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Quality Assessments of Aflatoxin M1 Residues in Milk and Some Milk Products in Dakahlia Governorate

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Abstract

This work was planned to qualitatively assess AFM1 residues in milk as well as milk product samples. Cow's raw milk, Aged Roomy cheese, Domiati and Kariesh cheese (50 of each type) were collected from various supermarkets in Mansoura governorate in Egypt during 2020-2022. Samples were tested using the TLC method, positive samples from TLC method were exposed to the HPLC method for detection of their levels and their concentrations were compared with the permissible limits. The obtained results showed high incidence in raw cow milk (36%) followed by fresh Kariesh cheese (28%), then aged roomy cheese (24%), and then Damietta cheese (12%) collected from the local markets in Mansoura by TLC. The obtained concentrations in analyzed dairy samples were compared with the Permissible Limits (PL) set by the concerning agents [US, EU] by quantitative detection using HPLC. The detectable concentration of AFM1 in raw milk and cheese by HPLC showed high concentration of aflatoxin M1 in aged roomy cheese (121.08) followed by raw cow milk (80.83), followed by fresh Kariesh cheese (50.14), and then Damietta cheese (35.33) collected from the local markets in in Dakahlia governorate. The current study's findings showed that raw milk and cheese sold in Mansoura city are occasionally contaminated with AFM1. As a result, it is strongly advised to effectively heat treat raw milk and give animals nutrition free of aflatoxins.

KEYWORDS
Aflatoxin M1, Kariesh cheese, Aged Roomy cheese, Damietta cheese Gamma irradiation, TLC, HPLC.

INTRODUCTION

The first identification of aflatoxins was in 1960 by discovery of *Aspergillus flavus* which was the toxin-producing mold and was named Aflatoxin in relation to its origin (Ayhan *et al*., 2010). Contamination of milk products with mycotoxins was first started in 1960 when the first case of AFM1 toxicity was recorded as a result of metabolism of AFB1 in the rumen and its excretion into milk (Duarte *et al*., 2013).

There are about twenty types of aflatoxins which are related to large group of toxic compounds, out of them only 4 types could induce natural contamination of foods which were AFB1, AFG1, AFB2, and AFG2. AFM1 could be existed in milk and its products as hydroxyl metabolites due to feeding dairy animals on feedstuffs contaminated with toxins (Dohnal *et al*., 2014). Detection of defined AFM1 as a milk toxin which was the main metabolite of aflatoxins in which ''M'' was referred to milk of mammals who consumed the feed contaminated with aflatoxins) Kara and Ince, 2014). The AFM1 was found to be higher than maximum residue limits of European Committee/Codex Alimentarius Commission by 36% in raw cow milk in comparison with 8% in raw buffalo milk. (Rahimi *et al*., 2010). Milk and its products might be contaminated by aflatoxins either directly by accidental growth of fungi and then toxins production or indirectly by ingestion of contaminated feed by AFB1 which excreted into milk as AFM1 (Sengun *et al*., 2008). Milk products might be directly contaminated by mycotoxins through colonization of fungi especially in cheese that its contamination could take place either from unhygienic media of processing or the usage of polluted fungal starter cultures that used in manufacturing of special milk products (Marin *et al*., 2013). Raw milk, soft, hard and processed cheese samples (fifty of each) were examined for the presence of AFM1 in Alex, Egypt by ELISA method (Amer and Ibrahim, 2010), the authors reported that AFM1 was detected in nineteen of raw milk, twelve of soft cheese, nineteen of hard cheese, and eleven of processed cheese samples.

The possibilities of human exposure to aflatoxins might be due to the consumption of foods of animal origin as contaminated milk, milk products as cheese, meat, and eggs (Bryden 2011). Milk products might be directly contaminated by mycotoxins through colonization of fungi especially in cheese that its contamination could take place either from unhygienic media of processing or the usage of polluted fungal starter cultures that used in manufacturing of special milk products (Marin *et al*., 2013). The highest levels and incidence of aflatoxins were reported to be in winter more than in summer as in the summer the animal fed on pasture, grass, weeds and green fodder while in the winter the fresh and green feed were low and unavailable so, the high consumption was from the concentrated feed stuffs as corn, wheat, and cotton seeds that might be exposed to improper storage conditions which enhance toxic strains of fungi to grow as Aspergillus spp., and to produce aflatoxins (Asi *et al*.,

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2012).

This work was planned to the qualitative assessment of AFM1 residues in milk as well as milk product samples that were being tested using the TLC method. The positive samples from TLC method are exposed to the HPLC method for detection of their levels and their concentrations were compared with the permissible limits.

MATERIALS AND METHODS

Samples

The examined samples were collected from various supermarkets in Mansoura governorate in Egypt during 2020-2022 that included cow's raw milk, Aged Roomy cheese, Domiati and Kariesh cheese (50 of each type), Each sample consisted of 1 liter of milk and 200–250 grams of cheese, and it was maintained in clean, dry, and sterile sampling vials. It was then quickly and efficiently transported in an ice box at 4°C to the laboratory of the Animal Health Research Institute, Dokki, in Giza for analysis. Each cheese sample was cut up in a mincer at high speed for two min. before analysis (Aygün *et al*., 1999).

Devices

Thin layer chromatography (TLC) consists of ultraviolet (UV) lamp with two hundred forty five and three hundred sixty four nm wavelengths (Philips), and silica gel plates (Merck and Fisher).

High Performance Liquid chromatography (HPLC), Agilent Series 1200, manufactured in the USA (chemistry department of Animal Health Research Institute, Dokki, Giza) with a fluorescence detector (Japan) are all components of the Agilent Series 1200, manufactured in the USA. Wavelengths for excitation and emission were 365 nm and 442 nm, respectively. Agilent's ChemStation software for data analysis.

SPE-C18 cartridges were used for clean-up procedure (Glasgow, United Kingdom).

Chemicals

Acetonitrile, acetone, and methanol of HPLC grade were purchased from Merck. Chloroform, hexane, bromide (KBr), phosphate buffered saline (PBS), anhydrous sodium sulfate, Di-ethyl ether, Potassium chloride, potassium di hydrogen phosphate, from (Merck, Darmstadt, Germany) and NaCl and anhydrous disodium hydrogen phosphate analytical grade will be obtained. De-ionized water (DW) obtained from a Milli-Q-system (Millipore, Mosheim, France).

Mobile phase for HPLC analysis

As shown in Table 1, solvents A and B were mixed at a flow rate of 1 ml/min each. Solvent A contained 80% methanol and 2% acetic acid in water. (The mobile phase was degassed with an ultrasonic bath for 15 minutes prior to use by passing it through a 0. 45 m nylon membrane filter (Brera *et al*., 2011).

Standard

Sigma-Aldrich Co. provided certified reference in stock solution of AFM1 (fifty mg/ml), which was made in a methanol/chloroform mixture (81:19, v/v) and stored at -20°C and diluted with methanol/chloroform (1/1, v/v) at the proper concentrations before use (Fallah, 2010a).

Derivatization of AFM1 was carried out with a post-column LC pump (LC pump Lab flow two thousand, Lab service Analytica, Bologna, Italy) at a flow rate of 0. 4 mL/min using a 0. 005% aqueous solution of Pyridine Hydrobromide Perbromide (PBPB).

Detection of AFM1 by TLC

The collected samples screened for presence of AFM1 according to Fallah (2010b). The positive AFM1 residues showed fluorescent spots which were visually Compared with that of standard AFM1spots under the long ultraviolet wave UV light (three hundred sixty four nanometer).

Quantification of AFM1 by HPLC

Extraction and clean-up of AFM1 according to Manetta *et al*. (2005) with little modification. The extraction was summarized in three steps (preparation, purification, and injection).

Milk

The milk was warmed to 37°C in a water bath before usage, and the fat layer was gently stirred out using a magnetic stirrer. After that, the thin top layer of fat was eliminated by centrifuging the mixture at 3000 rpm. Every sample was prepared at room temperature. Dilution to 10 m1 of the aqueous phase with deionized Water 1:1 before SPE purification (10 ml).

Acetonitrile (five ml) and (ten ml) deionized water was used for conditioning the A SPE-C18 cartridge. After that, 20 ml acetonitrile/water (20:80, v/v), 10 ml water, and 10 ml n-hexane were used to wash the diluted samples. Elution of AFM1 was done by using six ml of dichloromethane/acetone (95:5, v/v)

Elute was evaporated under a gentle stream of nitrogen, then the residue was dissolved in acetonitrile (200 µl). An aliquot (ten µl) of the extract was injected into the HPLC system.

Cheese

Ten grams of cheese were divided into tiny particles, its extraction was occurred by using (50 ml) dichloromethane/acetone (1:1, v/v) depending on UltraTurrax apparatus, ten grams of NaCl was added, and finally the mixture was centrifuged at 3000 rpm for 10 min. Via a gentle nitrogen stream, ten ml of the organic extract was dehydrated. The residue that had evaporated was diluted in 0.5 ml of methanol, 20 ml of 0.01 mol/l PBS, and 10 ml of n-hexane. The mixture was blended for five minutes then vertically set for five minutes. The bottom layer was exposed to purification and cleaned up on C18-SPE as mentioned in milk extraction. An aliquot (ten µl) of the extract was injected into the HPLC system.

Statistical analysis

Using the SPSS tool, the collected results were statistically evaluated. (Statistical Package for Social Sciences, version IBM 23) (Verma, 2012). Series of 3 analyses were conducted for each sample in a particular matrix, and the variability is represented by the coefficient SD.

RESULTS

TLC results

The obtained results were summarized in Table 1 showed

high incidence in raw cow milk (36%) followed by fresh Kariesh cheese (28%), then aged roomy cheese (24%), and then Damietta cheese (12%) collected from the local markets in Mansoura by TLC.

Table 1. Occurrence of AFM1 in examined raw milk and cheese samples which were collected from the local markets in Mansoura by TLC.

Dairy products*	Positive samples no	%
Raw cow milk	18	36
Aged roomy cheese	12	24
Fresh Kariesh cheese	14	28
Damietta cheese	6	12

*n= 50 for each product

Concentrations of AFM1 in analyzed samples by HPLC

The obtained concentrations in analyzed dairy samples by HPLC. showed high concentration of aflatoxin M1 in aged roomy cheese (121.08±124.27) followed by raw cow milk (80.83±82.30), followed by fresh Kariesh cheese (50.14±32.90), and then Damietta cheese (35.33±21.00).

A comparison between Permissible Limits (PL) set by the concerning agents [US, EU] and the detectable concentration of AFM1 in raw milk and cheese sample was summarized in Table 2.

Table 2. Comparing the detected levels of AFM1 (ppt) in samples of raw cow milk and cheese samples to the permissible limits of set by EU and US.

Milk product	Higher than EU regulation (0.05 µg/l or µg/Kg)		Higher than US regulation (0.50 µg/l or µg/Kg)	
	N (%)	Range (ppt)	N (%)	Range (ppt)
Raw cow milk	6 (12 %)	75- 297	-	-
Aged roomy cheese	5 (10 %)	98- 311	-	-
Fresh Kariesh cheese	6 (12 %)	55- 110	-	-
Damietta cheese	1 (2 %)	75	-	-

DISCUSSION

Samples in this study were collected from various supermarkets in Mansoura governorate in Egypt that included Cow's raw milk, Aged Roomy, Domiati and Kareish cheese (50 of each type) during a period extended from 2020-2022. The collected samples were screened for the presence of AFM1 by using TLC assay.

The obtained data reflected that the existence of AFM1 in 18 samples of raw cow milk at 36%, 14 samples of fresh kareish cheese at 28%, 12 samples of aged roomy cheese at 24%, and 6 samples of Damietta cheese at 12%.

Keeping with the same ground the results obtained by Younis *et al.* (2016) showed that the existence of AFM1 in 6 raw milk samples out 10 samples at 60%, in 8 samples of roomy cheese and in 6 samples of kareish cheese from 10 samples of each at 80% and 60% respectively. By matching the obtained data by that obtained by previous studies, our results were reflected the lower incidence of contamination.

Data from the present study agreed with the results obtained by Motawee *et al.* (2004) who recorded the incidence of AFM1 in raw cow's milk in 4 samples from 10 samples at 40%. The results also agree with that obtained by Aiad and Aboelmakarem (2013) who recordedAFM1 in 40% of raw milk and lower than its existence in kareish, Damietta and Ras cheese samples that were 46%,53% and 56% respectively.

Other investigation by Shundo and Sabino (2006) that detected the existence of AFM1 in raw milk at Brazil and they concluded that was 59.1% from collected samples which was higher than our results. Keeping with the same ground the higher incidence of AFM1 in white cheese that recorded by Fallah (2010a) were 81.9% from collected samples in Iran. On the other side of view, the data obtained by Atanda *et al.* (2007) recorded the existence of AFM1 by 0.05% in Nigeria.

The positive samples for AFM1 from milk and cheese were examined by HPLC for quantitative detection of the residual value and our data reflected that the presence of AFM1 in milk and its products by different concentrations and determined a definite number of samples exceeded the permissible limits that set by EU regulation (0.05 µg/l or µg/Kg) as mentioned in Table 2 and its value were 6 (12%), 5 (10%), 6 (12%) and 1 (2%) for raw milk, aged roomy, fresh kariesh and Damietta cheese respectively.

The HPLC detection for the positive samples showed high variations for AFM1 concentrations that ranged 14-297 ppt (mean 80.83 ppt), 11-311 ppt (mean 121.08 ppt), 15-110 ppt (mean 50.143 ppt) and 17-75 ppt (mean 35.33 ppt) for raw milk, aged roomy, fresh Kareish and Damietta cheese respectively.

By matching of our data with that obtained by Egyptian studies for detection of AFM1in milk and milk products the study by Motawee *et al.* (2004) concluded that the concentration of AFM1 in raw cow's milk was 0.25 ppb which is higher than the mean of AFM1 obtained by this study. Moreover, the concentration of AFM1 in cheese samples obtained in the present study was 1.25, 0.76 and 1.9 ppb for Kariesh cheese, fresh Domiati cheese and aged roomy respectively.

The data obtained by Nassib *et al.* (2005) recorded the presence of AFM1 in concentrations 0.144, 5.100, 3.3 and 2.34 ng/g in raw cow's milk, aged Roomy, fresh Domiati and Kariesh cheese respectively which were higher than obtained by our data. On the other side of view, the data obtained by Amer and Ibrahim (2010) showed the lower ranges of AFM1 in cow's milk, hard and soft cheese in concentration 23-73, 52-87.6 and 51.6-182 ng/l. Also, the data obtained by Awad *et al.* (2014) concluded the existence of AFM1 in Damietta and kariesh cheese by range of concentrations 1.95-6.11 and 1.54-14.73 ppb which were higher than our results.

CONCLUSION

The obtained results of the current study revealed the contamination of the raw milk and cheese retailed in Mansoura city with AFM1 at variable rates. Therefore, efficient heat treatment of raw milk, and providing animals with feed free from aflatoxins are highly recommended.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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