Asian Journal of Chemistry Vol. 19, No. 5 (2007), 4075-4082

Aflatoxin B_1, Total Aflatoxin and Ochratoxin A Levels in Wheat Flour

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In this study, 100 wheat flour specimens were analyzed with the ELISA technique regarding the amounts of total aflatoxin (AF), aflatoxin B_1 (AFB1) and ochratoxin A (OTA). The AF, AFB1 and OTA contaminations in the specimens were found to be 71, 57 and 87 %, respectively. The specimens with AF and AFB1 levels exceeding the maximum limits defined by the Turkish Food Codex and European Commission constituted 5 % of all, while the OTA contents did not exceed the allowed limits in any of the specimens that have been examined.

Key Words: Total aflatoxin, Aflatoxin B_1, Ochratoxin A, Wheat flour, ELISA.

INTRODUCTION

Mycotoxins are toxic metabolites with low molecular weight produced by certain types of mold genera such as *Aspergillus*, *Penicillium* and *Fusarium* during their growth on food and feed[1,2]. Exposure to toxins occurs predominantly by the ingestion of contaminated food, especially cereals and grains such as corn, wheat and peanut, among others[3]. Devegowda *et al.*[4] has reported that *ca.* 25 % of the cereals consumed in the world are contaminated by mycotoxins. Toxigenic fungi may contaminate food products at different phases of production and processing, mainly in favourable humidity and temperature conditions. Many mycotoxins also have significant chemical stability, which enables their persistence in food products even after the removal of the fungi by means of the usual manufacturing and packaging processes[5]. Mycotoxins, when present at high levels in the diet, have been shown to cause acute and/or chronic adverse health effects in animals and humans. These chemicals may affect many target organs and systems, notably the liver, kidney, nervous system, endocrine system and immune system[1].

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Aflatoxins are toxic compounds which are produced as secondary metabolites by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* growing on a variety of food products and are known to be carcinogenic, mutagenic, teratogenic and immunosuppressive. Among 18 different types of aflatoxins identified, the major ones are aflatoxin B_1 (AFB1), AFB2, AFG1 and AFG2[6,7]. AFB1 is the most potent hepatocarcinogen known in mammals and is classified by the International Agency of Research on Cancer (IARC) as a Group I carcinogen[8]. The primary target organ for toxicity and carcinogenicity is liver in human and animals and the toxicity is known to be mediated by metabolism in the liver by cytochrome P450 system to the highly reactive aflatoxin B_1-8,9-epoxide[9-11]. There are large number of reports that suggest intoxication of humans by the consumption of aflatoxin-contaminated agricultural products[11-14]. Additionally, the food contamination by aflatoxins causes serious economic losses[15]. The European Committee Regulations (ECR) establish the maximum acceptable level of AFB1 in cereals, peanuts and dried fruits either for direct human consumption, or as an ingredient in foods: 4 ppb for total aflatoxins (AFB1, AFG1, AFB2 and AFG2) and 2 ppb for AFB1 alone[6,16].

Ochratoxin A (OTA), a phenylalanyl derivative of a substituted isocoumarin, is mainly produced by *Penicillium verrucosum* and by *Aspergillus ochraceus*, but also by isolates of closely related *Aspergillus niger*[17]. It can be nephrotoxic, hepatotoxic, immunosuppressive, genotoxic and teratogenic[18]. The International Agency of Research on Cancer (IARC) has classified OTA in group 2B as a possible carcinogenic compound for humans[19]. OTA contamination has been reported in cereals, coffee, wines, dried fruits and animal feeds, as well as in tissues and blood of animals and human beings[20,21]. OTA contamination of grain is most prominent, although highly variable and directly influenced by storage conditions after harvest[22]. The main contributors to OTA intake in humans are cereals and cereal-derived products because of the resistance to technological processes by this mycotoxin[23,24]. In the EU, regulations list the maximum tolerable levels of OTA in cereals (5 μg/kg), cereal-derived products (3 μg/kg), dried vine fruits (10 μg/kg)[16,25] and in food for infants and young children (0.5 μg/kg)[26].

Many analytical methods including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), fluorescence, mass spectrometry or amperometric detection, immunoassay and combined methods have been employed to determination aflatoxin and OTA[27-30]. However, these methods require well-equipped laboratories, trained personnel, harmful solvents and several hours to complete an assay. Enzyme linked immunosorbent assays (ELISA) methods potentially have advantages over the other procedures because of their simplicity, sensitivity, low cost and the use of safe reagents[6].

Wheat, which is susceptible to these fungal infections through its growth, harvest, transport and storage, is the most staple food in Turkish Republic. Turkish Republic is one of the major wheat producing countries in the world. The average daily consumption of wheat and wheat products by and average Turkish person is about twice as high as most western countries. Daily consumption of these products is estimated to be approximately 400 g and this amount corresponds to almost 50 % of daily diet[31]. However, limited studies exist on the levels of mycotoxins in cereal and cereal products consumed and produced in Turkey[15,32-36].

The purpose of this study was to determine the amounts of total aflatoxin (AF), AFB1 and ochratoxin A in wheat flour specimens in order to demonstrate the potential community health hazards.

EXPERIMENTAL

Between January and July 2006, 500g weighing specimens of wheat flour sold in Erzurum, Agri, Igdir, Dogubeyazit and Patnos cities and their vicinity were taken into sterile pouches and brought immediately to the Kontrollab Food/Water Environment Analyse laboratories for analysis. Each specimen was kept in cold conditions until analysis and the analyses were conducted in parallel to each other.

The assessments of AF, AFB1 and ochratoxin A were conducted using the RIDASCREEN® Aflatoxin Total (Art. No. R 4701), RIDASCREEN® Aflatoxin B_1 (Art. No. R 1201) and RIDASCREEN® Ochratoxin A (Art. No. R 1301) ELISA test kits, respectively.

The levels of detection for each AF, AFB1 and ochratoxin A were found to be 1.75 μg/kg (ppb), 1.0 μg/kg (ppb) and 0.625 μg/kg (ppb), respectively. The values of average recoveries of these mycotoxins were 85, 56 and 85 %, respectively.

Preparation of the specimens for total aflatoxin analysis and the test procedure

2 g of a granulated specimen was weighed and placed in a container, followed by addition of 10 mL of methanol 70 % and mixing over a mixer for 10 min at room temperature. The complete specimen solution was filtered through a folded filter paper and 100 μL of the filtrate was diluted with a 600 μL of a specimen dilution buffer and 50 μL of this final diluted filtrate was used for the test.

Microtiter strips sufficient in amount for the standard and specimens were placed on the plate and 50 μL each of all standards or prepared specimens was placed in the wells. Then, 50 μL of diluted enzyme conjugate was added on each of them, followed by the addition of 50 μL of diluted antibody into each well and incubation at room temperature for 0.5 h. At the end of the incubation, the liquids in the wells were evacuated and the

wells were washed 3 times with washing solution in an automated washer. Following this step, 50 µL of substrate and 50 µL of chromogen were placed in each well and incubated at room temperature at dark for 0.5 h. At the end of the incubation, 100 µL of stopping solution was added and the absorbance was assessed at 450 nm. The results obtained *via* reading over a calibration curve were multiplied with the dilution factor, which were 35.

Preparation of the specimens for aflatoxin B_1 analysis and the test procedure

2 Of granulated specimen was weighed two times and placed in a screw-lidded glass container, followed by addition of 3 mL of PBS-buffer or distilled water and 0.2 mL of amilase solution and gentle mixing at room temperature for 20 min. Then, 7 mL of methanol (100 %) was added and shaken for 10 min for extraction. The specimen solution was filtered through a filter paper and 2 mL of distilled water and 3 mL of dichloromethane were added over 2 mL of filtrate. This was shaken thoroughly for 5 min, followed by centrifuging for 10 min at 3500 rpm at 10-15°C temperature. The supernatant liquid was removed and mixed in vortex for a short duration. The mixture was suspended for *ca.* 0.5 h at 50-60°C temperature for complete drying. The dry remnant was re-dissolved with 1 mL of methanol (100 %), followed by the addition of 1 mL of distilled water and 1.5 mL of *n*-heptane and shaking for 5 min. This was centrifuged for 5 min at 3500 rpm at 10-15°C temperature. The overlying *n*-heptane layer was completely removed and 100 µL of the underlying methanolic layer was taken, which was then diluted with 400 µL of specimen dilution buffer. During the test, 50 µL of this mixture was used.

Microtiter strips sufficient in amount for the standard and specimens were placed on the plate and 50 µL each of all standards or prepared specimens was placed in the wells. Then, 50 µL of diluted enzyme conjugate was added on each, followed by incubation at room temperature for 2 h. At the end of the incubation, the liquids within the wells were evacuated and the wells were washed with washing solution in an automated washer 3 times. Later, they were incubated for 0.5 h. At the end of the incubation, 100 µL of stopping solution was placed in each well and the absorbance at 450 nm was assessed. The results were obtained over a calibration curve and multiplied with the dilution factor, which were 25.

Preparation of the specimens for ochratoxin A analysis and the test procedure

2 Of granulated specimen was weighed two times and placed in a screw-lidded glass container, followed by addition of 3 mL of PBS-buffer or distilled water and 0.2 mL of amilase solution and gentle mixing at room temperature for 20 min. This was mixed with 1 mL of 5 N HCl for 5 min,

followed by the addition of 10 mL dichloromethane and mixing for 15 min. This step was followed by centrifuging for 15 min at 3500 rpm at 15°C. The overlying layer was removed and the remainder was filtered. Then, 0.13 M of sodium hydrogen carbonate at a volume equal to that of the filtrate was added and they were mixed for 15 min and re-centrifuged for 15 min at 3500 rpm at 15°C, followed by removal of 100 µL of the upper phase of the diluted filtrate, over which 400 µL of 0.13 M sodium hydrogen carbonate was added for additional dilution. Finally, 50 µL of this final diluted material was taken for testing.

Microtiter strips sufficient in amount for the standard and specimens were placed on the plate and 50 µL each of all standards or prepared specimens was placed in the wells. Then, 50 µL of diluted enzyme conjugate was added on each, followed by incubation at room temperature for 2 h. At the end of the incubation, the liquids within the wells were evacuated and the wells were washed with washing solution in an automated washer 3 times. Then, 50 µL of substrate and 50 µL of chromogen were placed in each well, followed by incubation for 0.5 h in dark at room temperature. At the end of the incubation, 100 µL of stopping solution was added and absorbance at 450 nm was assessed. The results were read over a calibration curve and multiplied with the dilution factor, which were 25.

RESULTS AND DISCUSSION

100 specimens of wheat flour commerced in Turkish Republic were analyzed using the ELISA method regarding amounts of AF, AFB1 and ochratoxin A. The amounts of AF, AFB1 and ochratoxin A in the specimens are displayed in Table-1 and whereas their contamination rates are displayed in Table-2.

TABLE-1
TOTAL AFLATOXIN, AFLATOXIN B1 AND OCHRATOXIN A CONCENTRATIONS IN THE WHEAT FLOUR SAMPLES*

Mycotoxin levels	Total aflatoxin	Aflatoxin B_1	Ochratoxin A
≤ 0.625 µg/kg	29/100 (29%) (< 1.75 µg/kg)**	43/100 (43%) (< 1.0 µg/kg)**	13/100 (13%) (< 0.625 µg/kg)**
0.625-1.00 µg/kg			12/100 (12%)
1.0-1.75 µg/kg		47/100 (47%)	23/100 (23%)
1.75-2.0 µg/kg	7/100 (7%)	5/100 (5%)	22/100 (22%)
2.0-3.0 µg/kg	45/100 (45%)	3/100 (3%)	30/100 (30%)
3.0-4.0 µg/kg	14/100 (14%)	0/100 (0%)	0/100 (0%)
> 4.0 µg/kg	5/100 (5%)	2/100 (2%)	0/100 (0%)

*Minimum detection limit of total aflatoxin, aflatoxin B1 and ochratoxin A are 1.75, 1.0 and 0.625 µg/kg, respectively; **Not detected.

TABLE-2
TOTAL AFLATOXIN, AFLATOXIN B1 AND OCHRATOXIN A CONTAMINATION IN THE WHEAT FLOUR SAMPLES AND THE SAMPLES EXCEEDING TURKISH, EC/CODEX AND US REGULATION

Mycotoxins	Samples analyzed	Positive samples (%)	Exceeding Turkish and EC/Codex regulations*		
			Number (%)	Range (µg/kg)	Mean (µg/kg) ± SE
Total Aflatoxin	100	71	5	5.08-7.30	5.952 ± 0.412
Aflatoxin B1	100	57	5	2.75-5.17	3.561 ± 0484
Ochratoxin A	100	87	–	–	–
Total	100	91	–	–	–

*Maximum limits of Turkish and EC/Codex regulation are 4, 2 and 3 µg/kg for total Aflatoxin, Aflatoxin B_1 and Ochratoxin A, respectively.

Among all specimens, only in 9 (9 %) did not have any of the analyzed mycotoxins above the lower limit of detection, whereas the other 91 (91%) had been contaminated with at least one of these mycotoxins. The AF and AFB1 contamination rates were found to be 71 and 57 %, respectively. The amounts of mycotoxins were found to be above the maximum allowed limits as determined by the Turkish Food Codex and Council of European Community, the AF levels (5.08-7.30 µg/kg) being excessive in 5 specimens and the AFB1 levels (2.75-5.17 µg/kg) being excessive in 5 specimens. Although the ochratoxin A contamination rate in the specimens of analysis was 87 %, none of these specimens had ochratoxin A levels above the maximum limit allowed.

Correlation analysis revealed an extremely significant correlation between the AF, AFB1 and ochratoxin A levels in the specimens ($p < 0.01$). The correlation coefficients were 0.871 between the AF and AFB1, 0.446 between AF and ochratoxin A and 0.381 between AFB1 and ochratoxin A.

Differing results have been reported regarding the contamination rates and amounts of wheat and wheat flour with AF and ochratoxin. Özturan *et al.*[37] have reported the AF, AFB1 and OTA contamination rates in 50 wheat flour specimens commerced in Erzurum as 37 (74 %), 37 (74 %) and 45 (90 %). In the same study, the amounts of specimens with levels above the maximum limits as determined by Turkish Food Codex were 9 (18 %) for AF, 8 (16 %) for AFB1 and 6 (12 %) for OTA. While Baydar *et al.*[38] have reported only 1 (4 %) of their specimens with levels found using the ELISA method above the maximum limits, Abdulkadar *et al.*[1] have conducted a study in Qatar using the HPLC method and have not found aflatoxin and ochratoxin in 4 specimens of wheat and 6 specimens of wheat flour. Giray *et al.*[15] have analyzed 41 specimens of wheat grown and consumed in different regions of Turkey regarding the levels of AFB1, AFB2, AFG1 and AFG2 by HPLC. The specimens were found to contain AFB1 (42 %) and AF (59 %) with levels of 10.4-144.2 and 10.4-643.5 ng/kg, respectively.

Zinedine *et al.*[17] have reported the ochratoxin contamination rates in Moroccan wheat specimens to be 40 %, while their mean contamination level was 0.42 µg/kg and maximum contamination level was 1.73 µg/kg. Wolff[39] has studied 6476 food specimens in Europe and reported the OTA contamination rate to be 57 %. The same author has reported the presence of OTA above 3 ppm in only 1.4 % of the 2374 specimens of grain and grain products (rye, wheat, barley, oats, maize, buckwheat and millet). Karagözlü and Karapinar[40] have analyzed 100 specimens of grain and reported OTA varying from 0.27 to 9.84 ppb in 4 specimens including 1 specimen of Noah's pudding wheat, 2 specimens of corn and 1 specimen of oatmeal. OTA was detected in 58 of the 115 specimens (limit of detection 0.066 µg/kg), with a mean OTA concentration of 0.219 µg/kg. One corn specimen contained a quantity of this mycotoxin surpassing the EU legal limit (5 µg/kget)[24].

The results of this study display lower values than those reported by Ozturan *et al.*[37], but above those reported by the other authors. The differences between reports may source from the variations of conditions during harvesting, transport, granulation and storage.

Wheat and wheat products are main source of food for Turkish population and daily intake of these products corresponds to at least 50 % of daily diet[15]. The contamination rates of AF, AFB1 and OTA detected in wheat flour specimens in this study are 71, 57 and 87 %, respectively and are quite high. Although the rates of specimens with levels exceeding the maximum legal limits are 5, 5 and 0 % for AF, AFB1 and OTA, respectively. It should be regarded that contaminations in wheat flour, which has an important place in the daily diet and is included within many nutrients, constitute a potential hazard for the community health. The producers and consumers should be informed about this hazard and the actions to be taken against it.

Implementation of food security and quality control systems and better agricultural and hygiene applications in all steps from harvest to consumption will provide reduction or abolishment of the community health hazards.

REFERENCES

1. A.H.W. Abdulkadar, A.A. Al-Ali, A.M. Al-Kildi and J.H. Al-Jedah, *J. H. Food Control*, **15**, 543 (2004).
2. M. Lobeau, S. De Saeger, L. Sibanda, I. Barna-Vetr´o and C. Van Peteghema, *Anal. Chim. Acta*, **538**, 57 (2005).
3. F.S. Chu, *Mutation Res.*, **259**, 291 (1991).
4. G. Devegowda, M.V.L.N. Raju, N. Afzali and H.V.L.N. Swami, Proceedings of the 14th Annual Symposium, Nottingham University Press, p. 241 (1998).
5. A.B.F. Bittencourt, C.A.F. Oliveira, P. Dilkin and B. Correa, *Food Control*, **16**, 117 (2005).

6. N.H.S. Ammidaa, L. Micheli and G. Palleschi, *Anal. Chim. Acta*, **520**, 159 (2004).
7. S. Xiulan, Z. Xiaolian, T. Jian, G. Xiaohong, Z. Jun and F.S. Chu, *Food Control*, **17**, 256 (2006).
8. International Agency for Research on Cancer-IARC. IARC monographs, 56, 359 (1993).
9. R.C. Garner and C.N. Martin, CRC Press, Boca Raton, p. 187 (1979).
10. M. Peraica, B. Radic, A. Lucic and M. Pavlovic, *Bull. World Hlth. Org.*, **77**, 754 (1999).
11. J.H. Williams, T.D. Phillips, P.E. Jolly, J.K. Stiles, C.M. Jolly and D. Aggarwal, *Am. J. Clin. Nutr.*, **80**, 1106 (2004).
12. K.V.A.R. Krishuamachari, R.V. Bhat, V. Nagarajan and T.B.G. Tihak, *Indian J. Med. Res.*, **63**, 1036 (1975).
13. A. Ngindu, P.R. Kenya and D.M. Ocheng, *Lancet*, **1**, 1346 (1982).
14. R.G. Hendrickse, J.B.S. Coulter, S.M. Lamplugh, S.B.J. Macfarlane, T.E. Williams, M.I. Omer and G.I. Suliman, *Br. Med. J.*, **285**, 843 (1982).
15. B. Giray, G. Girgin, A.B. Engin, S. Aydin and G. Sahin, *Food Control*, **18**, 23 (2007).
16. Turkish Food Codex, Official Gazzette 23.09.2002/24885, Ankara (2002).
17. A. Zinedine, C. Brera, S. Elakhdari, C. Catano, F. Debegnach, S. Angelini, B. De Santis, M. Faid, M. Benlemlih, V. Minardi and M. Miraglia, *Food Control*, **17**, 868 (2006).
18. R. Liu, Z. Yu, Q. He and Y. Xu, *Food Control*, **18**, 872 (2007).
19. International Agency for Research on Cancer-IARC. IARC monographs, 73, 1 (1999).
20. S. MacDonald, T.J. Prickett, K.B. Wildey and D. Chan, *Food Add. Contamin.*, **2**, 172 (2004).
21. G.A. Lombaert, P. Pellaers, G. Neumann, D. Kitchen, V. Huzel, R. Trelka, S. Kotello and P.M. Scott, *Food Add. Contamin.*, **6**, 578 (2004).
22. E. Petzinger and A. Weidenbach, *Livestock Production Sci.*, **76**, 245 (2002).
23. A.J. Alldrick, *Food Add. Contamin.*, **13**, 27 (1996).
24. C. Araguas, E. Gonzalez-Penas and A. Lopez de Cerain, *Food Chem.*, **92**, 459 (2005).
25. European Commission, Official Journal of the European Communities, L75/18-20, (2002).
26. European Commission, Official Journal of the European Union, L106/3-5, (2004).
27. L.M. Soares and D.B. Rodriguez-Amaya, *J. Assoc. Off. Anal. Chem.*, **6**, 1128 (1985).
28. C. Dall'Asta, G. Galaverna and A. Dossena, *R. J. Chromatogr. A*, **1-2**, 275 (2004).
29. L. Solti, F. Salamon, I. Barna-Vetro, A. Gyongyosi, E. Szabo and A. Wolfling, *J. Anal. Toxicol.*, **1**, 44 (1997).
30. R.J. Garcia-Villanova, C. Cordon, A.M.G. Paramas, P. Aparicio, M.E.G. Rosales, *J. Agric. Food Chem.*, **24**, 7235 (2004).
31. O. Koksal, National Nutrient-Health and Food Consumption Survey of Turkey, Ankara, Turkey (1990).
32. S. Duru and G. Sahin, *Microbiol. Bull.*, **13**, 371 (1979).
33. S.E. Simsek and N. Bozkurt, *Tur. Bull. Hygiene Exp. Bio.*, **49**, 139 (1992).
34. G.Z. Omurtag and D. Yazicioglu, *J. Environ. Sci. Health*, **35**, 797 (2000).
35. F. Nizam and H. Oguz, *Food Add. Contamin.*, **20**, 654 (2003).
36. G.Z. Omurtag and D. Beyoglu, *Food Add. Contamin.*, **20**, 405 (2003).
37. K. Özturan, C. Unsal, Y. Karakaya, M. Atasever, Z.G. Ceylan, M. Atasever and K. Demirkaya, 2006. 2. Ulusal Veteriner Gida Hijyeni Kogresi, Istanbul, Turkey (2006).
38. T. Baydar, A.B. Engin, S. Aydin, G. Girgin and G. Sahin, Abstract book of 5th International Congress of Turkish Society of Toxicology, Antalya, Turkey, p. 179 (2003).
39. J. Wolff, *Arch. Lebensmittelhyg.*, **51**, 85 (2000).
40. N. Karagözlü and M. Karapinar, *Turk. J. Biol.*, **24**, 561 (2000).

(*Received*: 11 December 2006; *Accepted*: 12 March 2007) AJC-5519