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AFLATOXIN M_1 IN MILK BY IMMUNOAFFINITY COLUMN CLEANUP WITH TLC/HPLC DETERMINATION

Luzia Shundo*; Myrna Sabino

Instituto Adolfo Lutz, Seção de Química Biológica, São Paulo, SP, Brasil

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ABSTRACT

During 2002 and 2003, a total of 107 samples of raw, pasteurized and ultrahigh treated temperature (UHT) milk commercialized in the cities of São Paulo and Marília (SP) were analyzed for the presence of aflatoxin M_1 (AFM_1). AFM_1 was detected in 79 (73.8%) of milk samples, ranging from <0.02 to 0.26 μg/L.The samples were analyzed using an immunoaffinity column for cleanup and a thin layer chromatography for determining AFM_1. The parameters, such as recovery, repeatibility, detection and quantification limit were evaluated to optimize this method (in-house). Based on spiked samples, the recovery values ranged from 85.83 to 73.86% at levels of 0.010-0.50 μg/L, respectively, and the relative standard deviation for repeatibility ranged from 7.73 to 2.08%. The quantification limit was 0.02 μg/L. The results of some samples analyzed by this method demonstrated a satisfatory correlation when compared with High Performance Liquid Chromatography (HPLC). In conclusion, immunoaffinity column cleanup gave excellent results for recovery, sensibility and sample through put. Despite the high rate of occurrence of AFM_1 in samples in both cities, the contamination level could not be considered a serious public health hazard, according to Brazilian legislation.

Key words: milk, aflatoxin M_1, thin layer chromatography, immunoaffinity column

INTRODUCTION

In October 2002, the Brazilian Ministry of Health (1) established the maximum acceptable limit of aflatoxin M_1 (AFM_1) in 0.5 μg/L for fluid milk and 5 μg/L for powder milk following the MERCOSUL Technical Regulations (12). Two groups of countries have adopted different regulatory limits: 0.05 μg/L and 0.5 μg/L respectively. The European Commission by the Regulation CE 1525/98 and Directive 199/29/CE has implemented a limit of 0.05 μg/L of AFM_1 in liquid milk, whereas the Food and Drug Administration (FDA) has established an action level of 0.5 μg/L in whole, low fat and skim milk.

Although AFM_1, the hydroxylated metabolite of Aflatoxin B_1 (AFB_1) is less carcinogenic and mutagenic than AFB_1, it exhibits a high level of genotoxic activity and certainly represents a health risk because of its possible accumulation and linkage to DNA. Monitoring of AFM_1 levels in animal studies has shown that the rate between the amount of AFB_1 ingested by cows and the quantity excreted in milk is usually 0.2 to 4% (8). Milk has the greatest demonstrated potencial for introducing AFM_1 into the human diet and the possible presence of AFM_1 in milk and their products represents a worldwide concern mainly because these products are widely consumed by children, the major consumers, who are more susceptible to the adverse effects of micotoxins.

The incidence of AFM_1 contamination is often higher in commercial milk than in raw farm milk because of uncontaminated bulk milk is often diluted with contaminated samples. However, as result of this dilution, high AFM_1 contamination levels in commercial milk seldom occur (15). Efficient control of AFM_1 in milk requires efficient and easily performed analytical methods, allowing low quantification which increases the percentage of positive samples.

In Brazil, there is little data in the literature on the occurrence of AFM_1 in raw and commercialized milk. The purpose of this work was to investigate of AFM_1 in raw, pasteurized and UHT

*Corresponding Author. Mailing address: Instituto Adolfo Lutz, Seção de Química Biológica, Av. Dr. Arnaldo, 355, Vila Clementino. 01246-902, São Paulo, SP, Brasil. Tel.: (+5511) 3068-2922, Fax: (+5511) 3062-5363. E-mail: lushundo@ial.sp.gov.br

milk commercialized in the cities of São Paulo and Marília (SP), Brazil, in 2002/2003. The samples were analyzed by thin layer chromatography (TLC), immunoaffinity column (IC) and some samples were also submitted to HPLC for results comparison.

MATERIALS AND METHODS

Samples

A total of 107 samples of milk were analyzed to determine AFM_1. Twenty-two samples of raw milk from grazing cows supplemented with animal feed were collected from farms across the region of Marília (SP), Brazil, in 2002 and 2003. In the same period, 42 samples of UHT milk and 43 samples of pasteurized milk were collected from supermarkets in the cities of São Paulo and Marília (SP). Both the raw and pasteurized milk were kept on the ice during the transportation and raw milk samples were analyzed immediately upon arrival at the laboratory. The pasteurized milk samples were stored at 5ºC and analyzed before expiration.

Determination of AFM_1

Milk samples were analized for the presence of AFM_1 using an immunoaffinity column for clean up and HPLC with fluorescence detection for determination based on the method of Grosso *et al.* (7) and Shundo *et al.* (21). The milk samples (100 mL) were centrifuged at 2000x*g* for 15 minutes and the upper fat layer was discarded. The skimmed milk was passed through an immunoaffinity column (AFLAPREP M, R-Biopharm Rhône Ltd). The column was washed with water (40 mL) to remove extraneous non-specific material. Following, the AFM_1 bound to the antibody was released by the elution with 2.5 mL acetonitrile-methanol (3:2;v/v) and 2.5 mL methanol. The eluate was evaporated to dryness using a stream of N_2.

The TLC procedure was developed, dissolving the AFM_1 residue in 150 µL of toluene-acetonitrile (9:1;v/v). On the TLC plate (TLC aluminum sheets, 20x20 cm, Silica gel 60), 50 µL of sample and spots of working standard were applied. The plate was developed in chloroform-acetone-isopropanol (87:10:3;v/v). After the plate had dried, it was examined under long wave light (366 nm). The concentration and chemical identity of AFM_1 was determined according to Scott (20). Recovery tests were performed to determine the efficacy of the analytical method by spiking aflatoxin raw milk samples with known amounts of AFM_1 (0.01, 0.02, 0.03, 0.05, 0.3, 0.5 µg/L) and submitting them to free extraction procedures in 5 replicates. AFM_1 standard was purchased from Sigma Chemical Co (St Louis, MO, USA).

HPLC was performed on GBC system (GBC, Dandenong, Victoria, Australia) equipped with a LC 1110 HPLC pump, a fluorescence detector (model LC 1255) at wavelengths of 360 and 430 nm for excitation and emission, respectively. The LC column was a LiChrosorb C_{18} (250 x 4 nm, 10 µm – Merck, Darmstadt, Germany). The mobile phase consisted of acetic acid 2% aqueous solution-acetonitrile-methanol (40:35:25;v/v) and the flow rate was 1 mL/min. (13). Linearity was determined to be in the range 0.01-0.5 µg/L of AFM_1. Recovery test were performed by spiking aflatoxin-free raw milk samples with known amounts of AFM_1 (0.01, 0.02, and 0.05 µg/L).

Some samples analyzed by IC/TLC were submitted to HPLC determination in order to investigate the relationship between the two analytical methods and evaluated the efficiency of TLC in determining AFM_1 at low levels. The statistical analysis applied to compare these methods (TLC and HPLC) was based on the "t" Student's test for paired samples, utilizing the Bioestat 3.0 software program.

RESULTS AND DISCUSSION

Recoveries of AFM_1 by TLC and HPLC

Based on spiked samples, values of recoveries by TLC were 85.8, 85.2, 85.8, 80.8, 80.0, 73.9% and the relative standard deviation (RSD) were 7.7, 6.9, 6.0, 6.0, 4.4 and 2.1% to levels 0.01, 0.02, 0.03, 0.05, 0.3 and 0.5 µg/L respectively. The quantification limit established was 0.02 µg/L and the detection limit was 0.01 µg/L. Quantitative TLC requires skilled visual interpretation so divergent visual acuity was taken into consideration in establishing the detection limit in this study.

For HPLC, the calibration curves obtained by least-squares linear regression were linear, in the range 0.010-0.5 µg/L, with correlation coefficient of 0.99987. The recoveries were 86.1, 83.7 and 84.5% in levels of 0.01, 0.02, 0.05 µg/L. The quantification limit was 0.01 µg/L.

The statistical analysis applied to compare these methods (TLC and HPLC) calculating a $p = 0.6059$ ($t_{calculated} = -0.5345$, $t_{table} = 2.2622$ and $\alpha = 0.05$) found no significative difference between the two methods compared (Table 1).

Table 1. Comparison between TLC and HPLC results for AFM_1 levels in contaminated milk.

Samples	TLC* (µg/L)	HPLC** (µg/L)
1	0.012	0.014
2	0.035	0.034
3	0.014	0.013
4	0.030	0.031
5	0.146	0.136
6	0.051	0.056
7	0.234	0.231
8	0.263	0.278
9	0.077	0.073
10	0.085	0.092

*TLC – Thin Layer Chromatography;
*HPLC – High Pressure Liquid Chromatography.

According Trucksess (24), the number of publications on TLC has declined, but this is not necessarily reflect the extent of its worldwide. Apparently, TLC methods are used routinely but information on their use is not published.

The TLC and HPLC methods employed in this study were optimized in-house and have been utilized as routine in our laboratory

Occurrence of AFM_1

The incidence and levels of AFM_1 are summarized in Table 2. From 107 samples analyzed, 79 (73.8%) were contaminated with AFM_1. AFM_1 was detected at low levels (<0.050 µg/L) in 72 (67.3%) of the samples, while 7 (6.5%) samples had the highest value of >0.05 µg/L and none of the samples exceeded the Brazilian legislation (0.5 µg/L for fluid milk)

In Brazil, Garrido *et al.* (6) and Prado *et al.* (16) found a high incidence of AFM_1 (79.9% and 82.0%, respectively) in low concentrations. Both studies were performed by HPLC. In others studies conducted on AFM_1 contamination (2,14,18, 22,23) the authors found low incidence with varied levels.

As Visconti *et al.* (26) observed in studies conducted in Italy, the poor sensitivity of the results in the first studies performed in Brazil should be attributed to poor sensitivity of the analytical methods used. In this study, the increased extraction efficacy provided by immunoaffinity column resulted in a high sensitivity to TLC determination.

In comparison with recent data reporting the incidence of AFM_1 reported by others investigators (3,4,5,9,10,11,15,17, 19,25) the results of this study are comparable with those presented in other countries, showing high incidence at low levels. According to Galvano *et al.* (4), in recent years the incidence of AFM_1 has been balanced on the one hand by the higher efficiency of analytical methods, and on the other hand by the setting of a stricter regulatory limit for aflatoxins in feed and milk.

According to Velasco *et al.* (25) and Markaki and Melissari (10) the present study revealed no significant seasonal differences among contaminated samples. In Brazil, pasture is the major source of cattle feed. The highest level of contamination was found in raw milk (0.26 µg/L), collected in winter. However, this originated from a single (farm) producer, and was thus an isolated case.

Based on the milk samples taken from the cities of São Paulo and Marília (SP), Brazil, during 2001-2002, the occurrence of AFM_1 does not appear to be a serious public health hazard, according to Brazilian legislation. However, aflatoxins are recurrent and their formation in foods and feeds may sometimes be difficult to avoid. For this reason, specific regulations to control AFB_1 in feeds and a systematic AFM_1 monitoring program performed by accurate and reliable analytical techniques constitutes an important strategy for protecting milk consumers.

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RESUMO

Determinação da aflatoxina M_1 em leite por coluna de imunoafinidade, CCD/CLAE

No período de 2002 e 2003, cento e sete amostras de leite cru, pasteurizado e UHT (ultrahigh treated temperature) comercializadas nas cidades de São Paulo e Marília (SP) foram analisadas para determinar Aflatoxina M_1 (AFM_1). A aflatoxina M_1 foi detectada em 79 (73,8%) amostras, variando de 0,02 – 0,26 µg/L. As amostras foram analisadas utilizando coluna de imunoafinidade para limpeza e cromatografia em camada delgada para determinação da AFM_1. Para a otimização do método, os parâmetros avaliados foram: recuperação, repetitividade, limite de detecção e limite de quantificação. Baseado em adição de padrão nas amostras, os valores das recuperações variaram de 85,83% e 73,86% em níveis de 0,01-0,5 µg/L, respectivamente, e o desvio padrão relativo para repetitividade variou de 7,73-2,08%. O limite de quantificação foi de 0,02 µg/L. Os resultados das amostras analisadas por este método tiveram boa correlação quando comparadas com a Cromatografia Líquida de Alta

Table 2. Occurrence of AFM_1 in raw, pasteurized and UHT milk samples.

Type of milk	Nº of samples	Contamination µg/L (%)	Frequency distribution of samples in (µg/L) (%)		
			> LD**	0.02-0.05	>0.05
Raw	22	0.013 (59.1)	05 (22.8)	06 (27.3)	02 (9.0)
Pasteurized	43	0.032 (74.4)	19 (44.2)	11 (25.6)	02 (7.2)
UHT*	42	0.034 (80.9)	11 (26.2)	20 (47.6)	03 (7.1)
Total	107	0.079 (73.8)	35 (32.7)	37 (34.6)	7 (6.5)

*UHT – Ultra Hight Temperature; **LD – Detection Limit.

Eficiência. Concluindo, a utilização de coluna de imunoafinidade fornece excelentes resultados na recuperação, sensibilidade e apresenta fácil operacionalidade. Apesar da alta incidência de aflatoxina M_1 em amostras em ambas as cidades, o nível de contaminação pode não ser considerado um sério problema de saúde pública, de acordo com a legislação brasileira.

Palavras-chave: leite, aflatoxina M_1, cromatografia em camada delgada, coluna de imunoafinidade

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