International Food Research Journal 20(6): 3351-3355 (2013)
Journal homepage: *http://www.ifrj.upm.edu.my*

# Aflatoxin M1 occurrence in pasteurized milk from various dairy factories in Iran

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**Article history**

*Received: 12 April 2013*
*Received in revised form: 1 August 2013*
*Accepted: 9 August 2013*

**Keywords**

*Aflatoxin M1*
*Pasteurized milk*
*Dairy factory*
*Tabriz*

**Abstract**

Several studies have shown evidence of the carcinogenic nature of aflatoxin M1, which passes to humans through dairy derivatives. The objective of this study was to determine the contamination and the concentrations of aflatoxin M1 in pasteurized milk in the spring season. A total of 80 pasteurized milk samples was collected from ten dairy factories in Tabriz, northwest Iran, during spring 2011. The quantity of aflatoxin M1 was determined by a competitive enzyme immunoassay test based on antigen–antibody reaction. The overall mean level of AFM1 in the entire set of samples was 27.8ng/l. Based on the results, with regard to the existence of aflatoxin M1, 3/80 (3.8%) cases were detected as negative, aflatoxin M1 was detected in 77/80 (96.3%) of the samples. Taking into consideration the European Commission limits for aflatoxin M1 in liquid milk, 16 (20.1%) of the samples had AFM1 in excess of the maximum tolerance limit. According to factor- by-factory data and statistical analysis, significant differences were observed between factories ($p < 0.05$). No significant differences between month of sampling and aflatoxin M1 levels could be found. Reducing the levels of aflatoxins in animal feedstuff by improved processes and storage practices could be a major step in the removal of this problem. Furthermore, it is important to check and control dairy products and animal feed for the presence of aflatoxins in a routine manner to evaluate levels of hygiene management. Moreover, providing milk from multiple & well supervised cattle lead to better quality dairy factories.



## Introduction

Aflatoxins are main secondary metabolites of fungi. These metabolites are produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius.* These species are prevalent in food harvests in tropical and subtropical regions worldwide (Bennet and Klich, 2003; Unusan, 2006). Aflatoxin B1 (AFB1) as more toxic and carcinogenic effects than other aflatoxin compounds is the most important compound among all aflatoxins that are naturally produced by fungi (Agnes and Akbarsha, 2003; Wangikar *et al.*, 2005; McKean *et al*., 2006).

After the ingestion of AFB1 by dairy cattle, AFB1 is transformed to a hydroxylated derivative in the liver, namely Aflatoxin M1 (AFM1) (Bakirci, 2001; Aycicek *et al*., 2005). AFM1 is excreted into the milk and is detectable after 12 h. But, 72 h after AFB1 intake, the AFM1 concentration in the milk decreases to an undetectable level (Kamkar *et al*., 2008; Rahimi and Karim, 2008; Fallah, 2010). On the other hand, the level of AFM1 in milk is dependent on AFB1 consumption by dairy animals and other studies have shown that <7% of AFB1 ingested by cattle is transformed to AFM1 in milk, however this is highly variable and dependent on certain conditions (Creppy, 2002; Unusan, 2006; Ayar *et al*., 2007). The presence of AFM1 in milk and dairy products is a problem worldwide, and various studies have shown evidence of the carcinogenic nature of AFM1, which passes to humans through dairy derivatives (Sassahara and Yanaka, 2005; Unusan, 2006; Oveisi *et al.*, 2007).

Although AFM1 is less mutagenic and carcinogenic than AFB1, WHO International Agency for Research on Cancer (IARC), is classified of AFM1 as a primary group of carcinogenic compounds (IARC, 2002). Furthermore, in 2002 the European

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Commission (EC) and Codex Alimentarus described an acceptable level of AFM1 in liquid milk as 50 ng/l. In some countries, the maximum admissible level of AFM1 for children's food is 10 ng/l (FAO, 2004). Previous studies conducted in Iran have shown the presence of AFM1 in milk and dairy products.

Kamkar (2002) showed that 82% of UHT milk samples were contaminated with AFM1 in Tehran, while 100% of contaminated samples had levels exceeding 50 ng/l (EC) (Kamkar, 2002). In the city of Shiraz, Alborzi *et al*. (2006) examined over six hundred pasteurized milk samples, in which AFM1 was found in 100% of the examined samples and 17.8% had an AFM1 level greater than the maximum tolerance limit accepted by the EC (Alborzi *et al*., 2006).

In further studies carried out by Gholampour Azizi *et al*. (2007) in Babol and Oveisi *et al*. (2007) in Tehran, 100% of milk samples were contaminated and the level of toxins in 78% of the examined samples was higher than the EC accepted limit. On the other hand, in Urmia all samples were lower than the EC standard (Tajik *et al*., 2007). Tajkarimi *et al.* (2007) analyzed aflatoxin levels of 319 raw milk samples from 14 regions in Iran. Of these samples, 54% were contaminated with AFM1and 33% were over 50 ng/l. In a recent study carried out in Tehran (Heshmati and Milani, 2010), in 55.2% of 210 UHT milk samples, AFM1 was detected in concentrations between 8 ng/l and 249 ng/l; and 33.3% of samples were higher than the maximum tolerance limit (0.05 mg/l) accepted by some European countries. The objective of this study was to determine the contamination and concentrations of AFM1 in pasteurized milk obtained from 10 dairy companies in the region of Tabriz, IRAN, during spring. Moreover, this is of vital importance to evaluate the effects of potential surveillance, control and inspection programmes for dairy production in this area.

## Material and Methods

*Milk sampling*

A total of 80 pasteurized milk samples was collected from 10 dairy factories in Tabriz, northwest Iran (including Tabriz, Azarshar, Khosroshah and Ilkhchi) during the spring of 2011 (April, May, June). The samples were transported to the laboratory under refrigerated conditions. As aflatoxins are water soluble milk samples were centrifuged at 10˚C for 10 min at 3500 g. After centrifugation, the upper cream layers were completely discarded and the lower phases were frozen for the assessment of AFM1 (Ghazani, 2009; Fallah, 2010).

*Assessment of AFM1 levels*

The quantity of AFM1 was determined by RIDASCREEN aflatoxin M1 test (R-biopharm, Germany), which is a competitive enzyme immunoassay based on antigen–antibody reaction. The wells in the micro titer strips were coated with specific antibodies to AFM1. The samples were prepared according to the manufacturer's instructions.

One hundred µl of samples, controls or standards were added to the wells to occupy the binding sites proportionately, then mixed gently and incubated for 60 min at room temperature (20–25˚C) in the dark. After the incubation period the solution was discarded from the wells and washed 3 times with rinsing buffer (250 µl). In the next stage, 100 µl of enzyme conjugate was added to occupy all used wells, except blanks, and sealed and incubated for 30 min at room temperature (20–25˚C) in the dark. In a further washing step, any unbounded enzyme conjugates were washed by rinsing buffer, after which 100 µl of substrate solution was pipetted into each well and incubated for 30 min. at room temperature. Bond enzyme conjugate converted the chromogen into a blue product. 100 µl of stop solution was then added to each well, which lead to yellow discoloration of the chromogen. The absorbance of each well was read at 450 nm against a blank by a micro plate reader and calibration curves of AFM1. The absorbance values were obtained for the standards and the AFM1 levels in the milk samples were subsequently determined.

*Statistical analysis*

Chi-square test in a single example and one-way ANOVA were used for the statistical analysis of data by SPSS18. P values of less than 0.05 were considered statistically significant.

## Results

All the results and statistical analysis are shown in Tables 1 and 2. The overall mean level of AFM1 in the entire set of samples was 27.8 ng/l. The standard error of the total of all the samples was 2.33 ng/l. Based on the results, with respect to the existence of AFM1, 3/80 (3.8%) cases were detected as negative, AFM1 being detected in 77/80 (96.3%) of the samples. 16 (20.1%) of the samples had AFM1 in excess of the maximum EC tolerance limit for AFM1 in liquid milk i.e. 50 ng/l (Table 1). Factory-by-factory data are shown in Figure 1 and based on statistical analysis, and significant differences were observed between some factories ($p < 0.05$). Based on the results shown in Table 2, we were unable to

Table 1. Statistical view of Aflatoxin M1 levels in milk samples, based on factories

| Dairy Factory | No sample | Mean | SD | SE | 95% Confidence Interval for Mean | | Undetected (%) | Up to 49 ng/l (%) | Over 50 ng/l (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | | |
| F1 | 9 | 25.1 | 11.1 | 3.7 | 16.5 | 33.6 | 0 | 100 | 0 |
| F2 | 8 | 54.2 | 28.3 | 10.0 | 30.5 | 77.9 | 0 | 37.5 | 62.5 |
| F3 | 8 | 10.9 | 11.7 | 4.1 | 1.1 | 20.6 | 0 | 100 | 0 |
| F4 | 6 | 13.7 | 9.3 | 4.1 | 2.1 | 25.1 | 16.7 | 83.3 | 0 |
| F5 | 8 | 38.3 | 35.3 | 12.5 | 8.8 | 67.8 | 12.5 | 37.5 | 50 |
| F6 | 8 | 34.1 | 28.2 | 9.9 | 10.4 | 57.6 | 0 | 75 | 25 |
| F7 | 8 | 13.1 | 11.9 | 4.2 | 3.1 | 23.1 | 0 | 100 | 0 |
| F8 | 8 | 41.1 | 32.2 | 11.4 | 14.1 | 68.0 | 12.5 | 37.5 | 50 |
| F9 | 8 | 25.9 | 22.4 | 7.9 | 7.1 | 44.6 | 0 | 87.5 | 12.5 |
| F10 | 9 | 18.1 | 7.4 | 2.5 | 12.3 | 23.7 | 0 | 100 | 0 |
| Total | 80 | 27.8 | 24.9 | 2.8 | 22.2 | 33.4 | 3.8(3) | 76.3(61) | 20(16) |

F: Factory, SD: Standard deviation, SE: standard error

Table 2. Statistical view of Aflatoxin M1 levels in milk samples, based on month of sampling

| Month | No of sample | Mean | SD | SE | 95% Confidence Interval for Mean | | Undetected (%) | Up to 49 ng/l (%) | Over 50 ng/l (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | | |
| April | 13 | 14.6 | 10.8 | 3.0 | 8.1 | 21.2 | 0 | 100 | 0 |
| May | 47 | 28.3 | 23.8 | 3.4 | 21.2 | 35.3 | 4.2 | 75 | 20.8 |
| June | 20 | 35.5 | 31.1 | 7.1 | 20.5 | 50.6 | 5.3 | 63.2 | 31.5 |
| Total | 80 | 27.8 | 24.9 | 2.8 | 22.2 | 33.4 | 3.6 | 76.3 | 20.1 |

SD: Standard deviation, SE: standard error

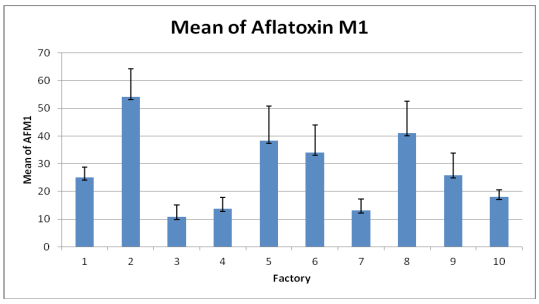


Figure 1. Comparative columnar diagram of contamination rate of pasteurized milks at different dairy factories to Aflatoxin M1

find significant differences between the month of sampling and AFM1 levels, however in the warmer months AFM1 levels were higher than in the colder months.

## Discussion

AFM1 has cytotoxic effects in human hepatocytes *in vitro* and it has been demonstrated that its acute toxicity in several species is similar to that of AFB1. AFM1 can also cause DNA damage, gene mutation, chromosomal anomalies and cell transformation in mammalian cells *in vitro* (Prandini *et al*., 2009). According to the present study, the occurrence of AFM1 in milk is highly prevalent and most of the samples were contaminated, however 20% of samples have unacceptable aflatoxin concentrations according to the EU. In agreement with the present study, previously published data have revealed a high frequency of AFM1 contamination in milk samples in Iran (Kamkar, 2002; Alborzi *et al.*, 2006; Oveisi *et al.*, 2007; Heshmati and Milani, 2010). This indicates that the milk used in these samples has been obtained from animals fed with AFB1 contaminated foodstuffs.

Based on the results, the percentage of contaminated samples were 96.3% and the percentage of samples over the EC limit was 20.1%. In some previous studies in Iran, the mean levels of AFM1 in spring were 43-76.5 ng/l (Kamkar, 2005; Tajkarimi *et al.*, 2007; Rahimi *et al.*, 2010; Panahi *et al.*, 2011). However, in studies carried out in the cities of Sanandaj and Sarab in recent years, the level of AFM1 was 13-37.2 ng/l, which is more similar to our results (Mohammadian *et al*., 2010; Davoudi and Nazeri, 2011;), presumably as a result of more surveillance, control and education for dairy production in these area. There is a more subtle point that we must also consider, which is that the wide variations in AFM1 levels across studies is not only attributable to geographic and climatic condition, but also to differences in control of feed supply, farm management practices and analytical methods used (Fallah, 2010).

In addition, compared to some European & Latin American countries, the AFM1 levels in our study are higher than those found in Turkey (Özdemir, 2007), Italy (Capei an Neri, 2002), Greece (Roussi *et al*., 2002), Portugal (Martins and Martins, 2000), Brazil (Garrido *et al*., 2003) and Argentina (Lopez *et al*., 2003). This may be due to improved storage practices carried out to control mycotoxins in feed supply in feed and milk in these countries. In Iran, control methods for AFM1 in milk and milk products are not strict.

According to results obtained in Iran and other

countries, incidence and contamination levels of AFM1 in Iran and other countries would appear to be a serious problem. This study has shown that contamination with AFM1 in Tabriz in some cases and in some factories is higher than standard levels. With regards to the levels of variation between factories, we found the dairy factories that had high levels of AFM1 (Factory 2 and 8) provided milk from private their cattle or one cattle. On the other hand, other factories provided milk from a variety of cattle and had stricter regulations on bought milk than previous factories. Also when milk is mixed from different cattle, the rate of contamination reduces.

Regarding the mean AFM1 levels over different months, we could not find any significant differences. Previous studies also failed to show any meaningful differences between months (Tajkarimi *et al.*, 2007; Panahi *et al.*, 2011; Sefidgar *et al*., 2011). However Tajkarimi *et al.* (2007) reported that high levels of AFM1 contamination can occur in the colder months. A comparison of the results of the present study and other studies during the spring season (April to June) of 2005- 2010 in Iran, in the same or other geographical areas, shows that AFM1 levels in milk have decreased (Kamkar, 2002; Alborzi *et al*., 2006; Azizi *et al*., 2007; Oveisi *et al*., 2007).

Considering the increasing rate of consumption of milk and dairy products by the Iranian people, aflatoxin contamination of food could also pose a serious risk for the public. However, decreasing AFM1 levels in the latest studies is inspiring and optimistic. It appears that aflatoxin levels should be determined at a continual time for dairy products and all those with levels of the upper maximum tolerance limit should impede to marketing.

## Conclusion

The results of the present study reveal information on the AFM1 contamination of pasteurized milk from factories in the region of Tabriz and the concentration of AFM1 during the spring season. In addition, the present study has shown that the level of AFM1 in some examined samples is high, and variation exists in the level of AFM1 between dairy factories. Reducing the levels of aflatoxin in animal feedstuff by improved processes and storage practices could be a major step to the removal of this problem. Furthermore, it is important to check and control dairy products and animal feed for the presence of aflatoxins in a routine manner to evaluate levels of hygiene management. Moreover, better quality dairy factories provide milk from multiple and well supervised cattle.

## Acknowledgements

The authors gratefully acknowledge the financial support from Tabriz University of Medical Sciences. We also thank Dr. Seyedi, Dr. Ghorbanpour and Mr. Khakpour for helping to collect and prepare the samples.

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