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Determination of Aflatoxin M1 in UHT, Pasteurized and GSM Milks in Ahvaz (South-West of Iran) Using ELISA

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Abstract: Aflatoxin M1 (AFM1) is a secondary metabolite of Aflatoxin B1 (AFB1) and can be found in milk as a result of the ingestion of feedstuffs contaminated with some fungi by ruminants. AFM1 is heat stable and so can be found in pasteurized and UHT milks. This study was conducted to elevate and compare the levels of AFM1 in UHT milk, pasteurized and Government Subsidize Milk (GSM) samples during different seasons in Ahvaz area. During July 2009 to June 2010, 178 milk samples were collected and analyzed for AFM1 by ELIZA technique. Based on Iranian standards (100 ng/L), 17 samples (9.5%) were exceeded the legal level, but 62 samples (34.8%) were contaminated with AFM1 higher than the maximum tolerance limit (50 ng/L) accepted by European countries. Results showed that the mean of AFM1 level in spring milk samples was less than the other seasons. Regarding the effect of type of milk, the mean of the AFM1 level in subsidizes milk was greater than normal pasteurized and sterilized milk samples but this differences was not significant ($P<0.05$). It can be concluded that there is no differences between milk types. The least level of AFM1 was found in UHT milk samples.

Key words: Aflatoxin M_1 · Pasteurized milk · UHT milk · ELIZA

INTRODUCTION

Aflatoxins are produced by certain strains of the fungi *Aspergillus flavous*, *Aspergillus parasiticus* and rarely *Aspergillus nomius*. They are both actually and chronically toxic, mutagenic, teratogenic and carcinogenic compounds for animals and humans [1, 2]. Aflatoxin M1 (AFM1) is the main hydroxylate derivative of aflatoxin B1 (AFB1) forming in the liver of ruminants and excrete to milk [3-5]. It was noticeable that neither storage of milk nor some processing techniques such as pasteurization or UHT do not affect AFM1 concentration because of its heat stability [6, 7]. Moreover, as milk is the main nutrient for young children whose vulnerability is noteworthy and potentially more sensitive than that of adults, the occurrence of AFM1 in commercially available milk is one of the serious problems of food hygiene [6]. According to the European Union and Codex Alimentarius the maximum level of AFM1 in liquid milk dried or processed milk products should not exceed 50 ng/L [8], but based on the US regulations it should not be higher than 500 ng/L [9]. Institute of Standards and Industrial Research of Iran has been accepted 100 ng/l AFM1 as maximum tolerance level in raw, pasteurized and UHT milk, ice-cream, butter and yogurt [10]. There are three kinds of commercial milk available in Iran. Pasteurized, sterilized by UHT technique and a kind of pasteurized milk called government subsidize milk (GSM). The last kind of milk is more favorable for people due to cheap price.

There is little information about the occurrence of AFM1 in milk and milk products and no information regarding presence and level of AFM1 in GSM milk in Iran. Accordingly, Alborzi *et al.* [11] reported in 624 samples of pasteurized milk in Shiraz (South of Iran), that the amount of AFM1 in 17.8% of the samples was higher than the maximum tolerance limit accepted by European Union [8]. In 52 UHT milk in Tehran it was found that the level of AFM1 in 79.9% of samples was higher than 50ng/L [12]. Also, the incidence rate of AFM1 in raw cow, water buffalo, camel, sheep and goat milk in Khuzestan (South-West of Iran) were 78.7, 38.7, 12.5, 37.3 and 27.1%, respectively [13].

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The purpose of this survey was to determine natural occurrence and levels of AFM1 in pasteurized, UHT and GSM milk samples available in Ahvaz city. This paper reported the data of the first survey on the presence of AFM1 in Commercial milk, especially GSM samples, in Ahvaz area.

MATERIALS AND METHODS

During July 2009 to June 2010, 178 milk samples (54 UHT, 45 pasteurized and 79 GSM milk samples) were purchased from local markets in Ahvaz city and analyzed for AFM1 by ELIZA kite (Tecna lab, Italy). The milk samples were centrifuged at 3000 g for 10 min. at 8°C. The upper creamy layer was removed by Pasteur pipette. Based on company instruction, the samples were diluted with the sample diluent 5 times (100µl of the sample + 400µl of sample diluent). Hundred microliters of the AFM1 standard solutions and test samples were added to the wells of micro-titer plate and shake the plate gently with rotatory motion for few seconds and then incubated for 45 min at room temperature (20-25°C) in the dark. The liquid was poured out of the wells and the wells were filed completely with working wash solution and poured out the liquid again. The remaining droplets were removed by tapping the microplate upside down vigorously against absorbent paper. The washing sequence was repeated 4 times. In the next stage, 100 µl of enzyme conjugate solution was added to the wells using multichannel pipette and shake the plate gently for few seconds and incubate for 15 minutes in the dark. 100 µl of developing solution was added to each well and mix for few seconds and incubate for 15 minutes. Then 50ul of stop solution was added to each well and mix for few seconds. Finally, absorbance was measured at 450 nm and calibration curve was made. To obtain the effective aflatoxin M1 concentration in samples, the concentration read from the calibration curve were multiplied by 5.

The results were analyzed by one way analysis of variance (ANOVA), two way analysis of variance, one sample T-test and least significant difference (LSD). Means were considered statistically different at 95% confidence levels.

RESULTS AND DICUSSION

Aflatoxin M1 was found in almost 100% of the examined milk samples. As it is shown in Table 1, 15 (8.42%) of sterilized milk, 18 (10.11%) pasteurized milk and 29 (16.29%) GSM milk samples were exceeded the legal level of AFM1 accepted by European Union.

Distribution of AFM1 concentration by season has been presented in Table 2. Statistic analysis (two way analysis of variance) showed that the only factor which can affect on aflatoxin level is season ($P<0.001$) and the type of milk and interaction between the type of milk and season does not affect on aflatoxin level ($P>0.05$). LSD test showed that the mean of AFM1 level in spring samples (24.89±2.15) is significantly ($P<0.01$) lower than summer samples (58.02±4.35), autumn samples (64.35±6.14) and winter samples (43.17±6.15). Data also showed that the level of the AFM1 in winter samples is significantly lower than autumn samples ($P<0.01$) and summer samples ($P<0.05$), but there was no significant differences was observed between summer and autumn. Our finding showed that although the mean of the AFM1 in GSM samples (51.33±4.7) and pasteurized samples (48.28±4.88) is higher than sterilized samples (40.97±3.97)

Table 1: Aflatoxin M1 concentration of Sterilized, Pasteurized and GSM milk samples

		Aflatoxin levels		
Type of sample	No of sample	0-50ng/L	50-100ng/L	>100ng/L
Sterilized	54	39	12	3
Pasteurized	45	27	17	1
GSM	79	50	16	13
Total	178	116	45	17

Table 2: Distribution of Aflatoxin M1 concentration by season

Season	No of sample	Mean concentration (ng/L)	Standard deviation	Maximum	Minimum
Spring (April-June)	44	24.89	14.58	80.00	10.00
Summer (July-September)	43	58.02	28.55	125.00	15.00
Autumn (October- December)	46	64.34	41.66	175.00	5.00
Winter (January- March)	45	43.16	41.27	225.00	10.00
Total	178	47.45	36.54	225.00	5.00

Table 3: The prevalence of milk contamination in other studies

Location	Type of milk samples	No of samples	Number of contaminated milk samples (>50ng/L)	Percent	Reference
Iran	Pasteurized	624	101	17.8	[11]
Iran	Raw cow milk	319	217	68.0	[16]
Iran	Pasteurized	50	31	62.0	[2]
Iran	Raw cow milk	75	27	36.0	[13]
Iran	Pasteurized	272	12	4.4	[17]
Pakistan	Raw cow milk	168	167	99.4	[18]
Turkey	UHT	100	31	31.0	[19]
Syria	Pasteurized	10	8	80.0	[20]
Taiwan	Pasteurized	144	1	0.7	[15]
Egypt	Raw cow milk	50	17	34.0	[21]

but this differences is not significant ($P<0.05$). One sample T-test showed that although the AFM1 level in sterilized samples is significantly ($P<0.05$) lower than the maximum level of AFM1 accepted by European Union (50 ng/L) but no significant differences ($P<0.05$) was observed between the AFM1 level in GSM and pasteurized samples with European standard.

One way analysis of variance showed that the mean and the standard error of the mean of AFM1 level in sterilized samples in spring (n=15), summer (n=15), autumn (n=11) and winter (n=13) were 22.17±1.74, 51.33±7.79, 65.91±10.27 and 29.62±5.52 respectively. This difference was significant ($P<0.001$) and the AFM1 level in spring and winter samples is lower than autumn and summer samples ($P<0.05$). There was no significantly differences ($P>0.05$) between the AFM1 level in spring and winter samples with summer samples.

The mean and the standard error of the mean of AFM1 level in pasteurized samples in spring (n=11), summer (n=10), autumn (n=13) and winter (n=11) were 29.09±3.87, 64.5±6.8, 58.85±13.25 and 40.23±6.8, respectively. Differences were significant ($P<0.05$) and the mean of AFM1 level in spring samples was lower than summer and autumn samples ($P<0.05$). Also, this amount in winter samples was lower than summer samples ($P<0.05$).

In GSM samples, the mean and the standard error of the mean of AFM1 level in spring (n=18), summer (n=18), autumn (n=22) and winter (n=21) were 24.62±4.29, 60±7.26, 66.82±9.13 and 53.09±12 respectively. This difference was significant ($P<0.01$) and the AFM1 level in spring samples was lower than summer, autumn and winter samples ($P<0.05$).

Our data showed that out of 178 samples, the AFM1 level in 62 samples (34.8%) was higher than the maximum tolerance level accepted by European Union, but base on Iranian standards (100ng/L), only 17 samples (9.5%) were exceeded the legal level. Season is the only factor which can affect on aflatoxin level in all kind of milk samples. The least level of AFM1 was found in sterilized milk samples and difference between AFM1 levels in GSM with other kind of milks was not significant.

According to season some researcher reported that the AFM1 level in winter and autumn is higher than in summer [14-16], but our study showed that the mean of AFM1 level in spring milk samples was less than the other seasons. This may due to the differences between climate, temperature and other geographical conditions. Table 3 shows few literature data on the occurrence of AFM1 levels in milk and milk products in Iran and some neighboring countries.

These studies have shown that the contamination of milk and dairy products and variations in the concentrations were related to geographic position, national development level and season. Institute of Standards & Industrial Research of Iran currently reduced the maximum tolerance level of AFM1 from 500 ng/L to 100 ng/L in milk, ice-cream, butter and yogurt to improve the public health standards but the magnitude of concentration of AFM1 in Iran milk samples is still high. Milk production in Iran is mainly done by two types of dairy farms, industrial and traditional. Also, Iran is an arid country where the pasture is not widely available and cows are fed with great amounts of concentrated feed and hay containing fungi. Therefore, it is important to constantly monitor the level of AFM1 in milk and AFB1 in feedstuffs of dairy animals, especially in traditional dairy farms. Education of dairy farmers by government authorities on potential health consequences of aflatoxin and creating stringent regulations regarding reducing of AFB1 in feedstuffs is recommended.

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