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Investigation of aflatoxin contamination in maize flour consumed in Giresun, Turkey

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RESEARCH ARTICLE

Abstract

Keeping mould away from food and agricultural products is one of the major difficulties encountered in cultivation areas. Studies on mycotoxin contamination in agricultural products consumed as food in Turkey have been accelerated by the detection of aflatoxin and aflatoxigenic mould in nuts, pistachios, red pepper and dried figs. However, not much research has been performed to reveal the problems related to aflatoxin in maize flour in Turkey. In this study, maize flour samples were obtained from different vendors at bazaars (marketplace) in the province of Giresun, and the aflatoxin level was determined by the high performance liquid chromatography. In 11 of the 69 (16%) samples that were analysed, aflatoxin was detected in the range of 0.379-24.54 μg/kg total aflatoxin. The total aflatoxin level in one sample and the aflatoxin B_1 level in two samples were found to be above the permissible toxin limits of Turkey and the European Union. The data obtained in this study are important in terms of human health, because aflatoxin B_1 and G_1 are powerful carcinogens known to cause liver cancer. Maize is known to be contaminated by aflatoxin. Moreover, as the prevalence of this in Turkish maize from the Black Sea region has not been previously studied, these results indicate the extent of human exposure from consuming locally produced Turkish maize.

Keywords: aflatoxin, maize flour, mycotoxin

1. Introduction

Among cereals that are some of the most important foodstuffs produced and consumed in Turkey, wheat is dominant and with barley has the largest acreage grown with maize coming third (Sahin, 2001). However, maize is widely consumed in Turkey both by animals and humans with the latter use being popular as flour in Northern part of the country.

The contamination of foodstuffs with aflatoxin is a major problem worldwide. Keeping food and feed ingredients away from fungal growth is one of the major difficulties encountered in cultivated areas, especially in humid regions. The Black Sea region is an area with high rainfall and a climate that is hot in summer and warm in winter. These conditions favour the growth of mould in food. The most important factors causing the growth of mould in food are pH, water activity (a_w), temperature and atmosphere conditions. These moulds may be capable of producing mycotoxins, which are natural secondary metabolites, which may be produced on agricultural commodities in the field under a wide range of climatic conditions and during storage (Krska, 2009). Aflatoxins are considered to be one of the main types of mycotoxins produced by mould of the genus *Aspergillus*, especially *Aspergillus flavus* and *Aspergillus parasiticus* (Milhome *et al*., 2014; Perrone *et al*., 2014; Torlak and Akan, 2013). Some factors such as production conditions, humidity, temperature, storage conditions, moisture, and physical damage accelerate the formation of aflatoxin in food (Coppock and Christian, 2007; Giorni *et al*., 2008; Ribeiro *et al*., 2006; Wu *et al*., 2009). The most important aflatoxin types are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2). In particular, they can be found in products such as maize, grain, flour, bread, species dried under poor conditions, figs, grapes, nuts, cotton, sunflower seeds, and carried over from animals in meat, milk and eggs. These toxins are stable compounds that do not completely degrade at high temperatures. Among them, AFB_1 shows the highest toxicity (Fallah *et al*., 2014; Ibáñez-Vea *et al*., 2011; Mateo

et al., 2011; Set and Erkmen, 2010) and is not only toxic but also carcinogenic to humans and animals. According to the European Union and Turkish Food Codex, the maximum aflatoxin limits permitted in maize are 10 µg/kg for total aflatoxins and 5 µg/kg AFB_1 (EC, 2006; Turkish Food Codex, 2002). Studies into contamination of mycotoxin in agricultural products consumed as food in Turkey have accelerated with the detection of aflatoxin and aflatoxigenic mould in nuts and nut products (Aycicek *et al*., 2005; Baltaci *et al*., 2012; Basaran and Ozcan, 2009; Bircan *et al*., 2008; Gurses, 2006), pistachios (Denizel *et al*., 1976; Gurses, 2006; Set and Erkmen, 2010), dried figs (Bircan *et al*., 2008; Heperkan *et al*., 2012; Isman and Biyik, 2009), red peppers (Alpsoy *et al*., 2013; Aydın *et al.*, 2007; Erdogan, 2004; Set and Erkmen, 2010), cheese (Aycicek *et al*., 2005; Aydin *et al.*, 2007; Gul and Dervisoglu, 2014; Kav *et al.*, 2011; Yaroglu *et al.*, 2005) and wheat flour (Aydın *et al.,* 2008; Giray *et al.*, 2007), but studies of maize (Giray *et al.*, 2009; Nizamlioğlu and Oguz, 2003; Oruc *et al*., 2006) and maize flour (Algül and Kara, 2014) are insufficient to ascertain if there is any problem in Turkey. Many researchers have also been performed to determine aflatoxin contamination levels in maize in different countries (Karami-Osboo *et al.*, 2012; Krout-Greenberg *et al*., 2013; Perrone *et al*., 2014; Pleadin *et al.*, 2014; Saima *et al.*, 2013; Trung *et al.*, 2008).

The fact that the coastal areas of the Black Sea region in Turkey receive abundant rainfall in summer has led to maize replacing the production of wheat. For this reason, maize flour is consumed as a staple food, especially bread (Şahin, 2001). The high incidence of cancer in the Black Sea region brings to our minds the question of whether it stems from the presence of aflatoxin in the products consumed in the region. In addition, there is very little information on the presence of aflatoxin in maize flour. Purpose of this study was to determine levels of aflatoxins in maize flour which taken from bazaars (marketplace) in the province of Giresun, Turkey.

2. Materials and methods

Maize flour samples

In this study, 69 maize flour samples were randomly collected as 5-6 samples monthly within a year from bazaars in the province of Giresun in the Black Sea region of Turkey. These samples consisted of homemade flour that had been grown, milled and sold in bazaars by peasant farmers. No particular preference was used in selecting samples or locations. Maize flour samples of at least 1 kg were transported to the laboratory in sterile polyethylene bags under cold conditions and preserved at -20 °C until the analyses. All the samples were analysed individually (without subsampling) for aflatoxin content tests.

Aflatoxin analysis

The analysis was performed according to AOAC official method 991.31 (AOAC, 1991), which has international validity in aflatoxin analysis.

Chemicals and reagents

Standard solution of aflatoxin dissolved in methanol (Supelco, Bellefonte, PA, USA) was used in the preparation of calibration curves. It consisted of AFB_1, AFB_2, AFG_1, and AFG_2 in concentrations of 1000, 300, 1000, and 300 ng/ml, respectively. Standard solution of aflatoxin (Aflastandard; R-Biopharm, Madrid, Spain) was used in sample recovery experiments. The stock standard of aflatoxin is sold as a 1000 ng/ml concentration of a methanol solution. It consists of 250 ng/ml AFG_1, AFG_2, AFB_1, and AFB_2 type aflatoxins. HPLC gradient grade methanol and acetonitrile (ACN), nitric acid 65%, potassium bromide and sodium chloride were purchased from Merck (Darmstadt, Germany). AflaTest®-P immunoaffinity columns (IAC) with 1 ml volume were purchased from VICAM (Milford, MA, USA) for cleanup and isolation of aflatoxins extracted from maize flour samples.

Extraction process

Maize flour samples (25 g) were carefully weighed and mixed with 5 g of sodium chloride and 125 ml of extracting agent ACN/H_2O (70:30, v/v) into a blender jar. After blending and mixing for 2 min at high speed, the extract was filtered through Whatman No. 4 filter paper (Whatman International, Maidstone, UK), 15 ml was removed and 30 ml of water was added. It was mixed thoroughly and the extract was filtered through Whatman No. 4 filter paper. Finally, 15 ml of the reconstituted extract were passed through a 'AflaTest®-P' IAC at a flow rate of 2 ml/min. The column was washed with two aliquots of 10 ml ultrapure water at a flow rate of 5 ml/min, and the aflatoxin were slowly released from the antibody using 1 ml of methanol and eluted with 1 ml ultrapure water in vials. Vital was fully mixed in a vortex and made suitable for the high performance liquid chromatography (HPLC).

Analysis of extract for aflatoxins

Analysis was performed using a HPLC 1100 series (Agilent Technologies, Barcelona, Spain) fitted with an auto-sampler and a fluorescence detector operated at an excitation wavelength, of 360 nm and an emission wavelength of 430 nm. The mobile phase was a mixture of water-acetonitrile-methanol (6:2:3, v/v/v) with a flow rate of 1.0 ml/min. The chromatographic separation was performed using a reverse phase ODS-2 column. The column temperature was 20-25 °C. The injection volume was 100 µl.

Validation of the analytical method

In the analysis of maize flour, the method of AOAC 991.31, which is a validated method for aflatoxin analysis was used. The retention times of AFB_1, AFB_2, AFG_1 and AFG_2 were 10.8, 9.3, 7.9, and 6.9 min, respectively. The peaks were found to be quite symmetrical and sharp. There was no interference.

Linearity

Linearity was estimated by injecting triplicate aflatoxin standard solutions at concentrations of 1.00, 2.00, 4.00, 8.00, 12.0, 16.0 and 20.0 ng/ml for AFB_1 and AFG_1, and 0.30, 0.60, 1.20, 2.40, 3.60, 4.80 and 6.00 ng/ml for AFB_2 and AFG_2, respectively. The concentration of the samples was within the range of calibration. Only one of the maize flour samples exceeded the calibration range and was reanalysed after a ten-fold dilution. Linear correlation coefficients were found to be >0.999 for all the aflatoxin types. The relative standard deviation (RSD) values were <1%. The values belonging to the calibration curves are shown in Table 1.

Accuracy and precision

Recovery and repeatability analyses were conducted in a maize sample that did not contain any toxin by the addition of an aflatoxin standard obtained from R-Biopharm (4 ng/ml) containing 1 ng/ml of each toxin (AFG_2, AFG_1, AFB2 and AFB_1). The spike samples were studied as 10 parallels and two injections each. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to visual evaluation (empirical methods) (ICH, 2005; Magnusson and Örnemark, 1998; NATA, 2013). LOD and LOQ values calculated by the analysis of spike samples containing 0.25 µg/kg from each toxin and totally 1 µg/kg aflatoxin are shown in Table 2. The recovery rates were in the range of 90.7-102.6% in the order of $AFG_1 > AFB_1 > AFG_2 > AFB_2$. These values are within the acceptable values of AOAC (2013) and the Codex Alimentarius (1993). The AOAC guideline for acceptable recovery at the 10 µg/kg level is 70-125% and the Codex guideline for acceptable recovery at the 10-100 µg/kg level is 70-110%. A total of 60-120% is the acceptable recovery rate at the 1-10 µg/kg level (AOAC, 2013; Codex Alimentarius, 1993). The RSD percentage values were quite low. These results show that this method is suitable for aflatoxin analysis in maize flour.

Table 1. Summary of calibration curve parameters.[1]

Aflatoxin	Regression equation	R^2	%RSD
B_1	$y = 2.50487x - 1.20302e^{-1}$	0.99993	0.10709
B_2	$y = 3.97424x - 2.21820e^{-2}$	0.99997	0.03409
G_1	$y = 1.34294x - 1.93232e^{-2}$	0.99997	0.03691
G_2	$y = 1.40545x - 2.66917e^{-2}$	0.99983	0.02796

[1] R^2 = linear correlation coefficient; RSD = relative standard deviation.

3. Results and discussion

In this study, a total of 69 maize flour samples were studied and in 11 (15.9%) samples, aflatoxin was detected above the detection limit. No toxin was found to be present in the remaining samples. The number of samples by month and the concentrations of each aflatoxin and aflatoxins detected in positive samples are shown in Table 3.

The aflatoxins levels varied in the range of 0.379-24.54 µg/kg. According to Turkish Food Codex and European limits, the maximum residue limits for AFB_1 and total aflatoxins are 5 and 10 µg/kg, respectively (EC, 2006; Turkish Food Codex, 2002). Total aflatoxins in one sample and AFB_1 in two samples were found to be above those limits. AFB_1 was determined in all contaminated samples and was found to be in the range of 0.280-22.65 µg/kg. In all samples, the AFB_1 levels were found to be higher than those of AFB_2. AFB_2 was found in only four samples in the range of 0.194-1.890 µg/kg. AFG_1 was found in only one sample at 0.46 µg/kg. This sample was found to contain both AFB_1 and AFG_1, with the AFG_1 levels being higher than those of AFB_1. AFG_2 was not found in any of the samples in which the aflatoxin analysis was performed. These results are consistent with that of previous studies on maize flour by Algül and Kara (2014) and Luo *et al.* (2014). Algül and Kara (2014) reported that AFB_1 and AFB_2 levels were increasing, that AFG_1 was found at lower levels and that AFG_2 was found at the lowest levels. In the other study, AFB_2, AFB_1 and AFG_1 types were all found in the maize flour, while AFG_2 was not detected (Luo *et al.*, 2014).

There are a small number of studies on the occurrence of aflatoxin in the maize flour and maize samples in Turkey. Oruc *et al.* (2006) determined that the AFB_1, AFB_2, $AFG_1$and AFG_2 in the maize samples were obtained from different regions of Turkey (n=19) and imported material from USA (n=7). The aflatoxin levels were in the range of

Table 2. Validation studies in maize flour analysis.[1]

Aflatoxin	Repeatability (RSD%, n=10)	Recovery (%) (mean ± SD)	LOD (µg/kg)	LOQ (µg/kg)
B_1	0.0741	93.6±1.912	0.198	0,262
B_2	0.0630	90.7±2.417	0.168	0.235
G_1	0.0883	102.6±2.924	0.176	0,263
G_2	0.1301	92.2±4.945	0.182	0.256

[1] LOD = limit of detection; LOQ = limit of quantitation; RSD = relative standard deviation; SD = standard deviation.

Table 3. The amount of aflatoxins (µg/kg) in positive samples and the number of the samples by months.[1]

Month	Number of negative samples	Number of positive samples	Amount of aflatoxins in positive samples (n=3)				
			G_2 (µg/kg)	G_1 (µg/kg)	B_2 (µg/kg)	B_1 (µg/kg)	Total aflatoxin (µg/kg)
January	5	1	<LOD	<LOD	<LOD	0.720	0.800
February	6	0					
March	4	2	<LOD	<LOD	<LOD	2.140	2.140
			<LOD	<LOD	<LOD	1.580	1.740
April	6	0					
May	6	0					
June	6	0					
July	5	1	<LOD	<LOD	0.194	1.580	1.774
August	4	1	<LOD	<LOD	0.613	6.596	7.209
September	4	2	<LOD	<LOD	1.890	22.65	24.54
			<LOD	<LOD	<LOD	0.379	0.379
October	4	2	<LOD	<LOD	0.420	3.931	4.351
			<LOD	<LOD	<LOD	0.669	0.669
November	5	0					
December	3	2	<LOD	<LOD	<LOD	0.400	0.400
				0.46	<LOD	0.28	0.74

[1] LOD = limit of detection.

0.01-32.30 µg/kg in maize obtained within Turkey (mean = 10.94) and 0.09-1.50 µg/kg in the imported maize (mean = 0.78) (Oruç *et al*., 2006). Nizamlioglu and Oguz (2003) conducted a study of 15 samples and showed that the aflatoxin contaminations were <5 µg/kg (50% within the positive samples). Two maize samples exceeded the Turkish Food Codex and European limits (i.e. 67.4 and 133 µg/kg) (Nizamlioğlu and Oguz, 2003). Similar results have been reported by Giray *et al.* (2009). In that study, 47 samples of maize were collected from various street bazaars and market outlets in different regions of Turkey. The total aflatoxins and OTA levels in the maize samples were in the range of 1.75-120.3 µg/kg and 1.08-8.57 µg/kg, respectively. Although 53% of the samples analysed had no detectable aflatoxins levels, 4% of similar samples contained aflatoxins levels above the acceptable limit of 10 µg/kg in Turkey.

The occurrence of aflatoxin in maize has also been reported by several authors from different countries. Perrone *et al*. (2014) analysed 91 maize samples from farms and markets in Nigeria and Ghana and found that AFB_1 and/or AFB_2 occurred in 36 out of the 91 samples and 3 samples from the farms also contained AFG_1 and/or AFG_2. The incidence of aflatoxin contamination was higher (54%) in farm samples than in market samples (30%) (Perrone *et al.*, 2014). Krout-Greenberg *et al.* (2013) have documented that 14 out of the 50 maize samples from California contained detectable aflatoxins levels and the average concentration of aflatoxins for the 13 samples below the regulatory action level was 8.69 µg/kg (range 4.67-13.82 µg/kg), with one sample containing a concentration of 41.3 µg/kg. Saima *et al*. (2013) reported that 37 out of the 105 samples of maize had been found to be contaminated with aflatoxins, with average AFB_1 levels and total aflatoxins, of 7.90 and 12.08 µg/kg, respectively. Pleadin *et al*. (2014) determined the AFB_1 levels in the maize (n=633) sampled from farms and feed factories situated in Northern, Central and Eastern Croatia. The mean AFB_1 value found in maize coming from all investigated regions equalled 81 µg/kg, with the maximal value being 2,072 µg/kg (Pleadin *et al.*, 2014). The presence of AFB_1 was detected in 17 samples (68%) out of the 25 samples analysed by Trung *et al.* (2008) and only five of those samples showed AFB_1 levels <10 µg/kg. Ten samples showed contamination levels in the range of 11.3-47.2 µg/kg, and two samples showed contamination levels in the range of 98.4-126.5 µg/kg, respectively (Trung *et al.*, 2008). Karami-Osboo *et al.* (2012) investigated AFB_1 contaminations in 373 samples collected from different agro-climatic regions of the major maize production area in Iran. They found that a total 43.6% of the samples were contaminated and that 22.5% contained more than 5 µg/kg AFB_1. The mean value of contamination levels was in the range of 0.9-154.13 µg/kg (Karami-Osboo *et al.*, 2012). The differences in aflatoxin results from different studies may be related to climate change implications. Magan *et al.* (2011) suggest that climate-change factors have a profound effect on both growth and relative mycotoxin production. Many of the recent reviews which have examined aspects of the

impact of climate change, have focused on plant breeding, plant diseases and mycotoxins in Europe, Australia, Africa, and USA (Medina *et al.*, 2014).

In many of these studies, the percentage of contaminated samples was found to be low. The results of the present study showed that AFB_1 is the most commonly found in the maize and maize flour. AFB_1 is also the most dangerous among aflatoxin types, and the International Agency for Research on Cancer has classified it as the first level of carcinogens for humans (IARC, 1993). Aflatoxin is an extremely toxic metabolite produced by *A. flavus* and *A. parasiticus*. *A. parasiticus* generally produces both B-group (AFB_1 and AFB_2) and G-group (AFG_1 and AFG_2) aflatoxins, whereas *A. flavus* produces only B-group aflatoxins (Sweeney and Dobson, 1998).

Although the data about monthly variability in aflatoxin concentration in maize flours are limited, they have been included in this study (Table 3). The results demonstrated that much higher levels of toxins are found in August, September and October than those in the other months. In the production of aflatoxin, the temperature, pH, water level (a_w), gases in the atmosphere and relative humidity of the environment have great importance. Aflatoxin grows well in the range of 19-35 °C with 28 °C being the optimum temperature. *A. flavus* can grow and produce mycotoxins down to a_w=0.73 and 0.85. This corresponds to 8-12% and 17-19% moisture content (Giorni *et al.*, 2008). In the province of Giresun, the summers are hot and humid. The average total annual rainfall is 1,264.6 kg/m^2 and the average humidity is 73.6%. Maize is planted in May and harvested in October in the Black Sea region of Turkey. Harvested maize is shucked and dried by hanging in cobs. When it is time to sell it, the grains are separated from the cobs before milling, after which it is sold in the bazaars as maize flour. As the fresh crop will only be ready for sale after October, it is likely that the maize flour found in the bazaars in August, September and October was harvested 10-11 months prior. If the post-harvest drying and storage conditions are inappropriate, aflatoxin growth will be observed. The Black Sea region is humid and the month of August is particularly the hottest and most humid with an average temperature of 19.4 °C and average humidity of 65.6% (TUIK, 2013). Accordingly, the climate in summer is favourable for mould growth. If stored maize is not dried to the recommended moisture levels, this may result in mould growth and insect activity. As such, the increase in aflatoxin levels during August, September and October is an expected result.

In maize flour, the formation of AFB_1 occurs easily and because it is the most dangerous form of aflatoxin, it is necessary that vendors of the maize flour take appropriate precautions. During interviews with the vendors, it was found that the maize is typically stored as grain in a moisture-free atmosphere, for as long as possible, and is then ground and delivered to the bazaar as per demand.

4. Conclusions

Product preservation strategies are necessary to produce and maintain high quality maize flour. The results of this study indicate that aflatoxin contamination occurs in maize flour, and data from this analysis of maize samples from bazaars support this claim. Despite the satisfactory levels of contamination found in the maize flour samples, it is essential to continue regular monitoring for aflatoxins at bazaars in order to safeguard consumer health. Therefore, the public needs to have an increased awareness about safe harvest, transport and storage techniques. Discussions about food issues, food hygiene and controls must be more frequent. This is important for both public health and the economy.

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