

Determination of Aflatoxin M₁ and B₁ in Egyptian Raw Milk, Soft Cheese and Table Eggs using ELISA Technique

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Abstract: This study was conducted to determine the AFM₁ levels in Egyptian raw milk, soft cheese and AFB₁ in table eggs marketed in Kafrelsheikh city markets. Aflatoxin content was measured using microtiter-plate enzyme-linked immunosorbent assay (ELISA). The Test Kits, RIDASCREEN ELISA of Biopharm AG that used is based on competitive enzyme immunoassay for the quantitative analysis of Aflatoxin AFM₁ and AFB₁ and with detection limit of 5ppt for AFM₁ and 1ppb for AFB₁. Tests and standards were performed according the instruction of manufacturer and the results were measured on standard curve of concentrations ranged from 5 to 80 ppt for AFM₁ and from 1000 to 2000 ppt for AFB₁. Of 90 milk samples, 86 (95.6%) samples contained aflatoxin M₁ with concentrations ranged from 5.3 to 43.5 ppt and the mean value was 10.4 ppt. 46 cheese samples (92%) contained AFM₁ with concentrations ranged from 50 to 97 ppb and average value of 71 ppb while 2 (4%) of table eggs examined contained AFB₁ with quantities ranged from 1100 to 1700 ppt. Data also indicated that AFM₁ residues concentrations detected in all the positive samples were above the permitted levels of ESO and below the tolerated levels foreign standards. So it could be concluded that contamination of AFM₁ in milk and cheese marketed in Kafrelsheikh city appear to be of serious public health problem at the moment while table eggs needs more further examination at large scale.

Key words: Aflatoxin M₁, AFB₁, Cheese, Table eggs, ELISA.

INTRODUCTION

Aflatoxins are a group of naturally occurring mycotoxins produced from growth of *Aspergillus flavus* and *A. parasiticus* on feeds and food. They are unavoidable food contaminants even when good agricultural practices are applied. Animals are considered the most group exposed to high concentration of aflatoxins through feedstuffs that develop several health problems and lead to large economic losses. These losses are pronouncing in milk, meat and eggs in terms of quality and quantity as a result of contamination with aflatoxins residues [1, 2, 3]. Grain contamination with aflatoxins is considered by FDA as an avoidable contaminant [4] and its contamination with the mycotoxins was estimated by 25% of the produced world's crops [5]. In Egypt where the weather is mostly warm all over the year, growth of moulds and aflatoxin production on the grains were reported [6, 7].

Milk and milk products are a major nutrient for human especially children, however at the same time these products may be contaminated with AFM₁ residues [8, 9] and its presence in milk and milk products is considered to be undesirable. The International Agency for Research on Cancer (IARC) [10] classified it as a class 2B toxin, a possible human carcinogen. In the assessment of cancer risk, the infants are more exposed to the risk because the milk is a major constituent of their diet and appears warranted if the biological endpoints involved are carcinogenicity and mutagenicity. During cheese making, AFM₁ can be decreased in cheese by increasing renneting temperature from 30 to 40°C, decreasing cutting size of curd and increasing press time from 1 to 2 h, which causes more loss of AFM₁ in the whey. Moreover, it becomes 2.5 to 3.3 times higher in soft cheese and in hard cheese, 3.9 to 5.8 times higher than in the milk from which the cheeses were made [11].

It is well known that raw milk is processed mostly before human consumption. In connection with this, [12]; [13] and [14] observed negligible losses of AFM₁ during storage or after heat treatment.

The literature available on the occurrence of aflatoxins in cheese indicates presence of variable levels of contamination however; the findings of [15, 16, 17, 18] are slightly in accordance with our results. Researchers from different countries have also investigated AFM₁ in cheese samples. Elkak et. al, [19] has studied the presence of AFM₁ in Lebanon processed cheese and Tavakoli, et al., [20] in Tehran, Iran on white soft cheese. The variable AFM₁ levels detected are significantly affected by

cheese manufacturing procedures, different milk contaminants, type of cheese, conditions of cheese ripening and the analytical methods employed [21].

Avian egg provides Egyptian consumers of all ages with a unique well balanced source of animal protein. Moreover, their high quality, low caloric value and ease of digestibility make eggs valuable in many therapeutic diets for adults [22, 23]. In parallel, its contamination with mould producing aflatoxins still exist and several investigators [24, 25, 26, 27, 28] could isolate moulds and detected aflatoxin B1 in table eggs.

Consequently, aflatoxins negative milk, milk products and table eggs are required by avoiding the ingestion of contaminated feed by dairy cattle and laying hens [29]. Many countries have regulated the amount of AFM1 in milk and cheese. The European regulations [30] set a maximum level of AFM1, should not exceed 50 ng kg⁻¹ for liquid milk and milk products and a limit of 0.025 ug/kg for M1 for infant foods. In the USA, the AFM1 level should not be greater than 500 ng kg⁻¹ in milk. In Turkey, according to the Turkish Food Codex [31] set limits for AFM1 of 50 ng L⁻¹ (0.05 ppb) and 500 ng L⁻¹ (0.5 ppb) for milk and cheese, respectively. The Serbian regulation [32] also set a limit of .5 ug/kg cheese. In Egypt, the Egyptian regulations [33] stipulate the freedom of fluid milk and dairy products from aflatoxin M1 and B1. Pourelmi et al., [27] stated that the level of AFB1 allowed in table eggs should be 12 ng/ml.

Therefore, the present study was undertaken to investigate aflatoxin M1 and B1 contamination in Egyptian raw milk, soft cheese and table eggs sold in Kafrelsheikh city, Egypt in order to throw a light about the health hazards of the aflatoxins and to undertake measures for avoiding these toxins in Egyptian food as possible.

MATERIALS AND METHODS

1-Samples

A total of 180 of raw milk and cheese samples, consisting of 90 samples each and 50 table eggs were investigated in this study. Samples were collected from markets in Kafrelsheikh region. Dairy samples were carried to laboratory in a cold cabinet for the detection of AFM1 whereas table eggs were examined for AFB1.

2-ELISA Kits

AFM1 was detected by using RIDASCREENR Aflatoxin M1 Art No. R1121 kits with lower detection limit of 5ppt. while AFB1 content was detected by using RIDASCREENR Aflatoxin B1 30/15 Art No. R1211 with lower detection limit of 1000 ppt.

The Kits were purchased from R-Biopharm AG Co.

3-Preparation of samples

The kits were stored at 4°C and all the reagents were brought to room temperature, 2 h before use and all reagents were returned to 4°C after use. Milk samples were refrigerated and centrifuged at 2-8°C for 10 min at 3000xg. The fat from the skimmed milk was separated and the skimmed milk was used.

The cheese samples were weighted and 2 g of the samples were used. About 15 mL of dichloromethane was added to the sample and extracted by shaking the vial for 15 min. The suspension was filtered and 3.75 mL of the suspension was transferred to a glass tube and evaporated at 60°C under a nitrogen stream.

4-Method of detection

The residue was dissolved in 750 µL of the extraction solution and mixed by vortex for 1 min. After then, 750 µL hexane was added and vortexed for 1 min. The suspension was centrifuged for 15 min at 2000xg. The upper hexane layer was removed and 50 µL of the methanolic/aqueous phase was taken and 200 µL dilution buffers were added. This final suspension was used in the test.

The AFM1 standards and milk samples were added to 96 wells microplate coated with AFM1 antibodies and incubated for 45 min at room temperature. The kits reagents consisting of the enzyme conjugate solution, developing solution and stop solutions were added to the wells, respectively and washed several times in the appropriate order and incubation times according to the instructions. The absorbance was measured at 450 nm.

RESULTS and DISCUSSION

TABLE 1: Concentrations of AFM1 in raw milk, soft cheese and table eggs sample

Type of samples	Type of Aflatoxin	Detection limit of the method	Positive samples		Aflatoxin Concentration (ppt)	
			No.	%	Range	Mean
Raw milk	M1	5ppt	86	95.6	5.3 – 43.5	30.7
Soft cheese	M1	5ppt	46	51.1	50.0 – 97.0	71.0
Table eggs	B1	1000ppt	2	4.0	1100 – 1700	1400

TABLE2: Number of positive samples exceeding the Egyptian, EU and US limits

Type of samples	No. of Positive Samples	ER ¹		EUR ²		USR ³		TFC ⁴		SR ⁵	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Raw milk	86	86	100	0	0	0	0	-	-	-	-
Soft cheese	46	46	100	0	0	0	0	0	0	0	0
Table eggs	2	2	100	0	0	0	0	-	-	-	-

¹Egyptian regulation, 1990, 0ppt of AFM1 and AFB1

²European regulations 466/2001 (the limit is fixed for AFM1 at 50 ng/L for raw and liquid milk)

³FDAUS regulations limit, 500ppt of AFM1

⁴Turkish Food Codex limit of 250 ng/kg white cheese.

⁵Serbian regulation (2013), 0.5ug/kg cheese.

The levels of AFM1 and AFB1 in raw milk, soft cheese and table egg samples were determined. Of 100 raw milk samples examined 86 (95.6%) samples contained Aflatoxin M1 with quantities ranged from 5.3 to 43.5 ppt and a mean value of 30.7 ppt (table 1). All positive milk samples contained AFM1 exceed the Egyptian levels while below the EU and the US levels (table 2). The aflatoxin M1 levels in the milk samples were differing from each other surveys performed in Egypt. AFM1 was detected in 17 raw milk samples out of 30 samples examined with levels ranged from 30 to 400 ppt . Aman [15] found AFM1 in 12 raw milk samples out 40 samples examined with levels ranged from 13.4 to 64.9 ppt with 7 samples contained AFM1 over 50ppt.which exceed both the Egyptian and EU levels. Amer and Ibrahim [16] found 19(38%) samples had AFM1 with quantities ranged from 23 to 73 ppt while Ghareeb et.al.,[34] detected AFM1 in 47 samples (97.92 %) with quantities ranged from 2 ng/L to 110 ng/L. The level of AFM1 in 53.19 % of raw milk samples had higher quantities (79.85 ± 17.30 ng/L) than the maximum tolerance limit (50 ng/L) established by European regulations [30] and all positive samples exceeded the Egyptian regulations [33]. Moreover, Shaker and Elsharkawy [35] found all 30 raw milk samples tested were positive for AFM1, with quantities above the Egyptian regulations limits and they added that, 93% of samples were above the permitted limit set by the European Commission (EC), whereas 3.3% of samples exceed the US Food and Drug Administration (US FDA) tolerance limit [36]. The variations recorded in quantities may be due to the samples were collected from different localities at different seasons and different methods of analysis.

The AFM1 level in milk samples has been investigated in several countries. Dashti et al. [37] have studied AFM1 in 321 milk samples in Kuwait. Of them 177 were fresh milk and they found that all fresh milk samples except one were contaminated with AFM1 ranging from 4.9-68.7 ng kg-1 and eight samples were reported to exceed the European Union's regulatory limit. Nuryono et al. [38] detected AFM1 in 113 fresh milk in Indonesia, 65 samples were found to be contaminated with AFM1 and none of the samples exceeded the European Limit levels. Tajik et al. [39] investigated 72 raw milk samples and detected 9 raw milk samples exceeded the level of European Union limit. Muhammad et al., [40] examined a total of 84 samples from Lahor area in Pakistan and eighty one percent milk samples contained AFM1 levels exceeding the American and European tolerance limits. The mean value of AFM1 was 17.38µg/L ranged from 0.69 to 100.04 µg/L. High levels of AFM1 in the raw milk samples is an enormous health risk factor for end consumers. There is need to improve storage conditions of feed ingredients that will mitigate the AFB1 production.

Regarding Egyptian soft cheese samples analyzed, 46(51.1%) positive samples had average AFM1 contents of 71.0 ppt with quantities ranged from 50.0 – 97.0 ppt (table 1). All positive cheese samples exceed the Egyptian limits for AFM1 in cheese while none of them exceed the European Union's regulatory limit, FDA US Guidance and Turkish Food Codex limits (table 2).

The literature available worldwide on the occurrence of aflatoxin M1 in cheese indicates higher levels of contamination than our findings. In Turkey, Dincoglu et al., [41]; Sarimehmetoglu et al. [21], and Elgerbi et al., [42]. In Egypt, results obtained by Amer and Ibrahim [16] detected quantities from 52 to 87 ng/kg cheese which are nearly similar to our results.

According to EUR, USR, TFC and SR standards, all examined samples of cheese were below the levels permitted by the mentioned standards, while all positive samples exceed the limit set by the Egyptian regulation.

The differences in the results obtained was declared by [21] who attributed these variations to the manufacturing procedures, different milk contaminants, type of cheese, conditions of cheese ripening and to the method of analysis employed to measure AFM1. Moreover, Anfossi et al., [43] added the factor of season on the contamination of Italian cheese with AFM1 and they stated that cheese made during summer and autumn, which belong to milk from grazing animals, would be less contaminated than cheese made during winter and spring, which belong to milk from animals fed with composite and stored fodder. So, according to the Egyptian limit for AFM1 in raw milk and soft cheese, we need for further and continuous control to preserve consumer health as all positive samples exceeded the limit.

Table eggs examined contained AFB1 in 2 (4%) samples with quantities of 1.1ppb and 1.7ppb (table 1) and the rest of the samples were below the detection limit (1ppb) of the method used. According to Pourelmi et al., [27] who stipulated a limit of 12 ng/ml. table egg, none of the positive samples exceeded their limit.

In Egypt, Marouf et al., [24] detected aflatoxin AFB1 contamination in 9 (45%) of table eggs "brown" samples examined. While, they detected AFB2 in (10% of balady table eggs examined. Higher concentrations were reported by Pourelmi et al [27] who studied the AFM1 contamination of industrial and local eggs in west region of Mazandaran in Iran and showed highest aflatoxin B1 contamination was seen in Chalous local eggs (0.107 ng /ml) and lowest contamination was seen in industrial egg from Sattari farm (0.050 ng /ml) respectively. The results which supported by (44) Fernandes Oliveiraa (2003) who reported excretion of aflatoxin residues in quail eggs fed feeds contaminated with various concentrations of AFB1 under conditions of long-term exposure but at relatively low concentrations and (45) Aman et al., (1993) who could isolate AFB1 secreting strains of *Asepergillus parasiticus* and *A. parasiticus* from table eggs.

From the above achieved findings, it could be noticed that table eggs examined have a good quality to some extent but, it needs more care during producing and handling to safeguard human from being infected.

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