

## OCCURRENCE OF AFLATOXINS M<sub>1</sub> AND B<sub>1</sub> IN MILK AND SOME MILK PRODUCTS IN MANSOURA, EGYPT.

Motawee, M.\*; K. Meyer\*\* and J. Bauer\*\*

\* National Organization for Drug Control and Research, P.O. 29 -Egypt

\*\* TU-München.Institut Für Tierhygiene- D-85354 freising-Germany

### ABSTRACT

Aflatoxins are toxic metabolites found in foods and feeds. Aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) appears in milk as direct result of the intake of Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) contaminated feed by dairy cows. Our aim was to carry out a study to determine the incidence of AFM<sub>1</sub> and AFB<sub>1</sub> in milk collected from farms and supermarkets in Mansoura -Egypt. 130 different milk samples were collected of which 25 samples of Buffalo's raw milk and 25 of Cow's raw milk during winter 2002-2003. 80 another milk samples were collected from supermarkets. The samples were analyzed for the presence of aflatoxins AFM<sub>1</sub> and AFB<sub>1</sub>. All samples were examined as duplicates by liquid chromatography mass spectrometry (LC-MS). The averaged concentration of AFM<sub>1</sub> was 0.288 ppb for buffalo's raw milk samples, and 0.312 ppb for cow's raw milk samples. One sample of Cow's milk out of twenty five contained above the maximum permissible limit of 0.5 ppb. Baby's milk, Butter milk, Dried whole milk and ultra high temperature milk (U.H.T) were completely free from aflatoxin M<sub>1</sub> and AFB<sub>1</sub>. On the other hand, all samples were found to be free from aflatoxin B<sub>1</sub>, except for one out of ten samples of Buffalo's milk, powdered milk and skim milk. All tested samples did not exceed the permissible limit of AFB<sub>1</sub>. In all cases, AFM<sub>1</sub> and AFB<sub>1</sub> levels were lower than the limit recommended for dairy products.

**Keywords:** Aflatoxin; Milk; LC-MS Method

### INTRODUCTION

Aflatoxin is a collective term that refers to a group of highly toxic and carcinogenic secondary metabolites produced by many strains of molds as *Aspergillus flavus* and *Aspergillus parasiticus* during their growth on foods and feeds or laboratory media (Marth, 1979, Rhona, *et al*, 1982, Wood, 1989 and Piva, *et al* 1995). Aflatoxins B<sub>1</sub> and M<sub>1</sub> are known as hepatotoxins and hepatocarcinogens and the deleterious effects in humans, especially children, of consuming AFM<sub>1</sub>-contaminated milk are of considerable concern (Qian, *et al.*, 1984, Chu, 1991). From published genetic studies, AFM<sub>1</sub> seems to produce DNA damage in cultured rodent cells and gene mutation in bacteria (Capei, Neri, 2002). Aflatoxin M<sub>1</sub> is a major metabolite of AFB<sub>1</sub> found in milk from animals that have consumed feeds contaminated with aflatoxin B<sub>1</sub> (Blanco *et al.*, 1993; Govaris, *et al.*, 2001 and Ciapara *et al.*, 1995). The carry-over of AFB<sub>1</sub> to AFM<sub>1</sub> in milk is linearly correlated with milk yield and the values as 6 % have been reported at µg daily intake levels of AFB<sub>1</sub> (Veldman, *et al*, 1992). The carry-over of AFB<sub>1</sub> 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 (FDA), AFM<sub>1</sub> should not exceed 0.5 ppb (Stoloff, 1980 and Van Egmond, 1989). The FDA has established also an action level of 0.5 ppb in whole, low fat, and skim milk (Govaris., *et al* (2001) The current permissible levels for AFM<sub>1</sub> in milk ranged from 0.05 to 0.5 ppb except for infant milk, for which lower levels exist (Maria and Herminia, 2000). The exposure of infants

to AFM<sub>1</sub> is something to worry about because milk is a main nutrient for all children (Lopez *et al.*, 2003). The European Union has established an acceptable limit of AFB<sub>1</sub> in feed for animals of 10 µg/kg (Moss, 1998). Milk is a good source of many nutrients such as proteins and calcium and are mainly consumed by children. Therefore, humans are potentially exposed to these toxic metabolites and it is generally assumed that neither storage nor processing determine a reduction of aflatoxin M<sub>1</sub> content (Galvano *et al.* 1996). Therefore, the present investigation was carried out to survey the occurrence of AFM<sub>1</sub> and AFB<sub>1</sub> in different milk samples collected from the market of Mansoura governorate in Egypt.

## MATERIALS AND METHODS

### Sampling

130 different samples were collected from different places of Mansoura -Egypt; during the period extended from winter 2002-2003. 50 samples of raw Buffalo's and Cow's milk, and 80 other samples of sterilized milk, powder milk, pasteurized milk, UHT milk, baby's milk, butter milk and dried whole milk were obtained from supermarkets. Representative milk samples were taken and kept frozen for analysis. The samples were analyzed for the presence of aflatoxins AFM<sub>1</sub> and AFB<sub>1</sub>. All samples were examined in duplicates by Liquid Chromatography Mass Spectrometry (LC-MS).

### Analytical methods

Aflatoxin M<sub>1</sub> and B<sub>1</sub> 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 prepared using a Millipore Milli-Q purification system (Millipore, Eschborn, Germany)

### Sample preparation

Since aflatoxins are subject to light degradation, sample preparation was carried out in the absence of daylight. Before analysis, laboratory glassware were soaked in 2 M Na<sub>2</sub>SO<sub>4</sub> for several hours and rinsed with distilled water to remove all traces of acid before use; since the use of non-acid-washed glassware for handling of aqueous solution may cause a loss of aflatoxin (Tuinstra *et al.* 1993).

### Extraction

#### 1-Fluid Milk

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 (CHCl<sub>3</sub>) in 250 ml separator funnel 60 s. The layer was separated after about 2 min. and the lower of (CHCl<sub>3</sub>) layer was drained into 150 ml Erlenmeyer. After that centrifugation of mixture was carried out to break emulsion. Then ca 10 g Na<sub>2</sub>SO<sub>4</sub> to CHCl<sub>3</sub>, 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-Powder milk.**

For powdered milk, 1g was mixed with 10 ml H<sub>2</sub>O and was reconstituted by shaking in 250 ml separator funnel. The sample was treated with salt solution and CHCl<sub>3</sub> as for fluid milks. The emulsion was broken by centrifugation at 4000 rpm. for 10 min.

### **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 (C<sub>18</sub>) Cartridge (100 mg/1ml, phenomenex, Aschffenburg, 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 MassLynx<sup>TM</sup> 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 C<sub>18</sub> 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 DISCUSSION**

From the results presented in table (1) it could be seen that all milk samples contained AFM<sub>1</sub> except for baby's milk, butter milk, dried whole milk and ultra high temperature milk were free of this toxin. The average of AFM<sub>1</sub> content in the different milk samples amounted from 0.04 to 0.312 ppb. The highest mean values of this toxin were 0.228 and 0.312 ppb in buffalo's and cow's milk respectively. About 30% of Buffalo's milk samples were contained AFM<sub>1</sub> and about 20% of Cow's milk samples, pasteurized milk samples and powdered milk samples were contaminated also with AFM<sub>1</sub>. On the other hand, about 10% of sterilized milk and skim milk samples contained also AFM<sub>1</sub>. One samples of Cow's milk out of twenty five samples contained above the maximum permissible limit of 0.5 ppb. Our results are in

agreement with Kim, et al. (2000), Roussi, et al. (2002) and Rodriguez Velasco et al. (2003).

However, the results differed from country to another country and also from time to time. Srivastava, et.al (2001) from Kuwait showed that, the highest level for three fresh cow's milk samples was 0.21(ppb). There was no contamination with AFM<sub>1</sub> in powdered milk and infant formulas Kiermeier and Mucke (1972) made a survey of commercial raw milk samples in West Germany and found that among 36 milk samples 12 samples were contaminated with AFM<sub>1</sub> in concentration 0.04- 0.25 ppb. Markaki and Melissari,(1997) stated that thirty- two samples of pasteurized milk contained aflatoxin M<sub>1</sub> at levels of 0.025-0.05 ppb. Furthermore Suarez (1988) studied the presence of AF M<sub>1</sub> in 47 samples of commercial UHT milk in northwest Spain and found 14 positive for AFM<sub>1</sub>. Fremy, (1982) noted that in France, milk contains between 0.05 and 0.5 ppb AFM<sub>1</sub>, with the amount varying according to season. On the other hand, Balata, et al., (1996) tested twenty four samples of raw camel's milk in part of north western coastal desert, Egypt. The collected samples were analyzed for detection of AFM<sub>1</sub> in 25% of camel's milk samples, with a mean value of 0.55 ppb (0.3-0.85).

**Table (1): Detection of Aflatoxins M<sub>1</sub> and B<sub>1</sub> in different milk samples**

Type of milk	No. of Examined samples	No. of positive samples		Average of Aflatoxins contents (ppb)	
		M <sub>1</sub>	B <sub>1</sub>	M <sub>1</sub>	B <sub>1</sub>
Buffalo's raw milk	25	8	1	0.228	0.084
Cow's raw milk	25	5	0.0	0.312	0.0
Sterilized milk	10	1	0.0	0.04	0.0
Powder milk	10	2	1	0.06	0.14
Pasteurized milk	10	2	0.0	0.15	0.0
* UHT milk	10	0.0	0.0	0.0	0.0
Skim milk	10	1	1	0.05	0.04
Baby's milk	10	0.0	0.0	0.0	0.0
Butter milk	10	0.0	0.0	0.0	0.0
Dried whole milk	10	0.0	0.0	0.0	0.0

\* Ultra High Temperature

They indicated that the incidence of AFM contamination is often higher in some countries where cows are fed with greater amount of dry feed specially in summer. Our results disagreed with those obtained by most authors as Panariti (2001), who found in Albania, thirteen percent of the winter samples resulted in above the 0.5 ppb level, as compared to 3% of the summer samples exceeding that level. Kriengsag Saitanu,(1997) found greater AFM<sub>1</sub> than 0.5 ppb in 18% (48) of the samples including raw milk, pasteurised milk (20/63), ultra high temperature milk (7/60), sterilised milk (3/30). All powdered milk samples were negative for aflatoxin M<sub>1</sub> except two samples with less than 0.1 ppb. Motawee, (2003) noted that survey in Giza- Egypt about 10% of different milk samples were contaminated with 0.162, 0.288, 0.324, 0.234, 0.144 and 0.162 ppb of AFM<sub>1</sub> for skim , pasteurized , sterilized, UHT, powder, and baby milk, respectively, by using TLC method the results

were found due to the affinity of the methods used for detection of the aflatoxin. Garrido, *et al* 2003 observed that 20.9% of the milk samples in Brazil exceeded the European Union limit of AFM<sub>1</sub>. On the other hand, in obtained results revealed that all milk samples were found to be completely free from aflatoxin B<sub>1</sub>, except one sample of Buffalo's milk, powdered milk and skim milk out ten samples of all of them. All samples, therefore, we found either absolutely free from aflatoxin B<sub>1</sub>, or contained considerably lower concentrations less than the permissible limit of AFB<sub>1</sub>. These results are in agreement with Motawee, (2003), who indicated that the existence of AFB<sub>1</sub> in milk might be due to contamination of feedstuff not completely metabolized by the liver of cow to AFM<sub>1</sub>, thus AFB<sub>1</sub> will be excreted in milk, or from recontamination of milk samples after milking by AFB<sub>1</sub>.

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## وجود الأفلاتوكسين ب 1 م 1 في اللبن وبعض منتجاته في المنصورة - جمهورية مصر العربية.

محمود محمد مطاوع و كاراستين ماير ويوهان باور  
الهيئة القومية للرقابة والبحوث الدوائية - القاهرة - جمهورية مصر العربية  
معهد صحة الحيوان - جامعة ميونخ - جمهورية ألمانيا الاتحادية .

من المعروف أن الأفلاتوكسينات ب 1 من السموم الفطرية الناتجة عن التمثيل الغذائي لبعض الفطريات المعروفة عند نموها في أعلاف الحيوانات وبالتالي يمكن لتلك السموم أن تتواجد في اللبن. في صورة أفلاتوكسين م 1 . والهدف الرئيسي من هذه الدراسة هو البحث عن وجود تلك السموم في اللبن المختلفة. تم فحص مائه وثلاثون عينة من اللبن المختلفة وبعض منتجاتها والتي جمعت من السوق التجاري وكذلك من المزارع المختلفة بمحافظة الدقهلية - مصر ، خمسة وعشرون عينة من اللبن الجاموسي الخام وخمسة وعشرون عينة من اللبن البقري الخام في الفترة من شتاء ٢٠٠٢-٢٠٠٣ وثمانون عينة أخرى من لبن مختلف جمعت من السوق التجاري بالدقهلية حيث أن جميع العينات مجال البحث أختبرت مرتين بواسطة جهاز LC-MS من بين ٢٥ عينة للبن الخام الجاموسي وجد أن ٨ عينات مرجحة لوجود الأفلاتوكسين م 1 حيث أن متوسط معدل التلوث ب ٢٢٨ و جزء في البليون وعينة واحدة فقط كانت موجبة ب 1 الأفلاتوكسين م 1 بمعدل متوسط ٣١٢ و جزء في البليون حيث وجد في اللبن الخام البقري عينة واحدة فقط تعدت الحد المسموح به دوليا وهو ٥٥ جزء في البليون من الأفلاتوكسين م 1 ومن ناحية أخرى وجد أن جميع عينات لبن الأطفال والذبد واللبن الفريز واللبن المعامل بالبيسترة الفوقية كانت خالية تماما من وجود الأفلاتوكسينات م 1 ، ب 1 .

جميع العينات مجال البحث والدراسة كانت خالية من الأفلاتوكسين ب 1 ماعدا عينة واحدة فقط من بين عشرة عينات لكل من اللبن الخام الجاموسي ولبن البودرة واللبن الفريز . على جميع الأحوال جميع العينات المختبرة لا تتعدى الحد المسموح به دوليا من الأفلاتوكسين بالنسبة لللبان ومنتجاتها .