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# Determination of Aflatoxin $M_1$ Levels in White Cheese Samples by ELISA in Gilan Province, Iran

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**Abstract:** Aflatoxin $M_1$ ($AFM_1$) in milk and milk products is considered to pose certain hygienic risks for human health. These metabolites are not destroyed during the pasteurization and heating process. This study was undertaken to determine the presence and levels of aflatoxin $M_1$ ($AFM_1$) in Iranian white cheese consumed in Gilan province (Northern Iran). A total of 90 cheese samples was randomly obtained from retail outlets. ELISA technique was used to determine the presence and the level of $AFM_1$. In 78 of the 90 cheese samples examined (86.66%), the presence of $AFM_1$ was detected in concentrations between 7.2 - 413ng/l. The mean level of $AFM_1$ in positive samples was 151.97 ng/l. $AFM_1$ levels in 21 samples (23.33%) were higher than the maximum tolerance limit (250 ng/l) accepted by the European countries. Aflatoxin high concentration in milk and milk products cause widespread negative impact on public health and demonstrate considerable economic losses for producers. Therefore, it is necessary to establish strategies for reducing aflatoxin levels in animal feed and milk products.

**Key words:** Aflatoxin $M_1$ • Cheese • ELISA

## INTRODUCTION

Mycotoxins are secondary metabolites of fungi which are associated with certain disorders in animals and humans [1]. Aflatoxins are a group of structurally-related toxic compounds produced by certain strains of the fungi *Aspergillus flavus* and *A. parasiticus* [2, 3]. Under favorable conditions of temperature and humidity, these fungi grow on certain foods and feeds, resulting in the production of aflatoxins [4, 5]. Aflatoxin contamination in milk and milk products is produced in two ways. Either toxins pass to milk with ingestion of feeds contaminated with aflatoxin, or it results from subsequent contamination of milk and milk products with fungi. The major aflatoxins of concern are designated $B_1$, $B_2$, $G_1$ and $G_2$, also $M_1$ and $M_2$ as metabolic products of AFB. $AFB_1$ to $G_2$ belong to Group 1 and $M_1$ belongs to Group 2B, according to IARC [6]. It has been stated, in fact, that the contamination of milk and milk products with $AFM_1$ display variations according to geography, country and season. The pollution level of $AFM_1$ is differentiated further by hot and cold seasons, due to the fact that grass, pasture, weed and rough feeds are found more commonly in spring and summer than in winter. At the end of summer, greens are consumed more than concentrated feed, causing a decreased level of $AFM_1$ in milk and milk products [6-8]. $AFM_1$ is not destroyed during the pasteurization process or in yoghurt and cheese making. As aflatoxins pose more serious risks for public health, certain limits of aflatoxins in foods are determined [6, 9, 10]. The limiting rates of $AFM_1$ were shown in Table 1 [6, 11, 12]. $AFM_1$ could be detected in milk 12-24 h after the $AFB_1$ ingestion, reaching a high level after a few days. When $AFB_1$ intake is stopped, the $AFM_1$ concentration in milk decreases to an undetectable level after 72 h. Milk and its products are a major nutrient for human especially children all over the world [1, 13, 14]. At the same time, these products may be contaminated with $AFM_1$ residues which extensively threaten the human health. For this reason, many countries have regulations to control the levels of $AFB_1$ in feeds and to propose maximum permissible levels of $AFM_1$ in milk to reduce this risk [9]. The European

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Table 1: Maximum limits for aflatoxin $M_1$ in milk and milk products in various countries

| Country | Maximum limit (μg/kg or μg/l) |
|---|---|
| France | 0.05 Adult's milk |
| | 0.03 Children's milk |
| Turkey | 0.05 Milk and products |
| | 0.25 Cheese |
| Czech Republic | 0.1 Children's milk |
| | 0.5 Adult's milk |
| Belgium | 0.050 Milk |
| USA | 0.50 Milk |
| Switzerland | 0.050 Milk and milk products |
| | 0.250 Cheese |
| Netherlands | 0.020 Butter |
| | 0.200 Cheese |
| Germany | 0.050 Milk |
| Austria | 0.050 Milk |

Commission (EC) has approved a maximum admissible level of 250 ng/l for $AFM_1$ in cheese [15]. Therefore, the aim of this study was to investigate the presence of $AFM_1$ in cheese samples consumed in Gilan province (Northern Iran) by ELISA method.

## MATERIALS AND METHODS

**Preparation of Samples:** A total of 90 cheese samples was obtained randomly from retail outlets during summer and autumn 2011 in Gilan province (Northern Iran). The samples were transported to the laboratory in insulated container at about 4°C. Two grams of cheese samples were homogenized and extracted with 40 ml dichloromethane. The suspension was filtered and then 10 ml of the extract was evaporated at 60°C. The residue was redissolved in 0.5 ml methanol, 0.5 ml phosphate buffer and 1 ml n-heptane and was mixed thoroughly. After centrifugation for 15 min at 2700 rpm, 100 μl of the methanolic phase was brought to 10% methanol content by addition of 400 μl of kit buffer. 100 μl (per well) of this solution was used in the test [1, 12, 16].

**ELISA Test Procedure:** Before starting the test, the reagents were brought up to room temperature. The $AFM_1$ standards and test samples (100 μl per well) in duplicate were added to the wells of a micro-titer plate pre-coated with antibodies for $AFM_1$ and incubated at room temperature in dark for 60 min. After the washing step, 100μl of peroxidase conjugate was added to the wells and plate was incubated again for 60 min at room temperature in dark. After the washing step, the unbound conjugate was removed during washing. Subsequently, 50 μl each substrate (urea peroxide) and chromogen (tetramethyl-benzidine) were added to the wells and incubated for 30 min in dark. Finally, 100 μl of stop solution were added to each well. The optical absorbance of each well was read at 450 nm with ELISA plate reader. Absorbance percentages were taken to the calibration curve performed with standards at different concentrations [1]. Statistical analyses were performed using SPSS software.

## RESULTS

A total of 90 white cheese samples was analyzed with competitive ELISA. The occurrence of $AFM_1$ was shown in Table 2. Of the 90 samples analyzed, 78 samples (86.66%) were found to be contaminated with $AFM_1$. 21 samples (23.33%) failed to reach the desired level of the European Communities and Codex, defined as 250 ng/l. The aflatoxin $M_1$ contamination levels were between 7.2 - 413ng/l with the mean of 151.97 ng/l.

**Percentage of $AFM_1$ Positive Samples:** The range of contamination levels varied in two seasons, Table 3. $AFM_1$ in summer and autumn samples ranged from 7.2-402 and 8 - 413 ng/l whit the mean values 139.90 and 162.32 ng/l respectively. The highest mean concentration of aflatoxin $M_1$ registered in autumn 413 ng/l, while in summer samples highest concentration of aflatoxin $M_1$ was 402 ng/l.

## DISCUSSION

Dairy products play a significant role in human diet since they are rich sources of bioavailable calcium and proteins. However, many of the previous studies has indicated the presence of $AFM_1$ at high concentrations in dairy products [9, 17]. Aflatoxins are highly toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds. The main target organ for their toxicity and carcinogenicity is the liver, Milk and milk products, are a major nutrient for humans, especially children. For this reason, $AFM_1$ in milk and dairy products should be controlled systematically [9, 18, 19]. Occurrence of $AFM_1$ in cheese can be due to three possible causes: (1) $AFM_1$ present in raw milk because of carryovers of $AFB_1$ from contaminated cow feed to milk, (2) Synthesis of AF ($B_1$, $B_2$, $G_1$ and $G_2$) by *A. flavus* and *A. parasiticus* growing on cheese and (3) Occurrence of these toxins in dried milk used to enrich the milk which is being used in the production of cheese [16].

Table 2: Occurrence of $AFM_1$ in white cheese samples from Northern Iran

| $AFM_1$ levels ng/l | Sample No. | (%)* | Range |
|---|---|---|---|
| Not detected | 12 | - | - |
| < 50 | 22 | 24.44 | 7.2-48 |
| 50-100 | 15 | 16.66 | 50.2-99.2 |
| 101-250 | 20 | 22.22 | 101.2-248.5 |
| • 250 | 21 | 23.33 | 250.1-413 |
| Total Samples | 90 | 86.66 | 7.2-413 |

Table 3: Distribution by season of cheese samples and aflatoxin $M_1$ concentration (ng/l)

| Season | Samples | Negative | Positive | Mean | Range |
|---|---|---|---|---|---|
| Summer | 45 | 9(20%) | 36(80%) | 139.90 | 7.2-402 |
| Autumn | 45 | 3(6.66%) | 42(93.33%) | 162.32 | 8-413 |

Thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and enzyme linked immunosorbent assay (ELISA) are the most common techniques for detecting $AFM_1$ in milk and dairy products. Therefore, differences between these techniques may affect the results of different studies carried out by different investigators [8]. Several surveys were performed in order to determine the $AFM_1$ levels in milk, cheeses and milk products. In Turkey, a study done by Oruc and Sonal [18], found $AFM_1$ in 89.5% of 57 cheese samples with ranges of 0-180 ng/l. In a study of Tekinsen and Eken [20], 132 kashar cheese samples were analyzed for $AFM_1$ and 82.6% of the samples contained $AFM_1$ (50-690 ng/l). In 2005, the occurrence of $AFM_1$ was studied in Portuguese soft cheese, revealing that in a total of 42 samples no one was contaminated with $AFM_1$ [21]. Sarimehmetoglu *et al.* [9], detected $AFM_1$ contamination in 327 (81.75%) of 400 cheese samples. The numbers of cheese samples that contained $AFM_1$ over the legal limits of 0.25µg/kg were 110 (27.5%). In another study 193 white cheese samples were analyzed by Ardic *et al.* [22] and $AFM_1$ was found in 82.4% of the samples (52-860 ng/l). In Kuwait, 54 samples of dairy products were analyzed for aflatoxin $M_1$, 28% were contaminated with $AFM_1$ [23]. Gunsen and Buyukyoruk [24], analyzed 130 cheese samples and determined an average of 0.142 µg/ kg $AFM_1$. Ayhan *et al.* [25] showed that out of a total of 110 cheese samples, 9 do not contain $AFM_1$, while 101 samples were found to be contaminated with $AFM_1$ in the range of 10-2000 ng/l. Kokkonen *et al.* [26] did not find $AFM_1$ in the examined cheese samples. Gurses *et al.* [27], analyzed 63 cheese samples and in 28(44.44%), $AFM_1$ was detected in concentrations between 7-202 ng/l. Martins and Martins [28] analyzed a total of 182 samples of national cheese (Portugal) by TLC, observing that all of them were not contaminated by $AFM_1$. In our study, Of the 90 samples analyzed, 78 samples (86.66%) were found to be contaminated with $AFM_1$. 21 samples (23.33%) failed to reach the desired level of the European Communities and Codex, defined as 250 ng/l and $AFM_1$ in summer and autumn samples ranged from 7.2 - 402 and 8 - 413ng/l. According to observations, the levels of contamination of cheese by $AFM_1$ seem to vary in many studies. These variations may be related to different reasons such as cheese manufacturing procedures, different milk contaminations, type of cheese, conditions of cheese ripening, geographical region, the country, the season and the analytical methods employed [12, 25, 29].

In conclusion, According to results obtained, incidence and contamination levels of $AFM_1$, seem to be a serious problem for public health. For this reason, milk and dairy products have to be inspected and controlled continuously for $AFM_1$ contamination and animal feeds should be checked regularly for $AFB_1$ and storage conditions of feeds must be taken under strict control.

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