Arch Lebensmittelhyg 70,
17–22 (2019)
DOI 10.2376/0003-925X-70-17

ISSN 0003-925X

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Determination of Seasonal Distribution of Aflatoxin M_1 Level in Cheese Production

Bestimmung der saisonalen Verteilung des Aflatoxin M_1-Gehalts in der Käseproduktion

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Summary

The aim of this study was to determine the Aflatoxin M_1 (AFM_1) quantities in raw milk samples and cheese samples produced from the same lot. The samples were obtained from six dairy plants in four different seasons. AFM_1 amounts of the samples were assessed by a Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) device with fluorescence detector, using a preliminary immune affinity column (IAC) for post-column derivatization and these values were compared with legal limits. Average of AFM_1 values of raw milk samples were determined to be 41.9 ng/l, 31.3 ng/l, 68.5 ng/l and 92.0 ng/l in spring, summer, autumn, and winter, respectively. 54.2 % of the investigated milk samples exceeded legal limits. AFM_1 concentrations were higher during the autumn and winter. Average values of AFM_1 in cheese samples were determined to be 92.7 ng/kg, 72.3 ng/kg, 190.6 ng/kg and 255.8 ng/kg in spring, summer, autumn, and winter, respectively. AFM_1 was at levels of 1.25 to 5.18-fold higher than those presented in the raw milk used for the cheese production. Based on the admissible limit, 16.7 % of the cheese samples were shown to have exceeded this valid maximum limit value. Consequently, it was found that seasonal change had a significant effect on the amount of AFM_1.

Keywords: Aflatoxin M_1, Mycotoxin, Raw milk, Cheese

Zusammenfassung

Ziel der Studie war es die Konzentrationen von Aflatoxin M_1 (AFM_1) in Rohmilch und daraus produziertem Käse zu bestimmen. Die entsprechenden Proben wurden von sechs verschiedenen Molkereien zu vier verschiedenen Jahreszeiten bezogen. Die Probenvorbereitung erfolgte über eine Immunaffinitätssäule (IAC) mit anschließender Bestimmung des AFM_1 Gehalt mittels Umkehrphasen-Hochleistungsflüssigkeits-Chromatographie (RP-HPLC) mit Fluoreszenz-Detektor. Die Ergebnisse wurden mit gesetzlichen Grenzwerten verglichen. Die durchschnittlichen AFM_1 Werte der Rohmilchproben lagen bei 41.9 ng/l (Frühling), 31.3 ng/l (Sommer), 68.5 ng/l (Herbst) und 92.0 ng/l (Winter). 54.2 % der untersuchten Milchproben überschritten den gesetzlichen Grenzwert. AFM_1 Konzentrationen waren im Herbst und Winter höher. Die durchschnittlichen AFM_1 Werte in den Käseproben lagen bei 92.7 ng/kg (Frühling), 72.3 ng/kg (Sommer), 190.6 ng/kg (Herbst) und 255.8 ng/kg (Winter). Der AFM_1 Gehalt in den Käseproben war um das 1.25 bis 5.18-fache höher als in der Rohmilch. 16.7 % der untersuchten Käseproben überschritten den gesetzlichen Grenzwert. Es konnte ein signifikanter saisonaler Effekt auf die AFM_1 Gehalte festgestellt werden.

Schlüsselwörter: Aflatoxin M_1, Mycotoxin, Rohmilch, Käse

Journal of Food Safety and Food Quality 70, Heft 1 (2019), Seiten 1–30

Introduction

Certain molds may produce various secondary metabolites known as "mycotoxins" under appropriate conditions with optimum temperature and moisture. Mycotoxins are part of a large family, comprising major groups including aflatoxins, ochratoxins, fumonisins, patulin, trichothecenes, and zearalenon and hazardous to human health (Iqbal et al. 2014). The most important mycotoxins that may be found in feed and food substances are naturally occurring aflatoxins produced by the fungi species *Aspergillus flavus, Aspergillus nomius* and *Aspergillus parasiticus* (Kav et al. 2011). Aflatoxins are coayamposed of four sub-types Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1) and Aflatoxin G_2 (AFG_2) categorized as acute and chronic toxins for animals and humans according to their fluorescence properties Figure 1 (da Rocha et al. 2014). AFB_1 is known to be the most toxic and most prevalent one. AFB_1 has been recognized as AFM_1 especially in the milk of animals fed with aflatoxin contaminated feed. If animals are fed with AFB_1 contaminated feed, it is bio-transformed to AFM_1 by the hepatic microsomal mixed-function oxidase system and gets absorbed in the milk of mammals. AFM_1 is one of the hydroxylated metabolites of the potential carcinogen, AFB_1 and called milk toxin (Ozkaya and Temiz 2003; Chiara et al. 2006). AFM_1 contamination of milk and dairy products usually results from AFB_1 contaminated animal feed (Galvano et al. 1996; Xiong et al. 2013). Humans become exposed to AFM_1 through the intake of contaminated milk and dairy products. International Agency for Research on Cancer (IARC) classified mycotoxins according to their carcinogenic potentials in 1993 and AFB_1 was included among "Proven human carcinogens" whereas AFM_1 belonged to the group of agents, "Probably carcinogenic to humans" (IARC 1993).

Milk and dairy products are among the most risky food products concerning the presence of aflatoxin residues. In case AFM_1, which shows carcinogenic activity, occurs in raw milk, cheese, whey and curd made from such milk also contain AFM_1 (Cavallarin et al. 2014). The maximum aflatoxin level has been determined in milk and dairy products from several countries in order to minimize the risk factor. While these levels range between 0 and 500 ng/kg for milk in most countries, 500 ng/kg has been defined as the admissible limit value for cheese in USA and several Asian and European countries. The approved maximum levels vary depending on the developmental and economical states of the countries (Kabak and Var 2004; Yaroglu et al. 2005; Cavallarin et al. 2014). Although a uniform European Union (EU) regulation for maximum limits of AFM_1 in cheese currently does not exist, some member states (e.g. The Netherlands, Austria, Switzerland) have established different tolerance limits for AFM_1 in cheeses: 200 ng/kg to 250 ng/kg (Food and Agriculture Organization 2004). In Turkey, According to Turkish Food Codex (TFC) Regulation concerning the Notification on Maximum Levels for Certain Contaminants in Foodstuffs, this level was determined to be 50 ng/L and 250 ng/kg for milk and cheese, respectively (TFC 2002). The current TFC Regulation on Contaminants approved the same legal level for milk; however, no limits were defined for AFM_1 levels in cheese (TFC 2011).

The possible presence of such mycotoxins in milk and dairy products is a public health concern. Several studies have been carried out with respect to presence of AFM_1 in milk and dairy products in Turkey and throughout the world (Sarimehmetoglu et al. 2004; Erkan et al. 2009; Atasever et al. 2010; Aydemir et al. 2010; Kav et al. 2011; Kabak and Ozbey 2012; Xiong et al. 2013; Cavallarin et al. 2014; Skrbic et al. 2014). Although heat treatments can reduce AFM_1 activity, AFM_1 is relatively stable during processing of food production (Govaris et al. 2001). There are several reports of AFM_1 contamination of cheeses produced from milk artificially contaminated milk samples. The AFM_1 levels in cheese seem to be variable, depending on the type of the product. For example, Elgerbi et al. (2006) found the presence of higher levels of AFM_1 in cheese than in the artificially contaminated milk. Furthermore, López et al. (2001) reported that fresh cheese produced in Argentina from artificially contaminated milk with AFM_1 at levels of 1.7–2.0 ng/ml had 40 % of AFM_1 in cheese. Although in Turkey previous studies indicated a higher frequency of AFM_1 in milk, there are no previous reports on the carry-over of AFM_1 from milk to cheese. In the present study, the AFM_1 levels were determined in both non-processed milk samples and the cheese samples made from the same lot of milk collected for four seasons from six small dairy plants in Kırklareli located in the Thrace region that is known to play a crucial role in the Turkish cheese industry.

Materials and Methods

Study design

A total of 24 raw milk samples and 24 Turkish white cheese samples produced from the same lot of milk collected from 6 small scale milk processing plants in Kırklareli province during four seasons constituted the study material. Cheese samples, after a 3-month-long maturation in brine period were collected in due form and transferred to the laboratory. All samples were stored at –20 °C until the analysis. The analysis was performed in duplicate.

Preparation of the Milk Samples

Milk samples were heated to 37 °C in a water bath and centrifuged at 1540 × g for 15 min. The top fat layer was

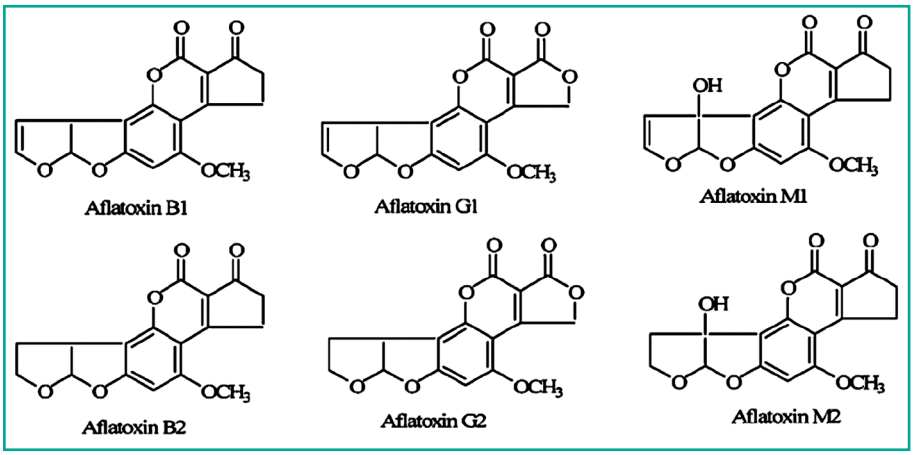

FIGURE 1: *Chemical structures of Aflatoxins B1, B2, G1, G2, M1, M2.*

removed and the remaining part was filtered through Whatman-4 paper. A 50 ml of the milk filtrate was passed through AflaTest™ immunoaffinity column at an approximate constant speed of 1–2 drops per sec. The column contained specific monoclonal antibodies linked to the source. When the specimen passed through the column selective antibodies were bound to AFM_1 forming an antigen-antibody complex. This procedure took approximately 20 min. Then 10 ml of pure water (twice) was passed through the column. Air was applied to clean up the remaining water in the column. In order to separate AFM_1 from the immunoaffinity column, 1.25 ml of acetonitrile/methanol (3/2) was added and the filtrate was collected into a separate vial. Then 1.25 ml of ultrapure water was passed through the column to a final volume of 2.5 ml in the vial.

Preparation of Cheese Samples

Cheese samples were homogenously mixed up with Celite 545 (10 g), saturated sodium chloride solution (2 ml, 6M) and chloroform (150 ml) were added to 40 g of the mixture and stirred in a homogenizer for 2–3 min and then filtered with Whatman-4. The filtrate was evaporated under vacuum at 30 °C by a rotary evaporator. 2 ml methanol, 98 ml phosphate buffered saline (PBS; 0.2 g KCl, 0.2 g KH_2PO_4, 1.16 g Na_2HPO_4, 8.0 g NaCl, pH 7.4 per liter) and 70 ml hexane were added to the residue, stirred and transferred to a separation funnel. Then, a 50 ml of the lower phase in the separation funnel was passed through the column. The column was rinsed with 20 ml of ultrapure water to remove all the components. AFM_1 entrapped in the column was transferred into a vial ready for injection like the milk samples (Cetin 2004; Vicam 2009).

HPLC analysis of AFM_1 in milk and cheese

AFM_1 analysis of pretreated milk and cheese samples were run by reverse phase high performance liquid chromatography (RP-HPLC) device (Agilent) with fluorescence detector (Ex: 360 nm; Em: 440 nm) by using Spherisorb S5ODS-2 column (4.6x250 mm i.d., 5 µm particle size). The isocratic mobile phase included 68 % ultrapure water, 24 % acetonitrile and 8 % methanol and the flow rate was 1 ml per min. Injection volume was 100 µl and the temperature of the column was 30 °C. Two repetitions were made for analysis. Under these conditions, the retention time for AFM_1 was approximately 10 minutes.

A calibration curves for AFM_1 were prepared using standard solutions of AFM_1 (10 µg/ml, Sigma®), diluted in acetonitrile at concentrations of 0.02 ng/ml, 0.06 ng/ml, 1.0 ng/ml, 2.0 ng/ml, 4.0 ng/ml and 8.0 ng/ml as previously described by Scott (1990). The coefficient of determination (R^2) for the AFM_1 curves ranged from 0.9816 to 0.9979 attesting to the repeatability (Horwitz 1982) of calibration curves for AFM_1. 100 µl of sample extracts were injected for AFM_1 determination.

All chemicals used were analytically pure (Merck, Germany). AflaTest™ immunoaffinity columns (Vicam L.P., Watertown, MA, ABD) and AFM_1 standard (Sigma C.C., ABD) were commercially available. AFM_1 stock solution (50 ng/ml) was prepared with acetonitrile and stored at –20 °C (Vicam 2009).

Statistical Analysis

Differences in amount of AFM_1 in the milk and cheese samples from four different seasons were statistically analyzed by one way analysis of variance (ANOVA) using SPSS version 18.0 software. The level of confidence required for significance was set at $p<0.05$.

Results and Discussion

The concentration of AFM_1 in milk samples was determined using the standard calibration graph plotted in the range of 0.2 to 8 ng/ml. Linear regression analysis was further used for the quantification of AFM_1 present within the milk samples. The standard calibration graph is presented in Figure 2, the HPLC chromatogram of standard solutions showed an excellent linearity with R^2 value of 0.999.

AFM_1 was detected in all 24 raw milk samples, with contamination range between 17.83–202.15 ng/l (58.43 ng/l average concentrations). The HPLC chromatogram of a milk sample was shown in Figure 3. AFM_1 levels of milk samples collected from dairy plants during four seasons were given in Table 1. Mean values of AFM_1 levels in milk samples were 41.9 ng/l, 31.3 ng/l, 68.5 ng/l and 92.0 ng/l for spring, summer, autumn and winter, respectively (Table 1). When average rate of change in AFM_1 content was evalua-

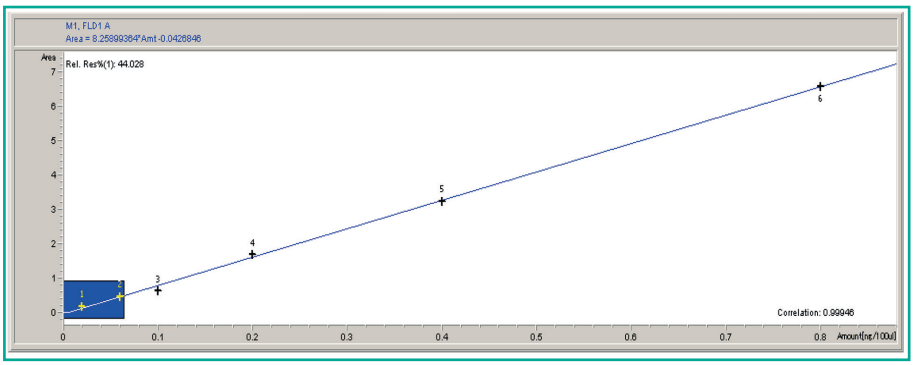

FIGURE 2: *Calibration curve of standard solutions of AFM_1 with concentrations of 0.2 to 8 ng/ml using HPLC.*

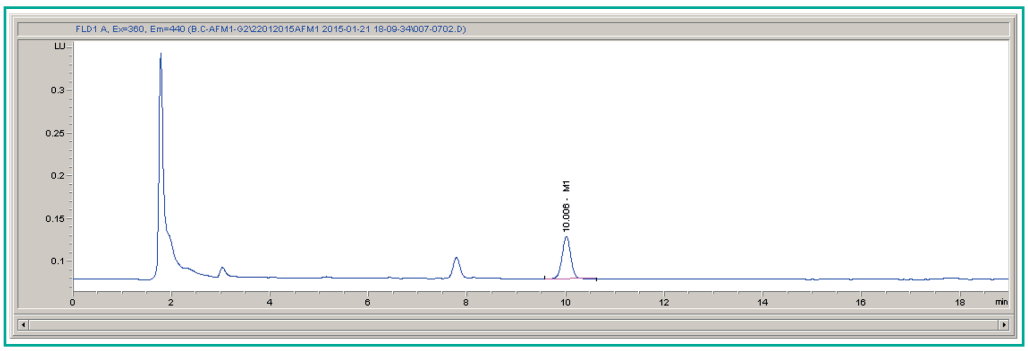

FIGURE 3: *Chromatogram of a milk sample, showing the retention time of AFM_1 (nearly 10 minutes).*

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TABLE 1: *AFM_1 concentrations in raw milk samples (ng/l) and cheese samples (ng/kg).*

Dairy plants	Milk (ng/l)[a]	Spring Cheese (ng/kg)[a]	Concentration factor[b]	Milk (ng/l)[a]	Summer Cheese (ng/kg)[a]	Concentration factor[b]	Milk (ng/l)[a]	Autumn Cheese (ng/kg)[a]	Concentration factor[b]	Milk (ng/l)[a]	Winter Cheese (ng/kg)[a]	Concentration factor[b]
A	55.2590±2.46^{cB}	136.6870±4.91^{cB}	2.47	54.0305±3.90^{dB}	105.8810±7.51^{cB}	1.96	56.9229±3.51^{bB}	191.4880±0^{bB}	3.36	77.6435±7.53^{aB}	197.8900±8.50^{aB}	2.55
B	18.8795±1.67^{cD}	54.2330±0^{cC}	2.87	20.1761±0.95^{dD}	47.3290±0^{cC}	2.35	38.5060±7.71^{bD}	83.2130±0^{bC}	2.16	71.3762±14.09^{aD}	151.1400±0^{aC}	2.12
C	39.1258±3.80^{cD}	99.5170±3.96^{cB}	2.54	17.8328±2.08^{dD}	45.3790±0.39^{cB}	2.54	50.6315±5.52^{bD}	198.5450±6.59^{bB}	3.92	56.8666±12.11^{aD}	294.4360±24.81^{aB}	5.18
D	64.1462±6.68^{cA}	80.3150±0^{cA}	1.25	29.4488±0.84^{dA}	77.7180±1.53^{cA}	2.64	173.3245±5.34^{bA}	357.4050±0^{bA}	2.06	202.1488±13.66^{aA}	422.6343±0^{aA}	2.10
E	24.0420±3.99^{cD}	77.9849±0.11^{cBC}	1.56	26.2245±5.49^{dD}	79.1753±2.58^{cBC}	3.02	34.5806±2.23^{bD}	106.8227±0.76^{bBC}	3.09	69.9151±6.31^{aD}	203.9020±0^{aBC}	2.92
F	49.9169±2.71^{cC}	107.2730±5.89^{cB}	2.15	40.0458±2.85^{dC}	78.4700±1.40^{cB}	1.96	56.9820±3.46^{bC}	206.0330±0.89^{bB}	3.62	74.2322±2.33^{aC}	264.6350±4.48^{aB}	3.56
Mean values	**41.8952**	**92.6683**	**2.14**	**31.2937**	**72.3254**	**2.41**	**68.4910**	**190.5845**	**3.03**	**92.0305**	**255.7724**	**3.07**

Different small letter depict the statistical difference between seasonal variations in milk and cheese samples (p<0.05). Different capital letter depict the statistical difference between milk and cheese samples of dairy plants (p<0.05), [a-d]: Results are expressed as mean ± standard deviation. n=3; [b]: Level of AFM_1 in cheese divided by the level in the original milk.

ted AFM_1 level in raw milk was detected to have increased in the autumn and winter months, yet all milk samples exceeded the legal limits (50 ng/l) in winter. The change in AFM_1 amounts compared to the seasons was found to be significant ($p<0.05$). The change in the type and the quality of feed due to seasonal conditions is recognized as the most important factor with respect to AFM_1 content in milk (Xiong et al. 2013). Animals are fed on dry forages of various types and particularly with silage instead of fresh grass especially in autumn and winter. Changes in the feeding regimen directly affect the content of AFM_1 and the quality of milk. Moreover, the occurrence of high levels of AFM_1 in every season in some dairy plants is considered to be associated with the processing conditions of milk.

In the present study, 54.2 % of raw milk samples assessed were determined to have exceeded the maximum residue level (50 ng/l). Studies have been carried out in Turkey with respect to the amount of AFM_1 in milk. In a study, AFM_1 analysis was performed by HPLC on a total of 27 milk samples (24 UHT and 3 pasteurized) collected in Ankara province. AFM_1 was detected in 59.3 % of all samples and only one of the samples was recorded to have exceeded the permissible limit of 50 ng/kg (Gürbay et al. 2006). Kabak and Özbey (2012) performed AFM_1 analysis on UHT milk collected from high capacity dairy plants by HPLC. The results revealed that 20 % of 40 milk samples contained AFM_1 and two samples exceeded the legal limits. It should be noted that ultra-high temperature processing must have contributed to the occurrence of high levels of AFM_1 only in such a small number of milk samples (Cavallarin et al. 2014). On the basis of the findings of researches performed, certain milk samples were determined to have exceeded the permissible limits regarding the levels of AFM_1 in Turkey (Bakirci 2001; Atasever et al. 2010; Kabak and Özbey 2012). This issue brings up the necessity to tighten up the control measures. In other studies worldwide, Skrbic et al. (2014) investigated the milk samples collected from the Serbian market between February and July and found out that 38 (76 %) of 50 milk samples exceeded the legal limits as regards to the level of AFM_1. In another study, Iha et al. (2013) in Brazil, analysis were performed on various types of milk samples in order to investigate the effects of production processes and storage on the content of AFM_1 and it was shown that these processes had little impact on the occurrence of AFM_1. Xiong et al. (2013) investigated the milk samples collected from different parts of China during 4 seasons in terms of the amount of AFM_1 and the levels of AFM_1 ranged between 10 and 420 ng/l in 43 (59.7 %) positive samples. On the basis of the findings, it was concluded that AFM_1 concentrations were higher in the milk samples collected in the winter time and there was no significant difference between spring and summer seasons concerning the mean concentrations of AFM_1.

The concentration of AFM_1 in cheese samples was determined from the standard calibration graph plotted in the range of 0.2 to 8 ng/ml using HPLC. Linear regression analysis was further used for the quantification of AFM_1 present within the cheese samples. The HPLC chromatogram of a cheese sample was shown in Figure 4. AFM_1 levels of cheese samples were given in Table 1. Mean values of AFM_1 levels in cheese samples were 92.7 ng/kg, 72.3 ng/kg, 190.6 ng/kg and 255.8 ng/kg for spring, summer, autumn and winter, respectively. AFM_1 concentrations were higher in the autumn and winter months. The change in AFM_1 amounts compared to the seasons was found to be significant ($p<0.05$). Products processed with AFM_1 contaminated milk also contained AFM_1 although the concentrations differed depending on the type of processing conditions. The type of cheese, processing technique applied and the water content removed all affect the levels of AFM_1. Therefore, it is crucial that AFM_1 content must not exceed the legal limits in raw milk (Cavallarin et a. 2014). Our findings also supported this situation. In TFC Regulation on Contaminants, no regulatory limits were declared for AFM_1 content in cheese (Turkish Food Codex 2011). Various permissible limits are implemented in different countries (Galvano et al. 1996; Xiong et al. 2013; Cavallarin et al. 2014). In TFC Regulation concerning the Notification on Maximum Levels for Certain Contaminants in Foodstuffs, this level was determined as 250 ng/kg for cheese (TFC 2002 and 2011). Based on the regulatory limit declared by the Notification, 16.6 % of the samples exceeded this limit. Moreover, comparable data regarding occurrence of AFM_1 in different cheese samples is also available in the literature. The concentration of AFM_1 determined in this study was supported by previous

studies carried out in Turkey and other countries (Kav et al. 2011; Erkan et al. 2009; Aydemir et al. 2010; Atasever et al. 2010; Torkar and Godic 2008).

In the present study, AFM_1 was at levels of 1.25 to 5.18-fold higher than those presented in the raw milk used for the cheese production (Table 1). There are several reports of AFM_1 contamination of cheeses produced from milk artificially treated with AFB_1; however, these reports refer to major cheeses from each country and the AFM_1 levels in cheese seem to be variable, depending on the type of the product (López et al. 2001). Recent studies including different types of cheeses demonstrated the presence of higher levels of AFM_1 in cheese than found in the artificially contaminated milk (Elgerbi et al. 2004; Oruc et al. 2006). For example, White cheeses from North Africa produced by using milk artificially contaminated with AFM_1 in concentrations of 10–300 ng/ml had AFM_1 concentrations ranging from 11 to 520 ng/kg (Elgerbi et al. 2004). White cheese from Iran had AFM_1 levels of 75 ng/kg in cheese, when milk was artificially contaminated with 50 ng/l AFM_1 (Kamkar et al. 2008). The concentration of AFM_1 in White cheese relative to milk was higher than those reported by researchers for other types of cheese. For example, fresh cheese produced in Argentina from artificially contaminated milk with AFM_1 at levels of 170–200 ng/l had 40 % of AFM_1 in the cheese (López et al. 2001). The AFM_1 levels in Parmesan and Mozzarella were 5.8- and 7.1-fold higher than in milk (Brackett and Marth 1982), while the level in Cottage cheese was 8.1-fold higher (Applebaum et al. 1982). Different types of cheese produced with milk artificially contaminated with AFM_1 have been reported to have concentrations 1.8 to 4.4 fold higher than in milk (Govaris et al. 2001; Oruc et al. 2006; Kamkar et al. 2008). Fremy et al. (1990) evaluated Camembert cheese produced with milk artificially contaminated with AFM_1 at levels as high as 30–750 ng/l and observed transfers of 35.6 and 57.7 % of AFM_1, respectively, from milk to cheese.

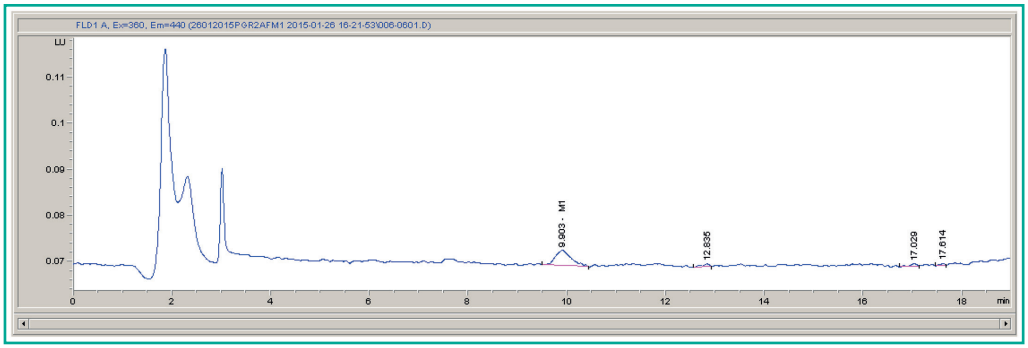

FIGURE 4: *Chromatogram of a cheese sample, showing the retention time of AFM_1 (nearly 10 minutes).*

In conclusion, the carry-over of AFM_1 from milk to cheese was 1.25–5.18 fold of the amount in the original milk. 54.2 % of dairy products examined exceeded the legal limits for AFM_1 in milk samples (50 ng/l) by Turkish regulations and when seasonal changes were evaluated, AFM_1 was shown to have increased during the autumn and winter months. Increased levels of AFM_1 pose a health threat to consumers. Therefore, factors that may lead to aflatoxin contamination such as feeding conditions and sanitation should be ameliorated and the authorities should be encouraged to increase the frequency of required controls. Especially, processing and feeding conditions in small dairy plants should be improved besides hygienic practice.

Acknowledgement

This work was supported by Kırklareli University Scientific Research Projects Coordinator (Project No: KLÜ-BAP-27)

Conflict of Interest

The authors declare that they have no conflict of interest.

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