0.5-1	1-1.5	>1.5
AFB1	225	2	1	-	3
AFB2	231	-	-	-	-
AFG1	231	-	-	-	-
AFG2	231	-	-	-	-
OTA	226	-	-	1	4
ZEA	231	-	-	-	-

*ND = Not Detected

Table 2: Analytical data for the AFs and OTA HPLC System

Compounds	t_R (min)	*Repeatability (Peak area)	Limit of detection (ng g^{-1})	Limit of quantification (ng g^{-1})	Recovery (%)
AFB1	9.98	1.3	0.27	0.91	91.1
AFB2	8.25	2.4	0.04	0.13	97.1
AFG1	7.48	1.5	0.23	0.75	85.2
AFG2	6.32	3.2	0.04	0.15	83.4
OTA	3.47	1.4	1.18	3.94	98.5
ZEA	6.94	2.1	21.56	71.86	97.3

*RSD %, n = 6

had a significant difference with the accepted limits by Iran regulations (5, 20 ng g^{-1} for AFB_1 and OTA, respectively). So, it seems that the present status of this mycotoxin is not at risk and don't be a serious problem for the public health. Therefore, it is need to routinely monitor this as a food quality control measure. The initial approach to control the occurrence of AFs and OTA in wheat had been to control the contamination with AFs and OTA in the field, however, is very difficult to control because it is influence primarily by climatic conditions such as relative humidity and temperature (Applebaum *et al*., 1982).

CONCLUSION

However, it is reported that the highest concentrations of AFs are associated with the post harvest growth of Aspergillus moulds on poorly stored stuffs (Jay, 1992).

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