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

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ABSTRACT

This study was designed to assess the total aflatoxin (total AF), aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) levels in 100 samples of wheat flour obtained from 7 different producing regions of Thrace by the microtitre plate enzyme-linked immunosorbent assay (ELISA). According to the EC Regulation and the Turkish Food Codex, the total AF, AFB_1, and OTA levels were between minimum detection limit (0.05, <1.0, 0.025 μg/kg) and the maximum tolerable limit (4, 2, 3 μg/kg) in 43, 18 and 53 samples, respectively. Whilst 2, 2 and 28 samples had unacceptable contamination levels higher than the maximum tolerable limits, respectively. This is the first study in Thrace, Turkey on account of OTA, and the presence of OTA in 81% of analysed samples and taking part in South-Eastern Europe of sampling area would be indirectly related to Balkan Endemic Nephropathy and also be of risk to public healthy.

Key words: total aflatoxin, aflatoxin B_1, ELISA, ochratoxin A, Turkey, wheat flour

INTRODUCTION

Invasion of cereal grain by fungi is frequently associated with a substantial risk of contamination by mycotoxins. Some mycotoxins known to exert toxic effect on human and animal health are constantly increasing so that the legislative provision is taken to control their presence in food and feed. Extensively mycotoxins are aflatoxins (AFs), OTA and *Fusarium* toxins[(1)].

AFs are toxic secondary metabolites produced by species of *Aspergilli*, especially *A. flavus*, *A. parasiticus* and *A. nomius*. *A. flavus* produces aflatoxin B_1 and B_2, while the two other species produce both aflatoxin B and G. AFs are acute toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic factors. Liver is the most affected organ by carcinogenic and toxic AFs[(2)]. AFB_1 is the most known potential hepatocarcinogen in mammals and it is classified by the International Agency of Research on Cancer (IARC) as Group 1 carcinogen[(3)]. The maximum tolerable limits for AFs allowed in cereals in EC Regulation[(4)] and Turkish Food Codex[(5)] have been set at 4 μg/kg for total AF and 2 μg/kg for AFB_1.

OTA is a naturally occurring mycotoxin which is produced by several species of the genera *Aspergillus* (e.g. *A. ochraceus*) and *Penicillum* (e.g. *P. verrucosum*). It has been shown to be hepatotoxic, nephrotoxic, teratogenic and carcinogenic to animals and has been classified as a possible human carcinogen (category 2B) by IARC[(3)]. Moreover, OTA is suspected to be the causing agent involved in Balkan Endemic Nephropathy (BEN), a kidney disease in South-Eastern Europe[(6)]. EC Regulations[(7)] and Turkish Food Codex[(5)] have set OTA maximum tolerable limits of 3 μg/kg for all products derived from cereals and 5 μg/kg in cereals.

Cereals have been grown in Anatolia (Turkey) for thousands of years and are a part of life in rural areas. Wheat is an important crop traditionally, covering 9.5 million hectare and has made it the eighth largest wheat producing area worldwide[(8)]. Likewise, it has significant impact in Turkish people's diet, with an annual consumption of about 250 kg/per head[(9)]. The major wheat growing regions are Central Anatolia, Thrace and South-Eastern Anatolia. Thrace Region of Turkey has a tremendous potential for agricultural facilities due to its suitable climate and soil features. The statistical results for 2005 showed that, 10-15% of wheat and 55-60% of rice of Turkey were produced in this region[(10)]. This region has also a geographical importance due to its location, southwest of Europe, and being a transpass corridor between Europe and Turkey.

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Commonly used methods for the determination of AFs are high performance liquid chromatography (HPLC) with fluorescence detection, thin layer chromatography (TLC) and immunochemical methods such as ELISA[11]. ELISA test kits are well-favored as high through-put assays with low sample volume requirements and often less sample clean-up procedures compared to conventional methods such as HPLC and TLC[12].

This study was aimed to determine the total AF, AFB_1 and OTA levels in wheat flour, in the respect of maximum tolerable limits in EC Regulation and Turkish Food Codex and to demonstrate the importance of contamination regarding the public health.

MATERIALS AND METHODS

I. *Material*

A total of 100 wheat flour samples obtained from 7 different producing points (Figure 1) in Thrace were analyzed in the summer 2006. The samples of wheat flour were obtained from the factories near to the producing points. One kilogram of each sample was brought to the laboratory at 4-6°C and analysed. Microtitre plate ELISA reader (ELX 800, Bio-tek Inst.) and total AF, AFB_1 and OTA test kit (Ridascreen, r-biopharm, Germany) were used to run ELISA analyses.

II. *Total AF Analysis*

The Ridascreen ® total AF test (Art. no: R4701) is a competitive enzyme immunoassay for the quantitative analysis of aflatoxin residues in cereals and feed.

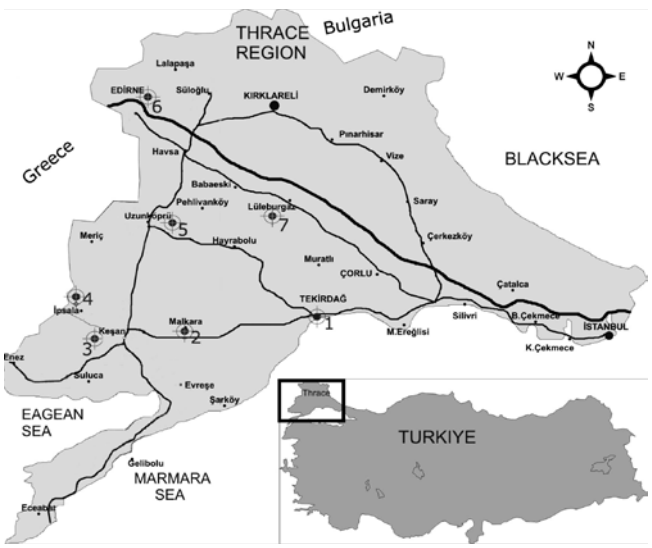

Figure 1. Location of study areas and wheat flour sampling points Thrace, Turkey. (1. Tekirdag; 2. Malkara; 3. Kesan; 4. Ipsala; 5. Uzunkopru; 6. Edirne; 7. Luleburgaz)

Ten grams of wheat flour sample was weighed into a screw-top glass vial and 50 mL of 70% (v/v) methanol/water was added and mixed using stomacher for 10 min at room temperature. Extract was filtered using filter paper (Whatman no. 1), 100 µL of the filtrate was diluted with 600 µL of sample dilution buffer, and 50 µL of diluted filtrate was transferred to each well in the microplate.

III. *ELISA of Total AF Test Procedure*

Total AF standards and the prepared sample extracts were added to wells in duplicate. The concentrated total AF enzyme conjugate was diluted 1:11 with the corresponding sample buffer. Fifty microliter of standard solutions (0, 0.5, 1.5, 4.5, 13.5 and 40.5 µg/kg AFB_1) and prepared samples in individual wells were added. Fifty microliter of diluted enzyme conjugate (urea peroxide) and 50 µL of diluted antibody solution (1:11 in buffer) were added to each well. The liquid was removed completely the wells. The washing procedure using 250 µL of water was repeated two times. Fifty microliter of urea peroxide and 50 µL tetramethyl-benzidine were added to each well and incubated for 30 min at room temperature in the dark. Then, 100 µL of the stop reagent (1 N H_2SO_4) was added to each well and the absorbance was measured at 450 nm in an ELISA reader. The detection limit of the total AF test was 0.05 µg/kg. The mean recovery rate was determined as 85%[13].

IV. *AFB_1 Analysis*

The Ridascreen® AFB_1 30/15 test (Art. no: R1211) is a competitive enzyme immunoassay for the quantitative analysis of AFB_1 in cereals and feed[14].

Ten grams of wheat flour sample and 50 mL of 70% methanol were mixed using stomacher (Lab-blender 400 Seward, London, UK) for 3 min. The extract was filtered (Whatman no. 1) and 1 mL of the filtrate was then diluted with 1 mL water. Fifty microliter of the diluted filtrate per well was used in the test.

V. *ELISA AFB_1 Test Procedure*

Fifty microliter AFB_1 standard solutions (1, 5, 10, 20 and 50 µg/kg AFB_1 in methanol/water) and 50 µL prepared test samples were added into individual well of micro-titer plate in duplicate. Subsequently, 50 µL enzyme conjugate (urea peroxide) and 50 µL anti-aflatoxin antibody solution were added to each well and incubated for 30 min at room temperature in the dark. The wells were washed with 250 µL washing buffer (10 mM PBS-0.05% Tween 20, pH 7.4) three times. One hundred microliter of the stop reagent (1 N H_2SO_4) was added to each well and the absorbance was measured at 450 nm in an ELISA reader. The lower detection limit of the AFB_1 30/15 test was 1 µg/kg and the recovery rate was 80-100%[14].

VI. *OTA Analysis*

The Ridascreen® OTA test (Art. no: R1301) is a competitive enzyme immunoassay for the quantitative analysis of OTA in cereals, feed, beer and pig serum[15].

Two grams of sample was putted into a centrifugal screw cap tube. Four milliliter of distilled water and 0.2 mL of α-amylase solution, by dissolving 0.5 g/mL of porcinepancrease (1.000.000 units, Sigma A-3176) in 1 mL PBS, were added. The solution was mixed for 20 min at room temperature and was added 5 mL of 1 N HCl. The solution was mixed for another 5 min and was added 10 mL of extra pure (≥ 99.0%) dichloromethane. The vial was centrifuged for 15 min (3500 ×g, 15°C). Then, the upper aqueous layer was removed completely up to the sample cake and then discarded. Entire dichlormethane layer was filtered by using a paper filter to separate the sample cake and the filtered dichlormethane was collected in a new centrifugal screw cap tube. The equivalent volume of 0.13 M NaHCO3 (pH 8.1) buffer was added and centrifuged vigorously for 15 min (3500 ×g, 15°C). One hundred microliter of upper aqueous phase was diluted with 400 µL of 0.13 M $NaHCO_3$ (pH 8.1) buffer. Fifty microliter was then used per well in the assay.

VII. *ELISA OTA Test Procedure*

Fifty-microliter standard solutions (0, 25, 75, 225, 675 and 2025 ng/kg OTA in aqueous solution) and prepared samples were added into individual well in duplicate. The concentrated OTA enzyme conjugate was diluted 1:11 with the corresponding sample buffer. Fifty microliter of diluted enzyme conjugate was added to each well. The mixture was mixed gently by rocking the plate manually and incubated for 120 min at room temperature in the dark. The wells were washed 3 times. Fifty microliter of urea peroxide and 50 µL of chromogen (tetramethyl-benzidine) were added to each well and incubated for 30 min at room temperature in the dark. One hundred microliter of the stop reagent (1 N H_2SO_4) was added to each well and the absorbance at 450 nm was measured against the blank. The recovery rate of OTA was 85%[15].

VIII. *Evaluation*

The mean values of the absorbances for the standards and the samples were evaluated according to the Rida® Soft Win program (RIDAVIN.EXE) distributed by Ridascreen (R-Biopharm).

RESULTS AND DISCUSSION

Turkey has been encountered the AF contamination problem in different foods exported and/or consumed in the country[16]. Although there are some studies on the level of mycotoxins in different foods consumed and produced in Turkey[17-20]. Wheat that is susceptible to these fungi infections through its growth, harvest, transport and storage, is the most staple food in our country.

Forty five percent of the wheat flour samples were found to contain total AF in the levels of 0.05-14.01 µg/kg (average level 0.79 ± 0.99 µg/kg) (Table 1). However, only 2% samples were exceeded to the maximum tolerable limits for total AF (4 µg/kg) of EU Regulation[4] and Turkish Food Codex[5].

Abdullah *et al.*[21] analysed 83 wheat flour samples in Malaysia and reported that 21.7% of the samples were contaminated with AFs, 1.2% of wheat flour samples were positive for AFB_1, at a concentration of 25.6 µg/kg, 4.8% of were positive for AFB_2, at the concentrations ranging from 11.3 to 252.5 µg/kg, 3.6% of were positive for AFG_1 at the concentrations ranging from 25.0 to 289.4 µg/kg and 13.3% of were positive for AFG_2 at the concentrations ranging from 16.3 to 436.3 µg/kg. In another study, Giray *et al.*[20] analysed 41 wheat samples in Turkey and 59% of samples were found to be contaminated with total AF and the percentage of positive samples for AFB_1,

Table 1. The total AF levels of wheat flour samples in Thrace

Total AF	The sampling points							General total samples (Mean ± standard error)[e]
	1 (Tekirdag)	2 (Malkara)	3 (Kesan)	4 (Ipsala)	5 (Uzunkopru)	6 (Edirne)	7 (Luleburgaz)	
< 0.05 µg/kg[b]	3	4	14	3	10	13	8	55 (0)[d]
0.05 - 4.0 µg/kg	12	6	6	1	9	7	2	43 (1.71 ± 0.17)
> 4.0 µg/kg[c]	-	-	-	1 (4.9)[a]	1 (14.0)[a]	-	-	2 (9.45 ± 4.55)
Total analysed samples	15	10	20	5	20	20	10	100 (0.79 ± 0.99)

[a]The highest of total AF level (µg/kg).
[b]Under the minimum detection limit.
[c]The maximum tolerable limit of total AF.
[d]The total Af values, which were determined under minimum detection limit, calculated as "0".
[e]Mean AF level and standard error.

AFB_2, AFG_1, and AFG_2 were 42, 12, 37 and 12%, respectively. In our study, the total AFs were lower than those reported by Giray *et al*.[20] and Abdullah *et al*.[21]. These results showed that total AF occurrence in wheat flour in Thrace (Turkey) could not be a relatively critical point, regarding quality of wheat flour.

AFB_1 levels in 20 (20%) of 100 samples were in higher levels than the detection limit (0.025 μg/kg) and AFB_1 levels in 2 (2%) wheat flour samples (Table 2) were found to be higher than the legal limits of EC Regulation[4] and Turkish Food Codex[5] (> 2 μg/kg). The highest AFB_1 level in the samples was 12.2 μg/kg (average level 0.48 ± 0.21 μg/kg). In a study performed in Croatia, AFB_1 were detected in a mean level of 16.3 μg/kg in 475 wheat grain samples and the mean level of 11.3 μg/kg in 238 wheat grain being milled to flour samples[22]. Ayalew *et al*.[23] analysed mycotoxins in 352 cereal samples (wheat, barley, sorghum, and teff grass). AFB_1 was detected in 8.8% of the 352 cereal samples at the concentrations ranging from trace to 26 μg/kg and 4.2% of 120 wheat samples were contaminated with AFB_1. The maximum AFB_1 levels in our study were lower than those reported by Giray *et al*.[20], Halt[22] and Ayalew *et al*.[23], but were higher than that of Abdullah *et al*.[21].

OTA is predominantly found in cereal grains, cereal products, legumes, oilseed, coffee beans and feed[24]. In our study, the levels of OTA were determined to be ranging from 0.025 to 10.5 μg/kg (average level 2.07 ± 0.08 μg/kg) (Table 3). Although these amounts are under the allowed level of 3 μg/kg for OTA in EC Regulation[4] and Turkish Food Codex[5] limits, it was seen that 81% of the samples were contaminated with OTA.

Zinedine *et al*.[25] reported that a total of 60 samples, consisting of 20 wheat, 20 corn, and 20 barley samples for contamination with OTA in Morocco and found that 40% of 20 wheat samples were contaminated with OTA and the maximum levels of contamination was 1.73 μg/kg. Ayalew *et al*.[23] reported that 23.4% of 107 wheat samples were contaminated with OTA in Ethiopia and the highest levels of OTA was 66.0 μg/kg. In another study, samples of cereals (wheat, barley and corn) obtained from Spain were analysed for OTA contamination and OTA was detected in 58 of the total 115 samples, in the mean concentration of OTA of 0.219 μg/kg[26]. Muscarella *et*

Table 2. The AFB1 levels of wheat flour samples in Thrace

AFB_1	The sampling points							General total samples (Mean ± standard error)[e]
	1 (Tekirdag)	2 (Malkara)	3 (Kesan)	4 (Ipsala)	5 (Uzunkopru)	6 (Edirne)	7 (Luleburgaz)	
< 1.0 μg/kg[b]	11	7	18	3	16	13	8	80 (0)[d]
1.0 - 2.0 μg/kg	3	3	2	2	3	7	2	18 (2.16 ± 0.73)
> 2.0 μg/kg[c]	1 (3.0)[a]	-	-	-	1 (12.2)[a]	-	-	2 (7.60 ± 4.60)
Total analysed samples	15	10	20	5	20	20	10	100 (0.48 ± 0.21)

[a]The highest of AFB1 level (μg/kg).
[b]Under the minimum detection limit.
[c]The maximum tolerable limit of AFB1.
[d]The AFB1 values, which were determined under minimum detection limit, calculated as "0".
[e]Mean AF level and standard error.

Table 3. The OTA levels of wheat flour samples in Thrace

OTA	The sampling points							General total samples (Mean ± standard error)[e]
	1 (Tekirdag)	2 (Malkara)	3 (Kesan)	4 (Ipsala)	5 (Uzunkopru)	6 (Edirne)	7 (Luleburgaz)	
< 0.025 μg/kg[b]	1	-	2	3	4	6	3	19(0)[d]
0.025 - 3.0 μg/kg	5	5	12	2	11	11	7	53 (1.39 ± 1.07)
> 3.0 μg/kg[c]	9 (6.9)[a]	5 (9.8)[a]	6 (10.5)[a]	-	5 (7.3)[a]	3 (4.4)[a]	-	28 (4.78 ± 0.38)
Total analysed samples	15	10	20	5	20	20	10	100 (2.07 ± 0.08)

[a]The highest of OTA level (μg/kg).
[b]Under the minimum detection limit.
[c]The maximum tolerable limit of OTA.
[d]The OTA values, which were determined under minimum detection limit, calculated as "0".
[e]Mean AF level and standard error.

al.[27] reported that 95 durum wheat, 80 maize and 85 barley samples were contaminated with OTA in Italy. At the same time, 15 out 95 wheat flour samples (15.8%) were also contaminated with OTA in the range of 0.2-3.9 μg/kg. It was seen that OTA percentages of wheat flour samples in our study were higher than those in the other studies[23, 25-27].

In several areas of Eastern Europe, where chronic exposure to OTA occurs, involvement of this mycotoxin in the cancer aetiology of the urinary system, and in kidney pathologies typical of BEN has been suspected[28]. Studies on the correlation between OTA and BEN have shown higher OTA contamination levels in cereals from endemic areas as compared to cereals from non-endemic areas[27-29]. In our study, detection of OTA in 81% of wheat flour samples potentially pose a risk to public healthy.

In our study, total AF levels of 2 samples obtained from 4th and 5th points in Thrace Region and AFB_1 levels of total 2 samples obtained from 1st and 5th points in Thrace exceeded the legal limits[4,5]. The highest total AF and AFB_1 levels in the samples were 14.0 μg/kg and 12.2 μg/kg (sampling point of 5th). OTA were detected in all of the sampling points. The maximum tolerable limit for OTA did not exceed in 4th and 7th points of sampling. The highest OTA level in the samples was 10.5 μg/kg (sampling point 3). Giray *et al.*[20] made a regional comparison about the presence of total AF and AFB_1 in wheat samples obtained from different regions of Turkey. The researchers reported that total AF and AFB_1 were detected in 38% of wheat samples obtained from Marmara (included Thrace) and Aegean Regions and the levels ranged 10.4-643.5 ng/kg and 10.4-135.9 ng/kg, respectively. The results were not in accordance with our findings. This could be related with that the number of samples analysed by them was insufficient for representing the region when the size of region was taken into consideration and that the wheat samples were obtained in different seasons.

ELISA is rapid, simple, specific, and sensitive and have become the most common quick methods for the detection of mycotoxins in food and feeds[12]. However, since the antibodies produced often cross-reactivity to compounds similar to mycotoxins, an extensive study on the accuracy and precision of the ELISA method over a range of commodities is essential and critical before commercially used[30]. In a study, ELISA test kit was validated an for the detection of total AF in grain and grain products (wheat, corn meal, milled rice, corn, etc.) by comparison with HPLC[30]. It is shown that the Agraquant® total AF ELISA test kit is effective in measuring total AF of several appropriate commodities with the its quantitation range of 4-40 μg/kg and good accuracy and precision for grain and grain products. In another study was validated a competitive direct ELISA with a reference HPLC and other methods including a minicolumn method and the VICAM Aflatest® system for AFB_1 in peanuts[31]. These researches clearly declared that ELISA was acceptable as an analytical method and despite the high expected sampling variation and an acceptable correlation between ELISA and HPLC for AFB_1 analysis was obtained when different sample extracts were used. More importantly, the accuracy of the ELISA was validated against a reference method applying HPLC/FLD, and showed an exceptionally good correlation between ELISA and HPLC when the same sample extracts were used.

OTA in high levels could cause the increase of BEN and urethra, renal and pelvis tumors in the region. Our study is the first report on the co-occurrence of OTA in cereals from Thrace of Turkey.

The data confirm that cereals, and cereal derived products from Mediterranean countries could be affected by mycotoxin contamination due to the climatic conditions, especially humidity and temperature of the region. Risk originated from mycotoxins should not be omitted in point of public healthy.

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