



Aflatoxin M₁ Concentration in Various Dairy Products: Evidence for Biologically Reduced Amount of AFM₁ in Yoghurt

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Abstract

Background: Aflatoxin M₁ (AFM₁), a carcinogenic substance is found in milk and dairy products. The effect of season and type of dairy products on AFM₁ level in northern Iran was investigated in this study.

Methods: Three hundred samples (each season 75 samples) including raw and pasteurized milk, yoghurt, cheese, and cream samples were collected from three distinct milk producing farms. The samples were subjected to chemical and solid phase extractions and were analyzed by using HPLC technique. Recovery percentages, limit of detection and limit of quantification values were determined.

Results: Seventy percent and 98% were the minimum and maximum recoveries for cheese and raw milk, respectively and 0.021 and 0.063 ppb were the limit of detection and limit of quantification values for AFM₁. We found that in autumn and winter the highest level (0.121 ppb) of AFM₁ in cheese and cream samples and failed to detect any AFM₁ in spring samples. Interestingly, our data showed that the yoghurt samples had the lowest level of AFM₁ in all seasons.

Conclusion: There are significant differences between the AFM₁ levels in dairy products in various seasons and also various types of products, suggesting spring and summer yoghurt samples as the safest products from AFM₁ level point of view.

Keywords: Aflatoxin M₁, Dairy products, Food safety, HPLC

Introduction

Aflatoxins (AFs) are known mycotoxins that threatening humans and animals health with causing disorders such as hepatocellular carcinoma (HCC). In addition to causing the certain cancers, AFs suppress the immune system and potentiate the other hepatotoxic compounds toxicity. The carcinogenicity and mutagenicity of AFs are mainly related to the conversion of AFB₁ into AFB₁ 8, 9-epoxide, which is able to bind the proteins and nucleic acids (1). Aflatoxins-induced reproductive disorders including disruption of testicular structure, secretion of androgens and generation of

meiotic micronucleate giant spermatocytes have been previously reported (2).

Aflatoxin M₁ (AFM₁) is the hydroxylated metabolite of aflatoxin B₁, which is found in milk and dairy products. Its weaker carcinogenicity than AFB₁ has been reported in laboratory animals. Besides, previous studies showed that there are potential hazardous human exposure to AFM₁ through the consumption of milk and milk products (3). Therefore, several countries have legislated action levels of AFM₁ in milk and dairy products (4). As AFM₁ is heat stable compound, there-

fore its level in contaminated foods remains unaffected by heating methods including pasteurization and sterilization (5). There are reports indicating that adding sequestering agents to the AFB₁-contaminated feeds, resulted in a significant reduction of AFM₁ level in milk (6). However, an effective sequester which be able to lower the AFM₁ level in milk and dairy products without negative impact on nutritional status has not been introduced yet. In addition of the diet level of AFB₁, ecological conditions, animal species and farm management strategy also affect the AFM₁ concentration in milk and dairy products (7). Tajkarami and his co-workers (2007) reported significant differences between 5 different regions of Iran, regarding the AFM₁ concentration in milk (8).

There are controversial and limited internationally published data about the effect of season and type of dairy products on the occurrence of AFM₁; therefore we aimed to investigate the effect of various seasons and different type of dairy products (raw and pasteurized milk, yoghurt, cheese, and cream) on AFM₁ level in Urmia, located in northern Iran.

Materials & Methods

Chemicals and standard solutions

The reference standard of AFM₁ (from *Aspergillus flavus*, 10 µg, A 6428) was purchased from Sigma-Aldrich (Germany). Acetonitrile, methanol, and water were of HPLC grade and were purchased from Baker (Deventer, the Netherlands).

Samples

Milk samples in each season (15 raw milk samples from three distinct milk producing farms) were collected in 2012 according to the Iranian national standard milk sampling method (INS No.419) and after taking raw milk (500 ml) the rest of samples were subjected to be pasteurized (30 min in 63 °C) and /or converted to the other dairy products including yoghurt, cheese, and cream. The samples were stored at 4 °C and analyzed within the 5 days.

Chemical and solid phase extraction

A 50 g milk sample or dairy products homogenate after addition of 20 ml deionized water was centrifuged at 10 °C in a polypropylene tube at 1500 × g for 15 min. Thereafter, once for milk samples and twice for cheese, yoghurt and cream samples defatting processes by using n-butanol was conducted. After defatting, 50 ml pure methanol was added to samples and was shaken vigorously for 10 min, followed by 15 min centrifugation at 1100 × g. The upper layers of the samples after addition of the same volume deionized water were subjected to solid phase extraction by using pre-conditioned immunoaffinity column (Aflastar M₁, Romer labs, Diagnostic GmbH, 3430, Tulln, Austria). The cartridges were washed with 10 mL water, dried by vacuum for 15 s and then eluted with 3 mL of methanol. The eluate was evaporated under a gentle stream of nitrogen. The residue was re-dissolved in 200 mL of mobile phase.

Method evaluation

To evaluate recoveries, the uncontaminated samples were spiked with 0.5, 0.8 and 1 ppb of standard AFM₁. The recovery test was performed five times at the concentration of 0.8 ppb. The limit of quantification level was determined by the addition of decreasing concentrations of the AFM₁ standard to uncontaminated samples. Standard curve for AFM₁, were constructed with 5 points (from 0.5– 10 ppb).

HPLC analyses

To analyze the prepared samples, we used a Waters Breeze 1525 HPLC system equipped with Waters 1525 Binary pump, on-line degasser, Waters 717 Plus Auto sampler and Waters 2475 fluorescence detector. To detect the AFM₁ by fluorescent detector, excitation was set at 360 nm and emission at 440 nm. The analytical column was an ODS 5 µm, 4.6 mm×250 mm C18 column (Nucleodur) and the mobile phase consisted of Acetonitrile: Methanol: H₂O (20:20:60, V/V/V), which was pumped at a flow rate of 1 ml/ min. Waters Breeze software also was used to analyze the data.

Statistical analyses

The statistical methods used in this study were based on normal confidence intervals and analysis of variance (ANOVA).

Results

The constructed standard curve demonstrated linearity within the concentration range and the calculated coefficients of correlation was found 0.9998. We found the retention time of 9.6 min for AFM₁ in standard and test samples.

To validate the method, which was applied to analyze the dairy products in this study, we determined the recovery percentages for AFM₁ at three concentrations in each single product. The obtained data indicated that the highest (98.5 %) and lowest (69 %) recoveries belong to raw milk and cheese samples, respectively. The recovery percentages for various dairy products are presented in Table 1. Moreover, the limit of detection (LOD) and limit of quantification (LOQ) levels of 0.021 and 0.063 ppb were estimated.

We found that there are significant differences ($P < 0.05$) between 3 distinct milk producing farms in terms of AFM₁ level in raw milk at summer time.

Figure 1 shows that in all three seasons the samples which were collected from farm II were contaminated with high level of AFM₁ when compared to two other farms. Additionally a season-dependent increase of AFM₁ level in raw milk and in all three farms was obtained.

We found the highest concentration of AFM₁ in the samples, which collected from farm B at autumn season and followed by samples, which ana-

lyzed during the winter season. None of the collected samples at spring time was found to be contaminated with AFM₁.

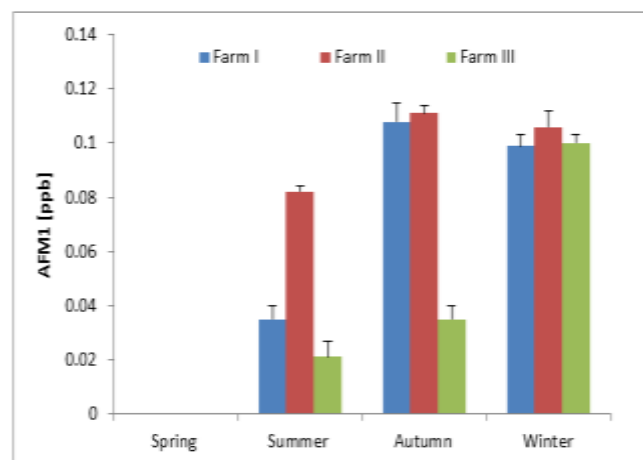


Fig. 1: Aflatoxin M₁ level in raw milks from three various farms at different seasons

Comparing the three farms samples revealed that 2 farms from 3 at autumn and all three farms at wintertime showed AFM₁ level exceeded from standard concentration of 0.05 ppb. There are no significant differences ($P > 0.05$) between raw and pasteurized milk samples. We found that AFM₁ concentration in the corresponding cheese and cream samples were slightly but significantly higher than that in raw and pasteurized milk samples (Table 2). Interestingly, analyses of yoghurt samples revealed that the AFM₁ level significantly ($P < 0.05$) was reduced when compared to the original raw milk and also to the other dairy products which made from the corresponding samples.

Table 1: Percentage of recovery for AFM₁ in various dairy products

AFM ₁	Raw Milk	Pasteurized Milk	Yoghurt	Cream	Primary cheese
0.5 (ppb)	98.5 ± 3.6	97 ± 14	88 ± 7	80 ± 2.3	74 ± 3.4
0.8	95 ± 7.2	91 ± 3.9	81 ± 4.1	72 ± 4.5	75 ± 7.1
1.0	88.1 ± 4.4	85.9 ± 3.2	85 ± 3.3	87 ± 7.2	69 ± 1.8

Table 2: AFM₁ Levels (ppb) in milk and dairy products in Urmia City

Season	Farm	Raw Milk	Pasteurized Milk	Yogurt	Cream	Primary Cheese
Spring	I	N.D.	N.D.	N.D.	N.D.	N.D.
	II	N.D.	N.D.	N.D.	N.D.	N.D.
	III	N.D.	N.D.	N.D.	N.D.	N.D.
Summer	I	0.035 ± 0.007	0.035 ± 0.003	0.029 ± 0.002	0.032 ± 0.007	0.031 ± 0.006
	II	0.082 ± 0.005	0.13 ± 0.04	0.029 ± 0.003	0.09 ± 0.008	0.098 ± 0.004
	III	0.029 ± 0.004	0.021 ± 0.005	0.021 ± 0.006	0.031 ± 0.005	0.031 ± 0.005
Autumn	I	0.108 ± 0.03	0.085 ± 0.002	0.032 ± 0.008	0.116 ± 0.007	0.121 ± 0.09
	II	0.111 ± 0.009	0.105 ± 0.04	0.033 ± 0.007	0.121 ± 0.06	0.112 ± 0.05
	III	0.035 ± 0.004	0.036 ± 0.007	0.032 ± 0.008	0.042 ± 0.005	0.047 ± 0.006
Winter	I	0.099 ± 0.008	0.097 ± 0.02	0.033 ± 0.003	0.110 ± 0.07	0.113 ± 0.02
	II	0.106 ± 0.06	0.102 ± 0.03	0.033 ± 0.007	0.113 ± 0.03	0.114 ± 0.04
	III	0.100 ± 0.04	0.098 ± 0.01	0.033 ± 0.005	0.113 ± 0.04	0.119 ± 0.07

N.D.: Not detected.

Discussion

This study showed that AFM₁ level in dairy products is affected by season and product type. The data indicated that the highest level of AFM₁ was detected in the cheese and cream samples which prepared at autumn and winter seasons. Moreover, the lowest concentration of AFM₁ was found in yoghurt samples in all seasons.

To evaluate the accuracy of the analytical method, we estimated the LOD, LOQ and recovery percentages for each single product. The values for LOD and LOQ were not different for dairy products, however, our results showed various recovery percentages for different products, suggesting the important role of food matrix in recovery of certain compound. The effect of various matrixes in the recovery of certain compounds has been well documented (9).

AFM₁ is the hydroxylated metabolite of AFB₁, which is produced by cytochrome P450 system in the liver and released mainly into milk and urine and in less extent into the feces. It has been reported that AFM₁ level in the milk has a direct relationship with the concentration of AFB₁ in consumed contaminated feed (10). Previous studies reported that consuming the contaminated feedstuffs with more than 70 mg AFB₁ will resulted in the release of AFM₁ at the higher concentration than standard level (0.05 ppb) in milk

(11). There are different regulations in various countries and in Iran. According to decision (5925 NSO) of the national standard organization, the AFM₁ level should not exceeded from 0.05 ppb in milk samples (12). Therefore, the first message of the current study might be warning about the out of range presence of AFM₁ in milk and dairy products in northwest of Iran at autumn and winter seasons. There are many factors that could affect the AFM₁ level in the milk and dairy products including: the diet source of lactating animals, ecological and economical factors and management policy.

Our findings in the current study indicate that there is no detectable AFM₁ in the milk and dairy products during the springtime. This finding may be explained with two facts: either due to using fresh forage during the springtime, there is no AFB₁ contamination, or the level of contamination is under the sensitivity of used method (13). It is well documented that the feedstuffs such as concentrate and silage due to having the favorite condition of fungi growth including high humidity and temperature and improper storage condition may result in the mycotoxins production (14-16). Aflatoxin B₁ has been found in various feed including cottonseed, barley, soy bran, pellet wheat, corn silage and sorghum silage in different countries (17). Exposure of lactating cattle to mycotoxins occurs mainly through the consumption of

contaminated feed and may cause mycotoxicosis. In case of exposure to AFB₁, dairy cattle not only may cause aflatoxicosis, but it may lead to the production of its metabolic product in milk and dairy products that consequently affect the public health (18).

We found no significant differences between the raw and pasteurized milk samples in terms of AFM₁ concentration and this finding is supported by previous reports indicating that the pasteurization processes do not affect the AFM₁ level in milk (19). One of the remarkable findings of the current study is that we detected the lowest range of the AFM₁ concentrations in yoghurt samples, corresponding to those raw milk samples, which the AFM₁ level at autumn and winter seasons exceeded from standard level. There could be several explanations for this finding including: the presence of various lactic acid bacteria in yoghurt samples which are able to destroy the AFM₁ (20). Up to 34% reduction of AFM₁ concentration in yoghurt samples compared to original raw milk samples has been previously reported. Moreover, it has been also demonstrated that the different strains of lactic acid bacteria do have various capability in the degrading of AFM₁, where *Lactobacillus acidophilus* and *L. rhamnosus* were able to reduce AFM₁ level by 18.5 and 49.6 %, respectively. Another reason for the AFM₁ reduction in yoghurt samples may be related to affinity of the present bacteria in binding to AFM₁ molecules, which by reduction of pH level would be able to degrade the bound molecules (21). Previous studies which have been performed in four Iranian large cities of Tehran, Esfahan, Shiraz and Yazd during winter and summer 2009 indicated that the AFM₁ concentration in yoghurt samples was significantly lower than in pasteurized milk samples (22). We also found that the AFM₁ concentration in cream and cheese samples were slightly higher than that in the corresponding milk samples. The high fat content of cream and lipophilic property of AFM₁ and high affinity of AFM₁ molecules to casein may explain the higher concentration of AFM₁ in cream and cheese samples, respectively (23).

Conclusion

Our data indicate that both season and type of dairy products could affect the AFM₁ level as the highest concentrations were found in autumn and winter seasons in cheese and cream samples, while the lowest level of AFM₁ was detected in yoghurt samples. These findings may help the dairy products producers to use the properly prepared/stored feedstuff during the cold seasons for milk producing animals and also might lead the food authorities to consider the knowledge-based use of probiotics to produce the safe foods.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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