



Aflatoxin M1 Contamination Levels in Cheeses Sold in Isfahan Province, Iran

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Objectives: Aflatoxin M1 (AFM1)-contaminated dairy products pose serious human health risks, causing liver and renal failure if consumed. They are also related to decreased milk and egg production in infected animals. This study investigated the AFM1 contamination levels in cheeses sold in Isfahan province, Iran, by enzyme-linked immunosorbent assay (ELISA).

Methods: A total of 100 white cheese samples were randomly collected from supermarkets in Isfahan province and after extraction using dichloromethane were prepared for the ELISA.

Results: Of the 100 samples, 52 (52%) were contaminated by AFM1, at levels ranging from 50.2 to 424.4 ng/kg. The remaining 48% of the samples had undetectable AFM1 levels (< 50 ng/kg). Based on the standard limit set by the European Commission and Iran, 8% (8/100) of the AFM1-positive samples (with concentrations between 250.2 and 424.4 ng/kg) had levels higher than the permissible value of 250 ng/kg.

Conclusion: Although the percentage of cheese samples in Isfahan province with AFM1 levels exceeding the national permissible limit was low, the examination of cheeses and the milk used for their production is nevertheless important for ensuring public health. Furthermore, optimum storage conditions of animal feed should be ensured, and livestock nutrition must be monitored for the presence of AFM1 and other aflatoxins.

Key Words: aflatoxin M1, cheese, enzyme-linked immunosorbent assay

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INTRODUCTION

Aflatoxins are highly toxic metabolites and the most carcinogenic substances produced by a certain group of *Aspergillus* species, namely, *A. flavus*, *A. parasiticus*, and *A. nomius* [1,2]. Foodstuffs and dairy products (e.g., infant milk formula, cheese, cream, butter, ice cream, and yogurt) that become contaminated by mycotoxins at various stages of the food-manufacturing process can be harmful to humans, once ingested [3–5]. In animals, aflatoxins exert acute and chronic effects, causing failure of various organs, especially the liver, kidney, and heart, as well as suppressed immunity and decreased milk and egg production [6]. There are six main aflatoxins: B1, B2, G1, G2, M1, and M2 [7]. The major aflatoxins M1 and M2 were first isolated from the milk of lactating animals that had been fed aflatoxin-contaminated feed. Aflatoxin M1



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(AFM1) is the principal hydroxylated AFB1 metabolite in mammals [8,9]. AFM1 contamination in dairy products, including cheese and butter, displays variations according to geographical region, development level of the country, and season [10,11], and represents a worldwide concern [12]. The levels of AFM1 are increased in the grass, pasture, weed, and rough feeds consumed by livestock in the spring and summer seasons, which causes enhancement of AFM1 pollution in milk and milk products [13,14].

The most common techniques used for detecting AFM1 in milk and dairy products are enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC) [15]. The maximum level of AFM1 in cheeses that is permissible by the European Commission (EC) is 250 ng/L [16]. The presence of AFM1 in milk and dairy products is undesirable and poses a serious risk to human health, especially to children [12,17]. In Iran, dairy products containing milk, including cheeses, are widely consumed. Thus, this work aimed to determine by ELISA the AFM1 contamination levels in cheese samples collected from supermarkets in Isfahan province of Iran.

MATERIALS AND METHODS

1. Sample preparation

Between January 2014 and March 2015, a total of 100 cheese samples were randomly collected from different supermarkets in the Isfahan province of Iran. All the samples (50–100 g slices) were immediately transported to the laboratory, where some were stored at 4°C before analysis and other fresh samples were immediately analyzed without freezing. The cheese samples (2 g each) were homogenized in 40 mL of dichloromethane for extraction by shaking for 15 minutes. Then, the suspension was filtered and 10 mL of the extract was evaporated at 60°C under nitrogen. The oily residue was redissolved and mixed thoroughly in 0.5 mL of methanol, 0.5 mL of phosphate-buffered saline (pH 7.2), and 1 mL of *n*-heptane. The mixture was then centrifuged for 15 minutes at 2,700 rpm. The upper heptane layer was completely removed, and 100 µL of the aliquot phase was brought to 10% methanol content and diluted in 400 µL of RIDASCREEN AFM1 kit buffer (R-Biopharm, Darmstadt, Germany). Finally, 100 µL of this solution was used for each well in the ELISA.

2. ELISA

The concentration of AFM1 in the cheese samples was determined using the RIDASCREEN AFM1 kit according to the manufacturer's recommendation. All reagents were first brought to room temperature (20°C–25°C) prior to use. The AFM1 kit reagents included AFM1 standard solutions (1.5 mL at levels of 0,

5, 10, 25, 50, 100, and 250 ng/L), peroxidase-conjugated AFM1, the substrate (urea peroxidase), the chromogen (tetramethylbenzidine), and the stop reagent containing 1 N sulfuric acid. In duplicate, test samples (100 µL for each well) were added alongside AFM1 standards into the wells of a microtiter plate that had been pre-coated with antibodies for AFM1. The plates were shaken gently on a rotator for a few seconds and then incubated at room temperature in the dark for 60 minutes. After washing with ELISA wash buffer (20×), 100 µL of peroxidase conjugate was added to the wells, and the plates were again incubated in the dark for 60 minutes at room temperature. At the end of the incubation period, the wells were washed with 250 µL of wash buffer to remove unbound conjugate. Subsequently, the substrate/chromogen (tetramethylbenzidine) was added to the wells and incubation was continued for 30 minutes in the dark. Finally, the stop solution (100 µL) was added to each well to terminate the reaction. The optical absorbance of each well was measured at 450 nm using a competitive Stat Fax-2100 ELISA plate reader (Awareness Technology, Palm City, FL, USA). The absorbance value of each AFM1 concentration was determined by interpolation from a linear calibration curve (Figure 1). The mean lower detection limit of the RIDASCREEN AFM1 test kit was 50 ng/kg.

3. Data analysis

The mean values and standard error of the mean were evaluated using IBM SPSS Statistics software, version 20.0 (IBM Co., Armonk, NY, USA).

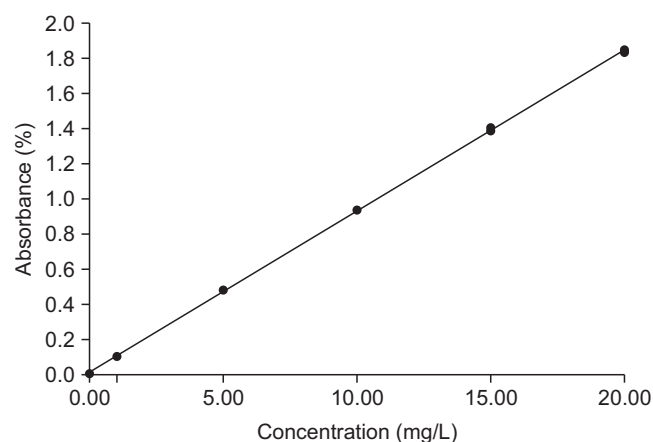


Figure 1. Calibration curve of the absorbance values for standard aflatoxin M1 concentrations. Linearization of the calibration curve was performed using logit-log transformation.

Table 1. Rate of aflatoxin M1 (AFM1) contamination in cheese samples sold in Isfahan province, Iran

AFM1 range (ng/kg)	Sample, n (%)	Mean	Range
Not detected ^a	48 (48)	-	-
50–100	29 (29)	62.4	50.2–99.6
100–250	15 (15)	156.8	101.6–248.2
> 250 ^b	8 (8)	288.2	250.2–424.4
Total	100 (100)	133.2	50.2–424.4

^aAFM1 level < 50 ng/kg; ^bAFM1 ranges higher than the permissible level (250 ng/kg) in cheese.

RESULTS

The contamination levels and distribution of AFM1 in the 100 cheese samples collected from different supermarkets in Isfahan province (Iran) are shown in Tables 1 and 2.

Among these 100 samples, 52 (52%) were confirmed to be contaminated by AFM1 at levels ranging from 50.2 to 424.4 ng/kg (Table 2). Of these, 8 (8%) samples (AFM1 levels between 250.2–424.4 ng/kg) had more AFM1 levels (250 ng/kg) than that allowed by the EC and Iran. In addition, 48% of the 100 samples were considered AFM1-negative, with levels assumed to be below the 50 ng/kg detection limit of the assay kit.

DISCUSSION

In Iran, special industrial dairy products and traditionally made cheeses play a significant role in the human diet. In different studies by many researchers, various levels of AFM1 contamination in cheese and dairy products have been reported. In the present study, the levels of AFM1 in Iranian cheeses obtained from different supermarkets in Isfahan province (Iran) were determined by ELISA. In this study, 52% of the cheeses from Isfahan province were found to have AFM1 contamination, with 8% of these carrying levels above the limit set by the EC and Iran. In a study by Rezaei et al. [18], 93.7% of the pasteurized milk, raw milk, and cheese samples in Arak, Iran were positive for the presence of AFM1, as measured by ELISA, with a total average concentration of 85.8 mg/L in milk and 30.39 mg/kg in cheese. In an HPLC study of dairy products from Punjab, Pakistan, AFM1 was detected in 61%, 78%, 59%, and 45% of the yogurt, white cheese, cheese cream, and butter samples, respectively, of which 47%, 15%, 11%, and 52%, respectively, were above the recommended limit set by the European Union [19]. In our present study of products in Isfahan, however, only 8% of AFM1-positive cheese samples exceeded the EC and nationally set permissible toxin

Table 2. Distribution level of aflatoxin M1 (AFM1) detected in cheese samples (Isfahan province, Iran)

Variable	Data
Sample	
Positive	52 (52)
Negative (ND)	48 (48)
Exceed legal limit (> 250 ng/kg)	8 (8)
Concentration (ng/kg) ^a	169.13 ± 32.4 (50.2–424.4)

Values are number (%) or mean ± standard deviation (range).

ND, no detectable AFM1.

^aFor positive samples.

level of 250 ng/kg. In Italian cheeses, 83% of samples showed detectable levels of AFM1 (> 25 ng/kg), most of them between 50 and 150 ng/kg [20], whereas only 52% of our Isfahan samples had detectable AFM1 (50.2–424.4 ng/kg). In a similar ELISA study on samples from Gilan province of Iran, AFM1 was detected in 23.33% (higher than the maximum permissible limit of 250 ng/L) and 63.33% (higher than 50 ng/L) of white cheeses and local yogurts, respectively [14,21]. Their findings on AFM1 contamination in white cheeses was less than that found in our present study (23.33% vs. 52%). ELISA showed 28.3% of white-brined Urfa cheese in Turkey contaminated by AFM1 (70.61–770.97 ng/kg), of which 10.2% exceeded the legal limit (250 ng/kg) established by the Turkish Food Codex [22]. Their results of cheeses exceeding the national permissible level closely resembled ours (10.2% vs. 8%). In an ELISA study on Turkish white Kashar and Tulum cheeses, 51.3% of all samples were contaminated with AFM1 in the range of 0.052–2.52 µg/kg [23], which was similar to the 52% contamination rate found in our present work.

The present study of Isfahan province white cheeses revealed a low-level presence of AFM1 (8%) that exceeded the national legal level for cheeses sold in Iran. Despite this low prevalence of cheese samples with exceeded AFM1 limit, the findings indicate the public health importance of examining for aflatoxin levels in milk, Iranian white cheese, and other dairy products. Moreover, checking for AFM1 levels in livestock feeds according to the national standards is essential, in addition to controlling the appropriate storage conditions of the animal feeds.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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