

Aflatoxin M₁ Contamination in Grated Parmesan Cheese Marketed in Rio de Janeiro - Brazil

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ABSTRACT

This study aimed to determine the occurrence of AFM₁ contamination in the samples of grated parmesan cheese marketed in the Metropolitan Region of Rio de Janeiro - Brazil. Thirty samples representing 10 major brands marketed in the region were analyzed by High Performance Liquid Chromatography with fluorescence detection (HPLC-FLD) after purification with immunoaffinity column. The method showed recovery values within the range of 70-90%, with RSD lower than 15% and limits of detection and quantification below the maximum level allowed by the European Commission for the presence of AFM₁ in cheeses. The mycotoxin was identified in 18 (60%) of the grated cheese samples tested. The highest value corresponded to 0.69 ± 0.02 µg/kg and the mean for all the analyzed samples was 0.16 µg/kg. All the samples were lower than the limit established by the Brazilian legislation (2.5 µg/kg) for AFM₁ in cheeses in general. However, eight samples (26.7%) presented AFM₁ levels above the tolerance limit of 0.25 µg/kg adopted by the European Commission. These results indicated that AFM₁ levels in the grated cheese consumed in Rio de Janeiro - Brazil were relatively high and it could provide a potential hazard for the public health.

Key words: mycotoxin, dairy products, HPLC-FLD

INTRODUCTION

Brazil is a country with a humid tropical climate, which is favorable for the growth of several fungi that produce mycotoxins. The aflatoxins B₁, B₂, G₁ and G₂ produced by *Aspergillus flavus* and *A. parasiticus* are considered the most relevant group in Brazil due to their high incidence and the toxic effects they have on humans and animals (Arana et al. 2011; Cardoso et al. 2011; Hoeltz et al. 2012). When ingested by an animal, AFB₁ is biotransformed in the liver and excreted in the milk as AFM₁ (Hussein and Brasel 2001). AFM₁ is classified by the International Agency for

Research on Cancer as a carcinogenic agent (IARC 2002). Oliveira et al. (2010) described levels of aflatoxin contamination in the feed for dairy cattle ranging from 1 to 19.5 µg/kg and aflatoxin M₁ in milk of these animals between 0.010 to 0.0645 µg/L in Brazil. Once present in milk, the toxin remains stable during the cheese-making process such as HTST and UHT pasteurization, coagulation, acidification, maturation and others (Deveci et al. 2007; Manetta et al. 2009; Fernandes et al. 2012).

Recent studies have reported the contamination of AFM₁ in Brazilian dairy products and reveal the high incidence of this mutagenic mycotoxin

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(Prado et al. 2008; Shundo et al. 2009; Iha et al. 2011). This represents a serious public health problem because cheeses are consumed by all age groups from infants to the aged (Sadeghi et al. 2009). In 2011, the National Agency of Sanitary Surveillance of Brazil (ANVISA) established the maximum tolerated limit for AFM₁ in cheeses in general as 2.5 µg/kg (Brazil 2011). The evaluation of contamination in cheese by AFM₁ is very important because cheese is an important product marketed in Brazil. In 2011, the formal production of cheese was 812,000 tonnes, which ranked the country as the sixth largest producer. However, the annual consumption of cheese by Brazilians is still small, with about 4 kg per person (ABIQ 2011). Among the different cheeses produced in the country, is the grated parmesan cheese, a ready-for-consumption product elaborated from the grating of one or up to four varieties of cheese and typically eaten with sauces and pastas (Brazil 1997).

The aim of this study was to quantify the levels of aflatoxin M₁ in the samples of grated parmesan cheese marketed in the Metropolitan Region of Rio de Janeiro.

MATERIALS AND METHODS

Sampling

Ninety 50 g packets from 10 major brands of grated parmesan cheese marketed in the Metropolitan Region of Rio de Janeiro – Brazil, between January and March 2011 (covering the cities of Niterói, Rio de Janeiro and Seropédica) were collected. Three packets were collected from three different batches of each brand evaluated (10 brands x 3 batches x 3 packets). The three packets of each batch were homogenized, making a total of 30 laboratory samples. The samples were stored in a domestic freezer at - 15°C until use. All the brands had the Federal Inspection Seal (SIF) and were within the expiry date established by the manufacturers.

AFM₁ determination

AFM₁ extraction was based on the method proposed by Mayes and MacDonalds (1995), adapted by Deveci (2007). Briefly, 10 g of sample were mixed with 150 mL of chloroform and 10 g of Celite (Sigma-Aldrich, USA) and 2.0 mL of saturated NaCl solution using a mixer (Omni, USA) at low speed for 15 min. The slurry obtained

was filtered through filter paper (14 µm). The filtrate was evaporated to dryness using a rotary evaporator (BÜCHI RE120, SUI) at 25°C. The residue obtained was dissolved in 60 mL of buffer solution (900 mL of water, 8 g NaCl, 1.16 g Na₂HPO₄) and 2.0 mL of methanol. The solution was defatted by partition using 100 mL of hexane, stirring vigorously for 1 min. The aqueous phase was collected and then passed through an immunoaffinity column (IAC Aflaprep M, R-Biopharma, GER). The IAC was washed twice with buffer solution (10 mL) and the toxin was slowly eluted with 2.0 mL of methanol: acetonitrile (2: 3, v/v). The final extract was evaporated to dryness under a nitrogen stream and redissolved in 1000 µL of acetonitrile: water (20: 80, v/v).

The AFM₁ was quantified in duplicate by external standardization using a chromatography system with a fluorescence detector (HPLC-FLD), autosampler Waters 717, Waters 600 Pump, Waters On-Line Degasser, Waters 2475 Multi Fluorescence Detector - excitation at 360 nm and emission at 430 nm, and a C₁₈ column Waters X-Terra (5 µm – 4. 6 x 250 mm). The mobile phase consisted of acetonitrile: water (20: 80, v/v) and the injection volume into HPLC was 40 µL at flow rate of 1 mL/min. All the solvents used were HPLC grade (Tedia, BRA). Recovery was carried out using two levels (0.5 µg/kg and 1 µg/kg) with two replicates for each level and the Limits of Detection (LOD) and Quantification (LOQ) were estimated (in µg/kg) graphically from the slope and intercept of the calibration curve as described by Frehse and Thier (1991) and INMETRO (2003).

RESULTS AND DISCUSSION

According to the results, the method used can be considered appropriate for the extraction of AFM₁ in the samples of grated parmesan cheese. The acceptable ranges of recovery for trace analysis are situated between 70 and 120% (AOAC 2002). The recovery values obtained for AFM₁ analyses were 72.2 and 89.4% when added at 0.5 and 1 µg/kg of AFM₁, respectively, with RSD lower than 20%. The linearity of the calibration curve was higher than 0.99 and the LOD and LOQ were 0.02 and 0.05 µg/kg, respectively. Under these experimental conditions, the retention time of AFM₁ was

approximately 11 min. Figures 1 and 2 demonstrated the high selectivity obtained where there was no interference near the AFM₁ retention time.

AFM₁ was detected in 60% (18) of the evaluated samples. The highest value was $0.69 \pm 0.02 \mu\text{g/kg}$ and the mean for all the analyzed samples was $0.16 \mu\text{g/kg}$. The results are shown in Table 1.

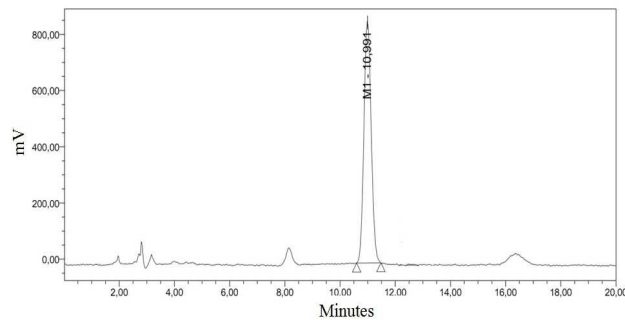


Figure 1 - Chromatogram obtained by HPLC-FLD in sample of grated parmesan cheese artificially contaminated with $1.0 \mu\text{g/kg}$ of AFM₁.

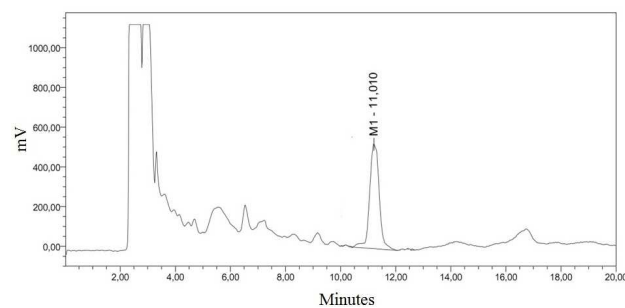


Figure 2 - Chromatogram obtained by HPLC-FLD in sample of grated parmesan cheese naturally contaminated with $0.42 \mu\text{g/kg}$ of AFM₁.

Table 1 - Aflatoxin M₁ in grated parmesan cheese marketed in the metropolitan region of Rio de Janeiro - Brazil in 2011.

| Range ($\mu\text{g/kg}$) | Number of samples | Percentage of samples |
|----------------------------|-------------------|-----------------------|
| ND* - 0.25 | 22 | 73.3 % |
| 0.25 - 0.5 | 5 | 16.6 % |
| 0.5 - 2.5 | 3 | 10.0 % |
| > 2.5 | 0 | 0 % |
| Total | 30 | 100% |

*ND: not detected ($< 0.05 \mu\text{g/kg}$)

All the evaluated samples were below the limit established by Brazilian legislation for cheeses in general ($2.5 \mu\text{g/kg}$) (Brazil 2011). However, 26.7% (8) could be considered above the limits set by the European Commission ($0.25 \mu\text{g/kg}$ of AFM₁ in cheese) (EC 2006). On the basis of the mean concentration of aflatoxin M₁ in grated parmesan cheese ($0.16 \mu\text{g/kg}$), the amount of this mycotoxin in a portion of the product (10 g) was estimated as 1.6 ng. This value, when added to the levels of AFM₁ in other dairy products, certainly indicated a risk to consumer health, since this mycotoxin has been genotoxic to humans and with un-safe level for its ingestion (IARC 2002). The results of this work were similar to other studies involving the quantification of AFM₁ in cheeses marketed in Brazil. Prado et al. (2008) evaluated 88 samples of parmesan cheese consumed in Minas Gerais state. The authors found values of the mycotoxin exceeding the permissible limit adopted by the European Commission ($0.25 \mu\text{g/kg}$) in two samples. Contamination in Minas Padrão cheese and Minas Frescal cheese was also described by Oliveira et al. (2011), who found the values of AFM₁ ranging from 0.04 to $0.31 \mu\text{g/kg}$.

The presence of AFM₁ in dairy products has also been described in other countries. In Turkey, Ertas et al. (2011) reported the presence of AFM₁ in 135 (64%) of 210 analyzed samples of various dairy products. In cheeses, the levels ranged from 0.01 to $0.37 \mu\text{g/kg}$. In Egypt, Amer and Ibrahim (2010) examined 150 samples of different types of cheeses commercialized in the country and found a maximum value of $0.25 \mu\text{g/kg}$ of AFM₁. In Italy, 41 cheese samples were evaluated and, about 10% were positive for AFM₁. The highest value found was $0.39 \mu\text{g/kg}$ (Viridis et al. 2008). AFM₁ contamination has also been recently described in other dairy products, including different varieties of cheeses by Elzupir and Elhussein (2010); Fallah et al. (2011); Elkak et al. (2012); Anfossi et al. (2012) and Tavakoli et al. (2012).

The results of this study indicated that there should be constant monitoring of AFM₁ levels in dairy products marketed in Brazil, especially because the country now has a specific limit for the mycotoxins in cheese. Other studies involving the quantification of chemical preservatives used in grated parmesan cheese must also be emphasized, since the abusive use of these and high fungal counts have been reported (Justus et al. 2011; Trombete et al. 2012).

CONCLUSION

From a total of 30 samples analyzed, 18 (60%) showed the presence of AFM₁. All the samples were below the maximum limit allowed by the Brazilian legislation for cheeses in general (2.5 µg/kg). However, eight samples (26.7%) presented AFM₁ levels above the tolerance limit adopted by the European Commission (0.25 µg/kg). The results indicated that AFM₁ levels in the grated cheese consumed in Rio de Janeiro - Brazil were relatively high and it could provide a potential hazard for public health.

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