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Detection of Aflatoxin M1 Concentrations in UHT Milk Consumed in Turkey Markets by ELISA

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Abstract: In this study, ELISA (Enzyme Linked Immunosorbent Assay) technique was used for detection of aflatoxin M1 in UHT milk sold in Bursa-TURKEY for consumption. A total of 50 samples of commercial UHT (Ultra High Temperature) whole milk were analyzed. Aflatoxin M1 residues were detected in all samples (100%) studied in different levels. The mean value was $101.2 \pm 53.8 \text{ ng L}^{-1}$. Although, 40 (80%) were below the limit, the remaining 10 (20%) were well above the limit permitted by European Community and Turkish Food Codex. Serious risks for public health exist from milk consumption. Therefore, milk has to be controlled periodically for AFM1 contamination. Also, dairy cow feeds should be stored in such a way that they do not become contaminated.

Key words: Aflatoxin M1, UHT milk, ELISA

INTRODUCTION

Aflatoxins produced by fungus such as *Aspergillus flavus* and *A. parasiticus* are genotoxic and cytotoxic carcinogens. When lactating mammals such as cows, sheep and goats are fed with feedstuffs containing aflatoxin B1 (AFB1), this metabolite can be converted to aflatoxin M1 (AFM1) (Cole and Cox, 1981). AFM1 stable in raw and processed milk products pasteurization and sterilization result in negligible destruction of AFM1 (Galvano *et al.*, 1996). It has been reported that AFM1 was a resistant to thermal inactivation food processing for procedures such as pasteurization and autoclaving (Bakirci, 2001; Park, 2002).

Many researcher from different countries have carried out studies about the incidence of AFM1 in milk samples. Martins and Martins (2000) studied the levels of AFM1 in 31 samples of raw and 70 samples of heat-processed milk and found that 80.6% of raw milk and 84.2% of heat processed milk samples were contaminated with AFM1. Among raw milk samples, 54.8% contained levels of AFM1 between $5\text{-}10 \mu\text{g L}^{-1}$ and 19.3% had levels between $21\text{-}50 \mu\text{g L}^{-1}$, while heat processed milk samples were in the range $11\text{-}50 \mu\text{g L}^{-1}$. Sassahara *et al.* (2005) showed that the incidence of AFM1 was 24% in Brazil. In a study in Korea, Kim *et al.* (2000) found that 76% of milk samples with a mean concentration of 18 pg g^{-1} . Unusan (2006) showed that the incidence of AFM1 in UHT

(Ultra High Temperature) milk was 58.1%, with a mean concentration of 108.17 ng L^{-1} in Turkey. In another study performed in Turkey, Tekinsen and Eken (2008) analyzed 100 UHT milk samples for AFM1 by ELISA and found that 67% milk samples were contaminated with AFM1, between $10\text{-}630 \text{ ng kg}^{-1}$.

To protect consumers from contaminated milk, several countries have established legislation to regulate the level of AFM1 in milk. The European Community (EC) and Turkish legal limit for AFM1 in milk are 50 ng L^{-1} .

Although, there is some information about the occurrence of AFM1 in raw milk and dairy products like cheese, not enough information about the occurrence of AFM1 in UHT milk in Turkey was reported. The present investigation was undertaken to detect AFM1 levels of UHT milk consumed in Bursa Region, Turkey and to compare the results with the maximum AFM1 tolerance limits which are accepted by Turkish Food Codex and European Community.

MATERIALS AND METHODS

Materials: A total of 50 samples of commercial whole milk (250 mL milk packet) were obtained randomly from markets between 1-15 April 2004 in Bursa, Turkey. All samples were analyzed before their expiry date.

Methods: The samples analyzed for AFM1 using the competitive ELISA (RIDASCREEN Aflatoxin M1, R-Biopharm, Product No: R1101) procedure as described by R-Biopharm GmbH. This method is quick, reliable and cost effective for estimation of AFM1 and has been included in the official collection of test procedures by the German Federal Board of Health.

Reagents: Most of reagents used such as microliter plate coated with capture antibodies, AFM1 standard solutions (1.3 mL each 0, 5, 10, 20, 40 and 80 ng L⁻¹), peroxidase conjugated AFM1, substrate (urea peroxidase), chromogen (tetramethylbenzidine) and stop reagent (1 N sulphuric acid) were provided included by RIDASCREEN test kit. Methanol, n-heptane and dichloromethane used were of analytical grade (Merck). Phosphate Buffer Solution (PBS) was prepared by mixing 0.55 g sodium dihydrogen phosphate with 2.85 g disodium hydrogen phosphate-2-hydrate and 9 g sodium chloride in 1000 mL distilled water.

Preparation of the milk samples: Preparation of samples was conducted according to the instructions of RIDASCREEN test kit. The pH of each sample was measured in order to ascertain its suitability (pH ≥ 6.5). The samples were centrifuged at 3500 g at +10°C for 10 min in order to separate fat, which was then removed with sterile spatulas. An aliquot (100 µL per well) of the lower oil-free phase was used directly in the test.

Test procedure: AFM1 standards, blank (controls air) and milk samples were analyzed in duplicates. They were added to microliter plates coated with antibodies for the AFM1 mixed gently by rocking the plate manually and incubated for 30 min at room temperature in the dark. At the end of incubation, the liquid in the wells was poured out and the microwell holder was tapped upside down on an absorbent paper to remove the remainder of the liquid. The wells were washed twice with 250 µL washing buffer. After washing, 100 µL of the conjugated-peroxidase AFM1 was added to microwells and these were incubated in the dark at room temperature for 15 min. At the end of incubation, the wells washed three times with 250 µL washing buffer. In the next step, 50 µL of enzyme substrate (urea peroxidase) and 50 µL of chromogene

tetramethylbenzidine) were added to each well and incubated 15 min at room temperature in the dark. Following the addition 100 µL of the stop reagent (1 M H₂SO₄) to each well, the absorbance was measured at 450 nm ELISA reader (ELX-800, Bio-Tek Instruments, USA).

Evaluation: The detection limit of the ELISA kit was 5 ng L⁻¹. AFM1 levels in samples were obtained by data analysis using RIDA SOFT WIN software (r-Biopharm AG, Germany) and interpolation from the standard curve obtained by analyzing five internal standards, ranging 5-80 ng L⁻¹. The yield and the coefficient of variation (CV%) of each internal standard was evaluated. The yield was expressed as the percentage relationship between the concentrations obtained and those expected. The CV% was expressed as the ratio between the SD and average.

RESULTS AND DISCUSSION

The mean value was 101.2±53.8 ng L⁻¹. There was a high incidence rate of AFM1, with 50 (100%) milk samples being contaminated. It was detected in 10 (20%) of the 50 samples in concentrations ranging from 50 to 244 ng L⁻¹. The concentrations of AFM1 in UHT milk samples were in the positive range 5-244 ng L⁻¹. Although, 40 (80%) were below the limit, the remaining 10 (20%) were above the limit permitted by the EC and Turkish Food Codex (Table 1).

Twenty percent of the sample was over the permissible level 50 ng L⁻¹ as accepted by EC and Turkish Food Codex. This contamination was found lower than earlier studies carried in Turkey in UHT milk samples (Bakirci, 2001; Gurbay *et al.*, 2006; Unusan, 2006; Tekinsen and Eken, 2008, Çelik *et al.*, 2005).

The contamination prevalence of AFM1 in UHT milk samples in present study was comparable to other European Countries such as Italy (Galvano *et al.*, 1998, 2001), Portugal (Markaki and Melissari, 1997) and Greece (Martin and Martin, 2000).

Although, some studies showed no AFM1 detected in milk samples examined in various countries, such as Argentina (Lopez *et al.*, 2003), Japan (Tabata *et al.*, 1993) and Turkey (Oruç and Sonal, 2001), many studies

Table 1: Occurrence of AFM1 in UHT milk samples

Sample	Range (ng L ⁻¹)						Positive samples			
	<5	10-May	25-Oct	26-50	51-80	>80	Mean (ng L ⁻¹)	Percentage	Min-max (ng L ⁻¹)	Exceed legal limit*(%)
UHT milk	0 (0)	1 (2)	13 (26)	26 (52)	9 (18)	1 (2)	101.2±53.8	100	5-244	20

<5: Range of negative samples, Values in brackets indicate percent, *EC and Turkish Food Codex limits in milk is 50 ng L⁻¹

indicated that AFM1 contamination in milk was an important risk to human health (Galvano *et al.*, 1998; Rastogi *et al.*, 2004). Lopez *et al.* (2003) also suggested that levels of AFM1 in samples of milk produced in Argentina were found to be very low and in no case did the levels exceed the recommended limits for milk products. Roussi *et al.* (2002) examined raw and market milk samples for AFM1 contamination over two periods. In the first sampling, the incidence rates of AFM1 contamination in pasteurized milk were 85.4%. In the second sampling, incidence rates were 79.6%. They found that none of the pasteurized milk samples exceeded the limit of 50 ng L⁻¹. Their finding was that the current regulatory status in Greece was effective. In present study, the contamination level of AFM1 was nearly, but the level of excess was determined at 20% of the samples examined.

Kamkar (2005) reported that in spring and summer, samples mean concentration of AFM1 were significantly lower than that of samples measured in winter and autumn. The investigator found that 40% of the samples exceeded the EC Limits, while none of the samples exceeded US limits in Iran. He suggested the importance of seasonal factors in AFM1 levels. The most important factor on the amount of AFB1 was temperature and moisture, since some moulds like *A. flavus* and *A. parasiticus* can easily grow in feeds having moisture 13-18% and environmental moisture between 50-60% (Jay, 2000).

Besides the type of analytic methodology used, the disagreement among the results found by these researchers and the ones verified in this work can also be justified by the differences in the level of contamination of the foodstuff destined for dairy cattle, as the same batch of raw material can present varied mycotoxin concentrations. The contamination of the foodstuff varies according to the area, climatic differences and temperature (Sassahara *et al.*, 2005).

Since, milk is one of the most important human foods and the main nutrient for growing young, who are notably vulnerable and potentially more sensitive than adults, it must therefore, be monitored for contaminants, including AFM1. The possible source of contamination by AFB1 for dairy cattle was the ingestion of contaminated feeds; therefore it is necessary to monitor the feeds and grains destined for animal consumption. It can be concluded from the present study that the level of AFM1 in commercial UHT milk in the Bursa region of Turkey could be higher than the permissible level of 50 ng L⁻¹. There is a need for further studies to cover other areas of the country and also to examine milk from other ruminants for AFM1.

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