

ORIGINAL ARTICLE

# Survey of Mycotoxins in Wheat from Iran by HPLC Using Immunoaffinity Column Cleanup

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## KEYWORDS

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**ABSTRACT:** Mycotoxins are small, toxic chemical products produced as secondary metabolites by a few fungal species 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<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>), 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. 2.67% and 2.21% of examined wheat samples contained AFB<sub>1</sub> 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. 2.60% and 2.16% of examined wheat samples contained AFB<sub>1</sub> and OTA. LOD was 0.27, 0.04, 0.23, 0.04, 1.18 and 21.56 for AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>, OTA and ZEA, respectively. Considering these results, these special products should not be a health concern for these collectives; however, special attention needs on foods distribution in retail shops.

## INTRODUCTION

Wheat is one of the most important grains consumed in the world. Food contamination with toxigenic moulds increased 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 [1]. Mycotoxins are secondary metabolites produced by

fungi when they grow on agricultural products before or after harvest or during transportation or storage. Currently, more than 400 mycotoxins have been identified in the world, but the most important groups of mycotoxins are the major health concern for humans and animals, and occur quite often in food including: AFs, OTA, trichothecenes (deoxynivalenol, nivalenol), ZEA and fumonisins. The International Agency for Research on Cancer [2] has classified naturally-

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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 occurred by mycotoxins in human and animals have prompted more than 100 countries to establish regulatory limits for some of the well-known mycotoxins, such as the AFs in the most frequently contaminated foods [3].

AFs (B1, B2, G1 and G2) are mycotoxins produced by the fungi *Aspergillus flavus* and *A. parasiticus*. These mycotoxins are hepatotoxic and carcinogenic in humans. *A. flavus* produces AFB1 and B2, while *A. parasiticus* gives rise to AFB1, B2, G1 and G2 [2]. 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 authors [4]. OTA has nephrotoxic and hepatotoxic effects and it is likely to have carcinogenic potential in humans [2]. 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 [5].

Because of potential health hazards on humans, regulatory levels have recently been documented. The range of worldwide regulations for AFB1 was from 0 to 30 ng g<sup>-1</sup> with a total from 0 to 50 µg kg<sup>-1</sup> [6]. Recently, the Codex Alimentarius Commission, Joint FAO/WHO Food Standards Program adopted a limit of 15 µg kg<sup>-1</sup> for total AFs [7]. 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. 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 [8]. 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 for raw cereal grains, 3 µg/kg for cereals and cereal products intended for human consumption, 0.5 µg/kg for baby food and cereal based food intended for young children [9]. There are currently no legal limits for OTA in spices; however, the European Commission has discussed a limit of 10 µg/kg for OTA in spices [10]. 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.

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 [9]. All samples were delivered to the Testa laboratory at ambient temperature and stored at +4 °C until primary sample preparation, and then they were stored at -20°C until prepared for analysis. Samples were defrosted to ambient temperature prior to analysis and returned to -20°C immediately after analysis. A minimum sample size (1000 g) was applied and samples were mixed for 10 min and 500 g of samples were ground to a fine powder with a blender and stored at -20 °C for 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, and UK) were used for the purification and preconcentration of AFLs, OTA and ZEA prior to the quantitative analysis. Double distilled water was used in all the experiments. Stock standard solution of AFLs, OTA and ZEA with concentrations of 100 µg ml<sup>-1</sup> 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.

### *AFLs*

Fifty 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. 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 and were eluted with 2 mL of methanol HPLC grade and 2 mL of milli-Q water.

### *OTA*

Twenty five g of ground sample was mixed with 100 mL of extractant solution (75% acetonitrile in water) and 0.3 g NaHCO<sub>3</sub> for 5 min and filtered. Ten 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*

Twenty five 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 was filtered. Ten 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 and were eluted with 2 mL of methanol grade HPLC and 2 mL of milli-Q water.

### *HPLC analysis*

HPLC analysis 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-10Ax1 fluorescence detector.

For aflatoxin and zearalenone analysis, Genesis RP C18 analytical column (150×4.6mm, 5µm) and for ochratoxin analysis, Chromolith® performance RP18 analytical column (100×4.6mm) 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<sup>-1</sup> 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 ( $r^2$ ) AFB1= 0.995 AFB2= 0.995, AFG1= 0.995, AFG2= 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 AFB1, B2, G1, G2, OTA and ZEA with HPLC. As it is shown in Table 1, AFB1 and OTA were detected in 2.60% and 2.16% of wheat samples with mean value 1.01 and 4.194 ng g<sup>-1</sup>, respectively. AFB2, G1 and G2 were not detected in wheat samples. All contaminated samples had a level of AFB1 and total AFs were below the Iranian National Standard No.5925. Based on Iranian National Standard No. 5925 the total AFs, AFB1 and OTA levels in wheat were 15, 5 and 20ng g<sup>-1</sup>, respectively [11]. Relevant data concerning the analytical system are summarized in Table 2. For

AFB1, B2, G1, G2 and OTA, LOD were 0.2, 0.1, 0.2, 0.1 and 0.2 ng g<sup>-1</sup> and LOQ were 0.6, 0.3, 0.9, 0.3 and 0.5 ng g<sup>-1</sup>, respectively. For the repeatability measurement a standard solution containing 10 ng ml<sup>-1</sup> 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 AFB1, B2, G1, G2 and OTA from samples spiked at 5 ng g<sup>-1</sup> for AFB1, AFG1 and OTA, and 1 ng g<sup>-1</sup> for AFB2 and AFG2 were quite good (Table 2). Relative standard deviations for within laboratory repeatability (RSD<sub>r</sub>, n=6) range from 1.3 to 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<sup>-1</sup> levels is 70-125% and Codex acceptable recovery range is 70-110% for a level of 10-100 µg kg<sup>-1</sup> for a level of 10-100 µg kg<sup>-1</sup>, and 60-120% for a level of 1-10 µg kg<sup>-1</sup> [12]. Our studies showed that out of 231 wheat samples, 6 samples contained AFB1 and 5 samples contained OTA with mean value 1.01 and 4.194 ng g<sup>-1</sup>, respectively. According to obtained results, the mean concentration of AFB1 and OTA contamination of wheat in Mashhad, Iran, had a significant difference with the accepted limits by Iran regulations (5, 20 ng g<sup>-1</sup> for AFB1 and OTA, respectively). So it seems that the present status of this mycotoxin is not at risk and doesn'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, it is very difficult to control because it is influenced primarily by climatic conditions such as

relative humidity and temperature [13]. However, it is reported that the highest concentrations of AFBs are

associated with the post harvest growth of *Aspergillus* moulds on poorly stored stuffs [14].

Table 1. Distribution of mycotoxins in wheat samples

Toxin	ND*	Frequency distribution			
		0.3-0.5	0.5-1	1-1.5	> 1.5
AFB <sub>1</sub>	225	2	1	-	3
AFB <sub>2</sub>	231	-	-	-	-
AFG <sub>1</sub>	231	-	-	-	-
AFG <sub>2</sub>	231	-	-	-	-
OTA	226	-	-	1	4
ZEA	231	-	-	-	-

ND not detected

Table 2. Analytical data for the AFBs and OTA HPLC system

Compound	t <sub>R</sub> (min)	Repeatability	Limit Of Detection	Limit Of Quantification	Recovery (%)
		(%RSD, n=6)			
		Peak Area	(ng g <sup>-1</sup> )	(ng g <sup>-1</sup> )	
AFB <sub>1</sub>	9.98	1.3	0.27	0.91	91.1
AFB <sub>2</sub>	8.25	2.4	0.04	0.13	97.1
AFG <sub>1</sub>	7.48	1.5	0.23	0.75	85.2
AFG <sub>2</sub>	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

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