The study of aflatoxin M_1 in UHT milk samples by ELISA

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Abstract: **Aflatoxin M_1 (AFM_1) levels in 52 samples of UHT milk that were produced by different plants in province of Tehran were analyzed by competitive ELIZA.AFM_1 was found in 100 percent of 52 of the UHT milk samples that were analyzed in this study. The range of contamination levels varied in two seasons of summer and autumn. AFM_1 in summer and autumn samples ranged from 22.40 to 84.80 and 19.40 to 93.60 ng/kg respectively, while the mean values were 69.22 and 65.50 ng /kg respectively. Statistical evaluation showed that there were not significant differences ($p>0.05$) between the concentrations of AFM_1 of UHT milk samples produced in summer and autumn. In other words, AFM_1 contents of UHT milk samples produced in summer were not lower than UHT milk samples produced in autumn. Almost 79.92% of the contaminated samples exceeded the maximum acceptable levels (50 ng/kg) that accepted by some of the European countries. It was, therefore, concluded that, high occurrence of AFM_1 in UHT milk samples was considered possible hazard for human health.**

Key words: aflatoxin M_1, UHT milk.

Introduction

Aflatoxins are actually toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds to animals and humans, and contamination of feed and food is a current problem (Piva *et al.*, 1995; Kotsonis, Burdock, and Flamm, 1996; Peraica *et al.*, 1999; Kocabas and Sekerel, 2003).

International Agency for Research on Cancer (IARC) of WHO included AFB_1 (Aflatoxin B_1) as primary and AFM_1 (Aflatoxin M_1) as secondary groups of carcinogenic compounds (Anonymous, 1993; Cathey *et al.*, 1994; Dragacci *et al.*, 1995).

Aflatoxins can be produced by three species of Aspergillus. A. flavus, A.parasiticus and the rare A.nomius. A. flavus only produces B aflatoxins, while the other two species produce both B and G aflatoxins. Aflatoxins M_1 (AFM_1) and M_2 (AFM_2) are hydroxylated metabolites of Aflatoxins B_1 (AFB_1) and B_2 (AFB_2), respectively, and may be found in milk products obtained from livestock that have ingested contaminated feed (Van Egmond, 1991; Wood, 1991; Cathey *et al.*, 1994; Creppy, 2002). Many researchers reported that there was a linear relationship between the amount of AFM_1 in milk and AFB_1 in feed consumed by animals (Wood, 1991; Dragacci *et al.*, 1995; Bakirici, 2001).

According to Stoloff (1980) milk has the greatest demonstrated potential for introducing AF residues from edible animal tissues into the human diet, and taking into account that pasteurization processes (even those using UHT, Ultra High Temperature, techniques) do not affect AFM_1 concentration because of its heat stability (Gelosa, and Buzzetti, 1994; Galvano, Galofaro, and Galvano, 1996), moreover, as milk is the main nutrient for growing young, whose vulnerability is noteworthy and potentially more sensitive than that of adults, the occurrence of AFM_1 in human breast milk, commercially available milk, and milk products is one of the most serious problems of food hygiene. For this reason, many countries have regulations to control the levels of Aflatoxins B_1 in feeds and to purpose maximum permissible levels of AFM_1 in milk to reduce this risk (Rastogi *et al.*, 2004;

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Sarimehmetoglo, Kuplulu, and Celik, 2004).

Regulatory limits throughout the world are highly variable, depending on the degree of development and economic involvement of countries, and may vary from one country to another (Van Egmond, 1989; Stahr *et al.*, 1990; Stoloff, Van Egmond, and Parks, 1991; Chen and Gao, 1993). The European Community and Codex Alimentarius prescribe that the maximum level of AFM_1 in liquid milk and dried or processed milk products should not exceed 50 ng/kg (Codex Alimentarius Commission, 2001). However, according to US regulations the level of AFM_1 in milk should not be higher than 500 ng/kg (Stoloff *et al.*, 1991). In Austria and Switzerland, the maximum level is further reduced to 10 ng/kg for infant food commodities (FAO, 1997). There are thus differences in maximum permissible limit of AFM_1 in various countries (Van Egmond, 1989), and many including Iran, have no legal limit for AFM_1 in milk and dairy products.

A number of survey and monitoring programs have been carried out in several countries attempting to obtain general pattern of extent of food contamination (Galvano *et al.*, 1996; Abdulkadar, Abdulla, and Jasim, 2000; Oruc and Sonal, 2001; Aycicek *et al.*, 2002; Gunsen and Buyukyörük, 2002; Rastogi *et al.*, 2004).

There is little information about the occurrence of AFM_1 in milk and milk products. In Tehran, of 73 samples taken in 1998, 60(82.2%) samples contained AFM_1, and all contaminated samples had a level of aflatoxin M_1 above the European countries standard (Karim *et al.*, 1998). In another study from 52 liquid milk samples that were analyzed, 48 (92.3%) were contaminated with aflatoxin M_1 at concentration between 23 and 3000 µg/l (Karim *et al.*, 1982).

The purpose of this survey was to determine natural occurrence and levels of AFM_1 in UHT milk which were produced and consumed in Tehran province of Iran, and to compare the obtained results with maximum AFM_1 tolerance limits (50 ng/kg) accepted by some European countries.

Materials and Methods

Fifty-two samples of UHT milk were taken randomly from markets located in the various districts of Tehran, and examined for the presence and levels of AFM_1. All samples were collected between the periods of May 2004 and November 2004, and aflatoxin M_1 concentration was determined before their expiration dates exceeded. Randomized block experiment was used to evaluate the differences between AFM_1 occurrence levels of the milk samples. Furthermore, Independent-samples t-test was used to obtain significant differences between sampling periods of all tests.

The milk samples were centrifuged 10min/3500 rpm/10°C. After centrifugation, the upper cream layer was completely removed by aspirating through a Pasteur pipette. The skimmed milk was used directly in the test (100 µl per well).A sufficient number of micro titer wells were inserted into the micro well holder for all standards and prepared samples. One hundred µl of standard solutions and prepared samples were added in separate wells and incubated for 60 min at room temperature in the dark.The liquid was poured off the wells and the micro well holder was tapped upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. All the wells were filled with 250 µl of washing buffer and emptied as described earlier. The washing procedure was repeated twice. One hundred µl of the enzyme conjugate was added and incubated for 60 min at room temperature in the dark.The washing sequence was repeated three times. Fifty µl of substrate and 50 µl of chromogen were added to each well and mixed thoroughly and incubated for 30 min at room temperature in dark. Then100 µl of the stop reagent was added to each well, mixed and absor bance measured at wave lgngth of 450 nm against air.

Results

In this study, 52 UHT milk samples were analyzed for AFM_1 with the competitive ELISA. The presence of AFM_1 in UHT milk samples is shown in tables 1 and 2.

The prevalence rate of AFM_1 contamination in UHT milk samples was 100 percent. In the other words, of 52 UHT milk, 52 samples (100%) were

Table 1: Occurrence of aflatoxin M_1 in UHT milk samples and concentration (ng/kg). Values in parentheses indicate percentages of positive samples.

		Number and percent of samples with AFM_1 in ng/kg ranges						Exceeding EC/Codex regulation (50 ng/kg)	
Samples Tested (n)	Level of positive samples (%)	ND	<2 0	20-40	40-60	60-80	80-100	Number	Range (ng/kg)
52	52(100)	0 (0)	1(1.92)	8(15.4)	7(13.5)	21(40.4)	15(28.8)	40(76.92)	52.7 - 93.66

contaminated with AFM_1 ranging from 19.40 to 93.60 ng/kg (table 1). One sample (1.92%) contained level <20 ng/kg. Eight samples (15.38%) were contaminated with AFM_1 levels ranging from 20 to 40 ng/ kg. Seven samples (13.46%) were contaminated with levels ranging from 40 to 60 ng/ kg. Twenty-one samples (40.38%) showed contamination levels from 60 to 80 ng/ kg and 15 samples (28.84%) presented high contaminations with levels from 80 to 100 ng/ kg. Table 2 shows distribution by season of UHT milk samples and aflatoxin M_1 (AFM_1) concentration. The range of contamination levels varied in two seasons. AFM_1 in summer and autumn samples ranged from 22.40 to 84.80 and 19.40 to 93.60 ng/kg while the mean values were 65.50 and 69.22 ng/kg respectively. The highest mean concentration of aflatoxin M_1 (AFM_1) registered in autumn (93.60), while in summer samples highest concentration of aflatoxin M_1 (AFM_1) was 84.80 ng/kg. Statistical evaluation showed no significant differences ($p>0.05$) between the mean concentration of AFM_1 of milk samples produced in summer and autumn. Almost 79.92% of contaminated samples exceeded the European Communities/ Codex Alimentarius recommended limits, while none of the samples exceeded the prescribed limits of US regulations.

Discussion

Since, milk is a major commodity for introducing aflatoxins in human diet, and several investigators (Galvano, Galofaro and Galvano, 1996; Stoloff, 1980) have showed evidence of hazardous human exposure to AFM_1 through dairy products, many countries have carried out studies about the incidence of AFM_1 in milk. In some of them, samples have been found which exceed the limit imposed by many countries (0.05 µg/l).

In Asia, high incidences and levels of aflatoxin M_1 contamination were found in In Thailand, of 310 liquid milk samples, more than 261 (>84%) were contaminated with aflatoxin M_1 concentrations > 0.05 µg/kg, and 58 samples (19%) contained >0.5 µg/kg, with a maximum of 6.6 µg/kg (Boriboon and suprasert, 1994; Saitanu, 1997).

In Philippine (Begino, 1998), indicated that 88% and 18% of 91 milk samples were contaminated with aflatoxin M_1 at > 0.05 µg/kg and >0.5 µg/kg, respectively. The data for Republic of Korea in 1995 and 1997 (Kim *et al.*, 2000; Shon, Lim and Lee, 1996), indicated that of 134 liquid milk samples, 50 (37%) contained aflatoxin M_1 at a concentration > 0.05 µg/kg, with a maximum of 0.28 µg/kg. Of 504 samples taken in India in 1995, 89 (15.6%) samples contained AFM_1 at concentration of 100 - 3500 µg/l (Rajan, Ismail and Radhakrishnon, 1995).

In another study in India, 87.3% of 87 samples that were analyzed showed to be contaminated with AFM_1. The range of contamination of AFM_1 in liquid milk was 28- 164 µg/l. Almost 99 percente of the contaminated samples exceeded the European Communities / Codex Alimentarius recommended limits (European Communities, 1992; Shipra *et al.*, 2004). In a Korean study, the incidence of AFM_1 in liquid milk was 76%, with a mean concentration of 18 pg/g (Kim *et al.*, 2000). In Kuwait, 54 samples of dairy products were analyzed for aflatoxin M_1 (AFM_1). Twenty-eight percet were contaminated

Table 2: Distribution by season of UHT milk samples and aflatoxin M_1 concentration (ng/kg). Values in parentheses indicate percentages of negative and positive samples.

Season							
	Tested	Negative*	Positive*	Minimum	Maximum	Mean	S. D.
Summer	22	0(0)	22(100)	22.40	84.80	65.50	12.54
Autumn	30	0(0)	30(100)	19.40	93.60	69.22	20.14

with AFM_1 with 6% being above the maximum permissible limit of 0.2 µg/l, (Srivastava *et al.*, 2001).

The prevalence rate of aflatoxin M_1 (AFM_1) contamination in the raw milk analyzed in Portugal was 80.6%, 17 samples (54.8%) contained low levels (0.005 - 0.010 µg/l), two samples (6.5%) had levels ranging from 0.011 to 0.02 µg/l and six samples (19.3%) had levels between 0.021 and 0.050 µg/l (Martins and Martins, 2000). During 1996, 161 samples of milk in Italy were checked for aflatoxin M_1 (AFM_1). AFM_1 was detected in 125 (78%) of milk samples (ranging from <1 ng/l to 23.5 ng/l; mean level: 6.28 µg/l) (Galvano *et al.*, 2001).

In Van province of Turkeys, AFM_1 found in 79 (87.77%) of 90 milk samples examined. AFM_1 concentration in 35 (44.30%) of the positive samples were higher than the maximum tolerance limit (0.05 µg/l) accepted by Turkey and some other countries (Bakirici, 2001).

There is little information about the occurrence of AFM_1 in milk products in Iran. In a study in Tehran, (Karim *et al* (1998), analyzed 73 milk samples delivered to Tehran milk pasteurization plants for the presence of aflatoxin M_1. AFM_1 was detected in 60 samples (82.2%). All contaminated samples had a level of aflatoxin M_1 above the European countries standard that is 50 µg/l. In another study, of 52 liquid milk samples, 48 (92.3%) were contaminated with aflatoxin M_1 at concentration between 23 to 3000 µg/l (Karim *et al.*, 1982).

According to results obtained in Iran and other countries, incidence and contamination levels of AFM_1 in these countries, (especially Asian countries) seem to be a serious problem for the public health. For this reason, milk and dairy products have to be inspected and controlled continuously for AFM_1 contamination. The most effective way of controlling aflatoxin M_1 in the food supply is to reduce contamination of raw material and supplementary feedstuffs for dairy cattle with aflatoxin B_1. Specific regulation exist in many countries (FAO, 1997), and practical programmes are being developed; e.g. the Codex Committee on Food Additives and Contaminants has developed, a code of practice for reducing aflatoxin B_1 in raw materials (Van Egmond *et al.*, 1997). Reduction can be achieved by good manufacturing practice and good storage practices. If preventive measures fail to reduce fungal growth and aflatoxin B_1 formation in agricultural commodities intended for use as animal feeds, the last means for avoiding or reducing the occurrence of aflatoxins in feed is to eliminate (part of) the toxins. Feeds that have higher concentrations of aflatoxin B_1 may be acceptable for feeding to dairy animals if they are blended with feeds that have lower concentrations, to make sure that the resultant aflatoxin M_1 concentration in milk does not exceed levels considered being safe.

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مطالعه آفلاتوکسین M_1 در شیرهای استریلیزه شده با روش الیزا

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در این مطالعه میزان آفلاتوکسین M_1 در تعداد ۵۲ نمونه شیر استریلیزه عرضه شده در شهر تهران که توسط کارخانجات مختلف تولید و روانه بازار مصرف شده بودند توسط روش ELISA رقابتی مورد ارزیابی قرار گرفت. نتایج حاصله نشان داد که صددرصد نمونه‌های مورد مطالعه به آفلاتوکسین M_1 آلوده بودند و محدوده آلودگی بین دو فصل تابستان و پاییز با یکدیگر تفاوت داشت بگونه ای که این محدوده در دو فصل تابستان و پاییز به ترتیب ۲۲/۴۰ تا ۸۷/۴۰ و ۱۹/۴۰ تا ۹۳/۶۰ نانوگرم در کیلوگرم بود در حالی که میانگین آلودگی در دو فصل سال به ترتیب ۶۹/۲۲ و ۶۵/۵۰ نانوگرم در کیلوگرم بود. محاسبات آماری نشان داد که تفاوت معنی‌داری بین غلظت آفلاتوکسین M_1 شیرهای استریلیزه تولید شده در تابستان با پاییز وجود ندارد ($p>0/05$) به عبارت دیگر میزان شیرهای AFM_1 استریلیزه تولید شده در تابستان پایین‌تر از پاییز نبود. تقریبا (۷۹/۹۲ درصد) نمونه‌های آلودگی بالاتر از حد استاندارد ۵۰ نانوگرم در کیلوگرم (استاندارد اتحادیه اروپا) را نشان می‌دادند. با توجه به آلودگی بیشتر شیرهای استریلیزه به آفلاتوکسین این شیرها برای سلامتی انسان می‌تواند خطرناک باشد.

واژه‌های کلیدی: آفلاتوکسین M1، شیر UTH.

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