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Research Article

A Research on the Determination of Aflatoxin M1 Levels in Milk and Dairy Products for Sale in Diyarbakır by ELISA

Ömer Faruk YEŞİL[1], Abdulkerim HATİPOĞLU [2*], Abdunnasır YILDIZ [3], Aydın VURAL [4], Mehmet Emin ERKAN [4]

[1] Food Technology Programme, Department of Food Processing, Diyarbakır Agricultural Vocational High School, Dicle University, 21280, Diyarbakır, Turkey
[2] Department of Nutrition and Dietetics, Health College, Mardin Artuklu University 47200, Mardin, Turkey
[3] Department of Biology, Faculty of Science, Dicle University, 21280, Diyarbakır, Turkey
[4] Department of Food and Nutrition Hygiene, Faculty of Veterinary Medicine, Dicle University, 21280, Diyarbakır, Turkey

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Abctract

In this study, the contamination levels of Aflatoxin M_1 of 248 sterilized milk, Beyaz, Cecil, Cokelek, Cream, Lor, Van Otlu (herbed), Van Otlu Lavash Cheese and butter samples randomly obtained from selected market and supermarkets from Diyarbakır province center were determined by ELISA method. The average AFM_1 contamination value of the cheese samples investigated was 138.65 ng/kg; while the minimum and maximum values were 50 and 595.31 ng/kg, respectively. On the other hand, the average AFM_1 value of 13 sterilized milk samples was 52.59 ng/kg; minimum and maximum values were determined as 25.97 and 80.0 ng/kg, respectively. In addition, the average AFM1 value of 18 butter samples was 97.54 ng/kg; the minimum and maximum values were calculated as 50 and 308.13 ng/kg. In the study, the level of AFM1 contamination in the 26 samples of cheese samples were exceeded the limits set by the European Commission for AFM1. In terms of Turkish Food Codex, only 2 cheese samples exceeded the AFM1 tolerance limit. According to the study, the level of AFM1 contamination in the 7 milk samples and 15 butter samples exceeded both the European Commission and Turkish Food Codex AFM1 tolerance limit.

Keywords: ELISA, AFM1, cheese, butter, milk

Diyarbakır’da Tüketime Sunulan Süt ve Ürünlerinde Aflatoksin M1 Düzeylerinin ELISA ile Belirlenmesi Üzerine Bir Araştırma

Öz

Bu çalışmada, Diyarbakır il merkezinde bulunan toplu ve perakende satış noktalarında tüketime arz edilen 248 adet sterilize süt, Beyaz Peynir, Çeçil, Çökelek, Krem, Lor, Van Otlu, Van Otlu Lavaş Peyniri ve tereyağı örneğinin Aflatoksin M_1 (AFM_1) bulaşma düzeyi ELISA yöntemi kullanılarak tespit edilmiştir. Araştırmaya konu olan peynir örneklerinin ortalama AFM_1 kontaminasyonu 138.65 ng/kg; minimum ve maksimum değerleri ise 50 ve 595.31 ng/kg olarak ortaya konmuştur. Diğer taraftan 13 adet sterilize süt örneğinin ortalama AFM_1 değeri 52.59 ng/kg; minimum ve maksimum değerleri ise 25.97 ve 80.0 ng/kg olarak tespit edilmiştir. Ayrıca 18 adet tereyağı örneğinin ortalama AFM1 değeri 97.54 ng/kg; minimum ve maksimum değerleri ise 50 ve 308.13 ng/kg olarak hesaplanmıştır. Araştırmada toplam 26 peynir örneğindeki AFM1 kontaminasyon düzeyi Avrupa Komisyonu’ nda AFM_1 için belirtilen limiti aşmıştır. Türk Gıda Kodeksi açısından ise sadece 2 peynir örneğinin AFM1 kontaminasyonu tolerans limitini aşmıştır. Çalışma sonuçlarına göre, 7 süt örneği ile 15 tereyağı örneğinde tespit edilen AFM1 düzeyi hem Avrupa Komisyonu hem de Türk Gıda Kodeksi AFM1 tolerans limitini aşmıştır.

Anahtar Kelimeler: ELISA, AFM1, peynir, tereyağı, süt

1. Introduction

Presence of aflatoxins (AFs) is one of the major food safety concerns in tropical and subtropical countries (Skrbic et al., 2015). The AFs among mycotoxins detected in dairy products are toxic, teratogenic, mutagenic, immunosuppressive compounds for human

***Corresponding Author:**
abdulkerimhatipoglu@artuklu.edu.tr

and animals (Fallah et al., 2009; Kamkar, 2008; Anfossi et al., 2012; Mohajeri et al., 2013). They cause especially on skin diseases and liver disorders (Rodas et al., 2018).

Afs are secondary metabolites produced by fungi, mainly *Aspergillus flavus, Aspergillus parasiticus* and rarely by *Aspergillus nomius* under suitable environmental conditions in animal feed and food. There are at least 20 different types of naturally-occurring AFs and the most recognized are B1, B2, G1 and G2 (Prodanov-Radulović et al., 2017). *A. parasiticus* and *A. nomius* produce both B and G Afs, while *A. flavus* only produces B Afs. Aflatoxin M1 (AFM1) and M2 (AFM2) are the hydroxylate metabolites of Aflatoxin B1 (AFB1) and B2 (AFB2), respectively. There are two main ways of aflatoxin contamination in the milk and dairy products. Firstly, when the animals in lactation period consume feeds contaminated with aflatoxin, aflatoxin B1 and B2 transform into AFM1/milk toxin and M2 by hepatic cytochrome p450 after metabolized in the animal body. These metabolized toxins pass to the milk produced from the animal and the contamination occurs. Secondly, the contamination occurs when the molds synthesizing aflatoxin pass to the milk and produce aflatoxin during transport, process and storage phases after milking (Torkar and Vengust, 2008; Fallah, 2010; Anfossi et al., 2012; Durakovic, 2012; Li et al., 2017, Berna et al., 2018, Hassan et al., 2018, Madali et al., 2018).

AFM1 is mainly soluble in the aqueous phase of milk or adsorbed to casein particles; a small ratio of AFM1 in milk is carried-over to cream, and yet a smaller proportion to butter. The remainder of AFM1 in milk, however, remains in skim milk and buttermilk (Atasever et al., 2010b). In other words, the stability of AFM1 determines its persistence in foodstuffs such as butter, yogurt, cheese, cream and ice cream. However, this toxin is not inactivated by the thermal processing (pasteurization and ultra-high-temperature (UHT) treatment) used in the dairy industry (Aksoy et al., 2016).

Human exposure to AFM1 is due to the consumption of contaminated milk and dairy products of which daily intake could be highly variable in the world. Infants represent the most susceptibly exposed population due to their high consumption of dairy products either as animal’s milk and related by-products in their diet or from breast milk where the mycotoxin can be excreted (Langat et al., 2016). The Joint FAO/WHO Expert Committee on Food Additives reported that the intake of AFM1 from milk was 6.8 ng/person/day for the European diet, 0.7 ng/person/day for the Middle Eastern diet, 12 ng/person/day for the Far Eastern diet, 3.5 ng/person/day for the Latin American diet and 0.1 ng/person/day for the African diet (Anonymous, 2001). Therefore, many countries have set legal regulations to control aflatoxin M1 level in milk and dairy products.

The present study, AFM1 contamination levels of White, Cecil, Cokelek, Cream, Lor, Van Otlu, Van Otlu Lavash Cheese, butter and sterilized / UHT milk samples (248 samples) sold in Diyarbakır were determined. The results were compared with the European Commission’s (Anonymous, 2010) and Turkish Food Codex’s AFM1 levels (Anonymous, 2008).

2. Material and Methods

2.1. Collecting of Samples

In our study, A total of 248 samples of Beyaz, Cecil, Cokelek, Cream, Lor, Van Otlu (herbed), Van Otlu Lavash Cheese, butter and sterilized/UHT milk samples (cheese and butter samples: about 250 g; milk samples: 1000 ml) between May and December 2013 were aceptically collected from randomly selected markets and super markets from different regions of Diyarbakır province. By the sampling procedure, attention was paid to collect different brands and lots. The samples were delivered to the laboratory by preserving cold chain. The samples (except sterilized milk) were stored at -18 °C in the laboratory until they were analyzed by ELISA method.

2.2. Preparation Of Milk Samples

Milk samples were centrifuged at 3500 rpm for 10 minutes at 10 °C. After centrifugation, the remaining oil layer top of the tube was removed using Pasteur pipette. 100 µl of the supernatant was used for direct analysis. Dilution factor was accepted as "1" for milk samples.

2.3. Preparation of Cheese Samples

40 ml of dichloromethane was added to 2 g of cheese, which had been thoroughly shredded and crushed into a stomacher bag, and homogenized on a stomacher for 2 minutes. After the obtained suspension was filtered (Whatman filter No:1, 125 mm), 10 ml of the extract was taken into the test tube and evaporated in a hot water bath at 60 °C. Then, it was evaporated at 60 °C by the weak nitrogen gas until the oil layer remained at the bottom of the tube. The residue, with the addition of 0.5 mL of methanol, 0.5 mL of Phosphate Buffered Saline (PBS) buffer and 1 mL of heptane, was centrifuged at 2700 rpm for 15 min (15 ºC). After removing the supernatant heptane layer with a pasteur pipette, 100 µl of the bottom methanolic liquid phase and added 400 µl Buffer 1. 100 µl of this mixture was used in the test. Dilution factor was accepted as "10" for cheese samples.

2.4. Preparation of Butter Samples

3 g of the butter samples were weighed into a 10 ml centrifuge tube and centrifuged (for 1 minute at 3000 rpm) at room temperature. Centrifuged samples were incubated at 40 °C in a hot water bath. 3 ml of n-hexane and 3 ml of 70 % methanol were added respectively to the samples removed from the water bath and mixed for 1 minute on a vortex stirrer. Mixing was continued for 15 minutes by reversing the tube. Then, the samples were centrifuged (for 10 minutes at 4000 rpm and 10 °C). 50 µl of the bottom liquid phase with buffer1 were diluted 1:17 in a new tube. 100 µl of this sample was used for analysis. Dilution factor was accepted as "10" for butter samples.

2.5. Aflatoxin M1 Test Procedure

100 µl of the prepared sample and the standards were incubated for 60 minutes. Plates were washed 2 times in the automatic washer, then 100 µl of enzyme conjugate was added and incubated for 60 minutes in room temperature. After 3 washes in the automatic washer, 100 µL of substrate/chromogen was added to each well, thoroughly mixed, and incubated for 30 minutes in the dark at room temperature. After adding 100 µL stock solution to each well, it was thoroughly mixed and read at 450 nm with a packet program. Dilution factor was accepted as "10" for cheese samples.

2.6. Determination of Aflatoxin M1 by ELISA

The presence of aflatoxin M1 was determined by ELISA method using Ridascreen® Aflatoxin M1 Art. No. ELX 50 Automatic Microplate Washer and ELX 800 Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) devices with the ELISA test kit R1101 (RBiopharm AG, Darmstadt, Germany). The results were evaluated in the RIDAWIN Package Program (RIDA®SOFT Win, RBiopharm AG, Darmstadt, Germany).

2.7. Statistical analysis

The results were analyzed by SPSS statistical program for Windows developed by IBM (IBM® SPSS© Statistics Version 21). Results were expressed as mean and also as minimum and maximum concentration of AFM1.

3. Results and Discussion

The AFM1 values of the cheese samples are presented in Table 1. According to the results, AFM1 was detected all of the samples analyzed. The average AFM1 value of cheese samples was 133.51 ng/kg; the minimum and maximum values were determined as 50 ng/kg and 595.31 ng/kg, respectively. 11.98% of the samples (26 samples of cheese) were found to exceed the limit for AFM1 (250 ng/kg) of the European Commission (EC) (Anonymous, 2010). On the other hand, 0.92% of the samples (2 samples of cheese) were found to exceed the tolerance limit for AFM1 (500 ng/kg) of the Turkish Food Codex (TFC)(Anonymous, 2008).

Table 1. Aflatoxin M1 levels of the cheese samples.

Samples	**The Number of Samples**						**Concentration (ng/kg)**	
	Total	**0-50 ng/kg**	**51-150 ng/kg**	**151-250 ng/kg**	**>250[a] (ng/kg)**	**>500[b]**	**Mean**	**Minimum - Maximum**
Beyaz Cheese	131 (60.37%)	27	63	26	14	1	132.94	50-595.31
Cecil Cheese	7 (3.2%)	0	1	2	4	0	249.84	70.06-406.76
Cokelek	6 (2.76%)	2	3	1	0	0	85	50-176.2
Cream Cheese	5 (2.30%)	0	4	1	0	0	136.96	99.46-181.14
Lor Cheese	12 (5.53%)	2	3	6	1	0	159.01	50-393.82
Van Otlu Cheese	43 (19.82%)	16	11	10	5	1	140.47	50-518.78
Van Otlu Lavash Cheese	13 (5.99%)	8	4	1	0	0	66.31	50-160.46
Total	217 (100%)	55 (25.35%)	89 (41.01%)	47 (21.66%)	24 (11.06%)	2 (0.92%)	138.65	50-595.31

[a]: European Commission limit (250 ng/kg), [b]: Turkish Food Codex limit: (500 ng/kg).

Some of the researchers found that the maximum AFM1 contamination in cheeses were between 17 and 19 ng/kg (Gürses et al., 2004; Özturk et al., 2014); while the others reported that were between 800 and 2000 ng/kg (Günşen and Büyükyörük, 2003; Alkan and Gönülalan, 2006; Atasever et al., 2010a; Filazi et al., 2010; Hatipoğlu et al., 2016).

A number of researchers reported that AFM_1 contamination rates did not exceed the tolerance limits (EC and TFC) in their studies on the Kashar, Hellim, Beyaz, Urfa Beyaz, Civil, Tulum and Lor cheese samples (Gürses et al., 2004; Kök, 2006; Atasever et al., 2010a; Dinçel et al., 2012; Öztürk et al., 2014). On the other hand, different investigators determined that AFM1 level in some Orgu, Kashar, Beyaz and Cream cheese samples exceeded the tolerance limit (Anoymous, 2010). The AFM1 contamination rates of the mentioned cheeses were reported as 14% (13), 17% (32); 4% (1), 17% (1), 30% (6), 33% (16), 44% (20), 35% (26), 33% (28); 2% (1), 20% (4), 10% (5), 21% (16), 27% (23), 28% (33); 17% (14), 19% (18), respectively (Günşen and Büyükyörük, 2003; Gürses et al., 2004; Çetin et al., 2005; Alkan and Gönülalan, 2006; Kök, 2006; Kireççi et al., 2007; Erkan et al., 2009; Fallah et al., 2009; Atasever et al., 2010a; Dinçoğlu et al., 2012; Filazi et al., 2010; Kögüstün, 2013; Hatipoğlu et al., 2016).

In this study, the total AFM1 rate of cheese samples (11.98%), which was exceeding the European Commission tolerance limit, was determined higher than the values reported by Çetin et al. (2005) in Kashar cheese (4%), Alkan and Gonulalan (2006) in Beyaz cheese (2%) and Filazi et al. (2010) in Beyaz cheese (10%). Conversely, the rate was determined lower than the values reported by Erkan et al. (2009) and Hatipoğlu et al. (2016) in Orgu cheese (14%, 17%); Günşen and Büyükyörük (2003), Kök (2006), Kireççi et al. (2007), Atasever et al. (2010a), Dinçoğlu et al. (2012) and Kögüstün (2013) in Kashar cheese (33%, 17%, 30%, 35%, 33%, 44%); Kireççi et al. (2007), Fallah et al. (2009), Atasever et al. (2010a) and Dinçoğlu et al. (2012) in Beyaz cheese (20%, 28%, 27%, 21%); Fallah et al. (2009) and Atasever et al. (2010a) in Cream cheese (19%, 17%).

Table 2. Aflatoxin M1 levels of sterilized/UHT milk and butter samples

Samples	The Number of Samples					Concentration (ng/kg)	
	Total	0-50 ng/kg	51-150[a] ng/kg	151-250 ng/kg	>250 (ng/kg)	Mean	Minimum-maximum
Sterilized /UHT milk	13 (100%)	6 (46.16%)	7 (53.84%)	0	0	52.59	25.97-80
Butter	18(100%)	3 (16.66 %)	12 (66.67 %)	2(11.11 %)	1 (5.56%)	97.54	50-308.13

[a]: European Commission and Turkish Food Codex limit for milk and butter:50 ng/kg

On the other hand, many investigators reported that almost all of the samples analyzed in their researches related to raw, pasteurized and sterilized milk exceeded the permitted limits (Anonymous, 2008; Anonymous, 2010; Bakırcı, 2001; Akdemir and Altıntaş, 2004; Çelik et al., 2005; Kök, 2006; Ünüsan, 2006; Özdemir, 2007; Kamkar, 2008; Elzupir and Elhussein, 2010; Fallah, 2010; Hazer, 2011; İşleyici et al., 2012; Yurt and Uluçay, 2016; Karadal et al., 2018). Some of the researchers determined that the maximum AFM_1 contamination in milk were between 37 and 94 ng/kg (Durakovic et al., 2012; İşleyici et al., 2012; Karadal et al., 2018), whereas the other part of the researchers determined were between 352 and 6900 ng/kg (Akdemir and Altıntaş, 2004; Ünüsan, 2006; Fallah, 2010; Elzupir and Elhussein, 2010; Yurt and Uluçay, 2016; Xiong et al. 2018). According to the results of our study (Table 2), it was revealed that 7 out of 13 sterilized milk samples (53.84%) exceeded the acceptance limits (Anonymous, 2008; Anonymous, 2010). The AFM1 contamination rates of the milk samples were determined higher than raw milks' values reported by Bakırcı (2001), Akdemir and Altıntaş (2004), Özdemir (2007), Hazer (2011), Rama et al. (2015), Mashak et al. (2016), Karadal et al. (2018), Quevedo-Garza (2018), Xiong et al. (2018) and Türkoğlu and Keyvan (2019). Likewise, the rates were determined higher than those in pasteurized and sterilized milks (Ünüsan, 2006; Fallah, 2010; İşleyici et al., 2012). On the other hand, the AFM1 contamination rates of the milk samples were lower than the values reported by Çelik et al. (2005), Kök (2006), Kamkar (2008), Elzupir and Elhussein (2010) and Yurt and Uluçay (2016) as 64% (48), 62% (8), 77% (40), 83% (35) and 80% (20), respectively. According to the results of a study with 520 milk samples in Pakistan, the lowest AFM1 levels in milk were observed in summer and the highest AFM1 level in winter months. The same researchers reported that AFM1 levels in the morning milk were higher than evening milk (İsmail et al., 2016).

The present study, the total AFM1 rate of butter samples (83.34%), which was exceeding the EC and TFC tolerance limits, was determined higher than the values reported by Atasever et al. (2010b) and Iqbal and Asi (2013) in the butter (16.3% and 22.97%). On the other hand, Aksoy et al. (2016) reported that they could not detect AFM1 in any of the 40 butter samples in their study by ELISA and HPLC. Additionaly, Hassan et al. (2018) reported that the level of AFM1 were below 50 ng/kg in 18 butter samples. Some researchers claimed the storage conditions of products including humidity and temperature were also important for toxin production in butter (Iqbal and Asi, 2013).

As seen above, different studies have been carried out to reveal the level of AFM1 contamination in milk and milk products. The differences in the results of the research can be attributed to the level of milk contamination due to seasonal changes, the geographical region, the milk/butter/cheese type or production technique, the cheese maturity level, the analysis methods used, the diet of dairy animals (rations) and the lactation period (Blanco et al., 1988; Barrios et al., 1996; Galvano et al., 1996; Pitter, 1998; Sarımehmetoğlu et al., 2004; Kamkar, 2008; Fallah, 2010; Rahimi et al. 2010; Tavakoli et al., 2012; Malissiova et al. 2013, İsmail et al., 2016).

4. Conclusion

As a result, 53.84% of milk samples and 83.34% of butter samples sold in Diyarbakır exceeded the limit for AFM1 of the European Commission and Turkish Food Codex. Additionaly, 11.98% of cheese samples sold in Diyarbakır exceeded the limit for AFM1 of the European Commision and 0.92% of cheese samples exceeded the tolerance limit for AFM1 of Turkish Food Codex. This situation poses a potential risk to public health and indicates the sporadic use of contaminated feedstuff at farms. For this reason, the following points should be noted: It is especially important to keep humidity and temperature levels under control in storage areas of animal feed (especially silage) from where raw milk/cheese/butter to prevent the risk. On the other hand, training of feed and dairy manufacturers/employees should be required about the health risks of aflatoxins and possible measures. Moreover, can be suggested the establishment of sustainable modern milk production farms. Finally, the competent authorities should take urgent precautions in these matters.

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