Occurrence of aflatoxins M_1 and M_2 in goat milk marketed commercialized in the region of Ribeirão Preto-SP, Brazil

Ocorrência de aflatoxinas M_1 e M_2 em leite de cabra comercializado na região de Ribeirão Preto-SP, Brasil

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ABSTRACT

The aim of the present study was to determine the occurrence of aflatoxins M_1 and M_2 in commercially available goat milk samples collected from supermarkets located in Ribeirão Preto region, SP, Brazil. A total of 39 goat milk samples manufactured in three Brazilian states, consisting of 15 Ultrahigh-temperature milk samples and 24 frozen pasteurized milk samples were analyzed following AOAC, 2000 protocol. None of the analyzed milk samples was contaminated with aflatoxin M_2**,** and aflatoxin M_1 was detected in 7 (18%) samples in a range of from 20 to 98ng/L. According to these findings both the occurrence and the level of aflatoxins M_1 are low**,** and the goat milk samples manufactured in three Brazilian states is devoid of aflatoxin M_2**.** Although, further surveillance studies on the occurrence of these toxins in goat milk are required, because these data are limited**.** On the other hand, the Brazilian climatic conditions may promote the occurrence of aflatoxin B_1 and B_2 in feeds**,** which can be metabolized into aflatoxin M_1 and M_2.

Key words. aflatoxin M_1, aflatoxin M_2, goat milk, mycotoxins.

RESUMO

O objetivo deste estudo foi de determinar a ocorrência e o nível de aflatoxinas M_1 e M_2 em amostras de leite de cabra coletadas em supermercados localizados na região de Ribeirão Preto, SP, Brasil**.** Foram analisadas 15 amostras de leite Ultra Alta Temperatura e 24 amostras pasteurizadas congeladas. As amostras foram analisadas de acordo com a AOAC, 2000. Nenhuma das amostras analisadas apresentou contaminação com aflatoxina M_2. Foi detectada M_1 em 7 (18%) amostras na faixa de 20 a 98ng/L. O resultado deste estudo mostra que a ocorrência e o nível de aflatoxinas M_1 são baixas e ausência de aflatoxina M_2 em amostras de leite de cabra produzidas por três estados do Brasil. Em função de escassas informações sobre esse tema, torna-se imprescindível o estudo dessas aflatoxinas em leite de cabra em outras regiões do Brasil, pois as condições climáticas do país favorecem a ocorrência da aflatoxinas B_1 e B_2 na ração dos animais**,** que conseqüentemente são metabolizadas em M_1 e M_2.

Palavra-chave. aflatoxina M_1, aflatoxina M_2, leite de cabra, micotoxinas.

INTRODUCTION

Aflatoxins are toxic metabolites produced by fungi, and are potent liver toxins and most animal species exposed to these mycotoxins show signs of liver disease ranging from acute to chronic. Mammals animals, like cow, goat, sheep and so on, fed rations containing aflatoxin B_1 and aflatoxin B_2 excrete in their milk metabolites, aflatoxin M_1 and aflatoxin M_2, respectively[1].

Milk and milk products are a good source of many nutrients such as proteins and calcium. Usually, the cow milk is the most consumed, however there are a significant percentage of the population that has cow milk allergies and gastro-intestinal disorders[2]. Goat milk could substitute cow milk because, study of comparison of goat milk infant formula versus cow milk infant formula shows that these two formula were similar in terms of protein quality[3] and growth of infants fed with these two formulas[4]. Besides these, researches demonstrated some beneficial effect of goat milk, comparing to cow milk: on the metabolism of iron and copper in control rats, especially those with malabsorption syndrome[5] and on the nutritive utilization of protein and on magnesium bioavailability, especially in animals with resection of the distal small intestine[6].

Fisberg et al.[7] studied the acceptability of goat milk in preschool children of municipal nurseries in São Paulo City and concluded that it represents an excellent alternative to substitute cow milk, after the first year of life, and there was no case of intolerance or allergy during the study.

Competent national authorities establish the tolerance limits for aflatoxins in milk, consequently, there are differences in tolerance levels among countries and many countries have no legal limit for aflatoxins in milk and dairy products. The *Codex alimentarius*[8] recommend and the MERCOSUR (Brazil, Argentina, Paraguay and Uruguay) has established a limit of 500ng/L of aflatoxins for milk[9].

There are several studies of incidence of aflatoxins in cow milk, but there are only few surveys focusing on goat milk. In Brazil, we have not been found literature on the incidence of these toxins in goat milk.

It is important to investigate the occurrence of alfatoxins M_1 and M_2 in this product, for the reason that, in Brazil, the goat milk consumption is increasing, mainly, by the children with cow milk allergies.

MATERIAL AND METHODS

Materials and reagents

(a) Aflatoxin M_1 standard, 2,3,6a,9a-tetrahydro-9a-hydroxy-4-methoxycyclopenta[c]furo[3',2',:4,5]furo[2,3-h][1]benzopyran-1,11-dione – (Sigma Chemical Company, USA).

(b) Aflatoxin M_2 standard, 2,3,6a,8,9,9a-hexahydro-9a-hydroxy-4-methoxycyclopenta[c]furo[3',2',:4,5]furo[2,3-h][1]benzopyran-1,11-dione – (Sigma Chemical Company, USA).

(c) Stock solution of M_1 was prepared in acetonitrile at concentration of 565 ng/mL, and concentrations determined according to Association of Official Analytical Chemists (AOAC), method 986.16, 971.22 and 970.44[10]. Working solutions were prepared by appropriate dilution in acetonitrile:benzene.

(d) Stock solution of M_2 was prepared in acetonitrile at concentration of 898 ng/mL, and concentrations determined according Association of Official Analytical Chemists, method 986.16, 971.22 and 970.44[10]. Working solutions were prepared by appropriate dilution in acetonitrile:benzene.

(e) Solvents – Isopropyl alcohol and acetonitrile (EM Science, USA) were LC grade;

(f) Water – distilled, deionized water was purified in a milli-Q purification system (Millipore, USA).

(g) Columns for solid phase extraction, RP-18 (500 mg), were obtained from Merck (Germany).

(h) Mobile phase consisted of water:isopropyl alcohol: acetonitrile (80:12:8).

(i) trifluoroacetic acid (TFA), analytical reagent grade.

(j) LC column – Shim-pack CLC-ODS (M), 4.6x250 mm, 5µm (Shimadzu, Japan).

(k) Guard column – Shim pack CLC G-ODS, 4x4mm, 5µm (Shimadzu, Japan).

(l) All other chemicals were analytical reagent grade.

Apparatus

(a) Liquid chromatograph – pump (model LC-10ADVP), RF-10AXL fluorescence detector (excitation and emission wavelengths were 365 nm and 400 nm, respectively), column oven (CTO-10AVP), system controller (SCL-10AVP), degasser (DGU-14A), software (CLASS VP version 6.12) all from Shimadzu Instruments (Japan), and Rheodyne LP injector with a 20µL loop from Rheodyne (USA).

(b) Spectrophotometer – Hach (USA).

(c) Vortex mixer – Fanem (Brazil).

Samples

Twenty-four samples of commercial pasteurized frozen goat milk and 15 Ultra High Temperature (UHT) goat milk were collected from market in the region of Ribeirão Preto, SP, Brazil. These samples milk were produced in different Brazilian States: 5 from Rio Grande do Sul, 10 from Rio de Janeiro, and 24 from São Paulo.

Procedures

The method used to analyse aflatoxins M_1 and M_2 in goat milk samples was AOAC[10] Official Method 986.16 Aflatoxins M_1 and M_2 in fluid milk and liquid chromatographic method, in duplicate. The extraction through cartridges, C_{18} Sep-Pak, was time consuming, lengthening the sample preparation. The problem was solved by increasing the quantity of hot water added to thin the milk from 20 to 40mL. This way, the sample preparation became faster. Aflatoxins M_1 and M_2 were extracted from warm, diluted milk on a C_{18} Sep-Pak cartridge, eluted with ether through a silica column, eluted with dichloromethane: alcohol, and derivatized with

TFA. Liquid chromatography peaks were detected fluorometrically and compared with the standard-TFA derivative. The chromatography analyses were carried out in an oven controlled temperature (30°C), the flow-rate was 0.5 mL/min.

All samples analyzed were within their expiry date. The pasteurized frozen milk samples were stored at -20°C until analysis.

Linearity, recovery, precision and determination limits

The linearity of the method was carried out in the range of 0.9 – 18 ng/mL (11-225 ng/L in milk) of M_1 and 0.926 – 18.52 (12 – 232 ng/L in milk) of M_2. To determine the efficacy of the analytical method used for the recovery tests, milk samples were spiked with known amount of aflatoxins M_1 and M_2 (concentration of 222 and 109 ng/L, respectively) and submitted to the extraction procedures in 8 replicates. The precision of the method was calculated as the relative standard deviation (RSD). The detection and quantitation limit were determined based on the standard deviation of the response and the slope[11].

RESULTS AND DISCUSSION

The results obtained show the chromatograms after derivatization with TFA, (a) of goat milk free from aflatoxins M_1 and M_2, (b) of a sample spiked with aflatoxins M_1 and M_2 and (c) of a naturally contaminated sample (Figure 1). The calibration curves obtained by least-squares linear regression were linear in range of 11 – 225ng/L (M_1) and 12 – 232ng/L (M_2) with correlation coefficients of 0.9997 (M_1) and 0.9992 (M_2). The recoveries were 98.0 and 89.8% for M_1 and M_2, respectively. The precision was adequate with a relative standard deviation of 4.6 (M_1) and 5.0% (M_2). The detection limit was defined as the minimum level at which the analyte can be reliably detected and was 6 for M_1 and 8ng/L for M_2. The quantification limit was established as the lowest concentration of an analyte, using an appropriate standard or sample, could be determined with an acceptable level of accuracy and precision[12]. It was 19 and 23ng/L for M_1 and M_2, respectively.

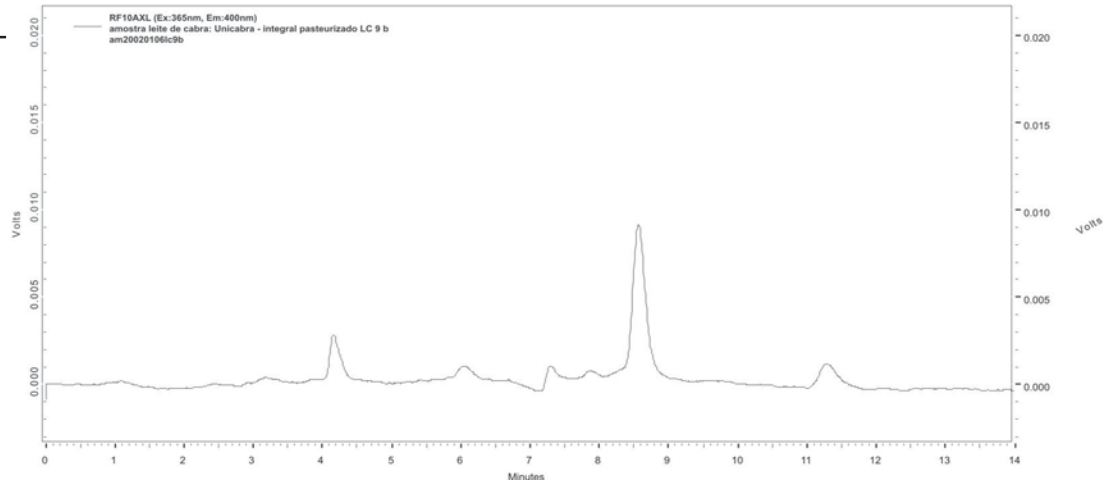
The results obtained in our laboratory, during the method validation, demonstrated that this method is efficient to analyze aflatoxins M_1 and M_2 in goat milk samples with good precision, accuracy. The limit of detection and quantification are low enough to be used to analyze aflatoxins M_1 and M_2 in small quantities. In addition to that, sample preparation is adequate to obtain an extract without interfering peaks, as we can see in the non-contaminated goat milk chromatogram (Figure 1).

The Table 1 shows the results of the levels of aflatoxins M_1 found in the samples analyzed. In all samples the level of aflatoxin M_1 were below the 500ng/L limit permitted by the MERCOSUR Technical Regulations[9] and recommended by the *Codex alimentarius*[8]. The results were compared with more regulations strict like the one adopted by the European Union. One (2.6%) sample of pasteurized milk was above the 50ng/L limit for milk[13]. Four samples of pasteurized milk (10.3%) were above 25ng/L limit for infant formulae and folow-on formulae, including infant milk and follow-on milk and dietary foods for special medical purposes intended specifically for infants[14]. In spite the fact of that four samples of pasteurized milk contained aflatoxin M_1 above 25ng/L and no UHT sample was contaminated there were no significant differences between their aflatoxin M_1 levels.

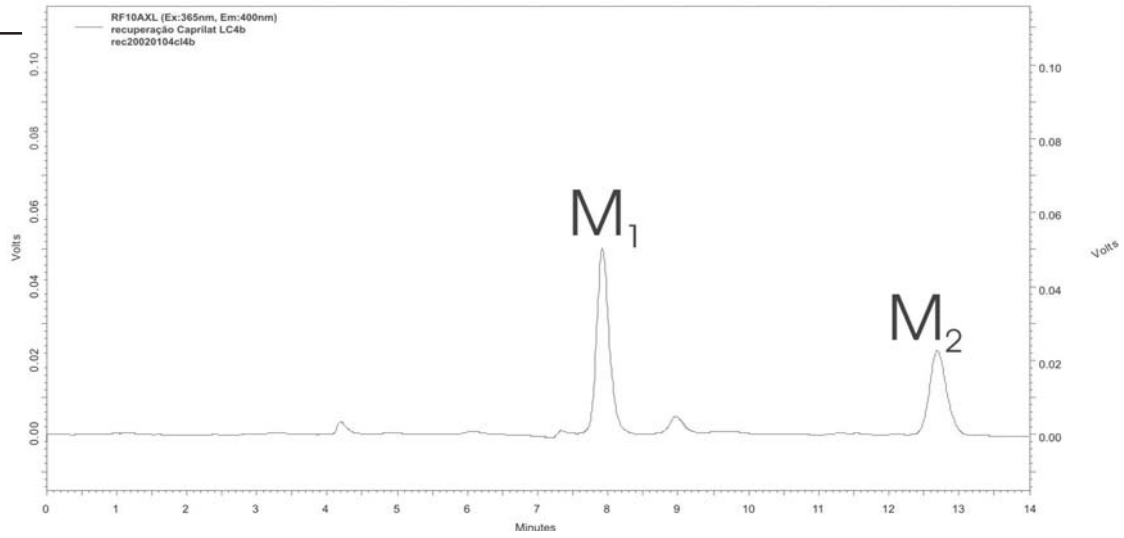
Table 1. Occurrence of aflatoxin M_1 in goat milk samples produced from trhee different states of Brazil and commercialized in the region of Ribeirão Preto, SP, Brazil.

Range of aflatoxin M_1 concentration (ng/L)	Type of Milk		Total (n= 39) n (%)
	Pasteurized (n= 24) n (%)	UHT (n= 15) n (%)	
Not detected	14 (58.3)	1 (6.7)	15 (38.5)
6 – 20	4 (16.7)	13 (86.7)	17 (43.6)
20 – 50	5 (20,8)	1 (6.7)	6 (15.4)
50 – 500	1 (4.2)	0 (0)	1 (2.6)
> 500	0 (0)	0 (0)	0 (0)

a)



b)



c)

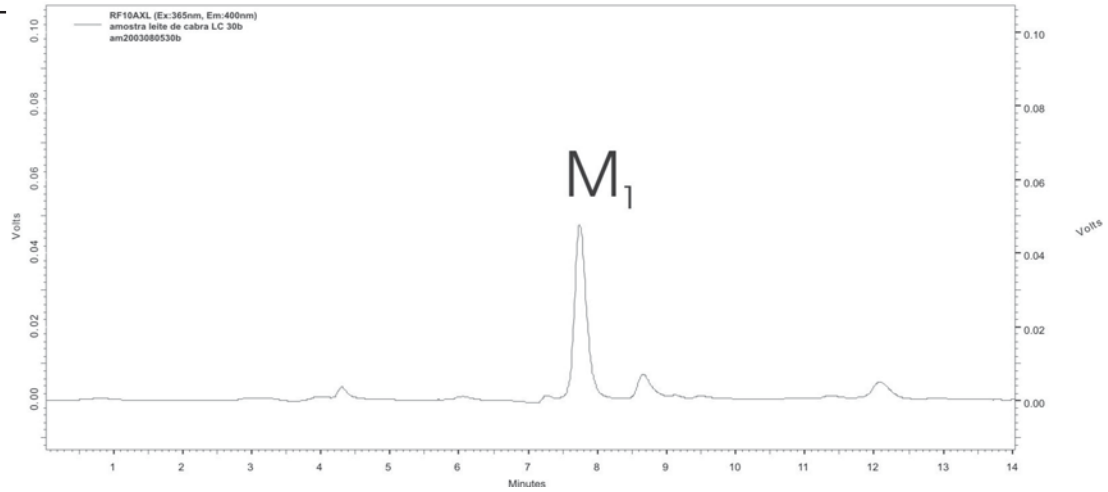


Figure 1. Chromatograms after derivatization with TFA, (a) of a non-contaminated goat milk (scale 5 time larger than b and c), (b) of a sample spiked with aflatoxin M_1 and M_2 and (c) of a naturaly contaminated sample. Chromatographic conditions: ODS column; mobile phase, water:isopropyl alcohol:acetonitrile (80:12:8); flow-rate, 0.5mL/min; fluorescence detection, λ_{ex} = 365nm, λ_{em} = 400nm.

There are several studies on the incidence of aflatoxins in cow milk in different countries like Korea[15], Colombia[16], Argentina[17], Greece[18], Brazil[19-25] and so on, but surveys on the occurrence of aflatoxins in goat milk are rare. Roussi et al[18] analyzed samples of goat, sheep and cow milk collected in Greece, compared the level of aflatoxin M_1 in these three types of milk, and observed that the contamination was lower in raw sheep and goat milk than cow milk. The incidence rate was limited to 66.7 and 40%, respectively, and none of the positive samples contained aflatoxin M_1 at levels > 50ng/L. They concluded that this might be due to the fact that during the winter and spring, lactating sheep and goats in Greece are grazing and are feeding consequently fedding less when compared with dairy cattle.

In Brazil, there isn't any published study about incidence of aflatoxin M_1 and M_2 in goat milk, only in cow milk that has been analysed and the results show low incidence and levels, like ours results for goat milk. Souza et al.[22] analyzed 110 milk samples and detected low levels of contamination with aflatoxin M_1 in 24.5% of them. Oliveira et al.[25] found M_1 in 77.1% (37) of the samples (0.011 – 0.251ng/mL). Garrido et al[24] studied the occurrence of aflatoxins M_1 in 139 samples of pasteurized and UHT cow milk commercialized in Ribeirão Preto, SP. They detected the toxin in 29 (20.9%) of the samples in the range of 50 – 240ng/L. Taveira and Midio[23] in São Paulo, SP and Sylos et al.[19] in Campinas, SP, also found low incidence and levels of the toxin in cow milk. Sylos et al.[19] concluded that a possible explanation for this is that cows in the area of Campinas graze all year round, and are rarely exposed to feed.

In this study the levels of aflatoxins in goat milk were found to be low, in spite of the fact that feed are used in same area where the goat milk samples originated, like cities of São Paulo state.

In our study, none of the milk samples analyzed were found to contain aflatoxin M_2 above the detection limit of the method used, 7 ng/L. Corrêa et al.[20] and Garrido et al.[24] analyzed 144 samples of raw cow milk and 139 samples of pasteurized and UHT milk, in Brazil, and didn't detect M_2 in any sample.

These results show that aflatoxins in goat milk in Brazil isn't a serious public health problem, but, it's necessary to continue the surveillance on the occurrence of these toxins in goat milk samples in order to achieve a more complete picture. Several reasons indicate this course of action: data are limited, the country is large, and its climatic conditions may favour the incidence of aflatoxin B_1 and B_2 in feeds that after being consumed by the goat is metabolized in M_1 and M_2.

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