

A Study of the Occurrence of Aflatoxin M₁ in Dairy Products Marketed in Urmia, Iran

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ABSTRACT

The purpose of this survey was to evaluate natural occurrence and content of Aflatoxin M₁ (AFM₁) in dairy products marketed in Urmia (Iran). During September 2007, 40 samples of pasteurized milk, 40 samples of Ultra High Temperature Treated (UHT) milk, 40 samples of creamy cheese and 40 samples of Iranian Feta cheese were collected from different supermarkets in Urmia city. AFM₁ contents were determined through competitive Enzyme Linked Immunosorbent Assay (ELISA) technique. All milk samples analyzed showed mean AFM₁ concentrations lower than the permissible level of 50 ng L⁻¹ (23.22±8.65, and 19.53±7.47 ng L⁻¹ in pasteurized milk, and UHT milk, respectively). The mean levels of AFM₁ contamination were 43.31±18.51 ng L⁻¹ in Feta cheeses, 21.96±3.23 ng L⁻¹ in creamy cheeses. The potential risk of human exposure to aflatoxin M₁ via consumption of milk and milk products is well known. Therefore, dairy products must be evaluated for aflatoxin and kept apart from fungal contamination as much as possible.

Keywords: AFM₁, Milk, Cheese, Enzyme Linked Immunosorbent Assay (ELISA).

INTRODUCTION

Aflatoxins are a group of naturally occurring toxins produced mainly by such moulds as *Aspergillus flavus*. When aflatoxin B₁ (AFB₁), the most toxic aflatoxin, is ingested, some animals are able to convert it into AFM₁, which, in turn, is transferred to such food products as milk, eggs or meat (Polan *et al.*, 1974; Trucksess and Stoloff, 1984). The conversion rate of aflatoxin B₁ to aflatoxin M₁ present in milk ranges between 0.5% and 5% for many mammals including dairy cattle and humans, yet values as high as 6% have been reported by Pipet (1998).

AFM₁ exhibits carcinogenic (IARC, 2002), genotoxic (Lafont *et al.*, 1989) and cytotoxic effects (Neal *et al.*, 1998). AFB₁ and AFM₁ have been classified by the International Agency for Research on Cancer as human carcinogens class 1 and 2B, respectively

(IARC, 2002). Because of their potential risks and also to minimize their hazard, the World Health Organization recommends the reduction in its consumption to a minimum, because there still is not enough information available to establish a tolerable exposure level (WHO, 2002).

The level of AFM₁ in milk should not exceed 500 pg ml⁻¹ according to US regulations but the level is set at 50 pg ml⁻¹ in most European countries and in the *Codex Alimentarius* (Van Egmond, 1995). In Iran, the action levels have been officially set at 50 pg ml⁻¹ for AFM₁ in milk (FAO, 2004).

As milk is the main nutrient for infants and children who are considered to be more susceptible to adverse effects of mycotoxins, the occurrence of AFM₁ in milk is a concern. On the other hand, milk is not only consumed as liquid milk, but also utilized in the preparation of infant formulas, yogurt,

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cheese, and milk-based confectioneries including chocolates, and pastry. Therefore, it is important and necessary to determine AFM₁ levels in milk and dairy products in order to protect consumers in various age groups, from its potential hazards.

Unfortunately, in Iran, in spite of the fact that the dairy industry has evolved a lot in the last few years, there are very few items of data concerning AFM₁ contamination in milk and milk products. In the present work, some milk and dairy product samples marketed in the city of Urmia, Iran, during the month of September 2007 were collected and analyzed for AFM₁ through direct competitive enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Sample Preparation

A total of 160 samples of commercial pasteurized milk and other dairy products were collected on a weekly basis from supermarkets located in Urmia, during September, 2007. The samples collected included 40 pasteurized, and 40 UHT milk samples, 40 samples of creamy cheeses, and 40 feta cheese. The samples were stored in a cool place and protected against light until the day of analysis. Pasteurized milk samples were collected on the production day and kept freezing cool before being analyzed later. Different brands of feta, creamy cheese and UHT milk samples, had been produced during the summer. Sampling was performed according to Iran standard (ISIRI, 2002); as regards the goal it was an assessment of average AFM₁ contamination in 4 types of dairy products.

Method employed for Analysis of Aflatoxin M₁

Determination of AFM₁ was based on an enzyme-linked immunoassay using the ELISA kit (I'screen AFM₁-Tecna, Italy). This method is quick, reliable and cost

effective for an estimation of AFM₁ (Kaniou-Grigoriadou *et al.*, 2005).

Most of the reagents used were available in the I'screen test kit. AFM₁ standard solutions used for the construction of the calibration curve were at levels of 0, 5, 10, 25, 50, 100 and 250 ng L⁻¹ and all included in the ELISA test kit. Detection limit in raw milk was 5 ng L⁻¹.

Preparation of samples was conducted according to the instructions of the I'screen kit. Briefly, milk samples were chilled and then centrifuged for 10 min at 4,800 rpm (Heraeus Megafuge 1.0). One hundred μ L of the skimmed milk was diluted with 400 μ L of the sample diluent. An aliquot (100 μ L per well) of this solution was used directly for analysis. Cheese samples (2 g each) were homogenized (Ultraturrax, IKA-Werke) and extractions obtained by shaking the cheese homogenate with 15 mL of dichloromethane for 15 minutes. The suspension was then filtered and 3.75 ml of the extract evaporated under nitrogen stream and at 60°C. The residue was redissolved in 750 μ L extraction solution and mixed thoroughly for one minute, then added 750 μ L of hexane and mixed for another one minute. After centrifugation for 15 minutes at 3,800 rpm, a 50 μ L aliquot of the methanolic/aqueous phase was diluted in a small tube (1.5 ml) with 200 μ L of dilution buffer and mixed gently. An aliquot of this solution was used in the test (100 μ L well⁻¹).

Aflatoxin M₁ standards or the prepared sample solutions were added to microtiter wells in duplicate. During incubation for 45 minutes at room temperature and in the dark, the antibody binding sites are occupied in proportion with the aflatoxin M₁ concentration. The liquid was then removed completely from the wells, which were subsequently washed four times with washing buffer. In the next step, any remaining free binding sites are occupied by the enzyme conjugate, which was added (100 μ L) and then incubated for 15 minutes at room temperature in the dark. Any unbound enzyme conjugate was then

Table 1. Occurrence of aflatoxin M₁ (ng L⁻¹ or ng kg⁻¹) in dairy products.

| Sample | Tested | Average contamination |
|------------------|--------|-----------------------|
| Pasteurized milk | 40 | 23.22±8.65 |
| UHT milk | 40 | 19.53±7.47 |
| Creamy cheese | 40 | 21.96±3.23 |
| Feta cheese | 40 | 43.31±18.51 |

Table 2. The prevalence of milk contamination as reported by some studies in Iran.

| Survey | Liquid milk samples (Number) | Contaminated milk samples > 50 ng L ⁻¹ |
|----------------|------------------------------|---|
| Tehran (1998) | 73 | 82.2 |
| Hamedan (2000) | 186 | 11.76 |
| Sarab (2003) | 111 | 40 |
| Shiraz (2004) | 624 | 17.8 |
| Tehran (2005) | 128 | 78 |

removed in a washing step. Developing solution (100 µL) was added to each well and incubated for 15 minutes at room temperature in the dark. Bound enzyme conjugate converts the colorless chromogen into a blue product. Then the addition of the stop reagent (50 µL per well) led to a color change from blue to yellow. The measurement was made photometrically at 450 nm.

In order to obtain AFM₁ actual sample concentration in ng L⁻¹ for milk and ng kg⁻¹ for cheese, the concentration read from the calibration curve was further multiplied by a dilution factor of 5 for milk and a dilution factor of 7.5 for cheese.

Data Analysis

The statistical analysis of the data was performed using the MINITAB Statistical Software, Release 13.1.

RESULTS AND DISCUSSION

In the present survey, the mean aflatoxins M₁ in pasteurized and UHT milk were 23.22 and 19.53 ng L⁻¹, respectively. The level of AFM₁ contamination in milk was not very high. Mean AFM₁ contamination in milk samples was not over the permissible level

of 50 ng L⁻¹ as accepted in most European countries (Table 1).

Cream separation can affect AFM₁ distribution, since 80% is partitioned in the skim milk portion (Grant and Carlson, 1971) and an amount of 30% is associated with the non-fat milk solids, particularly casein. The behavior of AFM₁ in the processes in which fat separation is involved may be explained by its semi polar character. It is a water soluble compound binding with hydrophobic sides of casein, thus leading to predominance in the non-fat fraction (Van Egmond and Paulsch, 1986). In cheese samples higher level of AFM₁ were detected, mean AFM₁ contaminations being 43.31 and 21.96 ng kg⁻¹ for Feta and creamy cheese, respectively (Table 1). Mean AFM₁ concentrations among the milk and Feta cheese samples were found to be different.

Some results about aflatoxin M₁ contamination in milk products have been reported in Iran (Table 2). In Tehran, 1982, the occurrence of AFM₁ in 52 liquid milk samples was studied. Contamination with AFM₁ was reported at concentrations between 23 and 3000 ng L⁻¹ (Karim *et al.*, 1982). In another study 73 milk samples delivered to Tehran milk pasteurization plants were analyzed for AFM₁. All the contaminated samples had a level of AFM₁ above the European countries' standard, which is 50 ng L⁻¹ (Karim *et al.*, 1998).



AFM₁ contamination of 624 pasteurized milk samples was studied in Shiraz (Alborzi *et al.*, 2006). AFM₁ was found in 100% of the samples and in some cases, it was greater than the maximum tolerance limit (50 ng L⁻¹) accepted by European Union. Recently, Oveisi *et al.* (2007) reported that the occurrence of contamination of aflatoxin M₁ in pasteurized liquid milk was 72.2 ng L⁻¹ in Tehran. There exists only one study about AFM₁ contamination in cheese in Iran. Kamkar found AFM₁ in 82.5% of 80 samples of Feta cheese examined. The range of contamination levels varied among different months, the mean value being was 0.41 µg kg⁻¹.

In many studies, the level of cheese contamination by AFM₁ seems to vary. These variations may be explained in part by different reasonings such as cheese manufacturing procedures, variation in the original milk contamination, type of cheese, condition of cheese ripening, and the analytical methods employed (Galvano *et al.*, 1996). In addition, AFM₁ level in milk was significantly affected by the geographical region, the country, and as well by the season. It is demonstrated that summer milk is less contaminated than milk produced in the winter.

CONCLUSIONS

The results of this study indicate that the occurrence of aflatoxin M₁ contamination in the samples were low in the study region, most probably because of uncontaminated food collections consumed by the milking cows. It can be concluded that the contamination of aflatoxin M₁ in dairy products produced and commercialized in Urmia does not appear to be a serious public health problem at the moment. However, more samples of the dairy products will have to be taken for analysis, and more surveys conducted over a wider and more extended period.

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بررسی وقوع آفلاتوکسین M₁ در محصولات لبنی مورد فروش در ارومیه، ایران

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چکیده

هدف از این مطالعه ارزیابی وقوع طبیعی و اندازه گیری محتوای آفلاتوکسین M₁ در محصولات لبنی واقع در شهر ارومیه بود. در طول سپتامبر ۲۰۰۷، ۴۰ نمونه از شیر پاستوریزه، ۴۰ نمونه از شیر استریلیزه (UHT)، ۴۰ نمونه پنیر خامه‌ای و ۴۰ نمونه از پنیر ایرانی فتا از سوپرمارکت‌های واقع در سطح شهر ارومیه جمع آوری شد. مقدار آفلاتوکسین M₁ نمونه‌ها با استفاده از روش ELISA مورد ارزیابی قرار گرفت. آنالیز نمونه‌های شیر نشان داد که میانگین آلودگی به AFM₁ در آنها کمتر از میزان مجاز مطابق قوانین اتحادیه اروپا (حداکثر ۵۰ نانوگرم در لیتر) است. میانگین آلودگی در نمونه‌های شیر پاستوریزه و استریلیزه به ترتیب ۲۳/۲۲±۸/۶۵ و ۱۹/۵۳±۷/۴۷ نانوگرم در لیتر بود. میانگین آلودگی در پنیر فتا برابر ۴۳/۳۱±۱۸/۵۱ و در پنیر خامه‌ای برابر ۲۱/۹۶±۳/۲۳ نانوگرم بر کیلوگرم بود. خطر بالقوه مصرف آفلاتوکسین از طریق محصولات لبنی شناخته شده است. بنابراین، محصولات لبنی باید برای آفلاتوکسین مورد ارزیابی قرار بگیرند و در حد امکان از آلودگی‌های قارچی در آنها جلوگیری شود.