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Seasonal variation of aflatoxin M_1 level in cow milk from Turkey

Saisonale Schwankungen des Aflatoxin M_1-Gehalts in Kuhmilch aus der Türkei

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Summary

This study aims to determine the presence of aflatoxin M_1 (AFM_1) in raw cow milk produced in the center and the surrounding villages of Çorum, Turkey. A total of 200 raw milk samples provided directly from the manufactures during four seasons have been analyzed using high performance liquid chromatography (HPLC). AFM_1 was found in 129 out of 200 cow's milk (64.5%) at levels from 22 to 401 ng/L. Moreover, 87 samples contained AFM_1 at levels higher than maximum level of 50 ng/L established by Turkish Food Codex. The contamination of AFM_1 level was in the range of 22–289 ng/L in autumn, 29–199 ng/L in winter, 30–177 ng/L in spring, and 26–401 ng/L in summer season. The results show that AFM_1 concentrations carry potential risk from the point of consumers.

Keywords: aflatoxin M_1, food safety, contamination, cow milk, seasonal variation, HPLC

Introduction

Milk is very rich in nutrients (enzymes, vitamins, minerals), which meets the needs of organism and is consumed by being loved in all age groups (Iqbal et al., 2016). Especially babies, children in the development period and the elderly are the biggest consumers of milk (Bilgin, 2014). The quality of milk can be affected not only microbiological hazards (*Salmonella, Escherichia coli* O157:H7, *Staphylococcus aureus, Bacillus cereus, Brucella* etc.) but also chemical hazards including detergent, veterinary drugs, pesticides, and mycotoxins.

Mycotoxins are produced by many different filementous fungi, mainly *Aspergillus, Penicillium, Fusarium, Alternaria* species (Ashiq et al., 2014; Bakirci, 2014). The most common mycotoxins in nature are aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FUM), trichothecenes (deoxynivalenol (DON) and T-2 toxin), zearalenone (ZEA) and patulin (Selvaraj et al., 2015; Anfossi et al., 2016; Basu et al., 2016; Kosicki et al., 2016).

Among the over 300 mycotoxins discovered, AFs are the most toxic and carcinogenic metabolites. AFs are produced predominantly by *Aspergillus flavus, A. parasiticus* and *A. nomius* (Pitt, 2000). The four main naturally produced AFs are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (HUSSEIN & BRASEL, 2001). However, AFB_1 can be converted into aflatoxin M_1 (AFM_1), also known as milk toxin, by as metabolizing in the liver in dairy animals (Pitt, 2000; Virdis et al., 2008; Öksüztepe & Erkan, 2016).

When lactating animals are fed with feedstuffs contaminated with AFB_1, this metabolite can be transferred into milk as AFM_1 in the ratio of 1–3% (Zentai et al., 2023). The amount of AFM_1 in milk depends on animal breed, lactation period, milking time and interval, and exposure of AFB_1 through the consumption of feed (Özdemir, 2007; Aksoy et al., 2010; Muhammad et al., 2010; Aliabadi et al., 2013). The International Agency for Research on Cancer (IARC) classified AFB_1 as human carcinogen (Group 1), while AFM1 was placed in group 2B (possible human carcinogen), based on adequate evidence on animals and insufficient evidence in humans (IARC,1993).

Due to AFM_1 is stable at high temperatures, it can be found not only raw milk but also heat-treated milk such as pasteurized and UHT milk. In order to protect consumer health's many countries have established regulations for AFs and many other toxins in susceptible foodstuffs. The European Commission has set a maximum level (ML) of 50 ng/l for raw milk, heat-treated milk, and milk for the manufacture of milk-based products. Turkish Food Codex is aligned with Commission Regulation (EC) No 1881/2006, which expresses MLs for certain contaminants including mycotoxins (TGK, 2011).

The aim of this study was to determine AFM_1 concentrations in raw cows' milks from Corum region, Turkey throughout four seasons in January – October 2016.

Material and Method

Samples

During January–October 2016, a total of 200 raw cow milk samples were collected from local producers in the center and surrounding villages in Çorum, Turkey. Animals from the same 50 dairy farmers were selected to reveal seasonal variation (autumn, winter, spring, summer). The number of animals in the personal barns of the farmers in the villages where the samples were collected varies between 15–20. Therefore, each sample taken consists of the milk of 15–20 cattle. Thus, the milk of the producers was examined seasonally. Milk samples (500 mL) were collected in steril plastic bottles and transported to Hitit University Food Engineering Laboratory under cool condition. The samples were stored in the refrigerator at 4°C and analyzed in the same day.

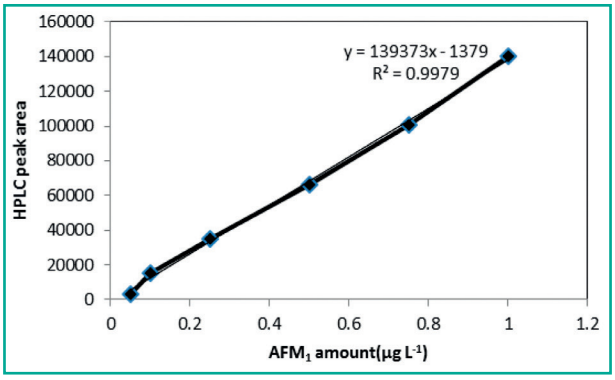

FIGURE 1: *Calibration curve generated.*

Aflatoxin M_1 standard

The AFM_1 standard solution, dissolved in one mL of acetonitrile at a concentration of 10 µg/mL in a glass ampoule, was commercially available (Supelco 46019-U, Bellefonte, PA, USA). The calibration standard solutions were prepared in six different concentrations at varying from 0.05 to 1 µg/L. The calibration curve was generated by marking the AFM1 peak area (A) against the AFM1 concentration value (Fig. 1). The prepared AFM1 standards were maintained at 4°C in the refrigerator.

Sample extraction and Immunoaffinity column (IAC) clean-up

AFM_1 analysis in dairy samples was performed according to TS EN ISO 14501 formal method (Anonim, 2002). In briefly, 40 mL milk samples were taken into disposable plastic containers and heated at 37°C for 15 minutes in water bath. Heated milk samples were centrifuged at 4000 x g for 15 min at 37°C (Sigma 3–30K, Germany). At the end of centrifugation, the oily cream layer was removed. Fat-free milk was passed through filter paper (FR101050, 40x40 cm). The remaining filtrate was passed (one-two drops per second) through AFla M_1™ HPLC immunoaffinity column (Vicam, MA, USA) containing specific antibody against AFM_1. Twenty mL of ultrapure water (2x10 mL) was passed through the column for washing and the column was dried with air. AFM_1 attached to antibodies in the column was collected in a glass tube using 3 mL of acetonitrile (Sigma Aldrich 34851, ≥99.9%). The collected eluate was evaporated under N_2 at 45 °C until 0.5 mL remained. Finally, the eluate was dissolved with 2 mL of mobile phase (water-acetonitrile, 75:25, v/v). Samples were passed through membrane filters with a pore diameter of 0.45 µm (Minisart® NY25, Sep-Pak Filter) and transferred to amber HPLC vials of 2 mL capacity. Vials were kept in the refrigerator at 4°C until injection onto the high-performance liquid chromatography (HPLC) system.

Determination of AFM_1 with HPLC

Chromatographic analysis was performed with a liquid chromatographic system of Shimadzu (Tokyo, Japan)

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with RF-20AXL fluorescence detector, LC-20AD pump system and SIL-20AHT automatic injection unit. Shimadzu LC solution software program was used to control and determine the data. ODS-3 column (150x4.6 mm, 5 µm) was used for chromatographic separations. The column temperature was set to 35°C. The mobile phase was formed from a mixture of water-acetonitrile (75:25, v/v) and the flow rate was adjusted to 1 mL/min. The amount of injection was 100 µl. An excitation wavelength of 365 nm and emission wavelength of 435 nm were used in fluorescence detector. The analysis time was 13 minutes and retention time was determined as 9.3 minutes.

TABLE 1: *AFM_1 content and seasonal distribution of raw milk samples (ng/L).*

Season	Number of Samples	Positive Samples (%)	Distribution of AFM_1 ≤ 50 ngL n (%)	Distribution of AFM_1 >50 ngL n (%)	AFM_1 Level (ng/L) Range Min.-Max.	AFM_1 Level (ng/L) Average[a]
Autumn	50	33 (66%)	15 (30%)	18 (36%)	22–289	66
Winter	50	38 (76%)	9 (18%)	29 (58%)	29–199	84
Spring	50	28 (56%)	10 (20%)	18 (36%)	30–177	64
Summer	50	30 (60%)	8 (16%)	22 (44%)	26–401	117

[a] Mean of positive samples

Statistical analysis

Experiments were performed using a completely randomized design. One-way analysis of variance (ANOVA) was performed using SPSS Statistics (22.0) software in order to analyse the data. Statistical differences between the samples means were determined by post hoc analysis using Tukey's multiple range test. The differences between the means are regarded as statistically significant if the p-value ≤ 0.05. Data were shown as mean ± standard deviation.

Results and discussion

The results of 200 samples analyzed by HPLC are shown in table 1. AFM1 was found in 64.5% of milk samples. The lowest and highest AFM_1 concentrations for a total of 129 positive samples were 22 ng/L and 401 ng/L, respectively. In the remaining 71 samples (35.5%), AFM_1 was not detected. The concentration of AFM_1 in 87 samples (43.5%) exceeded the ML of 50 ng/L specified in the Turkish Food Codex. The mean values of AFM_1 concentrations in milk for autumn, winter, spring and summer were 66, 84, 64 and 117 ng/L, respectively.

AFM_1 was found in 33 of 50 raw milk samples (66%) from autumn season at levels ranging from 22 to 289 ng/L, with a mean concentration of 66 ng/L. In autumn season, 18 samples exceeded the ML of 50 ng/L. The mean concentration of these samples, which exceeded the maximum limit, was determined to be 88 ng/L. The chromatograms of naturally contaminated milk samples containing 74 and 139 ng/L AFM_1 are shown in Figs. 2. and 3. respectively.

In winter season, AFM_1 was recorded in 38 out of 50 raw milk samples (76%). In amounts ranging from 29 to 199 ng/L with a mean level of 84 ng/L. In 29 samples, the concentration of AFM_1 was higher than ML. The mean concentration of these samples, which exceeded the maximum limit, was determined to be 98 ng/l. The ML exceedance rate for milk from winter season was increased when compared to milk from autumn season.

In spring season, AFM_1 was detected in 28 out of 50 raw milk samples (56%). The concentration of AFM_1 in milk from spring season varied from 30 to 177 ng/L (mean= 64 ng/L).

18 (36%) of the samples were contained AFM_1 in amounts ranging from 52 to 177 ng/L. The mean concentration of these samples, which exceeded the maximum limit, was determined to be 79 ng/L.

In autumn season, 30 of 50 raw milk samples (66%) contained AFM_1 with levels of 26–401 ng/L. The mean level of AFM_1 in milk from autumn season was 117 ng/L.

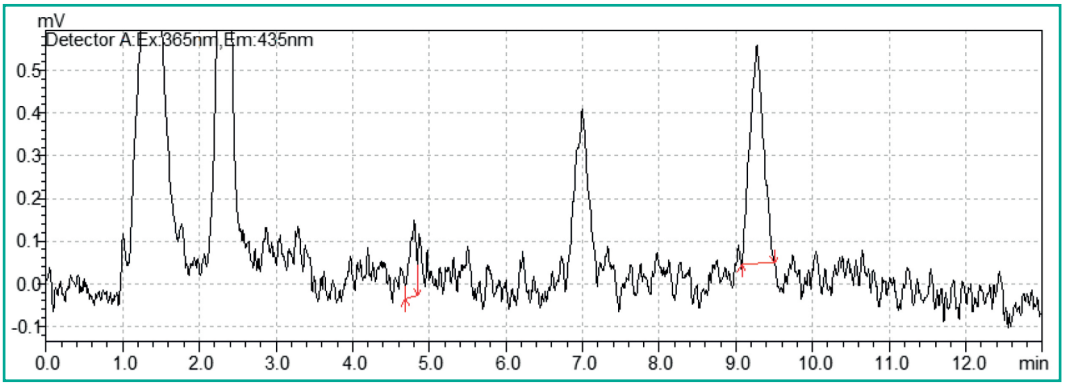

FIGURE 2: *HPLC chromatogram of a naturally contaminated raw milk sample with AFM_1 (74 ng/l).*

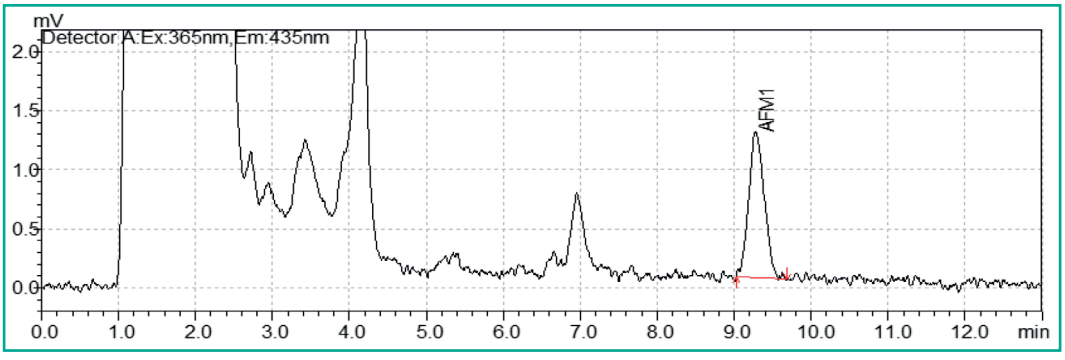

FIGURE 3: *HPLC chromatogram of a naturally contaminated raw milk sample with AFM_1 (139 ng/l).*

Twenty-two samples from autumn season exceeded ML of 50 ng/L.

The average AFM_1 values for all the four seasons were above the legal limit. This could be explained by the fact that dairy animals were fed with AFB_1-contaminated feed, which was kept in unstable warehouses in terms of temperature and humidity. Being an average level of AFM_1 in milk samples collected in summer is high can be due to the limited pasture areas, shepherd problem in rural areas, feed content (silage, mixed feed, pulp, roughage), limited time to release into the pasture, storage humidity and temperature.

As a result of the variance analysis performed for the statistical evaluation of the data, it was determined that the difference between the amounts of AFM_1 in milk samples was insignificant between samples ($p > 0.05$). However, in autumn and spring seasons, a low level of correlation was determined in AFM_1 levels, as a result of the statistical evaluation. In addition, it was determined that the differences in AFM_1 concentrations in the spring-summer, winter-spring and autumn-winter seasons were significant ($p<0.05$). Statistical analyses have shown a significant difference in the levels of AFM among raw milk samples based on the seasons. This suggests that the AFM levels vary depending on the time of the year. This result could be attributed to increase in AFM_1 level in summer compared to other seasons was largely associated with increased storage humidity and temperature, as well as not grazing milking animals.

In this study, feed samples were not examined for the presence of aflatoxins. However, according to the information received from the producers, the feed storage areas are not modern and are not carried out temperature and humidity control. In addition, studies have shown that this metabolite passes in milk in the form of AFM_1 within 12–24 hours as a result of animals consuming feed contaminated with AFB_1. The rate of transfer of AFB_1 amount in bovine animals to milk in AFM_1 form varies between 1–3% (Zentai et al., 2023).

Humidity and temperature are the most important factors affecting the amount of AFB_1 in feed. Toxin-producing molds such as *Aspergillus flavus* and *Aspergillus parasiticus* grow rapidly in feeds containing 13 to 18% water and in environments with 50 to 60% humidity. In addition, these molds can grow under 25°C and 85–95% relative humidity conditions. The precise effect of seasons on aflatoxin concentrations in food and feedstuffs has been the subject of debate. However, it has been proven that environmental conditions, moist environments, nutrient content, storage environment, water activity increase toxin activity (Bakırcı 2001).

Although many studies have been conducted on AFM1 in raw milk from different regions of Turkey, this is the first detailed study on cow's milk produced in Çorum province. In a previous study, İşleyici and co-workers (2015) determined AFM_1 in 53 of the 100 raw milk (53%) collected in summer season in Van province, Turkey at levels of <5–>80 ng/L. In another study, Bakirdere and co-workers (2014) found AFM_1 in 61 out of 77 raw milk samples (79,2%) at concentrations ranging from 5 to 410 ng/L. In the study conducted by Gölge (2014) in Adana, AFM_1 was detected in 53 of 176 raw milk (30,1%) at levels of 42–552, 33–1010, 47–150 and 25–102 ng/L in autumn, winter, spring, and summer seasons, respectively. In another work, 60 out of 92 milk samples (65.2%) collected from Mersin contained AFM_1 in the range of 2–867 ng/L (Aslan et al., 2010). The impact of seasons on aflatoxin concentrations in food and feedstuffs is a matter of debate. Raw milk, which is also used as raw material, is processed on different products (cheese, yoghurt, ice cream, butter). Thus, high concentrations of AFM1 in the final product have been reported by different investigators (Alkan & Gönülalan 2006; Tekinşen & Eken 2008; Ayyildiz 2012). Other studies on the subject support our findings.

Conclusions

In the study, the fact that the average results for the four seasons are above the legal limit is considered as an indication that the dairy farm animals are fed with AFB_1 contaminated feed kept in warehouses that are not suitable in terms of temperature and humidity. The concentration of AFM_1 exceeded EU ML in 43.5% of raw cow's milk. There was a significant difference in AFM_1 levels between the seasons. The highest AFM_1 concentration was detected in the summer season. The amount of AFB_1 in animal feed should be routinely analyzed to reduce AFM_1 contamination in milk. In order to protect public health, training should be given to direct producers, associations and cooperatives active in the field of milk and products. Conducting controls, awareness of the manufacturerer's and increasing the audits are of great importance to prevent potential dangers.

It should be aimed to reduce the exposure of aflatoxin, which is the riskiest mycotoxin with its proven toxic effect on public health, to protect the health of humans and animals, and to prevent economic losses.

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Authors' Contributions

Ezgi Yıkıcı contributed to the application of the analysis and the acquisition of data, writing, editing and visualization of the article. Fatih Özbey contributed to the methodology and arrangement of the study as a consultant. Bülent Kabak contributed to the methodology of the study and the implementation of the analyses.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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