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Original Article

Quantitative Determination of Aflatoxin by High Performance Liquid Chromatography in Wheat Silos in Golestan Province, North of Iran

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Abstract

Background: Aflatoxins are the most common mycotoxins that contaminate crops. They are produced by fungi such as *Aspergillus flavus* and *Aspergillus parasiticus*. Wheat (*Tricitumaestivum*) is one of the most important staple foods used in Iran, and the environmental conditions in the north of Iran are favorable to fungal growth. This study was designed in order to determine the aflatoxin concentration in wheat samples from silos in Golestan Province north of Iran.

Methods: Samples were collected from three silos of Golestan province. First, aflatoxins were isolated using immunoaffinity chromatography. Then the aflatoxin concentrations were determined by High performance liquid chromatography (HPLC) method and fluorescence detector.

Results: Ten out of 34 samples (29.4% of samples) were contaminated by aflatoxins.No concentration was found above permitted aflatoxin levels in Iran (15 ng/g). In one sample (2.9%), aflatoxin B_1 was seen over the permissible limits in Iran. The highest level found in samples for total aflatoxin, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2 were 7.08 ng/g, 6.91 ng/g, 0.29 ng/g, 1.37 ng/g and 0.23 ng/g, respectively. No correlation was found between humidity levels in wheat samples contained aflatoxin and wheat samples without aflatoxin.

Conclusion: Despite the total aflatoxins determined in samples were below the permissible limits in Iran, the 29% aflatoxin contamination rate can negatively affect health factors and it should not be neglected. So, it is predictable that if the storage duration of samples increases, the aflatoxin contamination levels will increase.

Keywords: Aflatoxin, Wheat, HPLC, Iran

Introduction

Mycotoxins, produced by fungi as secondary metabolites, can infect crops at pre- and post- harvest stages and adversely affect quality of food that destined for human and livestock. Fungal infection occurs chiefly at the latitudes between 40°N-40°S of the equator, where environmental conditions such as humidity and temperature are favorable to fungal activity. Common mycotoxins contaminating foods are aflatoxins, ochratoxin A, fumonisin that produced by *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* (1,2).

Aflatoxin is one of the most prevalent mycotoxins that contaminate foods. They are difuranocumarine derivatives that mainly produced by *Aspergillus flavus*, *A. parasitic* fungi, and refer to a group of four mycotoxins, aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2)(3,4). AFB_1 is the most po-

tent toxin of these mycotoxins. Aflatoxins have various negative effects on the organs, especially liver. Studies have shown that exposure to aflatoxin can lead to primary hepatocellular carcinoma. In addition, aflatoxins may result in an acute toxicity at higher concentration (5).

In terms of production and consumption, wheat is the most pivotal cereal in Iran, which can be contaminated by fungi. Since agricultural commodities are kept in silos before consumption, in addition, the environmental conditions such as damp and warm weather in Golestan province, located the southeast of the Caspian Sea, are favorable to fungal activity, they are highly vulnerable to fungal contamination (6).

Gastrointestinal tract cancer, particularly the esophagus cancer is common in Golestan province. The incidence of cancer in East and West of this province is significantly different. The incidence of esophageal cancer according to the ASR (age-standardized incidence) in 100,000 persons-yr in 2008,in the East of the area (KalalehCity) was 67.2 and in the West (Gorgan city) was only 11.3 (7). The aflatoxins particularly B_1 is a risk factor in this area, therefore, assay of them is valuable.

The aim of this survey was to determine aflatoxins in wheat from silos in Golestan province according to the locations of sampling. Besides, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2 were individually quantified.

Material and Methods

Sample Sources

Sampling was performed according to Institute Standard and Industrial Research of Iran (ISIRI)'s protocol No. 2581 in 2009-2010. Wheat samples were collected from 3 active silos in the Golestan province, North of Iran including 14 reservoirs in Gorgan (west of province) (Latitude: 36°50′N; Longitude: 54°44′E), 11 reservoirs in Gonband (Latitude: 34°33′N; Longitude: 48°71′S), 17 reservoirs in Galikesh (east of province) (Latitude: 37°25′N; Longitude: 55°48′E).

Since temperature and humidity were unified in all parts of silos, one sample was obtained from each silo. Samples weighing 1 kg were collected from the bottom the silos. Humidity and temperature of the samples were measured. The samples were refrigerated at 4 °C until the isolation of aflatoxin.

Sample Analysis

Sample Preparation

One hundred g of sample was thoroughly grounded. Then aflatoxins were extracted from 50 g of sample using methanol: deionized water (80:20 v/v) and they mixed for 30 min at 120 RPM using a shaker. The solution was passed through filter paper.Twenty ml of filtered solution was diluted 4:1 with Phosphate Buffer Solution (PBS), pH=7. 4. The diluent was centrifuged at 3400 RPM for 15 min, and filtered using a nylon membrane filter (pore size, 0.45µm).

Affinity Chromatography

In the next step, aflatoxins were isolated from the extract by affinity chromatography column, Aflaclean (LCTech, Germany). Fifty ml of the extract was applied onto the Aflaclean column, and allowed to flow at a rate of 1 ml/min. After the aflatoxin molecules were bound the column was washed with diagnosed water in order to remove unbound materials. Then, aflatoxins were eluted using 2 ml methanol. The eluate was incubated at 50 °C and the solvent evaporated using Nitrogen. The sample was injected into the HPLC for quantitative determination of Aflatoxin B_1, B_2, G_1 and G_2.

HPLC Analysis of Aflatoxins

Aflatoxin quantities of standards and samples were determined using HPLC with fluorescent detection. An HPLC system consisted of a pump (Knaur, Germany) and a fluorescence detector (Knauer, Germany). Aflatoxins were separated in HPLC column with a mobile phase of water: methanol: acetonitrile (60:30:15, v/v/v). Fluorescence detection was at an excitation wavelength of 365 nm an emission wavelength of 440 nm. Aflatoxin retention times with 1.2 ml/min flow rate were 8-9 min for AFG_2, 10.5-11.5 min for

AFG_1, 13-14 min for AFB_2 and 16-17 min for AFB_2. Total run time was 25 min.

Spiked sample was injected10 times. The results obtained for B_2, B_1, G_2 and G_1 12.03 ± 1.04, 53.74 ± 2.15, 16.26 ± 3.14 and 64.46 ± 7.26 ng/ml, respectively. The total recovery of B_2, B_1, G_2, and G_1 obtained 95.2, 91.6, 32.8 and 68.7%, respectively. The Afla-clean column recovery of B_2, B_1, G_2, and G_1 obtained 96, 101, 83 and 100%, respectively. RSD (relative standard deviation) for B_1 obtained 9.19%.

Results

Aflatoxin was detected in 10 out of 34 samples (29.4% of whole samples). The analysis of wheat samples showed no aflatoxin levels over the permissible limits (15 ngg^{-1} for aflatoxin). In one sample (2.9%), AFB_1 was seen over the permissible limits. The highest levels found in samples for total aflatoxin and AFB_1, AFB_2, AFG_1, and AFG_2 were 7.08 ngg^{-1}, 6.91 ngg^{-1}, 0.29 ngg^{-1}, 1.37 ngg^{-1} and 0.23 ngg^{-1}, respectively.

The mean levels for AFB_1, AFB_2, AFG_1, AFG_2 and total aflatoxins in all samples were: 1.4228±0.4703 ngg^{-1}, 0.0871±0.03030 ngg^{-1}, 0.27928±0.0920 ngg^{-1}, 0.04426±0.0151 ngg^{-1} and 1.8321±0.677 ngg^{-1}, respectively.

In samples contained at least one type of aflatoxin, the mean of levels for AFB_1, AFB_2, AFG_1, AFG_2 and total aflatoxins are 2.33774±1.599 ngg^{-1}, 0.12473±0.1030 ngg^{-1}, 0.4573±0.3130 ngg^{-1}, 0.716±0.0511 ngg^{-1} and 2.6600±2.41710 ngg^{-1}, respectively.

Nine of 34 samples were contaminated by AFB_1, and ninety percent of samples contained at least one type of aflatoxins showed contamination by AFB_1.

Amounts of humidity in samples contained at least one type of aflatoxins ranged from 10.3 to 12.40 percent, and the mean humidity in these samples was 11.47± 0.77 percent. The mean humidity in samples without any aflatoxin contamination was 11.6±0.8 percent. No correlation was found between humidity levels in wheat samples contained aflatoxin and wheat samples without aflatoxin. Comparison wheat aflatoxins in the west and east of Golestan Province are summarized in Table 1.

Table 1: Comparison wheat aflatoxin in the west and east of Golestan Province, northern Iran

Concentration (ng/gr)	Mean ± Sd	Maximum (ng/gr)	*P*-value
G_1West	0.0865 ± 0.226	0.92	0.699
East	0.976 ± 0.331	1.37	
G_2West	0.0196 ± 0.057	0.23	0.229
East	0.0104 ± 0.025	0.09	
B_1West	0.103 ± 0.284	1.16	0.003
East	0.837 ± 1.951	6.91	
B_2West	0.0315 ± 0.089	0.36	0.834
East	0.0291 ± 0.074	0.29	

Discussion

In the present study, the samples collected from active silos in Golestan province were analyzed for aflatoxin. 29.4% of samples were positive to aflatoxin, and the aflatoxin levels were below permissible limits for aflatoxin in Iran. In Mazandaran province, located near Golestan province, in 2002, 63.7% of wheat samples were contaminated with *Aspergillus* species (8). Aflatoxin and aflatoxin B_1 were found in 2.54% and 3.39% of samples at concentration ranged between 1.3-1.7 ngg^{-1} and 1.36- 1.76 ngg^{-1}. In this study, the samples analyzed using Thin Layer Chromatography (TLC); whereas, in our study the samples were analyzed by affinity chromatography and HPLC. Differences in analytical technique might be responsible for differences in mycotoxin concen-

tration and frequency in wheat samples among two adjacent provinces (8).

In Morocco, Zinedine et al. Analyzed corn flour and wheat flour using immunoaffinity chromatography, HPLC and fluorimetric detection. Fifty percent of corn flour, 17.6% of wheat flour samples were contaminated with aflatoxins. Aflatoxin B_1 levels in corn flour and wheat flour ranged between (0.03- 0.15 ngg^{-1}) and (0.23- 11.2 ngg^{-1}), respectively (9). Frequency of aflatoxin contamination in this study was approximately similar to those found in our study; however, aflatoxin levels in our samples were higher than aflatoxin levels in Morocco (9). It can result from differences in type of grain, storage condition, agricultural practices, different food processing and sampling method (10). In Turkey, 25 samples of cereals (including 12 wheat samples) were analyzed for aflatoxin using HPLC. Aflatoxin and aflatoxin B_1 were detected in 12 samples (100%) and 8 samples (66.7%), respectively (11). Forty-two percent of wheat samples were contaminated by aflatoxin and AFB_1, respectively (12). Despite aflatoxin levels were below the permissible limits of Turkey's regulation (4 ngg^{-1} for aflatoxin B_1), the possible long-term exposure to low level of aflatoxins can be health- threatening in human and livestock. In the present study, aflatoxin levels were over standard limits of EU regulation for aflatoxin (4 ngg^{-1}), and below permissible limits for aflatoxin Iran (15 ngg^{-1}).

This has raised concern about the effects on human of the long-term intake of small amount of aflatoxin. Meeting the developed countries' standard limits seems impractical in developing countries due to lack of basic infrastructure for aflatoxin monitoring and limited availability of food (13). However an appropriate policy for fulfilling the dual task of lowering health risks and guaranteeing sufficient food supply should be adopted in high-risk areas during high risk seasons. Aflatoxin contamination of crops is most prevalent in tropical and subtropical conditions, because molds occur more frequently in these areas, in addition, the appropriate temperature and humidity conditions for cereal production are the optimum conditions for *Aspergillus* growth and aflatoxin production. Since the Golestan province located in the subtropical area with favorable environmental conditions in cereal production as well as mold growth, it is considered as a high-risk area. Moreover, the storage duration in 2009, which the study was carried out, was shorter than previous yr due to less wheat production. So, it is expected longer storage duration results in the greater risk of aflatoxin contamination. In regard to various factors can affect aflatoxin levels such as storage conditions, transportation, grain types, temperature, environmental humidity and O_2 availability, risk mitigation and enhancing food safety are achieved by implementing process-based standards throughout the production, handling and processing chain (14-16). These standard limits should be cost-effective in order to minimize the negative effect on the food supply.

Stringent food safety regulations in developed countries lead to minimize aflatoxin contamination. In Japan, using HPLC, TLC and LC-MS, aflatoxin was detected in ten of twenty peanut samples. The maximum concentration was detected for aflatoxin B_1 at concentration 2.59 ngg^{-1}. No aflatoxin was detected in maize, wheat flour and sesame oil (17). In Canada 50% of 349 samples included rice-, soy-, barely-based and mixed grain infant cereals, corn, wheat and mixed grains and breakfast cereals were positive to aflatoxin B_1. The levels of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2 ranged between 0.002-1.00 ngg^{-1}, 0.002-0.014 ngg^{-1}, 0.008-0.27 and 0.008-0.048 ngg^{-1}, respectively (18).

Yazdanpanah et al. assessed aflatoxin B_1 (AFB_1) in several food stuffs such as rice, bread, puffed corn snack, wheat flour and peanut. The results showed that although the mean concentration of AFB_1 in all food samples was lower than maximum tolerated level in iran, the mean intake of AFB_1 from rice was estimated 3.49 times higher than the guidance value of 1 ng AFB_1/kg body weight/day (19).

Alborzi et al. evaluated aflatoxin M_1 (AFM_1) contamination in pasteurized milk samples. AFM_1 was found in 100% of the examined milk samples. 17.8% of the samples had AFM_1 greater than the

maximum tolerance limit (50 ng/l) accepted by European Union (20).

Several studies about aflatoxin contamination of various food stuffs in the north of iran have been done. The levels of aflatoxins in flour samples were within acceptable limits set by Iran Standard Institute (21). There was a positive relationship between aflatoxin level of wheat flour samples and the risk of esophageal cancer (EC) (22). Aflatoxin contamination may be a possible risk factor for EC in our region. aflatoxin B_1 was significantly differentinthe Eastand the West. The incidence of the gastrointestinal tract cancers are significant differences between the two regions. It seems the weather condition is suitable for fungal growth and aflatoxin productionin this area. The toxins can increase the risk and incidence rate of cancer. Wheat flour total aflatoxin in Golestan province was significantly higher in high risk esophageal cancer (22).

Conclusion

Despite the total aflatoxins determined in samples were below the permissible limits in Iran, the 29% aflatoxin contamination rate can negatively affect health factors and it should not be neglected. So, it is predictable that if the storage duration of samples increases, the aflatoxin contamination levels will increase.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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