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Occurrence of aflatoxin M_1 in bovine milk samples consumed in different regions of Brazil

Ocorrência de aflatoxina M_1 em amostras de leite bovino consumido em diferentes regiões do Brasil

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ABSTRACT

Two hundred and fifty-seven samples of milk proceeding from different geographical regions of Brazil were analyzed for determining the presence of aflatoxin M_1 (AFM_1). The AFM_1 extraction was carried out using immunoaffinity column, separated by reversed-phase (C-18) high performance liquid chromatography (HPLC), and quantified by fluorescence detector. The Limits of Quantification (LOQ) were 0.008 µg/kg and 0.080 µg/kg to the fluid and the powder milk, respectively. AFM_1 were detected in 209 (81.3 %) samples, being 26 (63.4 %), 105 (84.0 %) and 78 (85.7 %) of pasteurized, UHT (Ultra-high Temperature) and powder milk, respectively. The highest concentration of AFM_1 in powder milk was found in one sample from Minas Gerais (1.210 µg/kg). In UHT and pasteurized milk, the highest levels were detected in one sample from Sergipe (0.120 µg/kg) and one sample from Goiás (0.050 µg/kg), respectively. None of the samples analyzed in this study exceeded the Brazilian legal limits for AFM_1.

Keywords. mycotoxins, immunoaffinity column, high performance liquid chromatography, exposure assessment.

RESUMO

Duzentas e cinquenta e sete amostras de leite provenientes das diferentes regiões geográficas do Brasil foram analisadas para realizar a determinação de aflatoxina M_1 (AFM_1). As AFM_1 foram extraídas por meio de colunas de imunoafinidade, separadas por cromatografia líquida de alta eficiência em fase reversa (C-18) e quantificadas por detector de fluorescência (CLAE-FL). Os limites de quantificação (LQ) foram de 0,008 µg/kg e 0,080 µg/kg para o leite fluido e em pó, respectivamente. AFM_1 foi detectada em 209 (81,3 %) amostras, sendo 26 (63,4 %), 105 (84,0 %) e 78 (85,7 %) para o leite pasteurizado, UHT (*Ultra-high Temperature*) e em pó, respectivamente. A maior concentração de AFM_1 no leite em pó foi encontrada em uma amostra proveniente de Minas Gerais (1,210 µg/kg). No leite UHT e pasteurizado, os maiores níveis foram encontrados em uma amostra de Sergipe (0,120 µg/kg) e Goiás (0,050 µg/kg), respectivamente. Nenhuma amostra analisada ultrapassou os limites da legislação brasileira em vigor para AFM_1.

Palavras-chaves. micotoxinas, coluna de imunoafinidade, cromatografia líquida de alta eficiência, estimativa de exposição.

INTRODUCTION

In the last decades, there is an increasing concern on quality of animal products consumed by Brazilian population. Among these products, dairy products represents an important segment in the agribusiness, once concerning milk and their products play an important role in the food supply, job generation and income for society. It is placed among the six most important products of Brazilian agriculture and livestock sector. Besides its economic relevance, the consumption of milk is of great importance in view of its high nutritional value and represents a natural source of carbohydrates, proteins, fats, vitamins and minerals in different dispersion forms. Milk production and consumption in Brazil is increasing at an annual rate of 4 % with a significant growth potential for the next years. Among the MERCOSUL member countries, the Brazilian milk production account for 66 % of the total volume production[1].

Even though the milk consumption offers health benefits, milk could also be a source of toxic substances such as Aflatoxin M_1 (AFM_1). Derived from Aflatoxin B_1 (AFB_1) presents in feed consumed by dairy cattle, the presence of AFM_1 is considered undesirable due to its carcinogenic properties[2,3]. Although AFM_1 is less carcinogenic and mutagenic than AFB_1, it exhibits similar genotoxic activity to AFB_1 demonstrates in studies conducted in animals and certainly represents health risk to population exposed to this mycotoxin[4]. According to IARC (International Agency for Research on Cancer), AFM_1 is classified as Group 2B agent[5].

The presence of AFM_1 in milk and milk products represents a worldwide concern, mainly because these products are widely consumed by children who are more susceptible to the adverse effects of mycotoxins. The maximum tolerable limits of AFM_1 established by Brazilian Ministry of Health[6] are 0.5 µg/kg and 5.0 µg/kg for fluid and powder milk, respectively.

The aim of this study was to investigate the occurrence of AFM_1 in milk samples proceeding from different Brazilian geographical regions, determined by high performance liquid chromatography (HPLC) with fluorescence detection (FL), using immunoaffinity column (IC) for clean-up.

MATERIAL AND METHODS

Samples

A total of 257 samples of ultra-high temperature (UHT) treated milk (n=125), powder milk (n=91) and pasteurized milk (n=41) proceeding from different geographical regions of Brazil, were analyzed for AFM_1, during 2010. The samples were collected at random according to market availability by local health offices (state and municipal) and sent to the laboratory properly cooled, when it is required. All information on samples was taken from the labels.

Reagents and standard

AFM_1 standard was purchased from Sigma Chemical (St. Louis, MO, USA) and prepared as described by Scott[7]. Acetonitrile and methanol were HPLC grade from Merck (Darmstadt, Germany) and others chemicals were analytical grade from Merck (Darmstadt, Germany). Water was ultrapurified by Synergy UV System (Millipore SAS, Molsheim, France).

Preparation of samples

Fluid milk: Homogenized milk samples were centrifuged 15 min/1780xg/4 ºC. After centrifugation the upper cream layer was discarded and the skimmed milk was used to be applied to immunoaffinity column extraction.

Powder milk: Ten grams of powder milk was dissolved with ultrapurified water and make up to 100 mL by stirring for 10 min/approximately 37 ºC. Following, these samples were treated as fluid milk (described previously).

Immunoaffinity column clean-up

An aliquot of 50 mL of skimmed milk at room temperature was passed through immunoaffinity column ($AflaM_1$ HPLC-VICAM). The proceedings of washing and elution steps were performed following the manufacturer's instructions. The eluate was evaporated to dryness using a N_2 stream, the residue was dissolved

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in 400 µL mobile phase and an aliquot (20 µL) was injected into the HPLC equipment[8].

Determination of AFM_1 by HPLC with FL

HPLC was performed on GBC system (GBC, Dandenong, Victoria, Australia) equipped with a LC 1110 HPLC pump, LC 1255 fluorescence detector. The HPLC column was a LiChrosorb C-18 (250 x 4 mm, 5 µm – Merck, Darmstadt, Germany) and guard column was Phenomenex C-18 (4 x 3 mm). The mobile phase consisted of acetic acid 2 % aqueous solution-acetonitrile-methanol (40:35:25; v/v/v) and flow rate was 0.6 mL/min. The excitation and emission wavelengths were 360 nm and 430 nm, respectively.

Linearity was expressed by the linear correlation coefficient (r) of analytical curve obtained from five different points (triplicate) of external standard calibration with concentration ranging from 1 - 10 ng/mL of AFM_1 solution, equivalent to 0.008 µg/kg – 0.080 µg/kg for fluid milk and to 0.080 µg/kg – 0.800 µg/kg for powder milk.

The Limits of Detection (LOD) and LOQ values were calculated as 3-fold and 10-fold, respectively of the standard deviation concentration plus the mean values of five replicate of blank matrix.

Recovery experiments were carried out in triplicate by spiking aflatoxin-free fluid milk with amounts of AFM_1 standard solution, resulting as final concentration 0.010 µg/kg, 0.020 µg/kg and 0.050 µg/kg.

To use the same units described in the legislation, the concentration of the samples taken in µg/L was expressed in µg/kg using the factor 0.971 (average density of milk) as the volume/weight compensation.

RESULTS AND DISCUSSION

The analytical curve obtained by least-squares regression were linear presenting linear in the range 1 - 10 ng/mL (equivalent to 0.008 µg/kg - 0.080 µg/kg for fluid milk and 0.080 µg/kg - 0.800 µg/kg for powder milk) with correlation coefficient of 0.9992. The recoveries were 87.2 %, 85.5 % and 80.7 % at levels of 0.010 µg/kg, 0.020 µg/kg and 0.050 µg/kg and the relative standard deviations were 5.5 %, 5.0 % and 4.2 %, respectively, for fluid milk. The LOD were 0.003 µg/kg and 0.030 µg/kg and the LOQ were 0.008 µg/kg and 0.080 µg/kg, respectively, for fluid and powder milk. **Figure 1** shows chromatograms of AFM_1 standard and a naturally contaminated UHT milk sample.

The incidence and the range of AFM_1 levels are presented in **Table 1**. From a total of 257 milk samples, AFM_1 was found in 209 (81.3 %) and none of samples exceeded the Brazilian legislation[6].

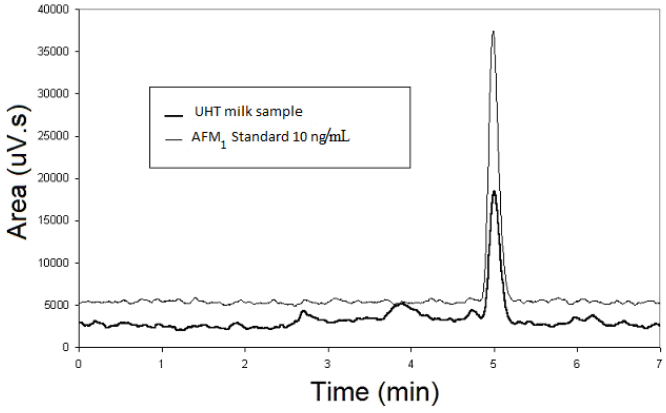

Figure 1. Chromatograms of AFM1 standard and a naturally contaminated UHT milk sample

Table 1. Occurrence of AFM_1 in pasteurized, UHT and powder samples

Type of milk	Nº of samples	Nº of contamined samples (%)	Frequency distribution of AFM_1 (µg/kg)		
			LOD*⊢0.05	0.05⊢0.10	≥0.10
Pasteurized	41	26 (63.4)	26	0	0
UHT	125	105 (84.0)	97	7	1
Powder	91	78 (85.7)	9	16	53
Total	**257**	**209 (81.3)**	**132**	**23**	**54**

* Limits of Detection (LOD) = 0.003 µg/kg and 0.030 µg/kg for fluid and powder milk, respectively

The frequency of AFM_1 in UHT and powder milk, as well as its distribution in different Brazilian geographic regions were summarized in **Tables 2** and **3**. AFM_1 determination in pasteurized milk was carried out in samples originating from Brazilian Southeast (n=36) and Central west (n=5) region. AFM_1 was found in 21 (58.3 %) and 5 (100 %) of samples, respectively.

The results of the present study indicate a high incidence of AFM_1 in all type of milk samples. Although the number of pasteurized milk samples might not be considered enough to comparison, in a general way the incidence of the AFM_1 in fluid milk presented major variations among the different regions when compared with powder milk.

Table 2. Distribution of UHT milk samples by geographic region, number of positive samples and range of AFM_1 concentration

Region	Nº of samples	≥ LOD* (%)	Range (µg/kg)
Southeast	29	28 (96.6)	LOD-0.100
Central west	30	25 (83.3)	LOD-0.041
Northeast	29	21 (72.4)	LOD-0.120
North	11	6 (54.5)	0.010-0.029
South	26	25 (96.2)	LOD-0.033
Total	**125**	**105 (84.0)**	

* Limit of Detection (LOD) = 0.003 µg/kg

Table 3. Distribution of powder milk samples by geographic region, number of positive samples and range of AFM_1 concentration

Region	Nº of samples	≥ LOD* (%)	Range (µg/kg)
Southeast	39	36 (92.3)	LOD-1.210
Central west	28	23 (82.1)	0.036-0.378
Northeast	10	7 (70.0)	LOD-0.140
North	7	6 (85.7)	LOD-0.760
South	7	6 (85.7)	0.117-0.420
Total	**91**	**78 (85.7)**	

*Limit of Detection (LOD) = 0.030 µg/kg

The highest concentration of AFM_1 was found in Northeast region (0.120 µg/kg) and in Southeast region (1.210 µg/kg) for fluid and powder milk, respectively. In Brazil, the largest producer states are located in the Southeast (Minas Gerais state), Central west (Goiás state) and South (Paraná and Rio Grande do Sul state) regions[9].

As summarized in **Table 4**, different studies carried out in Brazil reported varied levels both in frequency of AFM_1 in milk and its levels of contamination.

The differences between results observed in these studies may be explained by the higher efficiency of analytical methods with better extraction and clean up steps, as well as improvement of separation and detection process occurred along the past years. In addition, ELISA (Enzyme Linked Immunosorbent Assay) techniques have been used to determine presence of AFM_1 in milk[25-27]. This method provides speed and high sensitivity although its considered a screening method.

In comparison with recent data, the results of this study are comparable with the incidence of AFM_1 reported by others investigators in different countries[25-31], showing high incidence and predominantly low levels.

The estimated daily intake (EDI) was calculated on UHT milk, considering its availability and increased consumption of this type of milk in the last decades[32]. On the basis of mean concentration of AFM_1 in UHT milk (0.021 µg/kg) and an intake of 400 mL of milk for 23 kg as body weigh (bw) for children[8], the ingestion of AFM_1 was 0.365 ng/kg bw. For adult, the EDI was 0.123 ng/kg bw, assuming a body weigh of 60 kg and milk consumption of 350 mL. The results found in this study was similar to Santili et al[10] which calculated EDI was 0.358 ng/kg bw and 0.120 ng/kg bw for children and adult, respectively, for fluid milk. Santos et al[12] found an EDI for fluid and powder milk of 0.468 ng/kg bw for adolescents and 0.384 ng/kg bw for adult. In a study conduced in 2009 Shundo et al[8] found an EDI of 0.23 ng/kg bw and 0.08 ng/kg bw for children and adult, respectively, for fluid milk.

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Table 4. Occurrence of AFM_1 in cattle milk in Brazil

Type of milk	Nº of samples	Nº of positive samples (%)	Range (µg/kg)	Method	Reference
raw	635	334 (52.6)	0.012-0.725	HPLC	10
UHT	152	133 (87.5)	----	HPLC	11
different types	42	42 (100.0)	0.010-0.810	ELISA	12
raw	30	11 (36.7)	0.010-0.645	HPLC	13
different types	125	119 (95.2)	0.010-0.200	HPLC	8
raw	50	21 (42.0)	0.010-0.645	HPLC	14
fluid	48	37 (77.1)	0.011-0.251	HPLC	15
fluid	139	111 (79.9)	-----	HPLC	16
different types	61	50 (82.0)	0.006-0.077	HPLC	17
raw	42	10 (23.8)	0.29-1.97	ELISA	18
different types	110	5 (4.5)	-----	ELISA/TLC	19
reconstituted	300	33 (11.0)	0.01-1.000	ELISA	20
different types	144	0 (0.0)	---	TLC	21
fluid	224	4 (1.8)	traces-2.000	TLC	22
different types	100	1 (1.0)	0.100-1.700	TLC	23
raw	50	9 (18.0)	0.100-1.700	TLC	23
fluid	6	3 (50.0)	0.025-0.500	Fluorodensitometry	24

HPLC: High performance liquid chromatography; ELISA: Enzyme linked immunosorbent assay; TLC: Thin-layer chromatography; traces: ‹ 0.025 µg/kg

Leblanc et al[33] estimated a daily intake in french population in 0.09 ng/kg bw for adult and 0.22 ng/kg bw per day for children. In Spain, Cano-Sancho et al[25] reported values of 0.305 ng/kg bw for the adult population and Duarte et al[34] described an EDI of 0.08 ng/kg bw per day for adult Portuguese citizen.

The mycotoxins especially the AFM_1 are usually present at low levels in food, and the chronic effects take the greatest role to health. In addition since it is a genotoxic carcinogens, no tolerable daily intake (TDI) can therefore be set. The JECFA (Joint Expert Committee on Food Additives)[4] concluded that daily exposure as low as <1 ng/kg bw does not contributes to the risk of liver cancer and recommended to reduce the levels of this type of substances to limits as low as reasonable achievable (ALARA principle)[35].

Data and information of AFM_1 occurrence available in literature are used by internationally recognized organization such as Codex Alimentarius Commission (CAC), Food Drug and Administration (FDA) and European Communities (EC) to carry out studies of exposure assessment and set the maximum tolerable limit of these substances in food. Relating to this mycotoxin regulation, the Commission of the European Communities[36] has set more strict limits (0.050 µg/kg) than established in our country. This low level in European countries has in turn resulted in the fairly stringent regulation of AFB_1 in complementary feed stuffs in dairy cattle in the EC.

CONCLUSION

Although none of samples exceeded the Brazilian legislation, the presence of AFM_1 in milk represents an important public health problem, mainly because it is consumed by the infant population, who are more susceptible to the toxic and carcinogenic effects.

Besides, the aflatoxins are recurrent and their formation in food and feed may sometimes be difficult to avoid due to the fact that these contamination have a directly relation with the climatic conditions such as temperature and humidity. For these reasons, an effective strategy to control AFB_1 in feed and a systematic AFM_1 monitoring program under obligatory mycotoxin regulation limits, altogether with an accurate and validated analytical technique constitutes an important strategy to reduce their health risk and economic loss.

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