

Aflatoxin M₁ in UHT Cow Milk samples Collected in Burdur, Turkey

Türkiye Burdur İlinden Toplanan UHT Süt Örneklerinde Aflatoxin M₁ Kontaminasyon Düzeyi

 Murat BAYEZİT¹,  Selinay Başak ERDEMLİ KÖSE^{2*},  Fatma ŞAHİNDOKUYUCU KOCASARI³

¹ Burdur Mehmet Akif Ersoy University, Faculty of Health Sciences, Department of Aid and Disaster Management, Burdur, Turkey

² Burdur Mehmet Akif Ersoy University, Faculty of Arts and Sciences, Department of Chemistry, Burdur, Turkey

³ Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Departement of Pharmacology and Toxicology, Burdur, Turkey

Abstract: Aflatoxins are secondary metabolites of toxigenic moulds of the *Aspergillus* species. Aflatoxin M₁, a metabolite of the potent carcinogen aflatoxin B₁ (AFB₁) occurs in milk of animals consuming feed contaminated with AFB₁. The aim of this study was to investigate the occurrence and levels of aflatoxin M₁ (AFM₁) in UHT milk samples consumed in Burdur city markets. In 2018, a total of 78 UHT milk samples were randomly collected from different markets of Burdur. The occurrence and contamination levels of AFM₁ in the samples were investigated by the competitive enzyme-linked immunosorbent assay (ELISA) method. Aflatoxin M₁ was detected in 24 of 78 samples (30.77%) in concentrations between 4.30-127.44 ng/L (mean level: 47.54 ng/L). AFM₁ levels in 11 of these 24 positive samples were above legal limits of Turkey which is 50 ng/L for milk samples. It is concluded that the occurrence of AFM₁ in milk samples in particular may be considered as a possible hazard for public health.

Keywords: Aflatoxin M₁, UHT milk, Burdur, contamination level.

Öz: Aflatoxinler, toksijenik küflerden *Aspergillus* türleri tarafından sentezlenen sekonder metabolitlerdir. Aflatoxin M₁ (AFM₁), potansiyel bir kanserojen olan aflatoxin B₁ (AFB₁) ile kontamine yemlerin hayvanlar tarafından tüketilmesi sonucunda, AFB₁'in bir metaboliti olarak süte geçer. Bu çalışmanın amacı, Burdur marketlerinden toplanan UHT süt örneklerinde AFM₁ varlığı ve düzeylerini araştırmaktır. Burdur'un farklı marketlerinden 2018 yılında rastgele toplam 78 UHT süt örneği toplanmıştır. Numunelerde AFM₁'in varlığı ve kontaminasyon düzeyleri, enzim bağlı immunoabsorbent assay (ELISA) metodu ile ölçülmüştür. Aflatoxin M₁ 78 örneğin 24'ünde (% 30.77) 4.30-127.44 ng/L (ortalama: 47.54 ng/L) düzeyleri arasında tespit edilmiştir. Pozitif örneklerin 11'inde AFM₁ düzeyleri, Türkiye'de süt örnekleri için 50 ng/L olarak belirlenen yasal sınırın üstünde bulunmuştur. Özellikle süt örneklerinde AFM₁ varlığının halk sağlığı için olası bir tehlike olarak kabul edilebileceği sonucuna varılmıştır.

Anahtar Kelimeler: Aflatoxin M₁, UHT süt, Burdur, kontaminasyon düzeyi.

*Corresponding author : Selinay Başak ERDEMLİ e-mail : sberdemli@mehmetakif.edu.tr

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Introduction

Aflatoxins are secondary metabolites of toxigenic moulds of the *Aspergillus* species including *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Creppy, 2002; Bilandzic et al., 2016). The term aflatoxin refers to six main

compounds known as Aflatoxin B₁ (AFB₁), Aflatoxin B₂ (AFB₂), Aflatoxin G₁ (AFG₁), Aflatoxin G₂ (AFG₂), Aflatoxin M₁ (AFM₁) and Aflatoxin M₂ (AFM₂). Among them, AFB₁ is the most synthesized and therefore the most abundant

and toxic compound in nutrients. This is followed by AFG₁, AFB₂, AFG₂. Aflatoxin M₁ and AFM₂ are the milk-extracted metabolites of AFB₁ and AFB₂, respectively. Aflatoxin M₁ is the major monohydroxylated derivative of AFB₁ formed in liver by cytochrome P450 enzymes (Price et al., 1993; Rustom, 1997; Oguz and Kurtoglu 2000; Zinedine et al., 2007).

Aflatoxin M₁ is passed into milk by feeding the animals in lactation with feed containing AFB₁ and it is also found in dairy products. Infection of aflatoxin into milk and milk products occurs in two ways. The first one is the passing of toxins into the milk of the animals eating the food contaminated with aflatoxin and the second one is the result of the milk and milk products being contaminated with molds (Marth, 1979; Whitlow et al., 2000). Approximately 0.3-6.2 % of the AFB₁ taken by the animals is discarded as AFM₁. It is reported that this ratio varies depending on the animal, the amount of AFB₁ taken by the feed, the lactation period and the milk quantity. In addition, it is stated that the contamination with AFM₁ in milk and dairy products varies according to geographical regions, countries and seasons (Van Egmond and Paulch, 1986; Galvano et al., 1996; Pittet, 1998). Aflatoxin M₁ is detected in milk 12-24 hours after AFB₁ is taken by animals. It has been reported that the amount of AFM₁ passed into the milk by stopping the removal of AFB₁ by animals is below the detectable level within 72 hours (Van Egmond, 1989).

Aflatoxins cause liver cancer in humans and animals and, particularly suppress the immune system leading to the emergence of many diseases. Both AFB₁ and AFM₁ cause DNA damage, gene mutation, and abnormal chromosomes in mammalian cells, insects, bacteria *in vitro* (Lin et al., 2004). International Agency for Research on Cancer (IARC) classifies AFB₁ in Group I and AFM₁ in the Group 2B carcinogens (IARC, 2002). For these reasons, AFM₁ levels, which are allowed to be found in milk and milk products in our country and in some countries, especially in the US and European countries, have been determined.

AFM₁ level which are allowed in milk is 500 ng/L, 50 ng/L and 50 ng/L in USA, EU and Turkey, respectively (Food and Drug Administration (FDA), 1996; European Commission (EC), 2006; Turkish Food Codex (TFC), 2011).

Milk and milk products are important sources of protein and calcium for humans, especially infants and children. Therefore, intensive studies have been conducted on the presence of AFM₁ in milk and dairy products. Infants and children are more sensitive to aflatoxins than adults are. The main reasons for this are the low body weight of infants and children, faster metabolism, poor detoxification ability and inadequate development of some tissues and organs. Excessive consumption of milk and milk products, especially by infants and children of developmental age increases the severity of the situation (Akdemir and Altintas, 2004; Lee et al., 2009; Sherif et al., 2009).

The aim of this study was to detect the levels of AFM₁ in ultra-high temperature (UHT) milk samples in Burdur and to compare the results with legal regulations for AFM₁.

Material and Methods

In 2018, 78 samples of UHT cow milk (the commercial serial numbers of the samples are not the same) were collected randomly from different markets of Burdur. All these samples were stored at +4 °C in a dark and dry place until analysis. The analyses of this research were performed in Department of Pharmacology and Toxicology.

The quantitative analysis of AFM₁ in the samples was performed by competitive enzyme-linked immunosorbent assay (ELISA) method according to the procedure described by EuroProxima B.V. The Netherlands (EuroProxima B.V. Aflatoxin M₁ Fast ELISA Cat No.: 5121AFMF).

Preparation of samples was conducted according to the instructions of the EuroProxima ELISA kit (EuroProxima B.V. The Netherlands).

Cold milk samples centrifuged at 2000g for 10 min at +4 °C. The upper fat layer was removed. Afterwards, the extracts were used in the assay.

ELISA test procedure

One hundred µL of the standard solutions and prepared samples in separate wells were added to each well mixed by priming pipettor at least 3 times. The microtiter plate was sealed and then incubated at room temperature in the dark for 30 min. At the end of incubation, the solution was discarded from the microtiter plate. The wells were washed three times with rinsing buffer. After washing steps, 100 µL of the conjugate (Aflatoxin M₁-HRP) was added to the wells (except blank wells) and incubated for 15 min at room temperature in the dark. At the end of incubation the solution was discarded from the microtiter plate, the wells were washed three times with rinsing buffer. Then, 100 µL of substrate solution

was added to each well and mixed thoroughly and incubated for 15 min at room temperature in the dark. Following this step, 100 µL of the stop solution was added to each well and mixed. The absorbance was measured at 450 nm by an ELISA (ELX-800, Bio-Tek Instruments Inc., Winooski, VT, USA) within 15 min.

The results were evaluated according to the computer program, prepared by EuroProxima B.V. The levels of aflatoxin standards used were 6.25, 12.5, 25, 50, 100 and 200 ng/L. The detection limit of this ELISA method was 5 ng/L.

Results

Aflatoxin M₁ was detected in 24 of 78 samples (30.77 %) in concentrations between 4.30-127.44 ng/L (mean level: 47.54 ng/L). AFM₁ levels in 11 of these 24 positive samples were above legal limits of Turkey which is 50 ng/L for milk samples (Table 1).

Table 1 Occurrence of AFM₁ in UHT milk samples.

Samples	Tested <i>n</i>	Positive <i>n</i> (%)	Contamination (ng/L)		Exceed regulation ^a <i>n</i> (%)
			Range	Mean±SD ^b	
UHT milk	78	24 (30.77)	4.30-127.44	47.54±35.14	11 (45.83)

^a The Turkish limit for AFM₁ is 50 ng/L in UHT milk.

^b SD: Standart deviation

Discussion

Many studies have been conducted about the existence of AFM₁ in various milk samples in different countries. In Turkey, researchers reported different levels of AFM₁ in UHT milk samples (Unusan, 2006; Tekinsen and Eken, 2008; Var and Kabak, 2008; Gundinc and Filazi, 2009; Aydemir-Atasever et al., 2010; Sahindokuyucu-Kocasari, 2014; Turkoglu and Keyvan, 2019).

Unusan et al. (2006) who analysed 129 milk samples, detected AFM₁ between 0-543.6 ng/L (mean value: 108.17 ng/L) concentrations in 75 (58.1 %) of samples and 61 of 129 (47 %) samples

were above the limit permitted by the EU. Tekinsen and Eken (2008) examined 100 milk samples and found 67 % of samples contaminated with AFM₁ in concentrations of 10-630 ng/kg and researchers stated that 31 (31%) of UHT milk samples contain AFM₁ above maximum tolerable limit of the EC and the TFC. Var and Kabak (2008) analysed 20 samples of milk and found AFM₁ in 100 % of the samples ranged from 10 to 80 ng/L and 3 of milk samples were found to contain AFM₁ higher than the tolerance of Turkish legal limits. Gundinc and Filazi (2009) observed that in all of 50 milk samples, AFM₁ was detected in a concentration 5-244 ng/L (mean value: 101.2

ng/L). Researchers found that in 10 (20 %) of 50 samples AFM₁ levels exceed the legal limits of EC and TFC. Aydemir-Atasever et al. (2010) evaluated the occurrence of AFM₁ in 59.3 % of 150 samples at levels of 5-185 ng/L and AFM₁ in 16 (10.7 %) of the samples were found to be higher than the maximum tolerable limits of EC and TFC. Sahindokuyucu-Kocasari (2014) analysed 30 samples of milk and found AFM₁ in 30 (73.2 %) of the samples ranged from 6.42 to 71.33 ng/L (mean value: 17.76 ng/L). Only in 3 (7.3 %) of UHT milk samples, AFM₁ levels were above the Turkish legal limit (50 ng/L). Turkoglu and Keyvan (2019) analysed 35 UHT milk samples and detected mean AFM₁ level as 20.29 ng/L in 97.14 % of samples and 8 of 34 positive samples were above the legal limits.

Some researchers from different countries also reported AFM₁ contamination in their studies (Martins and Martins, 2000; Roussi et al., 2002; Shundo and Sabino, 2006; Shundo et al., 2009; Rahimi et al., 2009; Fallah, 2010; Heshmati and Milani, 2010; Movassagh, 2011; Rahimi et al., 2011; Oliveira et al., 2013; Zheng et al., 2013; Silva et al., 2015; Bilandžić et al., 2016).

In Portugal, Martins and Martins (2000) analysed 70 UHT milk samples and detected AFM₁ in 84.2 % of the samples ranged from 5 to 61 ng/L. Researchers found AFM₁ which were higher than the legal limits (50 ng/L) in only 2 of the samples. In Greece, Roussi et al. (2002) detected AFM₁ in 82.3 % of 17 milk samples at levels 5-50 ng/L. None of the UHT milk samples had AFM₁ level above the legal limits. In Saudi Arabia, Abdallah et al. (2012) analysed 96 milk samples and detected AFM₁ in 79 (82.3 %) of the samples ranged from 10 to 190 ng/L. In all the positive samples, levels of AFM₁ were below the tolerated limits. In India, Siddappa et al. (2012) detected AFM₁ in 64.4 of 45 milk samples at levels 60-700 ng/L and 38% of these positive levels were above the maximum permitted limits. Many studies have been conducted about the existence of AFM₁ in various milk samples in Iran (Rahimi et al., 2009; Fallah, 2010; Heshmati and Milani, 2010; Movassagh,

2011; Rahimi et al., 2011). Rahimi et al. (2009) observed that in all of 48 milk samples AFM₁ was detected in a concentration 10->100 ng/L (mean value: 65 ng/L). Fallah (2010) found the toxin in 68 (62.4 %) of 109 milk samples contained in the range of 5.6-515.9 ng/L and researcher stated that 3 (2.7%) of UHT milk samples had levels above the maximum tolerance limit. Heshmati and Milani (2010) detected AFM₁ in 116 (55.2 %) of 210 samples ranged from 8-249 ng/L and the levels of AFM₁ in 70 (33.3%) samples were higher than the maximum tolerance limit. Movassagh (2011) observed AFM₁ in all of 49 milk samples at levels between 0 to 259 ng/L and 83.67 % of the samples had AFM₁ greater than the accepted limit of EC. Rahimi et al. (2011) analysed 59 milk samples and detected AFM₁ in 91.5 % of samples ranged from 10->100 ng/L. In Brazil, Shundo and Sabino (2006) detected 80.9 % of 42 UHT milk samples ranged from 20-206 ng/L, Shundo et al. (2009) analysed 40 milk samples and all of the samples were contaminated with AFM₁ at levels 10-500 ng/L, Oliveira et al. (2013) analysed 75 milk samples and AFM₁ in 30.7 % of the samples ranged from 1000-4100 ng/L which were above the tolerance limit of Brazilian regulations (500 ng/L) and Silva et al. (2015) detected AFM₁ in 87.5 % of 152 samples at levels 1.8-121 ng/L and levels of AFM₁ were below the tolerated limits. In China, Zheng et al. (2013) analysed 153 milk samples and detected AFM₁ in 54.9 % of samples ranged from 6 to 160 ng/L and none of the samples exceed the tolerated limit (500 ng/L). In Bosnia and Herzegovina and Croatia, Bilandžić et al. (2016) analysed 214 samples and found AFM₁ in samples at levels 2.29-21.4 ng/kg, levels of AFM₁ were below the tolerated limits (50 ng/L).

In comparison with previous studies, the incidence and contamination levels of AFM₁ in UHT milk in our study were higher than Var and Kabak (2008), and lower than Unusan et al. (2006), Shundo and Sabino (2006), Tekinsen and Eken (2008), Shundo et al. (2009), Gundinc and Filazi (2009), Fallah (2010), Heshmati and Milani (2010), Movassagh (2011), Siddappa et al. (2012). The incidence of AFM₁ in UHT milk was lower, but the

contamination levels were higher than Martins and Martins (2000), Rossi et al. (2002), Sahindokuyucu et al. (2014) and Turkoglu and Keyvan (2019). The incidence of AFM₁ in UHT milk was lower and the contamination levels were similar to Rahimi et al. (2009), Aydemir et al. (2010), Rahimi et al. (2011), Abdallah et al. (2012), Zheng et al. (2013) and Silva et al. (2015). In Oliveira et al. (2013), the incidence of AFM₁ in UHT milk was similar, but the contamination levels were much higher than our study.

Previous studies have reported different levels of AFM₁ in UHT milk samples. On the other hand, Srivastava et al. (2001) reported that AFM₁ were not detected in any of the samples. Different analytical methods, geographical region, climatic factors and seasonal variability may change results of researches (Var and Kabak 2008; Fallah 2010).

In conclusion, in UHT milk samples which are available for consumption in Burdur region, AFM₁ contamination prevalence is 30.77 % and 11 (14.10 %) of samples were above the maximum limit that allowed to be present in UHT milk samples in Turkey. The presence of AFM₁ in milk and dairy products is a major risk factor for public health, especially for children and infants. For this reason, it is necessary to prevent the growth of fungi and especially the synthesis of AFB₁ in feed and feed raw materials both in field and in storage conditions.

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