

ORIGINAL ARTICLE

NATURAL OCCURRENCE OF MYCOTOXINS IN CEREALS FROM MAZANDARAN AND GOLESTAN PROVINCES

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Abstract

Background-Aflatoxins (AF) and ochratoxin A (OA) are two toxic substances produced by specific types of fungi. Many reports consider them as carcinogens and nephropathogens, respectively.

Objective-The aim of this study was to present the natural occurrence of AF and OA in some important foodstuffs in the southern Caspian littoral.

Methods-Fourteen barley and nine corn samples, intended for animal feed, were collected from Golestan and Mazandaran provinces, northern Iran, and were analyzed for AF and OA by high performance liquid chromatography (HPLC).

Results-In corn samples, aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2) were detected in 8 (88.8%) and 6 (66.6%) samples at a mean level of 15.83 and 2.99 ppb (median 1.72 and 1 ppb) respectively. Only one of the AF-contaminated corn samples was co-contaminated with OA at a concentration of 0.35 ppb. Neither AF nor OA were found in any of the barley samples.

Conclusion-This study was the first report on natural occurrence of OA and its co-occurrence with AF in the corn samples of northern Iran. Although our findings showed a low level of AF and OA, we recommend a periodical survey to be conducted on the natural occurrence of mycotoxins in cereals from the Caspian littoral.

Keywords • Mycotoxin(s) • aflatoxin(s) • ochratoxin • Caspian littoral • Iran

Introduction

Aflatoxins (AF) are highly toxic, mutagenic, carcinogenic and teratogenic metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* and have been implicated as causative agents in human hepatic and extrahepatic carcinomas. They contaminate various agriculturally important commodities including corn. Aflatoxin B1 (AFB1) was evaluated as a Class 1 human carcinogen.¹ The incidence of AF in foods and feeds is relatively high in tropical and subtropical regions, where the

warm and humid climate provides optimal conditions for the growth of moulds.² A number of surveys and monitoring programs have been conducted in several countries attempting to obtain an overall pattern of the extent of food and/or feed contamination by AF.²⁻⁹ Nevertheless, 77 countries are known to have some regulations on AF and other mycotoxins. Most existing mycotoxin regulations concern AF and all countries with mycotoxin regulations have tolerances for AF in food and/or feed. Accordingly, medians and ranges of maximum tolerated levels (MTL) of AFB1 and aflatoxin total (AFT) in feedstuffs are 20 and 5-1000 ppb, and 50 and 0-1000 ppb, respectively.¹⁰

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Ochratoxin A (OA) is a nephrotoxic and nephrocarcinogenic mycotoxin produced by *Penicillium verrucosum* in temperate or cold climates and by a number of species of *Aspergillus* such as *A. ochraceus* in warmer and tropical areas of the world. OA may be involved in the etiology of Balkan Endemic Nephropathy in the Balkan countries.^{11,12} It is commonly found in both human food and animal feed, especially in temperate areas including Denmark, Germany^{13,14}, Canada¹¹, United Kingdom¹⁵ and Sweden.¹⁶ Currently eight countries have specific regulations for OA in one or more food commodities. In addition, a few countries have proposals for OA regulations. The current proposed limits range from 1 to 5 µg/kg for children and infant foods, from 2 to 50 µg/kg for food and from 5 to 300 µg/kg for animal feed.¹⁷ In Europe, tolerance levels for OA have been suggested at 1 µg/kg for infant food and 5 µg/kg for cereals.¹⁸ Regulations for OA in some food commodities including cereals has been recently set at 3 µg/kg in Italy.¹⁹

The high humidity and moderate temperatures in the subtropical Caspian littoral of northern Iran could promote the growth of these toxigenic fungi and the production of mycotoxins. In this regard, several toxigenic fungi such as the *Aspergillus*, *Penicillium* and *Fusarium* species have been isolated from cereals produced in these regions.^{20,24} Furthermore, it has been shown that these fungi could produce mycotoxins in culture.^{20,24,25} There are some reports on natural occurrence of mycotoxins in certain Iranian food commodities.^{26,27} Surveys have shown natural occurrence of AF in bread^{28,29} and corn.³⁰ With regard to OA, its natural occurrence in red wheat has been reported.²⁰

At present, establishment of regulations for some mycotoxins is underway by the Iranian Standards Organization in collaboration with the Ministry of Health and Medical Education.

No information is so far available in the literature on the natural occurrence of AF in barley and OA in barley and corn samples of northern Iran. The present study was therefore undertaken to determine the natural contamination levels and possible co-occurrence of AF and OA in corn and barley in northern Iran.

Materials and Methods

Samples

During the year 1998, 9 and 14 samples of

farmers' corn and barley lots, intended for animal feed, were collected at random from consignments sold to the Iranian Agriculture Office in Mazandaran and Golestan provinces, respectively. Samples (0.5 to 5 kg) were collected from the lots, personally carried to Italy, and individually stored in zip-lock plastic bags at 4°C until analysis in which each sample was ground to a meal and after thorough mixing, a 25-50 g subsample was withdrawn for analysis.

Analysis of AF

Extraction and immunoaffinity clean-up:

1) Barley

Samples were analyzed using the method of Stroka.³¹ Ground sample (50 g) was weighed into an Erlenmeyer flask and after adding 5 g of sodium chloride and 300 mL of methanol: water (8:2), the flask was shaken on a wrist-action shaker set at medium speed for 45 minutes. After filtration through a filter paper, 10 mL of the filtrate was diluted with 60 mL of phosphate buffer solution (PBS) (pH 7.4). Afla Test immunoaffinity column (Vicam, Watertown, MA) was conditioned with 10 mL PBS at a flow rate of 2-3 mL/min.

Whole-diluted extract was then applied to the column, at a steady flow rate of 3 mL/min. After washing the column with 15 mL of bidistilled water, air was drawn through the column until dry. AF was eluted from the column into a 3-mL calibrated volumetric flask in a 2-step procedure. At first, 0.5-mL methanol was applied on the column and passed by gravity. After 1 min, a second portion of 0.75 mL was applied and most of the applied evasive solvent collected by forcing air through. Finally, it was filled to the mark with water and mixed.

2) Corn

Samples were analyzed using the Afla Test procedure.³² In brief, 50 g of ground sample (plus 5 g of sodium chloride) was extracted with 100 mL of methanol: water (8:2). Then, 10 mL of filtered extract was diluted with 40 mL of purified water, mixed well and filtered through glass microfiber filter into a clean vessel. Ten milliliters of filtered diluted extract was passed through Afla Test immunoaffinity column. The column was then washed with 10 mL purified water. AF were eluted from the column using 1 mL HPLC grade methanol. One mL purified water was then added to the eluate.

Since the recovery of AFB1 in the two highest level AF-contaminated samples was about 70%,

we tried to verify the reason for this matter. In this regard, the method of analysis of AF in barley³¹ was modified for analysis of these samples as follows: after extraction and filtration, 10 mL of filtered extract was diluted with 60 mL of PBS, mixed well and filtered through glass microfiber filter. For extract clean-up, two different immunoaffinity columns including Afla Test and AFLAPREP (Rhone-Diagnostics Technologies Ltd., UK) were used for each sample. Sixty mL of filtered diluted extract was passed through immunoaffinity column and AF were eluted as described for barley.

HPLC analysis

Samples were analyzed by HPLC equipped with a JASCO AS-950-10 autosampler and a JASCO FP-920 fluorescence detector after post-column derivatization with bromine. The analytical column used was C18 (Alltech), 5 mcm, 250 mm × 4.6 mm i.d. Mobile phase was water: methanol: acetonitrile (54:29:17) at a flow rate of 1 mL/min. The post-column pump was Waters 510 and the post-column reagent was saturated bromine in water at a flow rate of 0.4 mL/min. The fluorescence detector was operated at an excitation wavelength of 365 nm and an emission wavelength of 435 nm.

Two different five-point calibration curves, with triplicate injections, covering the range of 0.07-0.65 µg/kg and 0.70-6.5 µg/kg for AFB1 and AFG1, 0.04-2.6 µg/kg and 0.2-1.3 µg/kg for AFG2 and 0.02-0.13 µg/kg and 0.2-1.3 µg/kg for AFB2 were built and checked for the linearity. For both calibration curves, correlation coefficients were higher than 0.999 for all four AFs. Quantification was based on an external standard of AF. Limits of detection for toxin peaks were defined as 3 times baseline signal and were 0.07 µg/kg for AFB1 and AFG1, 0.04 µg/kg for AFG2 and 0.02 µg/kg for AFB2.

Analysis of OA

Extraction and immunoaffinity clean-up:

1) Barley

Samples were analyzed using an in-house validated method. Ground sample (25 g) was extracted with 100 mL of acetonitrile: water (60:40) by a wrist-action shaker set at medium speed for 45 minutes. Four mL of the filtrate was diluted with 44 mL of PBS and filtered diluted extract was then added to the Ochra Test immunoaffinity column followed by 10 mL water

wash. OA was eluted from the column using four 1 mL portions of methanol. The eluate was evaporated under a stream of nitrogen at 40°C and then redissolved in 1-mL mobile phase.

2) Corn

Samples were analyzed using the Ochra Test (Vicam, Watertown, MA) procedure.³³ The extraction of ground sample (50 g) and extract dilution were carried out as described for analysis of AF in corn using the Afla Test procedure. Ten mL of filtered diluted extract was passed through Ochra Test immunoaffinity column. The column was then washed with 10 mL mycotoxin wash buffer solution followed by 10 mL purified water. OA was eluted from the column using 1.5 mL HPLC grade methanol. Finally, 1.5 mL purified water was added to eluate.

HPLC analysis

Samples were analyzed by HPLC equipped with a JASCO AS-950-10 autosampler and a JASCO FP-920 fluorescence detector. The analytical column used was C18 (Alltech), 5 mcm, 250 mm × 4.6 mm i.d. Mobile phase was acetic acid 2%: acetonitrile (49:51) at a flow rate of 1 mL/min. The fluorescence detector was operated at an excitation wavelength of 333 nm and an emission wavelength of 470 nm.

A five-point calibration curve, with triplicate injections, covering the range of 0.24-20 µg/kg was built and checked for the linearity. Correlation coefficient was 0.9999 for OA. Quantification was based on an external standard of OA. Limit of detection for OA was 0.24 µg/kg (signal/noise: 3/1).

Spiking of AF and OA for recovery studies

One spiked sample was analyzed with each series of samples (usually 5-7) to check percentage of recovery of OA and AFG1, AFG2, AFB1 and AFB2. After spiking of samples with known amounts of mycotoxins, the solvent was allowed to evaporate overnight. Then the sample was simultaneously analyzed together with other samples and the recovery calculated. All analytical results were corrected for recovery. The obtained recovery factors are shown in Table 1.

Results

Performance of the analytical method

Statistical analyses of the performance of analytical methods used for analysis of AF and OA

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Table 1. Statistical analysis of the performance of analysis methods of aflatoxins (AF) and ochratoxin A (OA) in corn and barley samples.

Cereal	Mycotoxin	Spiking level (µg/kg)	Average recovery (%)	Standard deviation	Coefficient of variation (%)*
Barley	OA	4	79.5	3.7	4.7
	AFG1	2	88.4	8.7	9.8
	AFG2	0.6	84.5	4.7	5.6
	AFB1	2	82.5	9	10.9
	AFB2	0.6	89.7	8.5	9.5
Corn	OA	2	72.6	1.5	2.1
	AFG1	4.8	74.1	2.1	2.8
		40**	72.4	2.4	3.3
		40***	73.2	2.8	3.8
	AFG2	0.125	66	14	21.2
		2.4	75.8	7.6	10
		5	70	4.1	5.9
	AFB1	0.25	60.9	9.0	14.8
		2	80.3	1.5	1.9
		40**	74.4	2.2	3
		40***	69.2	1.1	1.6
	AFB2	0.125	89.8	1.3	1.4
		2.4	76.8	9.2	12
		5	70.9	0.57	0.8
		20**	73.1	2.2	3
20***		70	2.3	3.3	

* Coefficient of variation reproducibility.

** Samples analyzed by modified method of analysis of AF in barley and Afla Test (Vicam, Watertown, MA) column.

*** Sample analyzed by modified method of analysis of AF in barley and AFLAPREP (Rhône-Diagnostic Technologies Ltd, UK) column.

in corn and barley samples are shown in Table 1.

The recovery of AF and OA in barley and corn samples were good and in the acceptable range (Table 1). The coefficient of variation for reproducibility of all spiked samples with AF or OA ranged from 0.8 to 21.2%.

The recovery of AF in corn samples spiked with 40 ppb of AFB1 and 40 ppb of AFG1 and analyzed with Afla Test procedure were between 65-68%. In order to verify the reason of this matter, spiked samples were analyzed with a modified method of analysis of AF in barley, using two different immunoaffinity columns including Afla Test and AFLAPREP. In this method, we increased the ratio of extraction solvent to corn from 2 to 6. In addition, we passed about 1.4 g of corn through the column in comparison to 1 g in the first analysis but the recoveries were not significantly changed (69.2-74.4%) (Table 1).

Mycotoxin results

1) Corn

The concentration, mean, median and incidence

of positive samples for AF in corn samples in northern Iran are given in Table 2.

On the whole, 88.8% of the samples were contaminated with AFB1 at concentrations ranging from 0.09 ppb to 73.10 ppb (mean and median level, 15.83 and 1.72 ppb, respectively). Furthermore, 66.6% of the samples were positive for AFB2 at concentrations ranging from 0.02 to 12.70 ppb (mean and median level 2.99 and 1ppb, respectively). AFG1 and AFG2 were not detected in any of the corn samples. The concentration of aflatoxin total (AFT) ranged from 0.09 to 85.30 ppb with a mean and median level of 18.82 and 1.83 ppb, respectively. Typical chromatograms relative to AF standards and corn sample are shown in Figure 1.

As shown in Table 2, no significant difference was observed between the AF concentrations of samples, which were analyzed with both Afla Test and AFLAPREP immunoaffinity columns.

On the co-occurrence of both carcinogenic mycotoxins, only one of the AF-contaminated corn samples (No. 6) was co-contaminated with OA.

Table 2. Natural occurrence of aflatoxins (AF) in corn samples of northern Iran, 1998.

Sample number	Immunoaffinity column	Mycotoxin concentration (µg/kg)				
		AFG1	AFG2	AFB1	AFB2	AFT
1	Vicam	ND ^a	ND	57.40	12.70	70.10
1	Rhone-Diagnostics	ND	ND	51.70	11.20	62.90
2	Vicam	ND	ND	0.22	ND	0.22
3	Vicam	ND	ND	8.00	1.80	9.80
4	Vicam	ND	ND	3.10	0.20	3.30
5	Vicam	ND	ND	0.09	ND	3.30
6	Vicam	ND	ND	73.10	12.20	85.30
6	Rhone-Diagnostics	ND	ND	78.80	12.10	90.90
7	Vicam	ND	ND	0.33	0.02	0.35
8	Vicam	ND	ND	0.28	0.02	0.30
9	Vicam	ND	ND	ND	ND	----
Mean (ppb)*		----	----	15.83	2.99	18.82
Median (ppb)*		----	----	1.72	1	1.83
Positive samples (%)		0	0	88.8	66.6	88.8

^a: Lower than limit of detection.

*Mean and median concentrations were calculated for samples analyzed by Afla Test columns (Vicam, Watertown, MA). AFG1: Aflatoxin G1; AFG2: Aflatoxin G2; AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFT: Aflatoxin Total.

The highest level of AFT-contaminated corn (85.30 ppb) was co-contaminated with OA at a concentration of 0.35 ppb. Typical chromatograms relative to the OA standard and corn sample are shown in Figure 2.

2) Barley

All of the samples showed results below detection limits.

Discussion

The acceptability of the coefficient of variation for reproducibility of all spiked samples with AF or OA was assessed on the basis of the Horwitz equation. The values of coefficient of variation, which were calculated from Horwitz equation for the spiking level of 1, 10 and 100 ppb, were 45.2,

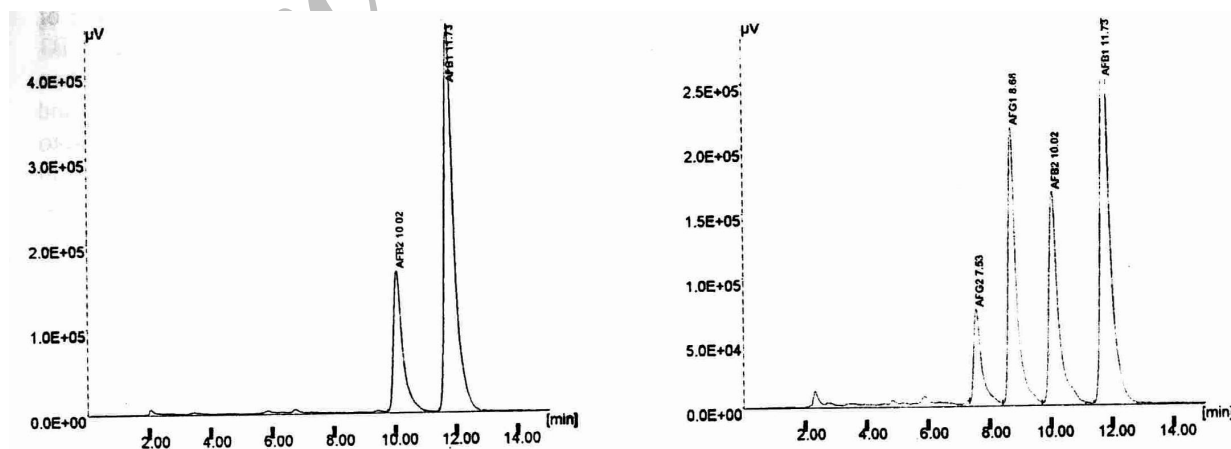


Figure 1. Traces of AF standards and naturally contaminated corn with AF: a) corn sample, b) standards.

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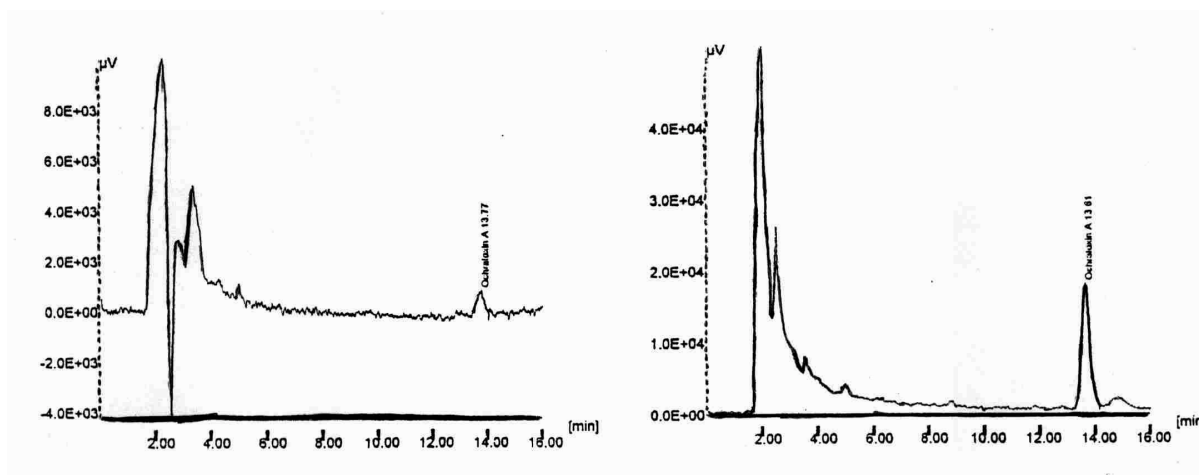


Figure 2. Traces of ochratoxin A (OA) standards and naturally contaminated corn with OA: a) corn sample, b) standards.

32 and 22.6%, respectively. All of the coefficients of variation values obtained from our experiments (0.8 to 21.2%) were lower than values calculated from the Horwitz equation.

Corn is among the commodities with the highest risk of AF contamination³⁴ whilst barley, wheat, soybeans, beans, sorghum, oats, pulses, cassava, millet and rice are resistant or only moderately susceptible to aflatoxin contamination in the field.^{35,36} The incidence of AF in food and feed is relatively high in tropical and subtropical regions.² In this regard, it has been reported that during 1983-1993, AF contamination in Indian animal feedstuffs, such as maize, was very high and 563 out of 862 maize samples contained AF at levels ranging from 3 to 8,260 ppb (mean level, 264 ppb).⁸ In the present study, we also found that only corn samples were contaminated with AF and these toxins were not detected in any of the barley samples. The median and ranges of concentrations of AFB1 in corn samples were 15.83 and 0.09-73.10 ppb, respectively, which was lower than median and were in the ranges of MTL of AFB1 in feedstuff (20 and 5-1000 ppb, respectively).¹⁰ Furthermore, the median and ranges of concentrations of AFT in corn samples were 18.82 and 0.09-85.30 ppb, respectively, which was also lower than median and were in the ranges of MTL of AFT in feedstuffs (50 and 0-1000 ppb, respectively).¹⁰ Tayebi and Esavi³⁰ reported that one (5%) out of every twenty corn samples collected from North of Iran in 1996, was contaminated with AFB1 and AFB2 at a level of

245 and 35 ppb, respectively. Paradoxically, Lacey²⁰ reported that among 141 wheat and barley samples which were collected at harvest and after different periods of storage in underground pits or in buildings from Northeast of Iran, AF were not detected in any of the samples. However, it seems there are some uncertainties concerning the results of mycotoxin analysis. Moreover, Hormozdiari²⁸ reported that analysis of food samples, which were consumed by people of some villages in the Caspian littoral of Iran, showed low levels of AF contamination. If mycotoxins are produced only periodically, regular sampling and analysis of mycotoxins over a number of years could be necessary to assess their frequency of occurrence.

Eight of the corn samples in this study (No 1-8), have been analyzed for the presence of fumonisins (FM) B1 (FB1), B2 (FB2) and B3 (FB3).²⁷ Regarding the co-occurrence of the two carcinogenic mycotoxins, fumonisins and aflatoxins, all of the samples (8/8) were found to be contaminated with both toxins and fumonisin levels were mostly higher than those of aflatoxins (Table 3). The highest and lowest levels of AFT-contaminated corn samples (85.3 ppb and 0.09 ppb) were co-contaminated with fumonisins at concentrations of 6115 ppb and 1635 ppb, respectively. This simultaneous contamination is significant from the standpoint of potential risks to human and animal health.

OA has been reported as naturally occurring in almost all cereals including corn, barley, wheat, sorghum, rye, oats and rice. It appears that barley,

Table 3. Fumonisin levels in corn samples from Mazandaran Province, Iran (Shephard, et al 2000)²⁷

Sample number	Mycotoxin concentration ($\mu\text{g}/\text{kg}$)			
	FB1	FB2	FB3	FM Total
1	3.160	0.870	0.490	4.520
2	1.565	0.310	0.225	2.100
3	2.960	0.665	0.450	4.075
4	1.975	0.335	0.190	2.500
5	1.270	0.210	0.155	1.635
6	3.980	1.175	0.960	6.115
7	3.360	0.940	0.550	4.850
8	1.890	0.345	0.260	2.495

oats, wheat and corn grown in Denmark and other Scandinavian countries as well as in the Balkan countries and India are particularly susceptible to high levels of OA contamination.³⁵⁻³⁷ In addition, in Canada, Europe and Australia, animal feed may also be highly contaminated with OA, sometimes at concentrations exceeding 5000 ppb.³⁵ In the present study, only 11 % of corn samples were contaminated with OA at very low concentrations and it was not detected in any of the barley samples. Clearly, the incidence of OA found in corn kernels from Mazandaran Province compare favorably with the incidence found in wheat reported by Lacey.²⁰ He reported that among 141 red wheat samples only one was contaminated with OA. Concerning the simultaneous occurrence of three carcinogenic mycotoxins, aflatoxins, fumonisins and ochratoxin A, the highest level of AFT-and FM-contaminated corn (sample No. 6) was co-contaminated with OA.

The present study reports the natural occurrence of OA and co-occurrence with AF in the corn of northern Iran for the first time. In addition, along with the results of analysis of FM in Mazandaran corn samples²⁷ this study described, for the first time the simultaneous occurrence of *Aspergillus* toxins (AFT and OA) together with fumonisins in corn from northern Iran. Although the reported levels of AF and especially OA were low (due to suitable climate of these areas for fungal contamination), a high level of contamination may occur. On the other hand, toxigenic fungi have also been isolated from cereals produced in other parts of Iran.^{21,22} Taking into consideration all of the above concerns, and with regard to the presence of some diseases with unknown etiologies (like esophageal cancer in Northeast of Iran),

comprehensive studies including a periodical survey on natural co-occurrence of mycotoxins in cereals is needed in order to have a broader perspective on the extent of mycotoxin problem in Iran.

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