AFLATOXIN M₁ IN MILK BY IMMUNOAFFINITY COLUMN CLEANUP WITH TLC/HPLC DETERMINATION

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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 M₁ (AFM₁). AFM₁ 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 AFM₁. The parameters, such as recovery, repeatability, 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 repeatability 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 satisfactory correlation when compared with High Performance Liquid Chromatography (HPLC). In conclusion, immunoaffinity column cleanup gave excellent results for recovery, sensitivity and sample throughput. Despite the high rate of occurrence of AFM₁ 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 M₁, thin layer chromatography, immunoaffinity column

INTRODUCTION

In October 2002, the Brazilian Ministry of Health (1) established the maximum acceptable limit of aflatoxin M₁ (AFM₁) 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 AFM₁ 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 AFM₁, the hydroxylated metabolite of Aflatoxin B₁ (AFB₁) is less carcinogenic and mutagenic than AFB₁, 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 AFM₁ levels in animal studies has shown that the rate between the amount of AFB₁ ingested by cows and the quantity excreted in milk is usually 0.2 to 4% (8). Milk has the greatest demonstrated potential for introducing AFM₁ into the human diet and the possible presence of AFM₁ 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 AFM₁ 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 AFM₁ contamination levels in commercial milk seldom occur (15). Efficient control of AFM₁ 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 AFM₁ in raw and commercialized milk. The purpose of this work was to investigate of AFM₁ in raw, pasteurized and UHT
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 AFM₁. 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 AFM₁
Milk samples were analyzed for the presence of AFM₁ 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 2000xg 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 AFM₁ 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 N₂.

The TLC procedure was developed, dissolving the AFM₁ 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 AFM₁ 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 AFM₁ (0.01, 0.02, 0.03, 0.05, 0.3, 0.5 μg/L) and submitting them to free extraction procedures in 5 replicates. AFM₁ 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 C₁₈ (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 AFM₁. Recovery tests were performed by spiking aflatoxin-free raw milk samples with known amounts of AFM₁ (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 AFM₁ 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 AFM₁ 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, ttable = 2.2622 and α = 0.05) found no significative difference between the two methods compared (Table 1).

Table 1. Comparison between TLC and HPLC results for AFM₁ levels in contaminated milk.

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

*TLC – Thin Layer Chromatography; 
HPLC – High Pressure Liquid Chromatography.
According to 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<sub>1</sub>**

The incidence and levels of AFM<sub>1</sub> are summarized in Table 2. From 107 samples analyzed, 79 (73.8%) were contaminated with AFM<sub>1</sub>. AFM<sub>1</sub> 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<sub>1</sub> (79.9% and 82.0%, respectively) in low concentrations. Both studies were performed by HPLC. In others studies conducted on AFM<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> has been balanced on the one hand by the better efficiency of analytical methods, and on the other hand by the setting of a stricter regulatory limit for aflatoxins in feed and milk.

As 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<sub>1</sub> 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<sub>1</sub> in feeds and a systematic AFM<sub>1</sub> monitoring program performed by accurate and reliable analytical techniques constitutes an important strategy for protecting milk consumers.

**ACKNOWLEDGMENTS**

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**RESUMO**

Determinação da aflatoxina M<sub>1</sub> 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<sub>1</sub> (AFM<sub>1</sub>). A aflatoxina M<sub>1</sub> 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<sub>1</sub>. 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<sub>1</sub> in raw, pasteurized and UHT milk samples.**

<table>
<thead>
<tr>
<th>Type of milk</th>
<th>Nº of samples</th>
<th>Contamination μg/L (%)</th>
<th>Frequency distribution of samples in (μg/L) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt; LD**</td>
</tr>
<tr>
<td>Raw</td>
<td>22</td>
<td>0.013 (59.1)</td>
<td>05 (22.8)</td>
</tr>
<tr>
<td>Pasteurized</td>
<td>43</td>
<td>0.032 (74.4)</td>
<td>19 (44.2)</td>
</tr>
<tr>
<td>UHT*</td>
<td>42</td>
<td>0.034 (80.9)</td>
<td>11 (26.2)</td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td>0.079 (73.8)</td>
<td>35 (32.7)</td>
</tr>
</tbody>
</table>

Efi ciência. Concluindo, a utilização de coluna de imunoaf inidade fornece excelentes resultados na recuperação, sensibilidade e apresenta fácil operacionalidade. Apesar da alta incidência de aflatoxina M1 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 M1, cromatografia em camada delgada, coluna de imunoaf inidade

REFERENCES


Aflatoxin M1 en milk