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Determination of aflatoxin M1 levels in Iranian white and cream cheese

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ABSTRACT

A screening survey on the occurrence of aflatoxin M1 (AFM1) was accomplished on 210 cheese samples composed of white cheese (116 samples) and cream cheese (94 samples) purchased from popular markets in central part of Iran (Esfahan and Yazd provinces). The quantitative analysis of AFM1 levels in the samples was performed by using the competitive enzyme-linked immunosorbent assay (ELISA) technique. Aflatoxin M1 at measurable level (50 ng/kg) was detected in 161 (76.6%) samples, consisting of 93 (80.1%) white and 68 (72.3%) cream cheese samples. The concentration of AFM1 in the samples ranged from 52.1 to 785.4 ng/kg. Comparing to legal regulation (250 ng/kg) accepted by some of the countries, 24.2% of the samples exceeded the accepted limit. Among these, the AFM1 levels in 28.4% of white and 19.1% of cream cheese samples were not in accordance with the safety limit. The results indicated that contamination of the samples with AFM1 in such a level appear to be a potential hazard for public health. This paper represents the data of the first survey on the occurrence of AFM1 in cheeses consumed in central part of Iran.

1. Introduction

Mycotoxins are the secondary toxic metabolites of fungi, synthesized during the end period of the logarithmic growth phase and have no obvious role in fungi metabolism or growth. They are mainly produced by certain moulds genera such as *Aspergillus, Fusarium* and *Penicillium* under particular climatic conditions in agricultural products and food (Jay, 2000; Razavilar, 2003).

Aflatoxins, the most studied mycotoxins, are acutely toxic, carcinogenic, mutagenic and teratogenic compounds generally produced by some competent mould strains of *Aspergillus flavus, A. parasiticus* and *A. nomius* in various food commodities. *A. flavus* produces only B aflatoxins, while the other two species produce both B and G ones (Creppy, 2002; Ardic et al., 2008). Among them, aflatoxin B1 (AFB1) is notoriously the most frequent produced mycotoxin and the one has been recognized as the most powerful natural carcinogen in mammals (Polychronaki et al., 2007; Rosi et al., 2007). The International Agency for Research on Cancer (IARC, 1993) of WHO categorized AFB1 as Group 1 human carcinogen.

Aflatoxin M1 (AFM1) is the monohydroxylated derivate of AFB1, metabolized by cytochrome *P450* enzyme system in liver and excreted into the milk of lactating livestock which consumed AFB1 contaminated diet (Murphy et al., 2006). It could be appeared in milk within 12 h after the first ingestion of AFB1. Following the withdrawal of contaminated source, AFM1 disappeared within 72 h. There is a linear relationship between the AFM1 content in milk and the consumption of AFB1 via foodstuffs (Sassahara et al., 2005). It has been estimated that about 0.3–6.2% of AFB1 present in animal feed pass as AFM1 in milk (Creppy, 2002). Although the toxicity of AFM1 is less than AFB1, its cytotoxic, genotoxic and carcinogenic effects is well demonstrated. Hence the IARC of WHO initially categorized AFM1 as a Group 2 human carcinogen (IARC, 1993), but has transferred it to Group 1 according to recent investigations (IARC, 2002).

Occurrence of AFM1 in milk and milk derivatives is a worldwide concern since these products are frequently consumed in market and therefore could be important vehicles for introducing aflatoxin residues into the human diet (Ardic et al., 2009). Consequently, several countries have regulated the maximum permissible levels of AFM1 in milk and dairy products to protect consumers specially children. These regulations vary in different countries by the fact of economic considerations (Stoloff et al., 1991). The European Commission (EC) has set a limit of 50 ng/l for AFM1 in milk (European Commission, 2001) while the US Food and Drug Administration (US FDA, 1996) and Institute of Standards and Industrial Research of Iran (ISIRI, 2005) prescribed the maximum level for AFM1 in milk 10-fold higher (500 ng/l) than the current level in the EC.

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Among the dairy products, cheese is the only product susceptible to growth of fungi and produce mycotoxins (Sengun et al. 2008). Presence of aflatoxins in cheese might be possibly due to following causes: (a) AFM1 residue in milk from which cheese are made, (b) growing the fungi like *Aspergillus* spp on cheese and produce aflatoxins (B1, B2, G1 and G2) and (c) presence of AFM1 in powdered milk enriching the milk used in cheese manufacture (Lopez et al., 2001; Kamkar et al., 2008). The stability of AFM1 is not affected appreciably neither by heat treatments used in dairy industry, i.e. pasteurization and sterilization nor during processing and storage of various dairy products (Gurbay et al., 2006; Unusan, 2006; Prandini et al., 2009). Several authors reported that AFM1 remain stable during ripening and storage of different kinds of cheese (Van Egmond et al., 1977; Brackett and Marth, 1982; Blanco et al., 1988; Govaris et al., 2001; Oruc et al., 2007); whilst Colak (2007) revealed a little reduction (9.8%) of AFM1 during the white cheese ripening period.

The most common analytical methods employed for AFM1 determination are thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA). Among them, ELISA is often used for routine screening due to its several advantages such as rapidity, simplicity and cost-effective (Rosi et al., 2007; Radoi et al., 2008). Colak et al. (2006) reported that competitive ELISA is a useful and reliable method for the determination of AFM1 in cheese.

Referring to scientific literature, very few data have been published on the occurrence of AFM1 in dairy products in Iran. Therefore, this study was aimed to ascertain the presence and levels of AFM1 in white and cream cheese consumed in central part of Iran for the first time; and to compare the results with the legal regulations for AFM1 legislated by some of the countries like Switzerland and Turkey.

2. Materials and methods

2.1. Samples

During October 2007 to May 2008, a total of 210 cheese samples consisted of 116 white cheese and 94 cream cheese samples were randomly purchased from supermarkets and retail outlets in Esfahan and Yazd provinces located in central part of Iran. The samples were transported to the laboratory inside a digital portable refrigerator, stored at 3 °C and examined for the presence of AFM1 within 72 h.

2.2. Method and reagents

The quantitative analysis of AFM1 in the samples was based on competitive enzyme immunoassay using RIDASCREEN® Aflatoxin M1 30/15 (Art. No.: R1111, R-Biopharm, Darmstadt, Germany) test kit. Most of the used reagents were provided by the kit manufacturer. The other chemicals such as chloroform, dichloromethane, methanol and *n*-heptane were purchased from Merck. Phosphate buffer solution (PBS) was formulated by mixing 0.55 g sodium dihydrogen phosphate hydrate with 2.85 g disodium hydrogen phosphate-2-hydrate and 9 g sodium chloride and filling up to 1000 ml with double-distilled water.

For conducting recovery study, AFM1 standard was obtained from Sigma (Sigma–Aldrich, 6428). Stock solution of AFM1 (50 mg/ml) was prepared in a methanol/chloroform mixture (81:19, *v*/*v*) and stored at −20 °C. Before using, it was diluted with methanol/chloroform (1:1, *v*/*v*) at appropriate concentrations (Lopez et al., 2001).

2.3. Preparation of cheese samples

A cheese sample was homogenized (Ultraturrax, IKA-Werke, Staufen, Germany) and mixed thoroughly without addition of liquid. Two grams of the homogenized sample was weighed and 40 ml dichloromethane added for extraction of AFM1. Extraction was done by shaking the sample for 15 min. Then, the suspension was filtered through Whatman No. 4 filter paper, and 10 ml of the filtrate was dried under a weak N_2-stream at 60 °C. The oily residue was redissolved in 0.5 ml methanol, 0.5 ml PBS buffer and 1 ml *n*-heptane and mixed thoroughly for 1 min. After centrifugation at 2700*g* for 15 min, the upper heptane-layer was removed completely and from the lower methanolic-aqueous phase, 100 μl were taken and diluted with 400 μl sample dilution buffer. From this solution, 100 μl was used per well in the test.

2.4. ELISA test procedure

One-hundred microliters of standard solutions and prepared samples were pipetted into separate microtitre wells to occupy the binding sites; and incubated in the dark at room temperature (25 °C) for 60 min. The liquid was then poured out and the wells were washed with washing buffer (250 μl) twice. In the next step, 100 μl of the diluted enzyme conjugate was added to occupy free binding sites and incubated in the dark at room temperature for 15 min. Again, the wells were washed twice in order to remove any unbound enzyme conjugate. Subsequently, 100 μl of substrate/chromogen was added and incubated in the dark at room temperature for 15 min. Bound enzyme conjugate transformed the colorless chromogen into a blue product. Finally, 100 μl of the stop reagent (1 N H_2SO_4) was added into the wells and the color changed from blue to yellow. The absorbance was measured at $\lambda = 450$ nm in ELISA plate reader (ELX800, Bio-Tek Instruments, USA) against air blank within 15 min. According to the RIDASCREEN kit instructions, the lower detection limit is 50 ng/kg for cheese.

2.5. Recovery study

In order to validate our method, recovery study was performed by spiking known amounts (50, 150, 250 and 450 ng/kg) of AFM1 into homogenized cheese samples (2 g each) just before the test. The preparation of the samples and ELISA test procedure were done as described above. All experiments were carried out using four samples per each treatment. Under these conditions, the mean recovery scores in spiked cheese samples were 100.3% with a coefficient of variation of 9.5%. According to the instructions of the kit, the recovery rate in cheese is approximately 102% with a mean coefficient of variation of 11%.

3. Results and discussion

Considering the preferential affinity of AFM1 for casein fraction of milk, a high concentration of the toxin may occur in the curd during cheese manufacture (Sengun et al., 2008). Studies demonstrated that the concentration of AFM1 is 3–5 times higher in cheese than in corresponding milk (Prandini et al., 2009). For these reasons, cheese could be the most potent source of aflatoxin among dairy products.

The occurrence and levels of AFM1 in Iranian cheese samples are depicted in Tables 1 and 2. Aflatoxin M1 was detected above measurable level (50 ng/kg) in 80.1% (93/116) and 72.3% (68/94) of white and cream cheese samples, respectively. Altogether, AFM1 was found in 161 cheese samples, corresponding to 76.6% of the total samples examined, ranging between 52.1 and 785.4 ng/kg. Aflatoxin M1 levels in 51 (24.2%) samples consisted of 33 (28.4%) samples of white cheese and 18 (19.1%) samples of cream cheese were found to exceed legal limit of 250 ng/kg legislated by some of the countries like Switzerland and Turkey (Creppy, 2002). As shown in Table 2, the distribution of the AFM1 concentration represented great variations among the samples. However, most of the samples (52.2%) contained AFM1 at the level of 50–250 ng/kg.

Our results are comparable to those reported in several countries around the world. In Libya, Elgerbi et al. (2004) verified the presence of AFM1 in 75% of 20 white soft cheese samples, ranging between 0.11 and 0.52 μg/kg. In Germany, commercial cheese samples were examined for AFM1 during 10 May to 9 August 1976. The toxin was detected in 69% (136 samples) out of 197 cheese samples with a mean concentration of 0.09 μg/kg in positive samples (Kiermeier et al., 1977). In 74.7% of 75 cheese samples collected from Minas Gerais of Brazil, AFM1 was detected in the range of 0.02–6.92 μg/kg and 20 (26.7%) samples represented levels higher than 0.25 μg/kg (Prado et al., 2000). In a study, the occurrence of AFM1 in 177 fresh milk and 40 cheese samples in Kuwait were determined by Dashti et al. (2009). The toxin was recorded in all fresh milk samples except one and 80% of cheese samples. The aforementioned data along with our findings revealed a high incidence of AFM1 in cheese samples. This indicates that the milk used in cheese manufacture has been obtained from animals fed with AFB1 contaminated feedstuffs.

Table 1
Occurrence of aflatoxin M1 in Iranian cheese samples.

Type of cheese	Samples tested *n*	Positive samples *n* (%)	Concentration (ng/kg)			Exceed regulation (>250 ng/kg) *n* (%)
			Total samples (mean ± SEM)	Positive samples		
				Mean ± SEM	Range[a]	
White cheese	116	93 (80.1)	198.6 ± 17.0	247.7 ± 17.9	52.1–744.5	33 (28.4)
Cream cheese	94	68 (72.3)	166.4 ± 18.6	230.1 ± 21.1	58.3–785.4	18 (19.1)
Total	210	161 (76.6)	184.2 ± 12.5	240.2 ± 13.6	52.1–785.4	51 (24.2)

[a] Minimum–maximum value.

Table 2
Levels of aflatoxin M1 in Iranian cheese samples.

Type of cheese	Distribution of samples *n* (%)					
	<50 ng/kg[a]	50–150 ng/kg	151–250 ng/kg	251–450 ng/kg	451–650 ng/kg	>650 ng/kg
White cheese	23 (19.8)	32 (27.5)	28 (24.1)	21 (18.1)	8 (6.8)	4 (3.4)
Cream cheese	26 (27.6)	23 (24.4)	27 (28.7)	10 (10.6)	5 (5.3)	3 (3.1)
Total	49 (23.3)	55 (26.1)	55 (26.1)	31 (14.7)	13 (6.1)	7 (3.3)

[a] Distribution of negative samples.

In previous studies carried out in Iran, Parvaneh et al. (1982) found that 97.5% of 80 cheese samples were contaminated with AFM1 in Tehran. In another study (Kamkar, 2006) conducted 24 years later in the same area, AFM1 was detected in 82.5% of cheese samples (66 out of 80 samples) with the average value of 0.41 μg/kg; and AFM1 concentration in 60.5% of these positive samples was higher than the maximum acceptable level (0.25 μg/kg). In a recent survey performed in Esfahan city, Rahimi et al. (2009) reported that in 47 (53.4%) out of 88 traditional cheese samples, AFM1 was detected in concentrations between 82 ng/kg and 1254 ng/kg; and 28 (31.8%) samples exceeded the maximum tolerance limit (250 ng/kg). The mean concentration of AFM1 was also significantly higher in autumn and winter than in spring and summer. In agreement with our study, these reports demonstrated a widespread occurrence of AFM1 in cheese samples prepared and consumed in Iran.

Comparing to some reports from other countries, our results revealed higher contamination. In the Apulia region of Italy, for example, AFM1 was found in 16.6% out of 265 unripened, medium-term ripened and long-term ripened cheese samples with an average of 88.6 ng/kg (Montagna et al., 2008). In Slovenia, Tokar and Vengust (2008) observed that in 6 (15%) out of 40 samples AFM1 was present. The contamination level above 50 ng/kg was detected in 10% of the samples. The incidence of AFM1 in 39 samples of cheese in Ankara, Turkey has been reported by Gurbay et al. (2006). They observed that 11 (28.2%) samples were contaminated with AFM1, ranging from <50 to 188.4 ng/kg. In a study performed by TLC technique, the absence of AFM1 at detectable level was reported in "wara" (locally produced unripened cheese) and yoghurt samples collected from Ogun State of Nigeria (Atanda et al., 2007). Similarly, in a six year survey (1999–2004) carried out by HPLC technique in Portugal, Martins et al. (2005) found that none of the samples of traditional fresh cheese and powder milk revealed to be contaminated with AFM1.

According to studies carried out in different regions of our adjacent country, Turkey (Oruc and Sonal, 2001; Aycicek et al., 2002; Sarimehmetoglu et al., 2004; Tekinsen and Eken, 2008; Tekinsen and Ucar, 2008; Var and Kabak, 2009; Ardic et al., 2009) a high incidence of AFM1 in different kinds of cheese (89.5%, 65%, 81.7%, 82.6%, 99%, 65% and 82.4%, respectively) has been reported. These studies performed by ELISA technique.

As seen above, the contamination levels of cheese by AFM1 vary from one study to another. This variability can be explained by different factors: cheese-making procedures, conditions of cheese ripening, variety of cheese studied, geographical region, analytical technique employed and degree of milk contamination according to seasonal changes (Sarimehmetoglu et al., 2004). It has been reported that the level of AFM1 contamination in milk produced during cold seasons is higher than that produced during hot ones (Ghiasian et al., 2007).

In conclusion, the results of the present study indicated that the AFM1 levels in cheese consumed in central part of Iran were relatively high and it can provide a potential hazard for public health. The best way to deal with this problem is reducing the AFB1 concentration in animal feed by improved processing and storage practices. At the same time, attention should be given to regular monitoring of aflatoxins in animal feed and dairy products. In addition, the governmental agencies should train the farmers, dairy companies and dairy product consumers on the potential health consequences of aflatoxins. Finally, milk and dairy products contaminated with high levels of AFM1 must be prohibited for human consumption by the public health authorities.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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