

Determination of Aflatoxin M₁ Levels in White Cheese Samples by ELISA in Gilan Province, Iran

¹Morteza Azizollahi Aliabadi, ¹Khosro Issazadeh, ²Reza Kazemi Darsanaki,
³Mahdiyeh Laleh Rokhi and ⁴Abolfazl Amini

¹Department of Microbiology, Faculty of Basic Science,
Lahijan Branch, Islamic Azad University, Lahijan, Iran

²Young Researchers Club, Lahijan Branch, Islamic Azad University, Lahijan, Iran

³Department of Microbiology, Isfahan Branch, Islamic Azad University, Isfahan, Iran

⁴Laboratory Science Research Center, Laboratory Sciences Department School of Paramedical,
Golestan University of Medical Sciences, Gorgan, Iran

Abstract: Aflatoxin M₁ (AFM₁) in milk and milk products is considered to pose certain hygienic risks for human health. These metabolites are not destroyed during the pasteurization and heating process. This study was undertaken to determine the presence and levels of aflatoxin M₁ (AFM₁) in Iranian white cheese consumed in Gilan province (Northern Iran). A total of 90 cheese samples was randomly obtained from retail outlets. ELISA technique was used to determine the presence and the level of AFM₁. In 78 of the 90 cheese samples examined (86.66%), the presence of AFM₁ was detected in concentrations between 7.2 - 413 ng/l. The mean level of AFM₁ in positive samples was 151.97 ng/l. AFM₁ levels in 21 samples (23.33%) were higher than the maximum tolerance limit (250 ng/l) accepted by the European countries. Aflatoxin high concentration in milk and milk products cause widespread negative impact on public health and demonstrate considerable economic losses for producers. Therefore, it is necessary to establish strategies for reducing aflatoxin levels in animal feed and milk products.

Key words: Aflatoxin M₁ • Cheese • ELISA

INTRODUCTION

Mycotoxins are secondary metabolites of fungi which are associated with certain disorders in animals and humans [1]. Aflatoxins are a group of structurally-related toxic compounds produced by certain strains of the fungi *Aspergillus flavus* and *A. parasiticus* [2, 3]. Under favorable conditions of temperature and humidity, these fungi grow on certain foods and feeds, resulting in the production of aflatoxins [4, 5]. Aflatoxin contamination in milk and milk products is produced in two ways. Either toxins pass to milk with ingestion of feeds contaminated with aflatoxin, or it results from subsequent contamination of milk and milk products with fungi. The major aflatoxins of concern are designated B₁, B₂, G₁ and G₂, also M₁ and M₂ as metabolic products of AFB. AFB₁ to G₂ belong to Group 1 and M₁ belongs to Group 2B, according to IARC [6]. It has been stated, in fact, that the contamination of milk and milk products with AFM₁ display variations according to geography, country and season. The pollution level of AFM₁ is differentiated further by hot

and cold seasons, due to the fact that grass, pasture, weed and rough feeds are found more commonly in spring and summer than in winter. At the end of summer, greens are consumed more than concentrated feed, causing a decreased level of AFM₁ in milk and milk products [6-8]. AFM₁ is not destroyed during the pasteurization process or in yoghurt and cheese making. As aflatoxins pose more serious risks for public health, certain limits of aflatoxins in foods are determined [6, 9, 10]. The limiting rates of AFM₁ were shown in Table 1 [6, 11, 12]. AFM₁ could be detected in milk 12-24 h after the AFB₁ ingestion, reaching a high level after a few days. When AFB₁ intake is stopped, the AFM₁ concentration in milk decreases to an undetectable level after 72 h. Milk and its products are a major nutrient for human especially children all over the world [1, 13, 14]. At the same time, these products may be contaminated with AFM₁ residues which extensively threaten the human health. For this reason, many countries have regulations to control the levels of AFB₁ in feeds and to propose maximum permissible levels of AFM₁ in milk to reduce this risk [9]. The European

Table 1: Maximum limits for aflatoxin M₁ in milk and milk products in various countries

Country	Maximum limit (µg/kg or µg/l)
France	0.05 Adult's milk
	0.03 Children's milk
Turkey	0.05 Milk and products
	0.25 Cheese
Czech Republic	0.1 Children's milk
	0.5 Adult's milk
Belgium	0.050 Milk
USA	0.50 Milk
Switzerland	0.050 Milk and milk products
	0.250 Cheese
Netherlands	0.020 Butter
	0.200 Cheese
Germany	0.050 Milk
Austria	0.050 Milk

Commission (EC) has approved a maximum admissible level of 250 ng/l for AFM₁ in cheese [15]. Therefore, the aim of this study was to investigate the presence of AFM₁ in cheese samples consumed in Gilan province (Northern Iran) by ELISA method.

MATERIALS AND METHODS

Preparation of Samples: A total of 90 cheese samples was obtained randomly from retail outlets during summer and autumn 2011 in Gilan province (Northern Iran). The samples were transported to the laboratory in insulated container at about 4°C. Two grams of cheese samples were homogenized and extracted with 40 ml dichloromethane. The suspension was filtered and then 10 ml of the extract was evaporated at 60°C. The residue was redissolved in 0.5 ml methanol, 0.5 ml phosphate buffer and 1 ml n-heptane and was mixed thoroughly. After centrifugation for 15 min at 2700 rpm, 100 µl of the methanolic phase was brought to 10% methanol content by addition of 400 µl of kit buffer. 100 µl (per well) of this solution was used in the test [1, 12, 16].

ELISA Test Procedure: Before starting the test, the reagents were brought up to room temperature. The AFM₁ standards and test samples (100 µl per well) in duplicate were added to the wells of a micro-titer plate pre-coated with antibodies for AFM₁ and incubated at room temperature in dark for 60 min. After the washing step, 100µl of peroxidase conjugate was added to the wells and plate was incubated again for 60 min at room temperature in dark. After the washing step, the unbound conjugate was removed during washing. Subsequently, 50 µl each

substrate (urea peroxide) and chromogen (tetramethylbenzidine) were added to the wells and incubated for 30 min in dark. Finally, 100 µl of stop solution were added to each well. The optical absorbance of each well was read at 450 nm with ELISA plate reader. Absorbance percentages were taken to the calibration curve performed with standards at different concentrations [1]. Statistical analyses were performed using SPSS software.

RESULTS

A total of 90 white cheese samples was analyzed with competitive ELISA. The occurrence of AFM₁ was shown in Table 2. Of the 90 samples analyzed, 78 samples (86.66%) were found to be contaminated with AFM₁. 21 samples (23.33%) failed to reach the desired level of the European Communities and Codex, defined as 250 ng/l. The aflatoxin M₁ contamination levels were between 7.2 - 413ng/l with the mean of 151.97 ng/l.

Percentage of AFM₁ Positive Samples: The range of contamination levels varied in two seasons, Table 3. AFM₁ in summer and autumn samples ranged from 7.2-402 and 8 - 413 ng/l whit the mean values 139.90 and 162.32 ng/l respectively. The highest mean concentration of aflatoxin M₁ registered in autumn 413 ng/l, while in summer samples highest concentration of aflatoxin M₁ was 402 ng/l.

DISCUSSION

Dairy products play a significant role in human diet since they are rich sources of bioavailable calcium and proteins. However, many of the previous studies has indicated the presence of AFM₁ at high concentrations in dairy products [9, 17]. Aflatoxins are highly toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds. The main target organ for their toxicity and carcinogenicity is the liver, Milk and milk products, are a major nutrient for humans, especially children. For this reason, AFM₁ in milk and dairy products should be controlled systematically [9, 18, 19]. Occurrence of AFM₁ in cheese can be due to three possible causes: (1) AFM₁ present in raw milk because of carryovers of AFB₁ from contaminated cow feed to milk, (2) Synthesis of AF (B₁, B₂, G₁ and G₂) by *A. flavus* and *A. parasiticus* growing on cheese and (3) Occurrence of these toxins in dried milk used to enrich the milk which is being used in the production of cheese [16].

Table 2: Occurrence of AFM₁ in white cheese samples from Northern Iran

AFM ₁ levels ng/l	Sample No.	(%)*	Range
Not detected	12	-	-
< 50	22	24.44	7.2-48
50-100	15	16.66	50.2-99.2
101-250	20	22.22	101.2-248.5
≥ 250	21	23.33	250.1-413
Total Samples	90	86.66	7.2-413

Table 3: Distribution by season of cheese samples and aflatoxin M₁ concentration (ng/l)

Season	Samples	Negative	Positive	Mean	Range
Summer	45	9(20%)	36(80%)	139.90	7.2-402
Autumn	45	3(6.66%)	42(93.33%)	162.32	8-413

Thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and enzyme linked immunosorbent assay (ELISA) are the most common techniques for detecting AFM₁ in milk and dairy products. Therefore, differences between these techniques may affect the results of different studies carried out by different investigators [8]. Several surveys were performed in order to determine the AFM₁ levels in milk, cheeses and milk products. In Turkey, a study done by Oruc and Sonal [18], found AFM₁ in 89.5% of 57 cheese samples with ranges of 0-180 ng/l. In a study of Tekinsen and Eken [20], 132 kashar cheese samples were analyzed for AFM₁ and 82.6% of the samples contained AFM₁ (50-690 ng/l). In 2005, the occurrence of AFM₁ was studied in Portuguese soft cheese, revealing that in a total of 42 samples no one was contaminated with AFM₁ [21]. Sarimehmetoglu *et al.* [9], detected AFM₁ contamination in 327 (81.75%) of 400 cheese samples. The numbers of cheese samples that contained AFM₁ over the legal limits of 0.25 µg/kg were 110 (27.5%). In another study 193 white cheese samples were analyzed by Ardıc *et al.* [22] and AFM₁ was found in 82.4% of the samples (52-860 ng/l). In Kuwait, 54 samples of dairy products were analyzed for aflatoxin M₁, 28% were contaminated with AFM₁ [23]. Gensen and Buyukyoruk [24], analyzed 130 cheese samples and determined an average of 0.142 µg/kg AFM₁. Ayhan *et al.* [25] showed that out of a total of 110 cheese samples, 9 do not contain AFM₁, while 101 samples were found to be contaminated with AFM₁ in the range of 10-2000 ng/l. Kokkonen *et al.* [26] did not find AFM₁ in the examined cheese samples. Gurses *et al.* [27], analyzed 63 cheese samples and in 28(44.44%), AFM₁ was detected in concentrations between 7-202 ng/l. Martins and Martins [28] analyzed a total of 182 samples of national cheese (Portugal) by TLC, observing that all of them were not contaminated by AFM₁. In our study, Of the

90 samples analyzed, 78 samples (86.66%) were found to be contaminated with AFM₁. 21 samples (23.33%) failed to reach the desired level of the European Communities and Codex, defined as 250 ng/l and AFM₁ in summer and autumn samples ranged from 7.2 - 402 and 8 - 413 ng/l. According to observations, the levels of contamination of cheese by AFM₁ seem to vary in many studies. These variations may be related to different reasons such as cheese manufacturing procedures, different milk contaminations, type of cheese, conditions of cheese ripening, geographical region, the country, the season and the analytical methods employed [12, 25, 29].

In conclusion, According to results obtained, incidence and contamination levels of AFM₁, seem to be a serious problem for public health. For this reason, milk and dairy products have to be inspected and controlled continuously for AFM₁ contamination and animal feeds should be checked regularly for AFB₁ and storage conditions of feeds must be taken under strict control.

REFERENCES

- Hampikyan, H., E. Baris Bingol, O. Cetin and H. Colak, 2010. Determination of aflatoxin M₁ levels in Turkish white, kashar and tulum cheeses. *J. Food Agric. and Environ.*, 8: 13-15.
- Chen, C.Y., W.J. Li and K.Y. Peng, 2005. Determination of Aflatoxin M₁ in Milk and Milk Powder Using High Flow Solid Phase Extraction and Liquid Chromatography Tandem Mass Spectrometry. *J. Agric. Food Chem.*, 53: 8474-8480.
- Colak, H., 2007. Determination of Aflatoxin M₁ Levels in Turkish White and Kashar Cheeses Made of Experimentally Contaminated Raw Milk. *J. Food and Drug Analysis*, 15: 163-168.
- Kamkar, A., G.H.R. Jahed Khaniki and S.A. Alavi, 2011. Occurrence of aflatoxin M₁ in raw milk produced in Ardabil of Iran. *Iran. J. Environ. Health, Sci. Eng.*, 8: 123-128.
- Creppy, E.E., 2002. Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicol. Letters*, 127: 19-28.
- Celik, T.H., B. Sarimehmetoglu and O. Kuplulu, 2005. Aflatoxin M₁ contamination in pasteurised milk. *Veterinarski Arhiv*, 75: 57-65.
- Tajkarimi, M., F. Shojaee Aliabadi, M. Salah Nejad, H. Pursoltani, A. Motallebi and H. Mahdavi, 2007. Seasonal study of aflatoxin M₁ contamination in milk in five regions in Iran. *International J. Food Microbiol.*, 116: 346-349.

8. Galvano, F., V. Galofaro and G. Galvano, 1996. Occurrence and stability of aflatoxin M₁ in milk and milk products: A worldwide review. *J. Food Protect*, 59: 1079-1090.
9. Sarimehmetoglu, B., O. Kuplulu and T.H. Celik, 2004. Detection of aflatoxin M₁ in cheese samples by ELISA. *Food Control*, 15: 45-49.
10. Panahi, P., S. Kasaei, A. Mokhtari, A. Sharifi and A. Jangjou, 2011. Assessment of Aflatoxin M₁ Contamination in Raw Milk by ELISA in Urmia, Iran. *American Eurasian J. Toxicological Sci.*, 3: 231-233.
11. Lin, L.C., F.M. Liu, Y.M. Fu and D.Y. Chih Shih, 2004. Survey of Aflatoxin M₁ Contamination of Dairy Products in Taiwan. *J. Food and Drug Analysis*, 12: 154-160.
12. Dashti, B., S. Al-Hamli, H. Alomirah, S. Al-Zenki, A. Bu Abbas and W. Sawaya, 2009. Levels of aflatoxin M₁ in milk, cheese consumed in Kuwait and occurrence of total aflatoxin in local and imported animal feed. *Food Control*, 20: 686-690.
13. Van Egmond, H.P., 1989. Current situation on regulations for mycotoxins. Overview of tolerances and status of standard methods of sampling and analysis. *Food Addit Contam*, 6: 139-188.
14. Sibanda, L.S. De Saeger and C. Van Petegham, 1999. Development of a portable field immunoassay for the detection of aflatoxin M₁ in milk. *Int. J. Food Microbiol.*, 48: 203-209.
15. European Commission (EC), 2006. No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off J. Eur. Union.*, 364: 5-24.
16. Amer, A.A. and M.A.E. Ibrahim, 2010. Determination of aflatoxin M₁ in raw milk and traditional cheeses retailed in Egyptian markets. *Journal of Toxicology and Environmental Health Sci.*, 2: 50-53.
17. Tekinsen, K.K. and O.C. Tekinsen, 2005. Aflatoxin M₁ in white pickle and *Van otlu* (herb) cheeses consumed in southeastern Turkey. *Food Control*, 16: 565-568.
18. Oruc, H.H. and S. Sonal, 2001. Determination of aflatoxin M₁ levels in cheese and milk consumed in Bursa, Turkey. *Vet. Human Toxic.*, 43: 292-293.
19. Akkaya, L., Y.O. Birdane, H. Oguz and M. Cemek, 2006. Occurrence of aflatoxin M₁ in yogurt samples from afyonkarahisar, Turkey. *Bull. Vet. Inst. Pulawy*, 50: 517-519.
20. Tekinsen, K.K. and H.S. Eken, 2008. Aflatoxin M₁ levels in UHT milk and kashar cheese consumed in Turkey. *Food and Chem. Toxicol.*, 46: 3287-3289.
21. Martins, H.M., M.M. Guerra and F. Bernardo, 2005. A six year survey (1999-2004) of the occurrence of aflatoxin M₁ in dairy products produced in Portugal. *Mycotoxin Res.*, 21: 192-195.
22. Ardic, M., Y. Karakaya, M. Atasever and G. Adiguzel, 2009. Aflatoxin M₁ levels of Turkish white brined cheese. *Food Control*, 20: 196-199.
23. Srivastava, V.P., A. Bu-Abbas, W. Alaa-Basuny Al-Johar, S. Al-Mufi and M.K. Siddiqui, 2001. Aflatoxin M₁ contamination in commercial samples of milk and dairy products in Kuwait. *Food Addit. Contam.*, 8: 993-997.
24. Gunsen, U. and I. Buyukyoruk, 2002. Aflatoxins in retail food products in Bursa, Turkey. *Vet. Human Toxicol.*, 44: 289-290.
25. Ayhan, F., I. Sinan and T. Fusun, 2010. Survey of the occurrence of aflatoxin M₁ in cheeses produced by dairy ewe's milk in Urfa city, Turkey. *Ankara Univ. Vet. Fak. Derg.*, 57: 197-199.
26. Kokkonen, M., M. Jestoi and A. Rizzo, 2005. Determination of selected mycotoxins in mould cheese with liquid chromatography coupled to tandem with mass spectrometry. *Food additives and Contaminants*, 22: 449-456.
27. Gurses, M., A. Erdogan and B. Cetin, 2004. Occurrence of aflatoxin M₁ in some cheese types sold in Erzurum, Turkey. *Turk. J. Vet. Anim. Sci.*, 28: 527-530.
28. Martins, M.L. and H.M. Martins, 2000. Aflatoxin M₁ in raw and high temperature treated milk commercialised in Portugal. *Food Additives and Contaminants*, 17: 871-874.
29. Khoshnevis, S.H., I. Gholampour Azizi, S. Shateri and M. Mousavizadeh, 2012. Determination of the Aflatoxin M₁ in Ice Cream in Babol City (Northern, Iran). *Global Veterinaria*, 8: 205-208.