

INCEDENCE OF AFLATOXINS M₁ AND B₁ IN RAW MILK AND SOME DAIRY PRODUCTS IN DAMIETTA -EGYPT.

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ABSTRACT

Aflatoxin M₁ appears to be associated with the protein fraction of milk, and hence the aflatoxin is present not only in fluid milk but also in dairy products made from contaminated milk. Two hundred and seventy of different milk samples and some dairy products were collected from Damietta governorate in Egypt in the period extended from summer 2002 to summer 2003. the samples were analysed for the presence of AFM₁ and AFB₁. All samples were examined in duplicates by liquid chromatography mass spectrometry Method (LCMS). 30% of buffalo's milk samples had an average of 0.22 ppb of AFM₁ and 40% of cow's milk samples had an average of 0.25 ppb of AFM₁. For different cheese samples, an average of 0.21, 0.222, 0.54, 0.64, 0.669, 0.76, 0.826, 1.25, 1.9, and 1.98 ppb of AFM₁ was detected in Feta cheese, Cheddar cheese, aged Domiati cheese, Requforti cheese, Processed cheese, Fresh Domiati cheese, Spread cheese, Karish cheese, aged Romi cheese and Fresh Romi cheese, respectively. An average of 0.045, 0.1 and 1.85 ppb of AFM₁ was also detected in Yoghurt, Ice cream and Mish, respectively. On the other hand, from all 20 samples of Karish cheese, Processed cheese, fresh Domiati cheese, aged Domiati cheese, fresh Romi cheese and yoghurt only 2 samples of each had an average of 0.107, 0.112, 0.061, 0.074, 0.416 and 0.045 ppb of AFB₁, respectively, while 3 samples out 20 samples of Mish, Spread cheese, aged Romi cheese and Feta cheese had an average of 0.262, 0.03, 1.6, and 0.111 ppb respectively. The highest content of AFB₁ was found in Requforti cheese and Cheddar cheese; whereas ice cream, buffalo's milk and cow's milk were completely free from AFB₁. Many countries have carried out these studies about the incidence of AFM₁ in milk and other dairy products in most of these, samples have been found to exceed the limit imposed by many countries. In all cases, all milk samples were below the maximum tolerated levels of AFM₁ in liquid milk. Unfortunately, in our country, in spite of the fact that the dairy industry has evolved a lot in the last years, as regards of production levels and technology, most dairy products being above maximum permissible limit of AFM₁ (0.25 ppb), except for Feta cheese, Cheddar cheese, Ice cream and Yoghurt, which were under permissible limit of AFM₁ (0.25 ppb). All milk samples and other dairy products were below the maximum tolerated levels of AFB₁ (5 ppb). It is quite difficult to compare the present results with those in literatures data because of the wide differences between the countries relation to feeding, animal and environmental factors, extraction and analysis procedures, and regulatory limit for aflatoxin in feeds and milk.

Keyword: Aflatoxin; Milk; Dairy products

INTRODUCTION

Dairy products are one of the most susceptible foodstuffs to contamination by mycotoxins, the chemicals substances produced by fungi which can be passed into the food chain when products containing them are digested by man or animals (Van Egmond 1991). Aflatoxins are toxic metabolites produced by many strains of *Aspergillus flavus* and *Aspergillus parasiticus* growing on many vegetable foods, notably peanuts, and cattle feeds. Aflatoxin M₁ (AFM₁, 'milk toxin') is the major hepatic carcinogenic

metabolite of aflatoxin B₁ (AFB₁) excreted in milk when lactating animals are fed with AFB₁-contaminated feeds (Blanco *et al.*, 1993; Govaris, *et al.*, 2001 and Galvano, *et al.*, 2001). Both AFM₁ and AFB₁ can cause DNA damage, gene mutation, chromosomal anomalies and cell transformation in mammalian cells *in vitro*, in insects, lower eukaryotes and bacteria (SCF) (Standard Committee of Foods, 1994 and Govaris, *et al.*, 2002). The occurrence of AFM₁ in commercially available milk and dairy products is a matter of interest for public health due to continuous and prolonged exposure to a carcinogenic substance. Occurrence of aflatoxin in milk and milk products can be due to three possible causes, AFM₁ present in raw milk as a consequence of carryover of AFB₁ from contaminated cow feeds to milk, synthesis of AF (B₁, B₂, and G₂) by *Aspergillus Flavus* and *Aspergillus parasiticus* growing on cheese (Applebaum, *et al.* 1982 and Zerfiridis, 1985) and occurrence of these toxin in milk and dried milk used to enrich the milk used to make cheese and other dairy products. (Blanco, *et al.* 1988). The carry-over of AFB₁ to AFM₁ is linearly correlated with milk yield and the values as 6 % have been reported at µg daily intake levels of AFB₁ (Veldman *et al.* 1992). The carry-over of AFB₁ also to milk may vary largely from animal to animal, from day to day, and from one milking to the next (Van Egmond and Dragacci 2001). According to the U.S. Food and Drug Administration, AFM₁ in milk should not exceed 0.5 ng/ml (Stoloff, 1980 and Van Egmond 1989). Several countries control contamination of milk and dairy products by AFM₁, and they have established regulations for AFM₁ in these products. The current permissible levels for AFM₁ in milk range from 0.05 to 0.5 µg/kg, except for infant milk for which lower levels exist (FAO, 1997 & Maria and Herminia 2000). Whereas the food and Drug Administration has established an action level of 0.5 µg/kg AFM₁ in whole, low fat, and skim milk (US Food and Drug Administration, 1996). In Austria and in Switzerland, the maximum level is reduced to 10 µg/ml for infant foods and 250 ng/kg of cheese. There are thus differences in the tolerance levels among countries, and many, including Egypt, have no legal limit for AFM₁ in milk and dairy products. In tropical and subtropical countries, especially in African countries, a particular attention should be used in monitoring milk and milk products other than those from cows, as well as feed. Furthermore, extensive and periodic surveys on the occurrence of aflatoxin and their metabolites in human breast milk should be performed, since a serious health hazard to mother, fetus, or infants could occur (Galvano, *et al.* 1996) therefore, the present study was carried out to determine the incidence of AFM₁ and AFB₁ in milk and different milk products which collected from Damietta governorate- Egypt.

MATERIALS AND METHODS

Sampling

All milk samples and dairy products were carried out during the period extended from summer 2002-2003. 20 of raw milk samples were collected from Damietta farms and 250 of different dairy products were collected from different places of Damietta governorate in Egypt, as Buffalo's raw milk, Cow's raw milk, Ice cream, Feta cheese, Cheddar cheese, Yoghurt, Requforti cheese, Aged Romi cheese, Fresh Romi cheese, Aged Domiati

cheese, Fresh Domiati cheese, Spread cheese, Processed cheese, Mish and Karish cheese. The samples were analysed for the presence of aflatoxins AFM₁ and AFB₁. All samples were examined in duplicates by Liquid Chromatography Mass Spectrometry Method. A representative milk and milk products samples were taken and kept frozen for analysis. Each sample was taken for analysis at the particular time interval.

Analytical methods:

Aflatoxin M₁ and B₁ Standards were obtained from SIGMA (Deisenhofen-Germany) and dissolved in 10 % MeOH/PBS (phosphate buffer saline). All solvents used for extraction, cleanup and liquid chromatography-mass spectrometry (LC-MS) were HPLC-grade. HPLC-grade water was provided using a Millipore Milli-Q purification system (Millipore, Eschborn, Germany)

1-Fluid Milk, Yoghurt and Ice Cream

According to AOAC Method (1995). 50 ml of fluid milk were shaken (at room temp.), by 10 ml of salt solution, and 120 ml of chloroform (CHCl₃) in 250 ml separator funnel 60 s. The layer was separated after about 2 min. and the lower of (CHCl₃) layer was drained into 150 ml Erlenmeyer. After that centrifugation of mixture was carried out to break emulsion. Then ca 10 g Na₂SO₄ to CHCl₃, were added and stirred occasionally 3 min., and filtered through paper whatman No:2 into 100ml graduate (volume was recorded). The final filtrate was saved for column chromatography.

2-Hard and soft cheeses

For hard and soft cheeses, 15 g were blended. Cheese was cut in to small pieces, with 1 ml of salt solution, 5 g diatomaceous earth, and 100 ml CHCl₃ 60 seconds in a 1 L blender Jar. The mixture was filtered through whatman (1) paper into 125 ml Erlenmeyer. Extract was treated as for fluid milk. Maximum filtrate was obtained from cheese extract by closing filter paper top and compressing entire paper and contents against funnel.

Column Clean up chromatography.

After evaporation of the remaining hexane by a slightly stream of nitrogen at 40 °C the extracts were applied to strong anion exchange (C₁₈) Cartridge (100) mg/1ml, phenomenex, Aschfenburg, Germany) which has been conditioned twice with 1 ml of aqua distilled water, following 1ml of acetonitrile (ACN). 1 ml from extracted sample was transferred to clean up then, the cartridge was washed twice with 1ml of aqua dist.water and 1 ml 10% ACN in aqua dist .water after that, column was washed and rinsed by 1 ml Aqua dist .water and dried by the use of vacuum for 2 min. Aflatoxin was eluted with 0.5 ml 30% ACN / aqua. dist. water .

The elute was evaporated under gentle steam of nitrogen at 40 °C and resolved in 200 µl mobile phase ACN /water (2/8 v/v).

LC-MS analysis.

Quantification and identification of aflatoxin was carried out using a HPLC-system (water 2690 Separations Module, Milford, AM) connected to a quadrupole mass spectrometer (VG platform 2) with electro spray ionisation source and a MassLynxTM data system (Micromass, Altrincham, UK). The conditions were as follows: Column temperature 35 °C, ionisation mode

ESP+, source temperature 60 °C, cone voltage 30 V. Separation was performed using a C₁₈ column (Xtra 150 X 2.1 i.d., 3.5 µm particle size (Waters, Eschborn, Germany) with guard column (1x 4 mm, phenomenex). The gradient started at 20% solvent A (acetonitrile), 70% solvent B (water), and 10% solvent C (water / formic acid 98-100%, 95/5 v/v) for 1 min. increased linearly to 80% A (10% B, 10% C, 18 min.), and was held for 2 min. at a flow rate of 0.2 ml/min. The injection volume was 7.5 µl., the eluent was split (1:20) and monitored in selected ion recording mode. Identification of aflatoxin was based on retention time and relative peak area of selected ions (m/z 722, 388, 300). For quantitation, the area of the quasi molecular ion peak (m/z 722) was compared to that of an external standard.

RESULTS AND DISCUSSIONS

The obtained results in table (1) revealed that, 30% of buffalo's milk samples had an average of 0.22 ppb of AFM₁ and 40% of cow's milk samples had an average of 0.25 ppb of AFM₁. for different cheese samples had an average of 0.21, 0.222, 0.54, 0.64, 0.669, 0.76, 0.826, 1.25, 1.9, and 1.98 ppb of AFM₁ was detected in Feta cheese, Cheddar cheese, aged Romi cheese, Requforti cheese, Processed cheese, Fresh Domiati cheese, Spread cheese, Karish cheese, aged Romi cheese and Fresh Romi cheese, respectively. There was also 0.045, 0.1 and 1.85 ppb of AFM₁ in Yoghurt, Ice cream and Mish, respectively.

Table (1): Aflatoxins M₁ and B₁ Detection in raw milk and Some Dairy products in Damietta-Egypt.

Type Dairy products	No. of Examined samples	NO. of possitive samples		Average of Aflatoxins contents (ppb)	
		M ₁	B ₁	M ₁	B ₁
Karish cheese	20	9	2	1.25	0.107
Mish	20	12	3	1.85	0.262
Processed cheese	20	9	2	0.669	0.112
Spread cheese	20	2	3	0.826	0.03
Fresh Domiati cheese	20	13	2	0.76	0.061
Aged Domiati cheese	20	11	2	0.54	0.074
Fresh Romi cheese	20	9	2	1.98	0.416
Aged Romi cheese	20	9	3	1.9	1.6
Requforti cheese	20	10	5	0.64	0.395
Yoghurt	20	3	2	0.045	0.045
Cheddar cheese	20	5	4	0.222	0.321
Feta cheese	20	5	3	0.21	0.111
Ice Cream	10	3	0.0	0.1	0.0
Buffalo's milk	10	3	0.0	0.22	0.0
Cow's milk	10	4	0.0	0.25	0.0

On the other hand, out of 20 samples of Karish cheese, processed cheese, fresh Domiati cheese, aged Domiati cheese, fresh Romi cheese and yoghurt only 2 samples of each had an average of 0.107, 0.112, 0.061, 0.074, 0.416 and 0.045 ppb of AFB₁ respectively . while 3 samples out 20 samples of Mish, Spread cheese, aged Romi cheese and Feta cheese had an average of 0.262, 0.03, 1.6, and 0.111 ppb respectively. The highest content of AFB₁

was found in Requforti cheese and Cheddar cheese; whereas ice cream, Buffalo's milk and cow's milk were found completely free from AFB₁. Many countries have carried out these studies about the incidence of AFM₁ in milk and other dairy products, in most of them, samples have been found to exceed the limit imposed by many countries. In all cases, all milk samples were below the maximum tolerated levels of AFM₁ in liquid milk. Unfortunately, in our country, in spite of the fact that the dairy industry has evolved a lot in the last years, as regards production levels and technology, most dairy products being above maximum permissible limit of AFM₁ (0.25 ppb) for cheese except for Feta cheese, Cheddar cheese, Ice cream and Yoghurt which were under permissible limit of AFM₁ (0.25 ppb). These results are in agreement with those mentioned by (Capei and Neri (2002), Garrido *et al.*, (2003) Rodriguez Velasco, *et al.* (2003) and Lopez, *et al.*, (2003).

All milk samples and other dairy products also were below the maximum tolerated levels of AFB₁ (5 ppb). Currently available research results further show that, the concentration of AFM₁ is 2.5 to 3.3 fold higher in many soft cheeses and 3.9 to 5.8 folds in hard cheeses than in milk from which these cheeses were made (Yousef and Marth, 1989). On the other hand, Karish cheese manufactured from milk contaminated with AFM₁ is not safe for human consumption, since the aflatoxin is not partially detoxified during processing and storage (Mashally, *et al.*, 1986). On the other hands, 200 samples of fresh Parmesan cheese were analysed by Barbieri *et al.*, (1994) and 18 showed presence of AFM₁ in low concentration (max. 0.190 ppb). Our results are in agreement with those mentioned by Sylos *et al.* (1996) who examined 152 samples of different milks, cheese and Yoghurt collected from supermarkets in Brazil during 1989-1990 and found that the average of AFM₁ contamination at 73-370 ppb. Similar survey in Kuwait of 54 samples of fresh full cream and skimmed milk, powdered milk, yoghurt, and infant formula were analysed for AFM₁ by HPLC. 28% were contaminated with AFM₁ with 6% being above the maximum permissible limit of 0.2 µg/l. Three fresh cow milk samples collected from a private local producer showed the highest level of 0.21 µg/l. (Srivastava, *et al.*, 2001). Our results disagreed with Cirilli *et al.*, (1989) who tested 66 samples of commercial Italian cheese (Mozzarella and Finites) for hydroxy-AFM₂ and found that, 18% of samples were contaminated with AFM₁ 280 – 1300 ppb and 45 with AFM₂ 340 – 870 ppb. Barrios, *et al.*, (1996) examined thirty five samples of commercial cheese, 9 fresh, 9 semi-cured or semi-ripened and 17 ripened made with different types of milk produced in the South of Spain for the presence of aflatoxin M₁ by HPLC. In 16 of 35 samples (45.71%) the presence of AFM₁ was detected in concentrations ranging between 20 and 200 ng/kg of cheese. In positive cases, the mean levels of AFM₁ were 105.33 ng/kg in ripened cheeses, 73.80 ng/kg in simi-ripened cheeses and 42.60 ng/kg in fresh cheeses. Panariti (2001) investigated the presents monitoring data on levels of aflatoxin M₁ in the farm-gate milk in Aizania. The monitoring included 120 evenly distributed samples collected in winter and summer from various farms all over the country. On average, the winter milk samples revealed higher concentrations of aflatoxin M₁ than the summer samples. Thirteen

percent of the winter samples resulted in above the 0.5 µg /kg level, as compared to 3% of the summer samples exceeding that level. One of the first survey in several surveys also have been conducted to determine if AFB₁ could be found in commercial cheese samples and to quantify the toxin by Polzhofer (1977), who showed that, no AFB₁ was found in 169 samples of 19 varieties of cheese. However, a later survey by (Kiermeir and Bohm 1971) of the same 19 varieties revealed that 34 of 222 samples were positive for AFB₁, in addition to natural cheeses, AFB₁ can also occur in process cheese. Kiermeier and Rumpf (1975) examined 115 samples of processed cheese and found two positive samples containing AFB₁. The existence of AFB₁ in milk may be due to contaminated feedstuffs not completely metabolised by cow to AFM₁, thus AFB₁ will be excreted in milk. Aflatoxin B₁ can get into dairy products from contaminated milk and from growth of the toxigenic *Aspergilli* on dairy products during the storage period. It is quite difficult to compare the present results with those mentioned in the literatures because of the wide differences between the countries in relation to feeding, animal and environmental factors, seasons, extraction and analysis procedures, and regulatory limit for aflatoxin in feeds and milk.

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الهيئة القومية للرقابة والبحوث الدوائية - القاهرة - جمهورية مصر العربية
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في هذه الدراسة جمعت ٢٧٠ عينة من الألبان ومنتجاتها من محافظة دمياط - مصر في الفترة التسدي لستدت ما بين صيف ٢٠٠٢ - ٢٠٠٣ للبحث عن مدى وجود الأفلاتوكسينات ب ١ ، م ١ حيث أن جميع العينات تم تحليلها مرتين بواسطة جهاز liquid chromatography mass spectrometry. ٣٠% من العينات المختبره كان متوسط محتواها من الأفلاتوكسين م ١ (٠.٢٢ و) جزء في البليون بينما ٤٠% من ألبان البقر كان متوسط محتواها من من الأفلاتوكسين م ١ (٠.٢٥ و) جزء في البليون . وبالنسبة لعينات الجبن المختلفة كان معدل وجود الأفلاتوكسين م ١ ٢١ ، ٢٢٢ ، ٥٤ ، ٦٤ ، ٦٦٩ ، ٧٦ ، ٨٢٦ ، ١٢٥ ، ١٠٩ ، ١٩٨ جزء في البليون لعينات جبن الفيتا ، تشيدر ، الدماطي القديم ، الريكفورت ، المطبوخ ، الدماطي الجديد ، الجبن الاسبريت ، الجبن القريش ، الرومي القديم وكذلك الجبن الرومي الجديد على التوالي كما كان معدل وجود الأفلاتوكسين م ١ ٤٥ ، ١٠ ، ١٨٥ جزء في البليون في كل من اليوجورت والاييس كريم والمش على التوالي ومن ناحية أخوى وجد أن في كل ٢٠ عينة تم فحصها من جبن القريش ، الجبن المطبوخ والجبن الدماطي الجديد والدماطي القديم والرومي الجديد واليوجورت وجد أن عينتان فقط لكل منهما كانت موجبة بمتوسط ١٠.٧ ، ١١٢ ، ١٠.٦١ ، ٠.٧٤ ، ٤١٦ ، ٠.٤٥ و جزء في البليون أفلاتوكسين ب ١ على التوالي بينما ٣ عينات من كل من ٢٠ عينة فحصت لكل من المش وجبن الاسبريت والجبن الرومي القديم وجبن الفيتا كانت موجبة بمعدل ٢٦٢ ، ٠.٣ ، ١٠ ، ١١ و جزء في البليون من الأفلاتوكسين ب ١ على التوالي . أعلى معدل وجود أفلاتوكسين ب ١ وجد في جبن الريكفورت وجبن تشيدر بينما الأييس كريم ولبن الجاموس والبقر الخام كان خاليا تماما منه . في كل الأحوال جميع الألبان مجال الدراره لم تتعدى الحد المسموح به دوليا من الأفلاتوكسين م ١ في اللبن . ولسوء الحظ على الرغم من التقدم الانتاجي والتكنولوجي في الصناعة في بلادنا في السنوات الأخيرة الا أن وجود الأفلاتوكسين م ١ في معظم منتجات الألبان كان أعلى من الحد المسموح به دوليا وهو ٢٥ جزء في البليون ما عدا جبن الفيتا وجبن تشيدر والاييس كريم واليوجورت هي التي كانت أقل من الحد المسموح به دوليا وهو ٢٥ جزء في البليون من الأفلاتوكسين م ١ . جميع الألبان ومنتجاتها مجال هذه الدراسة كانت أقل بكثير من الحد المسموح به دوليا في مستوى الأفلاتوكسين م ١ وهو ٥ جزء في البليون من م ١ . ومن الصعب مقارنة هذه النتائج مع نتائج معظم الباحثين في دول العالم لاختلاف الظروف البيئية ومعدل تلوث الأعلاف ونوع وطرق التغذية وطرق التصنيع وطرق التحليل والاستخلاص للأفلاتوكسين .