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Aflatoxin M_1 Determination in Traditional Küp Cheese Samples of Turkey Using Immunoaffinity Column and High-Performance Liquid Chromatography

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ABSTRACT

Mycotoxin occurrence in foods, especially in uncontrolled produced traditional foods causes serious health problems. In this study, traditional Küp cheese samples were collected from different part of Anatolian region in Turkey (Ankara, Nevşehir and Yozgat) and analyzed to determine Aflatoxin M_1 (AFM_1) level. AFM_1 analysis was carried out by, immunoaffinity column (IAC) clean-up and high performance liquid chromatography (HPLC) attached with fluorescence detector (FL) The level of AFM_1 in all samples was in the range of 16 and 136 ng/kg which is lower than the maximum tolerance limit of the Turkish Codex Regulations (250 ng/kg). The levels of contamination indicated that more detailed and continuous monitoring is required to increase the public health conscious and reduce consumers' exposure to AFM1.

Introduction

Aflatoxins are secondary fungal metabolites of toxigenic strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* and one of the most hazardous mycotoxin, since they are associated with various diseases (Mortazavi and Tabatabai 1998). There have been widespread concerns about Aflatoxins because of their carcinogenic, teratogenic, and mutagenic properties (Peraica et al., 1999). Aflatoxins have become a major threat to human health and therefore detection methods of them have gained tremendous importance. Among Aflatoxins, Aflatoxin B_1 (AFB_1) is the most toxic and the most abundant one in naturally contaminated foods and feeds, especially in tropical and warm regions (Scudamore et al., 1998). When AFB_1 contaminates the feed of lactating animals, milk from these animals contains Aflatoxin M_1 (AFM_1), the monohydroxylated metabolite of AFB1 in liver and it is secreted into milk (Gürbay et al., 2006). AFM_1 content of milk is directly related to AFB_1 content of feed consumed by animals. It has been reported that 0.5-6.0% of AFB_1 converted into the AFM_1 (Galvano et al., 1996). Although AFM_1 was classified as possible carcinogen (class 2B substance) by IARC till (2002), it was moved to class I compound after studies indicated its harmful effects on human health (IARC 2002), AFM_1 level should be under 50 ng/kg as European Commission (2001) stated in raw milk, pasteurized milk and milk processed for dairy products however this limit is 250 ng/kg for cheese products according to Turkish Codex (Turkish Food Codex 2002). Gurbay et al. (2006) recommended the continuous control for not only milk but also dairy products including cheese since milk and dairy products are the main part of Turkish people' diet.

Turkey has tremendous type of cheese and many of them produced traditionally. Küp cheese is one of the traditional and artisanal cheeses which are very common in provinces of Ankara, Nevşehir and Yozgat. Traditional Küp cheese has its name because of packaging method. Küp cheese is usually produced in small farms which have their own cattle and produce their own milk and the cheese are kept in soil packs under the ground for ripening process. The use of pottery for ripening of cheese in the past was probably due to the absence of alternative materials for preserving and ripening, however, these practices are still being continued in order to preserve the taste and flavor of Küp cheese. Traditional cheese should require more importance because of uncontrolled process conditions. These products have been highly demanded in the local markets and consumed by especially kids including infants who are more sensitive to hazardous effects of Aflatoxins, therefore consumption of mycotoxin contaminated milk and dairy products create a worldwide concern.

Detection of AFM_1 concentration can be performed with different methods. Thin layer chromatography (TLC) (Fallah et al., 2011), enzyme-linked immunoassays (ELISA) (Atasever et al., 2010; Tekinşen and Eken, 2008) high-performance liquid chromatography (HPLC) with fluorescence detection, HPLC with tandem mass spectrometric detection (Aguilera-Luiz et al., 2011; Chen et al., 2012), were some of them used for Aflatoxin detection. Since HPLC methods are specific and sensitive, it is more preferable than other methods. Moreover, by the help of immumoaffinity column which has specific antibodies for toxin of interest, toxin analyses have become more rapid, simple and sensitive.

The consumption of cheese especially traditional cheeses is widespread in Turkey. For this purpose, this study was designed to determine the presence and levels of AFM_1 in Küp cheese samples produced traditionally. The AFM_1 concentration was determined by HPLC equipped with FL detector.

Materials and Methods

Samples Collection

Küp cheese samples were collected from shops selling traditional foods, local markets and villages in Ankara, Nevşehir and Yozgat. 20 of 1 kg cheese sample from each city (total 60 kg) were brought to the laboratory without breaking cold chain and stored at 4°C.

Chemicals and Reagents

HPLC grade acetonitrile and methanol were supplied by Sigma-Aldrich (Brøndby, Denmark). The IACs were AFLAPREP® columns purchased from R-Biopharm Rhône Ltd (Glasgow, Scotland). Ultrapure water, produced by Merck Millipore Milli-Q purification system (Molsheim, France) was used for the HPLC mobile phase and all analytical steps.

Standard Solutions

The AFM_1 standard was supplied by R-Biopharm Rhone (Glasgow, Scotland). For the quantification of AFM_1 in the samples analysed, a five-point calibration curve was constructed with the use of AFM_1 standard solutions in acetonitrile at 50, 100, 150, 200, and 250 ng/kg.

Methods

The AFM_1 detection analysis composed of two main steps: AFM_1 extraction from sample and HPLC-FLD analysis of the extract. In particular, extraction and purification of samples was performed by the help of immunoaffinity columns.

Cheese Sample Preparation

Cheese samples were prepared according to manual of Easy-Extract Aflatoxin (R-Biopharm RP71/70N) for clean-up. Briefly, 10 g of each sample were mixed with 40 mL of acetonitrile/methanol/water (60/10/30) mixture and then homogenized for 5 min. Mixture was centrifuged for 10 min at 4000rpm. 10 mL of aliquot was transferred and diluted with phosphate buffer saline (PBS) solution. This mixture was filtered through microfiber filter paper and filtrate adjusted to have 2.5 g of sample. The filtrate was passed through the immunoaffinity column at a flow rate of 2 mL/min. After that column was washed with 10 mL water and PBS solution with a speed of 5 mL/min to remove interferents and dried. The AFM_1 bound to the antibody was released by elution with 100% acetonitrile (1 drop/sec). The elute was evaporated at 60°C under nitrogen atmosphere, and reconstituted with water: acetonitrile (80/20, v/v). A volume of 100 µL was injected into the chromatograph.

High-Performance Liquid Chromatography Analysis

HPLC analysis was performed with Schimadzu LC 20 AT. Inertsil ODS-3V 4.6x150 mm column was used with a flow rate of 1 mL/min, and the column temperature was maintained at 25°C. The mobile phase consisted of the mixed solution of water: acetonitrile: methanol (70/24/6, v/v/v). The injection volume into HPLC system for both standard and sample was 100 µL.

Results and Discussion

Method Validation Parameters

The linearity was assessed by constructing five-point calibration curves over the concentration range of 50-250 ng/mL (Figure 1). Linear regression lines were plotted using the peak area versus the analyte of determination (R^2). f(x)=2.90618e-005*x+0.031996, r^2 = 0.9991082 (n=5). The limits of detection (LOD) and quantification (LOQ) were calculated using a signal-to-noise ratio of 3 and 10, respectively. The method's LOD was found to be 0.0062 ng/mL, while the Limit of Quantification (LOQ) was calculated as 0.0210 ng/mL. Recovery analysis was carried by performing test on cheese samples spiked with AFM_1 at concentration 100 ng/mL. Mean recovery of AFM_1 in cheese samples (n=5) was found as 88% (Table 1)

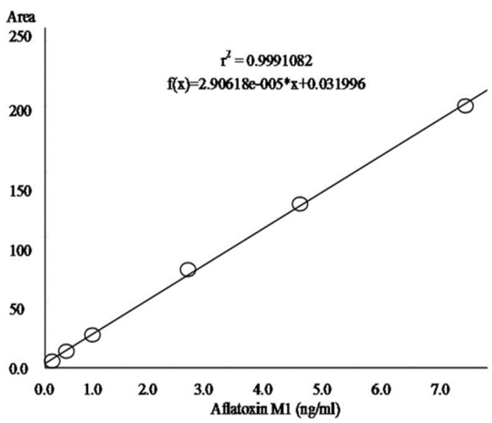

Figure 1 Calibration curve of standard solutions of AFM_1

Aflatoxins Analysis in Küp Cheese Samples

The occurrence of AFM_1 contamination in cheese samples was indicated in Table 2. The contamination levels of the AFM_1 toxin in cheese samples ranged from 16 to136 ng/kg, with higher levels detected in samples from Ankara. AFM_1 was found in 25 cheese samples, corresponding to 41.7% of the total samples examined. The AFM_1 content come out as undetectable in 35 of 60 samples. 45% of cheese from Nevşehir and 30% of cheese from Yozgat were contaminated with AFM_1, while 50% of cheese collected from Ankara was positive for AFM_1 contamination. The incidence of AFM_1 contamination in cheese was high but, all contamination levels were under the Turkish Codex regulation limit (250 ng/kg). However, 20% of the positive cheese samples exceed the limit of European Commission regulation (50 ng/kg) (European Communities 2001).

Results of this study were comparable with other studies. Akrami Mohajeri et al. (2013) reported that AFM_1 level in cheese samples ranged 93-309 ng/kg in Iran and another study done in Iran stated that similar AFM_1 level (41-374 ng/kg) in cheese samples. (Tavakoli et al., 2012). Both studies have indicated that there was higher contamination of AFM_1 in cheese samples in Iran when compared this result.

There were some studies done in Turkey to indicate incidence of AFM_1 contamination in cheese. Tekinşen and Eken (2008) analyzed various cheese samples and found contamination with mean concentration 194 ± 15 ng/kg. They explained the reason of high contamination level as the high affinity of toxin for casein in cheese. Gürbay et al. (2006) found out that 28% of cheese samples analyzed were contaminated with AFM_1. Furthermore 99 cheese samples in Turkey were investigated and all were contaminated with mean 330 ± 55 ng/kg (Tekinşen and Uçar, 2008). Another aflatoxin survey of dairy products done by Sarimehmetoglu et al. (2004) showed that 81.75% of cheese samples were positive for AFM_1 contamination. There have been more research about cheese in Turkey (Ayçiçek et al., 2005; Bakırcı, 2001; Buket et al., 2010; Çolak, 2007; Deveci, 2007; Gürbay et al., 2006; Oruç et al., 2006; Yaroglu et al., 2005).

All revealed the presence of AFM_1 in cheese samples. Studies indicated that AFM_1 contamination level in cheese samples is 3–5 times higher than in same amount of milk (Prandini et al., 2009). Regarding those studies, cheese might have high potential risk for aflatoxin contamination.

Table 1 Performance characterıstıcs of methods for the determınation of aflatoxin M_1 (AFM_1) in samples of Küp cheeses.

Analyte	Matrix	LOD (ng/mL)	LOQ (ng/mL)	RR[a]	RSD (%) (n = 5)	Accreditation
AFM_1	Küp Cheese	0.0062	0.0210	87.8-88.5	5.2-5.7	No

Notes: [a]Spiking levels: 0.200 and 0.500 ng/g.; LOD: Limit of determination; LOQ: Limit of quantification; RR: Recovery range; RSD: Relative standard deviation.

Table 2 AFM_1 occurrence and distribution in Küp cheeses samples collected from Yozgat, Nevşehir and Ankara.

Region	Number of Sample Range (ng/kg)							Positive Samples	
	Ss	<10	10-25	26-50	51-100	>100	*Mean ±SD*	%	Min-Max (ng/kg)
Yozgat	20	14(70)	3 (15)	3 (15)	ND	ND	24±4.5	30	20-32
Nevşehir	20	11(55)	4 (20)	5 (25)	ND	ND	28±5.9	45	23-39
Ankara	20	10(50)	3 (15)	2 (10)	4 (20)	1 (5)	54±36	50	16-136

<10: range of negative samples, (): indicates percent, ND: Not determined; Ss: Sample Size

The reason of the high occurrence of AFM_1 contamination in cheese might be transfer of the toxin into the curd. Since AFM_1 is not soluble in milk fat and has high affinity for casein, the AFM_1 contamination levels can differ based on the cheese production methods, whey separation process and milk quality (Blanco et al. 1988). Pasteurization of AFM_1 contaminated milk does not alter the contamination risk in cheese because of the heat stability of toxins. Also the maturation and storage conditions do not change the degree of AFM_1 contamination (Gürbay et al., 2006; Unusan 2006). If milk is initially contaminated, there is great possibility of AFM_1 appearance in cheese.

Conclusions

Many of the studies investigated the AFM_1 levels have been done in industrially produced cheese. This study indicated that traditionally produced Küp cheese has similar or less AFM_1 level when compared the cheese produced commercially. It proved that the original source of the contamination is milk, since AFM_1 contamination in cheese results from indirect milk contamination. Therefore, it is important to pay attention into the raw milk quality which depends on the quality of feed consumed by animals. The governmental agencies should monitor the Aflatoxins in animal feed to control the risk of Aflatoxins contamination along the feed supply chain by implementing good agricultural practices. Moreover, farmers, and dairy producers should be trained to increase the awareness of potential health risks of Aflatoxins.

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Conflict of interest

None

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects

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