DOI: 10.19113/sdufenbed.442395

Assessment of Aflatoxin M_1 Concentrations During Production and Long Storage of Salted (Tuzlu) Yogurt

Zehra ALBAY*[1], Bedia ŞİMŞEK[2]

[1,2]Suleyman Demirel University, Faculty of Engineering, Department of Food Engineering, 32260, Isparta, Turkey
[1](ORCID: https://orcid.org/0000-0002-5090-8151)
[2](ORCID: https://orcid.org/0000-0002-7497-1542)

(Alınış / Received: 10.07.2018, Kabul / Accepted: 29.01.2019, Online Yayınlanma/ Published Online: 17.04.2019)

Keywords
Aflatoxin M_1,
HPLC,
Salted (tuzlu) yogurt,
Stability,
Storage

Abstract: In this survey, it was aimed to determine the concentration of aflatoxin M_1 (AFM_1) in milk during the production and storage of salted (tuzlu) yogurt using High Performance Liquid Chromatography (HPLC). Salted (tuzlu) yogurt was produced artificially from contaminated milk with AFM_1 at two different levels (0.05 µg/l and 0.1 µg/l). Yogurt and strained yogurt production caused losses of AFM_1 about 65%, 70.25% and 73.75%, 81.12% respectively, in milk contaminated with 0.05 µg/l AFM_1, and 0.1 µg/l AFM_1. Also, it was determined that the storage process of the salted (tuzlu) yogurt (90 days) decreases the AFM_1 content of the salted (tuzlu) yogurt by 0.019 and 0.027 µg/l (0.05 µg/l and 0.1 µg/l AFM_1 respectively). Difference among dates in storage period was found to be statistically significant ($P<0.01$). Salted (tuzlu) yogurt has long shelf life and high heat processing, and AFM_1 is not completely lost in both levels.

Tuzlu Yoğurdun Üretimi ve Uzun Süre Depolanması Sırasında Aflatoksin M_1 Konsantrasyonundaki Değişiklikler

AnahtarKelimeler
Aflatoksin M_1,
HPLC,
Tuzlu yoğurt,
Stabilite,
Depolama

Özet: Bu çalışmada, yüksek performanslı sıvı kromatografisi (HPLC) kullanılarak tuzlu yoğurdun üretimi ve depolanması sırasında sütteki aflatoksin M_1 (AFM_1) konsantrasyonunun belirlenmesi amaçlanmıştır. Yapay yolla iki farklı düzeyde (0.05 µg/l ve 0.1 µg/l) aflatoksin M_1 (AFM_1) ile kontamine edilmiş sütlerden tuzlu yoğurt üretilmiştir. 0.05 µg/l AFM_1 ve 0.1 µg/l AFM_1 ile kontamine edilmiş sütlerden yoğurt ve süzme yoğurt üretimi sırasıyla % 65, % 70.25 ve % 73.75, % 81.12 düzeyinde AFM_1 kaybına neden olmuştur. Ayrıca tuzlu yoğurda uygulanan depolama işleminin (90 gün), tuzlu yoğurdun AFM_1 içeriğini sırasıyla 0.019 ve 0.027 µg/l değerlerine azalttığı tespit edilmiştir (0.05 µg/l ve 0.1 µg/l AFM_1). Depolama periyodundaki zamanlar arasındaki fark istatistiksel olarak önemli bulunmuştur ($P<0.01$). Tuzlu yoğurt uzun raf ömrüne ve yüksek ısıl işleme sahiptir ve AFM_1 her iki seviyede de tamamen kaybolmamıştır.

1. Introduction

Aflatoxins are synthesized, especially by the *Aspergillus parasiticus, Aspergillusnomius* and *Aspergillus flavus* species [1,2], and rarely by other *Aspergillus*, *Penicillium* and *Rhizopus* species [3,4]. Up to now, almost 19 different toxic differentiation of aflatoxins have been declared [5]. *A. parasiticus* produces both B and G aflatoxins, while *Aspergillus flavus* only produces B aflatoxins [6]. International agency for research on cancer (IARC) categorized AFB_1 as Group I of carcinogen and AFM_1 as Group 2B of carcinogenic compounds [1,7].

AFB_1 is thought to be the most potent toxic aflatoxin and metabolically produces the monohydroxy derivative AFM_1 [8,9,10]. AFM_1 is almost as acutely toxic as AFB_1, while its mutagenic and carcinogenic potential seems to be lower [11,12,13]. Aflatoxins metabolized to the 8-9-epoxide connect macromolecules and cause cancer, hepatopathy and immunosuppression [9,14].

The United States Food and Drug Administration has defined a limit of 500 ng/l for AFM_1 in milk and dairy products [15], while the European Commission has defined a limit of 50 ng/l for AFM_1 in these products

*Correspondingauthor: zehraalbay32@gmail.com

[16]. The Turkish Food Codex legal limits for AFM_1 in milk is 0.05 μg/kg [17]. Aflatoxins cause cancer, slow down child development, suppress the immune system, and may cause death [18]. Therefore, it is significant to assessment of aflatoxin M_1 concentrations in milk and dairy products since it poses a potential health hazard.

The most significant problem caused by milk and dairy products in terms of AFM_1 is that it is stable against heat process such as UHT, sterilization and pasteurization. This is the reason why AFM_1 does not decrease in amount during the manufacture of dairy products [19]. Salted (tuzlu) yogurt, which is prepared by heating (second pasteurized at 90°C) of strained yogurt, is a traditional milk product that has a high amount of dry matter and long shelf-life [20,21]. 1-5% of salt is added into the salted (tuzlu) yogurt during the heating process to eliminate microbial development, and to decrease water activity [22,23,24].

The aim of this study was to investigate the dispersion and stability of AFM_1 during the manufacture and the storage of salted (tuzlu) yogurt. In this study, by adding AFM_1 in two doses (0.05 μg/l and 0.1 μg/l) into the milk used for producing salted (tuzlu) yogurt, the effects of straining, heat treatments (applied to milk and strained yogurt) and storage on the change of the initial concentration of AFM_1 were investigated. The changes in the AFM_1 content during manufacturing and storage were determined by the immunoaffinity column, High Pressure Liquid Chromatographic method.

2. Material and Method

2.1. Experimental design and preparation of salted (tuzlu) yogurt samples

During the manufacturing of the salted (tuzlu) yogurt, a total of 24 liters of raw cow milk was used. The milk was divided into three equal 8 liter measurements. A standard aflatoxin M_1 (Sigma-Aldrich, CAS 6795-23-9; C17H1207; FW 238.3; Co., 3050 Spruce Street, MO 63103, St Louis, USA) was spiked to raw milk at the levels of 0.05 μg/l (A) and 0.1 μg/l (B). The last measurement of milk was taken as the control group (C) and no aflatoxin M_1 was added. After the samples were pasteurized at 95°C for 20 min, the 3 groups of milk were cooled to 43±1°C [23]. The yogurt culture was inoculated into the milk (2.5%) (YO-MIX, Real 500 and 600 series, DANISCO), and the samples were incubated at 42±1°C for 2.5-3 hours at pH 4.7. The yogurt samples were cooled by keeping them at room temperature for 15 min. Then, whey of the yogurt was drained. The strained yogurt was pasteurized at 90°C for 90 min (second pasteurization). At this point, salt was added about 1 g/100 g to sample A and B. The samples were cooled to 20°C. The salted (tuzlu) yogurt samples were placed into plastic-originated vacuum bags [25]. The samples were then transferred into a refrigerator, and stored at 4°C for 90 days (Figure 1). The samples were analyzed at storage days 1, 30, 60, and 90, at 4°C. All the analyses were replicated three times.

Raw milk
↓
Addition Standard Aflatoxin M_1
(A: 0.05 μg/l, B:0.1 μg/l, C: control)
↓
Pasteurization at 95°C for 20 minutes, cooling to 43±1°C
↓
Inoculation (2.5%) and incubation
at 42±1°C for 2.5-3 hours
↓
Drainage of yogurt whey in a cloth bag
↓
Cooking stage of drained yogurt for
90 min at 90 °C(second pasteurization)
↓
Salting about 1 g/100 g
↓
Packaging (PP bag)
↓
Storage 4±1°C, for 90 days

Figure 1. Schematic diagram of the manufacture of salted (tuzlu) yogurt

2.2. Aflatoxin M_1 analyses

The aflatoxin M_1 analysis was realized by High Performance Liquid Chromatography (HPLC) and using an immuno-affinity column (Afla M_1 HPLC, Vicam, USA) [26]. The AFM_1 standard was supplied from the Sigma company (Sigma-Aldrich, CAS 6795-23-9; C17H1207; FW 238.3; Co., 3050 Spruce Street, MO 63103, St Louis, USA), and prepared according to Anonymous [26]. The aflatoxin M_1 concentrations of samples was determinated in Shimadzu HPLC system. C18 Lichrospher column (25x4.6 mm, 5 μm, Waters Spherisorb ODS-2, Germany) were used as analytical columns. The chromatographic separation composition was carried out using a fluorescence detector (an excitation wavelength of 360 nm and an emission wavelength of 430 nm) with a mobile phase (at a flow rate of 1 ml / min) containing acetonitrile: water (25:75, v / v). Under these circumstances the AFM_1 was eluted from the column at around 5 minutes.

The pure aflatoxin M_1 standard in a crystal form was dissolved in chloroform to prepare the stock solution. A series of calibration solutions were prepared at different concentrations (μg /ml) AFM_1 using the prepared stock solution (Figure 2). Calibration curves are arranged by plotting the peak area for each calibration solution against the mass of injected AFM_1. The detection limit of AFM_1 was 0.01 μg/kg. Their AFM_1 contents were calculated and the recovery of the AFM_1 was found to be 99.72%. The analytical results were not corrected for the recovery (Figure 2).

Sample preparation: The milk samples were heated to 37°C, and then filtered through Whatman No 4 filter paper [27]. The filtered milk (50 ml) was passed through an immuno-affinity column (3 ml/min). After, 1.25 ml methanol: acetonytrile (20:30) was collected in a vial by passing it through a column. 100 µl of prepared vial content was injected into the HPLC.

This process was conducted by modifying the method given by Govaris et al. [25], and Martins and Martins [28]. The yogurt samples were homogenized by stirring, and a 20 g sample was weighed. Chloroform (75 ml), saturated NaCl solution (1 ml) and diatome soil (5 g) were homogenized at a high speed for 2-3 minutes. Hexane (50 ml), distilled water (30 ml) and methanol (1 ml) were added into the evaporated sample. The bottom phase was passed through an immunoaffinity column (3 ml/min). 100 µl of prepared vial content was injected into the HPLC.

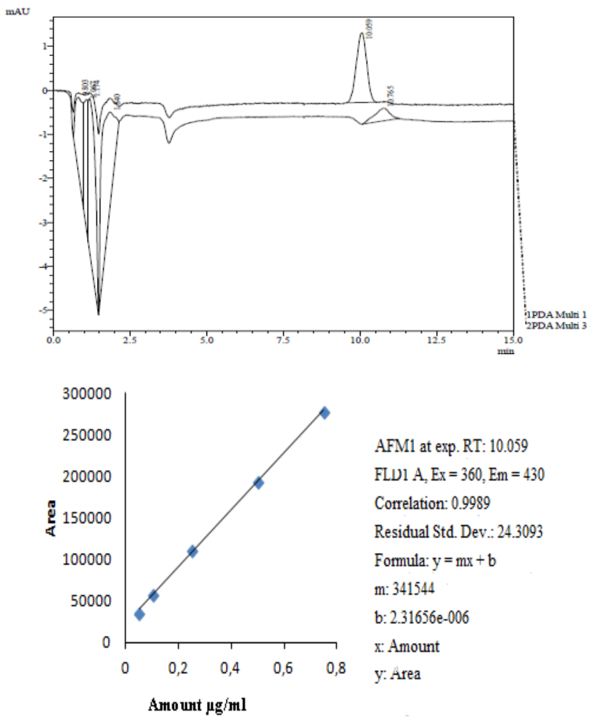

Figure 2. Chromatograms of AFM_1 and the calibration curve of AFM_1

2.3. Statistical analyses

The obtained data were evaluated by the variance (ANOVA). The Tukey Tukey's multiple range test in the general linear model of the SPSS statistical package (SPSS 15.0 SPSS Ltd. Working UK) test was applied to see the difference between the samples. The differences between the averages were regarded significant at P<0.05 and P<0.01.

3. Results

It was determined that there was 0.047 and 0.098 µg AFM_1 per liter of pasteurized milk respectively (Table 1). In this study, it was found that 0.05 µg/l AFM_1 (A) and 0.1 µg/l AFM_1 (B) added to the milk decreased to 0.020 µg and to 0.034 µg through the yogurt production. AFM_1 was not detected in the sample C (Control). After the filtration of yogurt serum, the AFM_1 content of the strained yogurt samples (A and B) was found to be 0.030 µg and 0.043 µg, respectively. AFM_1 was determined as 0.005 µg and 0.012 µg in the serum of yogurt. To emphasize the AFM_1 losses, the total AFM_1 content of the raw milk contaminated with AFM_1 was considered as 100%. The total aflatoxin M_1 losses in the products produced from this contaminated milk were shown in Table 1. In this study, it was determined that the AFM_1 content in the strained yogurt was higher than yogurt samples, due to an increase of the dry matter.

In fermentation of yogurt, pH decreases, organic acids and some other fermentation by-products (such as volatile fatty acids, amino acids, peptides or aldehydes) occur. These compounds formed in yogurt and decreased pH may cause a reduction in the amount of AFM1 [29]. In addition, it is reported that the lactic acid bacteria used in fermentation reduce the amount of AFM1. In a recent study, AFM_1 binding ability of lactic acid bacteria (*Lactobacillus plantarum, Lactobacillus helveticus* and *Lactococcus lactis*) and *Saccharomyces cerevisiae* strain were investigated in milk samples containing AFM1 at concentrations of 0.05 µg/l and 0.1 µg/l. As a result of these research, *Lactobacillus helveticus* and *Saccharomyces cerevisiae* strains were found to be 100% bound to AFM1. In addition, it was determined that *Lactobacillus helveticus* had higher binding potential than other lactic acid bacteria [30]. Some researchers reported levels of AFM_1 in four milk samples ranging from 53.7 to 123.8 ng/ kg were found to exceed the EU MRL of 50 ng/ kg, whereas levels of AFM_1 in 214 samples of processed UHT milk ranged from 2.29 to 21.4 ng/kg were found to all below the LOQ value [31]. In another recent study from China also reports AFM_1 content of UHT milk samples in 2014 and 2015 found to be 88.6% and 59.6%, respectively [32]. AFM_1 in the milk is comparatively stable, and it is not exterminated by pasteurization or heat treatments, therefore causes a serious health risk [10].

Nadira et al. [33] declared that 4/53 of dairy product samples had the contamination level greater than the European Commission (EC) limit (>50 ng/l). Iqbal and Asi [34] reported that AFM_1 was detected in 61% of yogurt samples. Approximately 47% of these yogurt samples were found above the EU recommended limit. A recent study in Iran also reported that the rate of cow milk and cheese samples exceeding the EU limit were 35.9% and 10%, respectively and also explained that there is a relationship between the season and aflatoxin M_1 content [35]. The reason for the decrease of the AFM_1 content after production of the yogurt could be based on a low pH, by-products of fermentation or lactic acid bacteria and organic acids. The change in the structure of the casein during the yogurt production and the by-products occurring after the fermentation such as aldehydes, amino acids and volatile fatty acids may play a role in the degradation of AFM_1.

Table 1. Effect of manufacture period on the AFM_1 levels of pasteurized milk, yogurt, strained yogurt produced from 0.05 and 0.1g/kg AFM_1 contaminated milk (n=3)

Samples	Sample amount (kg)	Concentrations of AFM_1 (µg/l)		Total AFM_1 (µg)		AFM_1 content during manufacture (%)*	
		A	B	A	B	A	B
Raw milk	8.0	0.05 ± 0.001	0.1 ± 0.001	0.4	0.8	100	100
Pasteurized milk	8.0	0.047 ± 0.003	0.098 ± 0.001	0.376	0.784	94	98
Yogurt	7.0	0.020 ± 0.003	0.034 ± 0.013	0.140	0.238	35	29.75
Strained yogurt	3.5	0.030 ± 0.007	0.043 ± 0.001	0.105	0.151	26.25	18.88
Serum	3.5	0.005±0.001	0.012± 0.001	0.017	0.042	4.25	5.25

A: 0.05 µg/l AFM_1 added salted (tuzlu) yogurt, B: 0.1 µg/l AFM_1 added salted (tuzlu) yogurt. * The total AFM_1 content of 0.05 and 0.1 µg/l AFM_1 added milks to be 100%.

Table 2. Effect of storage period on the AFM_1 levels of salted (tuzlu) yogurt produced from 0.05 and 0.1g/kg AFM_1 contaminated milk (n=3)

Salted (tuzlu) yogurt samples*	Concentrations of AFM_1 (µg/l)		Total AFM_1 (µg)		AFM_1 content during manufacture (%)**	
	A	B	A	B	A	B
1st day	0.025 ± 0.002^a	0.044 ± 0.002^a	0.045	0.078	11.25	9.75
30th day	0.026 ± 0.000^{ab}	0.034 ± 0.006^{ab}	0.046	0.061	11.5	7.63
60th day	0.022 ± 0.003^{ab}	0.027 ± 0.006^{ab}	0.039	0.048	9.75	6
90th day	0.019 ± 0.003^b	0.027 ± 0.003^b	0.034	0.048	8.5	6

A: 0.05 µg/l AFM_1 added salted (tuzlu) yogurt, B: 0.1 µg/l AFM_1 added salted (tuzlu) yogurt. a, b: Difference among dates in storage period has been found to be statistically significant (P<0.01). * The amount of salted (tuzlu) yogurt samples are 1.78 kg. ** The total AFM_1 content of 0.05 and 0.1 µg/l AFM_1 added milks to be 100%.

A decrease in the aflatoxin concentration has also been defined in some acidified milk [36]. Hassanin [37] reported lactic acid that develops in yogurt during fermentation could cause the degradation of AFM_1. The AFM_1 levels of yogurt samples were found to be 0.043 and 0.075 µg/l, respectively and these AFM_1 values become 0.052 and 0.088 µg/l after filtration have been reported by Govaris et al. [25].

It was determined that AFM_1 content became 0.026 µg at 30th day and 0.022 µg on the 60th day in salted (tuzlu) yogurt (A). Also, the AFM_1 content of the sample A was reported to decrease to 0.019 µg in 90 days of storage. It was found that the AFM_1 content of salted (tuzlu) yogurt (B) was 0.034 µg/l, 0.027 µg/l, 0.027 µg/l on the 30th, 60th, 90th day, respectively. The difference among samples was found to be significant statistically (P<0.01) (Table 2).

Iha et al. [38] decated that the effects on AFM_1 of yogurt production and storage were minimal and total AFM_1 mass in milk was reduced by 6% in yogurt. Another research that aflatoxin M_1 in yogurt was reduced to around 59% of the level in milk during refrigerated storage at 4°C [37]. During the production and storage of yoghurt, changes in aflatoxin M_1 levels may be caused by factors such as the pH, the concentrations of aflatoxin M_1 in the milk [39]. The other most important reason for that decrease may besecond pasteurized and salting of strained yogurt during salted (tuzlu) yogurt manufacturing.

Motawee [40] reported that the losses of AFM_1 were 20.5%, 21.4%, 22% for cheese curd prepared with 6%, 8%, and 10% salt. However, the salt ratio (about 1%) of salted (tuzlu) yogurt is lower than cheese curd. It is believed that the effect on amount of AFM_1 of salt ratio is very little.

4. Discussion and Conclusion

In this study, two different levels of AFM_1 (0.05 µg/l and 0.1 µg/l) were added in to milk during the manufacturing process of salted (tuzlu) yogurt. It was found to decrease the initial amount in both two concentration levels in all of the samples. This indicates that the production phases of the salted (tuzlu) yogurt and the 90 day storage decreased the initial AFM_1 contents. The factors that are effective in the reduction of AFM_1 are on the following; pasteurizing the milk, the filtering of the yogurt serum, the pasteurizing of the yogurt. However, even though some processes such as heat treatment (first pasteurization at 95°C for 20 min in milk and second pasteurization at 90°C for 90 min in strained yogurt), salt addition (about 1 g/100 g) in the production of salted (tuzlu) yogurt and 90 days of storage had been carried out, none of the samples had been completely removed from the AFM_1. According to these findings, contamination should be prevented for the safe consumption of milk and milk products.

Acknowledgment

This research was supported by Suleyman Demirel University, Project No: 2303 YL-10.

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