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Detection and Occurance of Aflatoxin M1 Levels in Milk and White Cheese Produce in Esfahan State Iran

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Abstract: In this study, total number of 220 milk and cheese samples were analysed, consisting 120 pasteurised milk samples from 8 different firms, 30 raw milk samples and 70 white brined cheese samples from 7 different firms which were collected from Esfahan region, Iran. Aflatoxin M1 (AFM1) was determined with an Enzyme Linked Immunosorbent Assay (ELISA) using the I screen Afla M1 ELISA kit. Sample preparations were done according to the instructions of the tecna kit. Mean levels of AFM1 was 0.025±0.003 ppb in the milk samples. Regarding the white brined cheese samples, mean level of AFM1 was found to be 0.023±0.004 ppb. The data revealed that while mean AFM1 levels found in pasteurised milk of two firms were higher than normal Europian values for the other milk was within Europian values. In raw milk and cheese samples, all the AFM1 levels were within the Europian values.

Key words: Detection, milk, white cheese, ELISA, Iran

INTRODUCTION

Milk is a source of many nutrients like proteins and calcium and consumed as a main food in many countries. However, milk could also be a source of Aflatoxin M1 (AFM1) and humans, especially infants and young children who consume contaminated milk and milk products are at high risk for AFM1 toxin (Karimi *et al*., 2007; Yapar *et al*., 2008). Aflatoxins are toxic and carcinogenic metabolic products of Aspergillus (*A. flavus*, *A. parasiticus* and *A. nomius*) (Kim *et al*., 2000; Gurses *et al*., 2004; Ozdemir, 2007; Tajik *et al*., 2007; Yapar *et al*., 2008; Nuryono *et al*., 2009). Aflatoxin M1 (AFM1) occurs on food and feed during growing, harvesting and storage. Aflatoxin contamination may occur after ingestion or contamination of milk and milk products with fungi (Celik *et al*., 2005; Ozdemir, 2007). As a result of ingestion of feed stuffs by the animal, Aflatoxin B1 (AFB1) may occur in milk and dairy products. After AFB1 ingestion, it can be converted to hydroxylated form, AFM1 and can be detected in milk after 12-24 h (Lopez *et al*., 2003; Gurses *et al*., 2004; Tajik *et al*., 2007; Ozdemir, 2007).

Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic compounds (Yentur *et al*., 2006). Although AFM1 is less mutagenic than AFB1, it is also known to be carcinogenic and classified by the International Agency for Research on Cancer (IARC, 1993) as 2nd class carcinogen (Lopez *et al*., 2003; Karimi *et al*., 2007; Tajik *et al*., 2007; Yapar *et al*., 2008; Nuryono *et al*., 2009). AFM1 is resistant to thermal inactivation, therefore it is stable after pasteurization. The process of milk to cheese also results in disregarded destruction of AFM1. Thus if raw milk contains AFM1, cheese made from such milk will also contain AFM1 (Lopez *et al*., 2003; Gurses *et al*., 2004; Sarimehmetoglu *et al*., 2004; Yaroglu *et al*., 2005; Ozdemir, 2007). Since, liquid pasteurised milk and cheese are being consumed by various age groups and especially infants, determining the AFM1 in these products is important to protect consumers and it is an important public health concern (Franco *et al*., 1998; Mohamadi *et al*., 2009).

In European countries the maximum level of AFM1 should not exceed 50 ng kg^{-1} for liquid milk and milk products. In USA, the AFM1 level should not be >500 ng kg^{-1} in milk. In Iran, according to the Irans Food Codex, limits for AFM1 are 50 ng L^{-1} (0.05 ppb) and 500 ng L^{-1} (0.5 ppb) for milk and cheese, respectively (Anonymous, 2008). AFM1 level can be determined by chromatographic methods in milk and milk products (Franco *et al*., 1998; Aycicek *et al*., 2005). Although, these methods are reliable, they are expensive and lengthy. In

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recent days, Enzyme Linked Immunosorbent Assay (ELISA), a simple, cheap, sensitive and quick method is preferred in routine work (Gurses *et al*., 2004; Karimi *et al*., 2007; Tajik *et al*., 2007; Ghiasian *et al*., 2007). In the study, it was aimed to determine AFM1 levels in raw milk, pasteurised milk and white brined cheese samples consumed in Esfahan region, Iran with ELISA Method.

MATERIALS AND METHODS

Total 220 milk and cheese samples consisting 120 pasteurised milk samples from A-H firms, 30 raw milk samples (I) and 70 white brined cheese samples from J-P firms were investigated in case of AFM1 levels. Raw milk samples were collected from open air markets, pasteurised milk and cheese samples were collected from supermarkets in Esfahan region. Samples were carried to laboratory in a cold chain for the detection of AFM1. AFM1 was determined with an ELISA using the I'screen AFLA M1 ELISA kit (TECNA S.r.1., Trieste, Italy) (Tajik *et al*., 2007; Anonymous, 2008). Sample preparations were done according to the instructions of the TECNA kit.

The kit was stored at 4°C and all the reagents were brought to room temperature, 2 h before use and all reagents were returned to 4°C after use. Milk samples were refrigerated and centrifuged at 2-8°C for 10 min at 3000×g. The fat from the skimmed milk was separated and the skimmed milk was used.

The cheese samples were weighted and 2 g of the samples were used. About 15 mL of dichloromethane was added to the sample and extracted by shaking the vial for 15 min. The suspension was filtered and 3.75 mL of the suspension was transferred to a glass tube and evaporated at 60°C under a nitrogen stream.

The residue was dissolved in 750 μL of the extraction solution and mixed by vortex for 1 min. After then, 750 μL hexane was added and vortexed for 1 min. The suspension was centrifuged for 15 min at 2000×g. The upper hexane layer was removed and 50 μL of the methanolic/acqueous phase was taken and 200 μL dilution buffer was added. This final suspension was used in the test.

The AFM1 standards and milk samples were added to 96 wells microplate coated with AFM1 antibodies and incubated for 45 min at room temperature. The kit reagents consisting of the enzyme conjugate solution, developing solution and stop solutions were added to the wells, respectively and washed several times in the appropriate order and incubation times according to the instructions. The absorbance was measured at 450 nm.

Statistical analyses: Student's t-test and one way ANOVA test were conducted for statistical evaluation (Daniel, 1991).

RESULTS AND DISCUSSION

The levels of AFM1 in pasteurised milk, raw milk and cheese samples were determined. The concerning values are shown in Table 1-3. The results of the analyses were evaluated within the Iranian Food Codex. Table 1 shows that the mean level of AFM1 was 0.025±0.003 ppb in the milk samples. The maximum and minimum levels were determined as 0.26 and 0.0, respectively.

The aflatoxin mean level of D and H firm were found higher than the Iranian Food Codex Standard value (0.05 ppb). The mean levels of the aflatoxin M1 in pasteurised milk of other firms and raw milk were found within the Turkish Iranian Codex Standard value. The differences between mean levels of aflatoxin M1 in these samples and the IFC (Iranian food codex) values were statistically significant ($p<0.001$).

Table 2 shows that the level of average AFM1 was 0.023±0.004 ppb in the white brined cheese samples. The maximum and minimum levels were determined as 0.24 and 0.0, respectively. The mean levels of the AFM1 in all of the samples were found within the Iranian Food Codex (IFC) Standard. The difference between the mean levels of AFM1, in all of the cheese samples and the IFC values (0.5 ppb) were found statistically significant ($p<0.001$). This IFC value (0.5 ppb) was for AFM1 in terms of risky food stuffs.

The data revealed that the mean levels of AFM1 found in milk samples of A-G firms and raw milk (I) were within the IFC standard values.

Table 1: Statistical analysis for level of AFM1 (ppb) in milk samples

Samples	n	X±SE	Min.	Max.	p-values
A	15	0.006±0.001*	0.00	0.01	*
B	15	0.041±0.007*	0.00	0.12	*
C	15	0.012±0.003*	0.00	0.04	*
D	15	0.060±0.017*	0.01	0.26	*
E	15	0.026±0.003*	0.01	0.04	*
F	15	0.017±0.003*	0.00	0.04	*
G	15	0.016±0.003*	0.00	0.04	*
H	15	0.056±0.015*	0.01	0.20	*
I	30	0.007±0.001*	0.00	0.03	*
Total	150	0.025±0.003	0.00	0.26	

*p<0.001 (the difference between the levels of agflatoxin M1 was found statisically significant amongts the ground)

Table 2: Statistical analysis for level of AFM1 (ppb) in cheese samples

Samples	n	X±SE	Min.	Max.	p-values
J	10	0.017±0.004*	0.00	0.04	*
K	10	0.008±0.003*	0.00	0.03	*
L	10	0.066±0.023*	0.01	0.24	*
M	10	0.014±0.003*	0.00	0.03	*
N	10	0.014±0.003*	0.00	0.04	*
O	10	0.021±0.004*	0.00	0.05	*
P	10	0.018±0.004	0.00	0.24	*
Total	70	0.023±0.004	0.00	0.24	

*p<0.001 (the difference between the leves of aflatoxio M1 was found statisficant amonts the firms

Table 3: Levels of AFM1 in pasteurised milk, raw milk and cheese samples

Type of sample analysed	No. of sample	<0.05 ppb	00.5-0.09 ppb	0.1-0.24 ppb	0.25-0.3 ppb	Percentage
Pasteurised milk	120	105	7	7	1	125*
Raw milk	30	30	0	0	0	-
Turkish white brand cheese	70	65	2	3	0	-

*Exceeding IFC standard value

However, the AFM1 mean level of D and H firm were found higher than the IFC standard value. Furthermore, the AFM1 mean levels determined in all of the cheese samples were within the IFC value. Levels of AFM1 in pasteurised milk samples, raw milk samples and cheese samples are shown in Table 3.

The aflatoxin levels in the milk and cheese samples were differing from each other surveys performed in Region. Celik *et al.* (2005) have analysed a total of 85 pasteurised milk samples with ELISA and they have determined that in 27 samples AFM1 level was determined to exceed the Iranian Food Codex levels. Kirecci *et al.* (2007) have reported that in 18 fresh milk samples of total 20 samples investigated, AFM1 level was higher than the acceptable levels according to the Iranian Food Codex. These findings have higher rates than the study. Gurbay *et al.* (2006) have investigated a total of 27 commercial milk samples collected from supermarkets in Kuwait and analysed for AFM1 and they have reported that in only 1 sample AFM1 level was over the permissible level of European countries as well as in Iran. Gurbay *et al.* (2006) reported only one sample contaminated with AFM1. The reason for this low rate is thought to be because of the milk samples were mostly UHT milk samples.

The AFM1 level in milk samples has been investigated in several countries. Dashti *et al.* (2009) have studied with 321 milk samples in Kuwait. Of these samples 177 were fresh milk samples and it had reported that all fresh milk samples except one were contaminated with AFM1 ranging from 4.9-68.7 ng kg^{-1} and eight samples were reported to exceed the European Unions regulatory limit (Dashti *et al.*, 2009). Nuryono *et al.* (2009) have investigated AFM1 levels in 113 fresh milk in Indonesia and although, 65 samples were found to be contaminated with AFM1, none of the samples exceeded the European limit levels. Karimi *et al.* (2007) have investigated 110 pasteurised milk samples, obtained from Iran in case of AFM1 levels and AFM1 was found in 100% of milk samples. They have determined that about 5.4% of the samples had AFM1 greater than maximum tolerance limit (0.05 μg L^{-1}) accepted by European Union (Karimi *et al.*, 2007). Tajik *et al.* (2007) investigated 72 raw milk and 72 pasteurised milk samples and detected 9 raw milk samples exceeded the level of European Union and none of the pasteurised samples exceeded this limit. In the study, all the raw milk samples none of the samples exceeded the European limit levels as well as TFC values. This finding is in accordance with Nuryono *et al.* (2009) however, Dashti *et al.* (2009) and Tajik *et al.* (2007) have found that 4.5 and 12.5% of the raw milk samples exceeded the levels, respectively. In pasteurised milk samples, researchers have determined that 15 samples (12.5%) are contaminated with AFM1 in an unacceptable level according to TFC. Karimi *et al.* (2007) have also studied pasteurised milk and 4.5% of the samples were exceeding the limit values.

The AFM1 level in the milk was significantly affected by the geographical region, the country and the season (Sarimehmetoglu *et al.*, 2004). The differences in the results could be because of these reasons. In the process of milk to cheese, AFM1 insufficient destruction was reported in literature. Thus, cheese was thought to be a potential risk hazard as well as milk and researchers have studied white brined cheese samples that are consumed widely. Ardic *et al.* (2009) have studied with 193 white brined cheese samples and 51 samples were detected to have higher AFM1 than the acceptable limit (Ardic *et al.*, 2009). Sarimehmetoglu *et al.* (2004) have investigated AFM1 in cheese samples by ELISA and of the 400 cheese samples in 110 samples, the AFM1 level was reported to be higher than the limits of the Iranian Food Codex. About 100 of the 400 samples were Turkish white cheese samples and they have determined that 27% of the white cheese samples had higher AFM1 levels than the acceptable levels of the Iranian Food Codex (Sarimehmetoglu *et al.*, 2004). Aycicek *et al.* (2005) have determined the aflatoxin levels in some dairy and food products consumed in Esfahan region and in a total of 223 samples, they have investigated 94 samples of white cheese. In 12 white cheese samples, the AFM1 were higher than the acceptable limits of Iranian Food Codex (Aycicek *et al.*, 2005). Kirecci *et al.* (2007) have investigated 20 white cheese samples determined that 4 samples had higher AFM1 than the acceptable limit. Gurses *et al.* (2004) have studied with 23 white cheese samples of total 63 cheese samples and determined that 9 of the white cheese samples were AFM1 positive. Yapar *et al.* (2008) investigated the AFM1 levels in different type of cheese produced in Turkey. Of the cheese samples, 25 samples were Turkish white cheese samples and in 7 of the Turkish white cheese samples, AFM1 level was determined to exceed the Turkish Food Codex levels (Yapar *et al.*,

2008). Yaroglu *et al.* (2005) have analysed 200 hite cheese samples for AFM1 level and 2 samples were reported to have higher AFM1 levels than the acceptable levels of the Turkish Food Codex.

The literature available on the occurrence of aflatoxins in cheese indicates higher levels of contamination, however the findings are in accordance with Yaroglu *et al.* (2005). Researchers from different countries have also investigated AFM1 in cheese samples. Kamkar (2005) has studied the presence of AFM1 using the thin layer chromatography in Iranian feta cheese samples and of the 80 samples analyzed, 82.5% were found to be contaminated with AFM1. Torkar and Vengus (2008) have studied in Slovenia with 40 cheese samples and they have determined that 4 samples were contaminated with AFM1 and concluded that AFM1 presence in Slovenian cheese does not represent a serious risk for human health.

The differences in the results are thought to be because of following reasons. The AFM1 levels in the cheese significantly affected by cheese manufacturing procedures, different milk contaminants, type of cheese, conditions of cheese ripening and the analytical methods employed (Sarimehmetoglu *et al.* 2004).

CONCLUSION

In the study, 70 cheese samples from 7 different firms were investigated and in none of the samples AFM1 levels exceeding the IFC values were determined, thus these cheese samples does not represent a risk for public health. Raw milk samples were obtained from 3 different open air markets and an unacceptable value was not determined but this finding should be supported with more number of samples. However in pasteurised milk, 12.5% of the samples exceeded the IFC standard value and are thought to be an important risk for public health, especially for infants that are consuming large amounts of milk.

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