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Immunoaffinity Column Clean-Up Techniques for Detection Aflatoxin M1 from Iranian Milk in Guilan Province

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Abstract: The aim of this study was to evaluate aflatoxin M_1 (AFM_1) contamination in milk samples in Guilan province in Iran. AFM_1 concentrations of 100 fresh milk samples, collected from farms in five different areas of the Guilan Province were analyzed. The occurrence and concentration range of AFM_1 in the samples were investigated by high performance liquid chromatography (HPLC) method. AFM1 was found in 100% of the examined milk samples by average concentration of 74.91ng/L. However, the level of aflatoxin M_1 in all samples was below the US tolerance limit of 0.5 μg/L. Just 80% of the samples had AFM_1 greater than the maximum tolerance limit (50ng/L) accepted by European Union, Codex Alimentarius Commission and Iranian national standard. The data were analyzed statistically by applying ANOVA. Results show that contamination with AFM1 is a serious problem for public health. To achieve a low level of AFM1 in milk, cows' feed samples from various cowsheds must be evaluated routinely for aflatoxin and kept away from fungal contamination as much as possible.

Key words: Aflatoxin M1 · Milk · High performance liquid chromatography (HPLC)

INTRODUCTION

Mycotoxins are secondary metabolites of molds which are associated with certain disorders in animals and humans. In addition to being acutely toxic, some mycotoxins are now linked with the incidence of certain types of cancer and it is this aspect which has evoked global concern over feed and food safety, especially for milk and milk products [1]. AFM_1 is a hepatocarcinogen found in milk of animals that have consumed feeds contaminated with aflatoxin B1 (AFB_1), the main metabolite produced by fungi of the genus *Aspergillus*, particularly A. flavus, A. parasiticus and A. nomius [2]. About 0.3-6.2% of AFB_1 in animal feed is transformed to AFM_1 in milk [3]. Although its potency of toxicity is less than those of its parent compound, it has been suggested as probable human carcinogens Group 2B by the International Agency for Research on Cancer (IARC), while AFB1 was classified as a group 1 carcinogen [4].

Due to serious health concerns, many countries have set maximum limits for aflatoxins, which vary from country to country [5]. The European Community prescribes that the maximum level of AFM_1 in liquid milk should not exceed 0.05μg/kg. However, according to the US standard, the level of AFM_1 in liquid milk should not be higher than 0.5μg/kg [5].

In Iran, consumption of milk and dairy products is increasing rapidly, particularly for infants, children and young people. The production processes do not essentially affect the concentration of AFM_1, due to its heat stability. Therefore, the main strategy to diminish exposure risk, both for animals and human beings, is an appropriate preventive monitoring program.

In the present work, analyses of AFM_1 in Iran were carried out and the incidence of AFM_1 contamination is discussed. This is the first report about the occurrence of AFM_1 in fresh milk from Iranian dairy cows.

Materials

Milk Samples: In this study the levels of AFM_1 and presence in raw milk samples were determined. A total of 100 fresh milk samples were sampled during 2009 in five different areas in Province of Guilan, Indonesia labeled as A, B, C, D and E, respectively. The fresh milk samples were stored at -18°C until analysis.

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Chemicals and Standards: Acetonitrile (HPLC grade) of Sigma-Aldrich (Steinheim, Germany) was used for AFM1 analysis. The immunoaffinity columns AflaM1 TM HPLC were obtained from VICAM (Watertown, MA, USA). The water was double distilled with Millipore water purification system (Bedford, MA, USA) and was used for analysis. Standard of AFM1 (10 µg/mL in acetonitrile) was purchased from Supelco (Bellifonte, PA, USA). All the other chemicals used were of Analar grade.

Methods

Determination of Aflatoxin M1: The method used for determination of AFM1 was the AOAC Official Method 2000.08 reported by Dragacci, Grosso and Gilbert [7].

Extraction Procedure: Milk samples, warmed at 37°C in water bath, were centrifuged at 2000 × g. The fat layer was removed completely and then milk was passed through filter paper (Whatman No. 4). Then 50 mL of this prepared test portion was taken in a syringe barrel which was attached with immunoaffinity columns (IAC).

The test portion was passed at the flow rate of 2-3 mL/min. The column was washed with 20 mL water and then blown to dryness. Acetonitrile (4 mL) was allowed to pass and to be in contact with the column for at least 60 seconds and consequently aflatoxin M1 was eluted. The gentle stream of nitrogen was passed to evaporate the eluate to dryness and it was diluted with the mobile phase at the time of LC determination.

LC Determination with Fluorescence Detection: The HPLC system of Agilent 1100 series (Agilent, USA), equipped with an auto sampler LAS G1313A and a fluorescence detector FLD G1321A with excitation and emission wavelength of 365 nm and 435 nm, respectively, was used for AFM1 determination. The ZORBAX Eclipse XDB-C18 (Octadecyl silane chemically bonded to porous silica) column (Agilent, USA), 150 mm with particle size 5 lm in diameter, was used. Acetonitrile/water (25/75, v/v) was used as mobile phase. The flow rate was set to be 0.8 mL/min. Standard solutions AFM1 with concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, 10.0 µg/L in acetonitrile were used to obtain the calibration curve. The retention time for aflatoxin AFM1 was 7 min.

Calculations: Calculations were made according to the following equation:

$$W_m = W_a \times (V_f/V_i) \times (1/V_s)$$

Where

W_m = Amount of AFM1 in the test sample in µg/L;
W_a = Amount of AFM1 corresponding to area of AFM1 peak of the test extract (ng);
V_f = The final volume of re-dissolved eluate (lL);
V_i = Volume of injected eluate (lL);
V_s = Volume of test portion (milk) passing through the column (mL).

Statistical Analysis: The AFM1 concentration results were statistically analyzed by applying one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Milk are good sources of bioavailable calcium and proteins; and presence of contaminants like aflatoxins in milk could be a serious health hazard for consumers specially children who are more sensitive to adverse effects of aflatoxins than adults.

In this study, AFM1 was analysed in 2009 in a total of 100 fresh milk samples, taken directly from farms from five different areas in Guilan, Iran. In the earlier effective methods for determination of aflatoxin M1 in fluid milk, water and methanol were used as extraction solvents. A more recent advancement in quantitative extraction of aflatoxin M1 and subsequent clean-up is the use of immunoaffinity columns. The first published method for AFM1 with immunoaffinity columns was that of Mortimer, Gilbert and Shepherd [8].

Modifications of the immunoaffinity-based methods for AFM1 were subsequently published and studied collaborately, under the auspices of the International Dairy Federation and AOAC International, by a group mainly of European laboratories that could determine AFM1 in milk at concentrations of 0.05 µg/L. A collaborative study resulted in approval of AOAC method 2000.08 [7].

The standard solutions of concentration from 0.05 µg/L to 10 µg/L AFM1 were used to find calibration/standard curve as described by the following regression equation: $y = 17.579x-1.520$, where y = area and x = amount of AFM1. The results showed the linearity of the standard curve over the range studied. The coefficient of determination (R^2) was 0.9998.

Table 1: Distribution by factory of milk samples and AFM1 concentration (ng/L)

Sample area	Samples tested	Minimum	Maximum	Mean	SD	SE
A	20[a]	46	111	75.10	20.87	4.52
B	20[b]	32	128	72.92	27.45	5.18
C	20[c]	19	125	77.46	26.02	4.91
D	20[d]	33	111	71.53	23.14	4.37
E	20[e]	19	121	77.66	25.31	4.98
Total	100	19	128	74.91	23.82	1.73

a: 18 contaminated samples (>50 ng/L) b: 10 contaminated samples (>50 ng/L)

c: 14 contaminated samples (>50 ng/L) d: 20 contaminated samples (>50 ng/L)

e: 18 contaminated samples (>50 ng/L)

To determine LOD, a series of standard solutions in the range of 50-0.01 μg/L AFM1 were injected into the HPLC equipment and observed the signal to noise ratio. The limit of determination (LOD) was determined to be 0.04μg/L. Recovery was studied by spiking the milk samples with AFM1 standard at the levels of 10, 20 and 50μg/L. The recoveries were found to be 88%, 90% and 96%, respectively.

Analytical results showed that the incidence of AFM1 contamination in milk samples was very high. Hundred percent of the samples were contaminated with AFM1. The Table shows the range of contamination level varied among different factories. Seventeen samples (17%) had contamination less than 50 ng/L AFM1 milk. Only three samples (3%) contained 50ng/L AFM1 milk, while the remaining 80 samples (80%) contained more than 50 ng/L AFM1 milk. The highest contamination level of AFM1 (128 ng/L) was found in milk. These results indicate that feeds for cows in Guilan province were contaminated with AFB1.

Occurrence of AFM1 in milk from two different provinces in Iran has been observed. Kamkar (2005) reported that in 85 (76.6%) of 111 samples in Sarab Province, AFM1 was detected at concentrations ranging between 15 ng/L and 280 ng/L and the AFM1 concentration in 40% of the positive samples was higher than the maximum tolerated limit (50 ng/L) accepted by some European countries [9]. In Shiraz province, AFM1 was found in 100% of the 640 milk samples examined and 17.8% of the samples had an AFM1 concentration greater than the maximum tolerated limit (50 ng/L) [10]. Tajkarimi *et al.,* 2007 reported contamination of AFM1 in 98 samples of raw milk from milk tanks in one dairy plant in each of five regions in Iran. Using a validated High Performance Liquid Chromatography (HPLC) system, 61 samples had 0.000-0.050μ/L, 29 samples were contaminated with 0.05-0.10 μg/L and the remaining 8 samples had 0.1-0.39 μg/L [11].

In comparison to other Asian countries, our results were significantly lower. In Korea, for example, AFM1 was detected in 79% (143 samples) of 180 samples with a mean concentration of 57 ng/L or ng/kg when determined by ELISA. By HPLC, 105 samples (58%) were contaminated with a mean concentration of 71 ng/kg [12]. In India, Rastogi, Dwivedi, Khanna and Das (2004) have found that 87% of 87 samples were contaminated. A mean concentration of 299 ± 21 ng/kg was noted and 99% of the samples were positive at a level higher than 50 ng/kg [13]. Occurrence of AFM1 in raw milk samples from 14 districts of the Punjab Province, Pakistan has been reported by Hussain and Anwar [14]. It was found that of all samples analysed were contaminated with aflatoxin M1 (AFM1) and the 99.4% samples exceeded the European Union limit, i.e. 0.05 μg/L.

It appears to be an urgent requirement to establish tolerance levels for AFM1 for milk and milk products in Asian countries, as well as in Iran.

CONCLUSION

The results of the present study provide information about AFM1 contamination of popular dairy products marketed in Iran; and reveal that the seasonal variations influence concentration of AFM1. Also, the present study showed that the level of the toxin in examined samples is a serious problem for public health. Reducing the levels of AFB1 in animal feedstuffs by improved processing and storage practices can be the initial approach to deal with this problem. Furthermore, it is important to inspect and control milk and animal feed for presence of aflatoxins in a regular manner to evaluate the hygienic managements.

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