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Aflatoxin M1 in cheese samples from the Amazon Region

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Aflatoxin M_1 (AFM_1) is a mycotoxin that can be found in cheeses and makes the monitoring of this food of public health interesting. In Brazil, the Amazon region has a cattle herd with dairy production of cheese, and an evaluation of the food and its risk to the population is required. The objective of this study was to evaluate the levels of AFM_1, moisture content (*mc*) and water activity (*aw*) in different cheeses types, inspected or not by the food safety authority in the Amazonas State in Brazil. All samples of coalho-type cheese were found to be in compliance with *mc* and *aw*. Both mozzarella cheeses and Minas frescal cheeses presented average *mc* below the acceptable limits. As for the samples of mozzarella cheese, these presented *aw* according to the values acceptable by the legislation, however the average of the cheese Minas frescal was inferior to the recommended values. None of the 25 cheeses samples showed AFM_1 contamination detectable by the HPLC method (LOQ = 0.0625 μg/mL). One possible explanation for the absence may be the lack of use of feed and confined animals, since in the state of Amazonas, for example, the milk-producing herd uses free grazing. Nevertheless, continuous monitoring is necessary since the consumption of cheeses produced in the state is relevant.

Key words: *Aspergillus*, water activity, moisture content.

INTRODUCTION

AFM1 is the hydroxylated metabolite of aflatoxin B1 (AFB1) and can be found in milk from animals that have ingested contaminated food by this group of aflatoxins and, consequently, in foods derived from contaminated milk (Caloni et al., 2006). It is estimated that 1 to 6% of

AFB1 present in animal feed persists as AFM1 in milk and can be detected 12 to 24 h after the first AFB1 intake and reaching a high level after a few days, probably associated with the protein fraction (Battacone et al., 2003). The objective of the present study was to evaluate

the effect of pasteurization, sterilization, preparation and storage of various dairy products (Oruc et al., 2006), and several studies have reported an increase in the concentration of this contaminant in cheeses and the elimination of water during the cheese processing (Baskaya et al., 2006; Kambar, 2006). Once formed, AFM1 is rapidly released into milk, urine and other body fluids from animals intoxicated with AFB1 (Battacone et al., 2003). The AFB1 conversion rate of food to AFM1 in milk generally ranges from 2 to 6% (Hassan et al., 2018). It can be found in milk within 12 h after the first ingestion of AFB1. After removal of the contaminated source, AFM1 disappears within 72 h. Thus, it is observed that the content of AFM1 in milk and consequently in cheese is strongly correlated with the level of AFB1 present in the raw food (Škrbić et al., 2015). AFM1 is found in milk when cattle and buffaloes receive feed contaminated with AFB1.

In several countries, monitoring and surveillance programs are being developed in order to make it possible to know the levels of contamination in milk and milk products. In Brazil, there are few studies on AFM1 in milk (Oliveira et al., 2006; Pereira et al., 2005; Garcia et al., 2003; Taveira, 2001) and derivatives such as cheeses and yoghurts (Prado et al., 2008, 2001, 2000). The occurrence of AFM1 in cheese may be due to the presence of AFM1 in milk (liquid or powder) used in the production of the product or dairy cattle may ingest food contaminated with mycotoxin or may be synthesized by fungi that grow on cheeses (López et al., 2001). In cheese manufacture, AFM1 is concentrated and retained in casein. The levels of this mycotoxin in cheeses appear to be higher in relation to milk, which is a problem of public health and worldwide concern, since cheese is a food intensively consumed by the population in general (Deveci, 2007). The fact that AFM1, a carcinogenic fungal metabolite, is present in cheeses is a fact of public health interest. In Brazil, there are about 72 types of cheeses, among which Minas Frescal cheese is the third most consumed, representing 9% of national production, followed by the so-called Mozzarella, 33% and "Prato", 24% (Marchiori, 2004). It is one of the most popular cheeses in Brazil, being consumed by all layers of the population throughout the year. It is a fresh product, for immediate consumption and of short durability. The coalho cheese is a food consumed by the local population, being part of the menu of the numerous regional cafes in the state of Amazonas. It is a source of income for some municipalities and also the food most incriminated in outbreaks that occur throughout the state, according to updated data from the Department of Epidemiological Surveillance (DVE), the Health Surveillance Foundation of Amazonas (FVS/AM). In the Amazon region in Brazil, there is production and consumption of cheeses and this requires the monitoring for future evaluation of the population risk. In the absence of studies in the North region, the objective of this study was to verify the presence and content of AFM1 in cheeses commercialized in the state of Amazonas.

MATERIALS AND METHODS

Sampling

Samples (25) of different types of cheeses (coalho, buffalo coalho, mozzarella, buffalo mozzarella and Minas frescal) were donated by the Health Surveillance Foundation (FVS). All of them came from dairy farms and cheeses from the municipalities of Autazes, Itacoatiara, Manicoré, Presidente Figueiredo and Silves, as well as samples of farms located on the AM-010 and BR-174 highways. They were sent to the capital and marketed by supermarkets of medium and large size and popular fairs of the city, from where the collections were carried out, from August to December 2017, by the fiscals of the surveillance agency. The samples were classified in two ways: (a) inspected (AI): that they had sanitary registration seal, being sold in supermarkets; and (b) not inspected (NI): samples without sanitary registration, handcrafted and sold at popular city fairs without any identification on the packaging. In total, 18 AI of coalho, buffalo coalho, mozzarella, buffalo mozzarella and Minas frescal and 07 NI all of coalho were analyzed. After being identified, they were cut into small pieces, crushed in an electric processor, stored in glass containers and refrigerated until the analyses were carried out.

Assays:

(a) Moisture content (*mc*): It was determined according to the gravimetric method by oven drying at approximately 104°C to constant weight (AOAC, 2016).
(b) Water activity (*aw*): It was determined using the AquaLab brand Water Activity Meter Dew Point 4TE by determining the dew point of the sample (AOAC, 2016).
(c) Aflatoxin M1: Separation and quantification of AFL were performed on a high performance liquid chromatography equipment (HPLC) (Shimadzu, Japan), coupled to a fluorescence detector configured with 360 nm excitation and 440 nm emission and Phenomenex® Gemini 5u C18 column 110A, 250 × 4.60 mm (5 μm). This was eluted isocratically with the mobile phase water: methanol: acetonitrile (650: 115: 270, v/v) at a flow rate of 1.0 ml/min. 50 μL of the filtered membrane filter sample (KASVI K18-430, 30 mm PES, 0.45 μm) was injected from a vial containing 500 μL. Under these conditions, the retention time was determined. All solvents used in this step were those recommended for liquid chromatography (CLAE grade) and water purified by the ultrafiltration system (MILLI - Q). From the calculation of the AFM1 peak area of the sample extract and standard solutions; it is possible to calculate the AFM1 content in the sample.
(d) Validation of the method for AFM1: Three different samples were fortified in duplicate with 1 mL of standard AFM1 solution at three concentrations: 1.0, 2.5 and 3.0 μg/mL. Subsequently, the extraction and purification were carried out with immunoaffinity column, and then the separation and quantification in HPLC. To determine the limit of detection, different concentrations of AFM1 standard solution were injected in a decreasing manner. The calibration curve was made from a 17.45 μg/mL AFM1 concentration solution in toluene and acetonitrile. From this, solutions were prepared in the following concentrations: 0.125, 0.5, 1.0, 2.5, 5.0 and 10.0 μg/mL, the range being defined. From each point of the curve, two injections were made (duplicate), obtaining the average of the readings of the same ones for the construction of

Table 1. *Aw* of the 18 samples inspected according to the type of cheese.

Cheese type	Aw[a] (mean±SD)	Limits[b]
Coalho	0.96 ± 0.00	0.91 - 0.97
Mozzarella and Buffalo Mozzarella	0.95 ± 0.01	0.93 - 0.96
Minas Frescal	0.97 ± 0.00	≥ 0.98

[a]Result obtained from the average of the samples inspected; b Limits established according to CRQ-IV (2008).
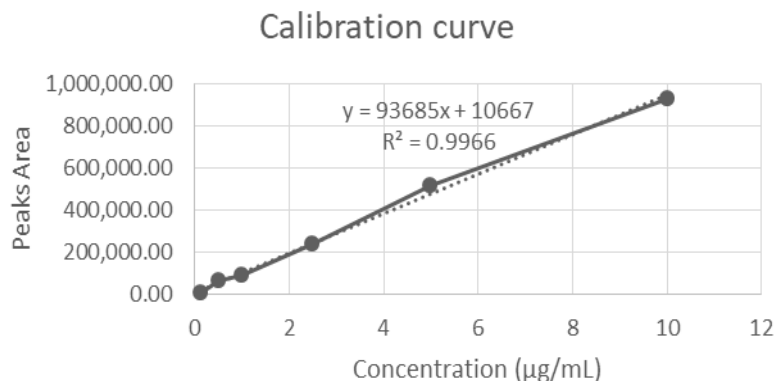
Figure 1. AFM1 calibration curve obtained by HPLC.

the calibration curve. The injection volume was 50 μL and was made from the highest to lowest concentration.

RESULTS AND DISCUSSION

Mc and Aw

Table 1 shows the results obtained in the analysis of the 18 samples of cheese inspected for aw and in Figure 1 the relation between the quantity of samples that comply with the legislation or not with respect to the *mc*.

Regarding the samples inspected, 11 of them were found in accordance with the legislation while the other 7 samples did not comply with the legislation regarding the *mc*. As for mozzarella cheese, the variation was 40.7 to 44.4%, with an average of 42.5%, being only products inspected, classifying them as medium and high *mc*. Fresh cheese showed a variation of 49 to 58.4%, with an average of 53.4%, being these products inspected, characterizing them as high and very high *mc*. Both had a mean *mc* below the acceptable limits and could be explained by the possibly inadequate transport of the samples, as high ambient temperatures could be exposed. These data are similar to those found in Matera et al. (2018) that showed Mc of 49 to 58.3%, with 60% of the samples of cheese minas frescal being outside the standard required by the legislation, that is, with a *mc* below the established levels. The *aw* of the mozzarella

cheeses varied from 0.94 to 0.96 with a mean of 0.95 and the cheeses Minas frescal was 0.96 to 0.97 with a mean of 0.97, both types being inspected. The mozzarella type cheeses were in agreement with the values acceptable by the legislation, however the aw average of the cheeses Minas frescal was inferior to the accepted values, although it is not aggravating since the smaller *aw* is difficult to be growth of microorganisms, being a problem only for the sensorial characteristics of the cheese. Similar results were found by Souza et al. (2017) in which 98% of the analyzed Minas frescal cheese samples presented *aw* from 0.91, which is below the recommended value for this type of cheese.

Regarding the uncoated samples of coalho cheese, the results of water activity and *mc* were found in Table 2.

Regarding the *mc*, the curd cheeses analyzed varied from 44.9 to 51.7% to the products with inspection, and from 38.5 to 45.2% for the ones without inspection, characterizing the cheeses as being of average and high Mc. These values of *mc* are similar to the results observed in the study by Silva et al. (2010), which obtained *mc* varying from 45.5 to 51.5%, being characterized as medium to high mc cheese (39% <Mc <55%). In this work, all the samples of coalho cheese were in agreement with the values of *mc* established in the Technical Regulation of Identity and Quality of Cheeses (Brasil, 2001). Regarding the *aw* of the coalho cheeses, the variation was 0.95 to 0.96 for the cheeses with inspection and 0.94 to 0.96 for the cheeses without

Table 2. *Aw* and *mc* of the 7 samples of coalho cheese were not inspected.

Type of cheese	Aw[a] (mean±SD)	Acceptable Value[b]	mc[a] (mean±SD)	Acceptable Value[c]
Coalhos	0.95 ± 0.00	0.91-0.97	42.6 ± 2.47	39-55%

[a]Result obtained from the average of the 7 samples not inspected, expressed in %; [b]There are no established values for coalho cheese. From the comparison with other works one can infer the result. c Value established according to Brasil (2001) for medium to high Mc cheeses.
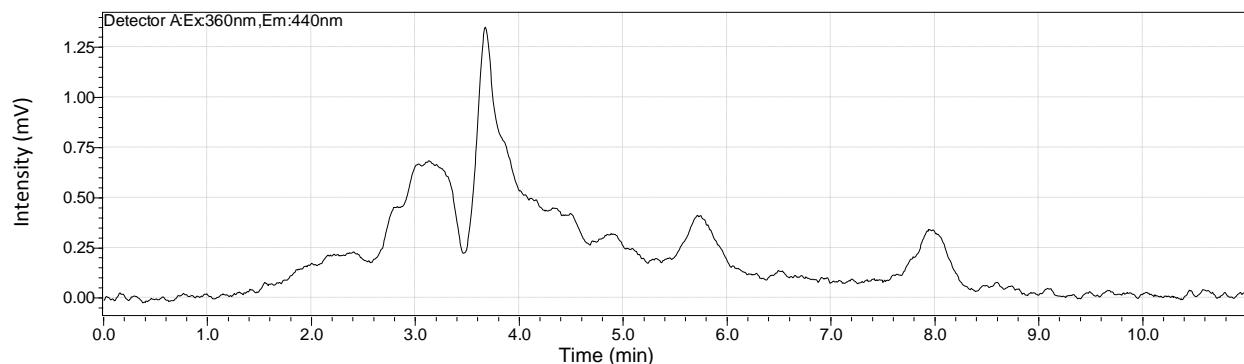
Figure 2. Chromatogram representing the limit of detection of the method for AFM1 with retention time in 7,977 min.

inspection. Andrade (2006) obtained similar aw values ranging from 0.94 to 0.97. The same author also verified that the average of *aw* for the inspected samples was superior to the one of the samples not inspected.

Determination of AFM1 in the samples

Validation of the methodology

The obtained curve correlated the area of the peaks of the chromatograms with the respective concentrations of AFM1 (Figure 1).

To determine the limit of detection, successive known concentrations of standard solution were injected decreasingly. The concentration of 0.0625 µg/mL was reached, in which the peak formation could still be observed, but it was not possible to calculate the exact area of the peak. Therefore, it was considered the detection limit of the method (Figure 2).

To determine the accuracy or recovery, the analytical methods employed in the experiment would be previously evaluated using cheeses experimentally fortified with standard AFM1 solutions at different levels. Samples were fortified with AFM1 at the required concentrations, extraction and purification were performed, and however, it was not possible to obtain recovery percentages, because during the analysis of the eluates obtained through the immunoaffinity column, there was a failure in the fluorescence module of the equipment that performs

the HPLC, which has to undergo maintenance. Considering that the limit of detection was 0.0625 µg/mL and the interval determined would be sufficient to detect levels of contamination below the limit allowed by current Brazilian legislation, which establishes as maximum limit of AFM1 in cheeses, a concentration of 2.5 µg/mL, it was concluded that of the 25 samples analyzed, none of them indicated the presence of AFM1 in values higher than 0.0625 µg/mL. Therefore, the analyzed samples are in compliance with the current legislation.

The following chromatograms indicate the peak concentration 2.5 µg/mL which corresponds to the maximum concentration allowed in cheeses by legislation compared to negative sample NI 18, respectively (Figures 3 and 4).

Sylos et al. (1996), analyzed 36 samples of Minas frescal, mozzarella and cheddar cheese in Campinas, São Paulo and also the presence of AFM1 was not detected in any of the samples, although the analytical method used was thin layer chromatography (CCD) which is less sensitive than high performance liquid chromatography (HPLC). One possible explanation for the absence in all samples is that the cows and buffaloes of the Amazon region graze year-round, rarely being fed with feed. In surveys from several countries, lower levels of AFM1 in milk were observed during the summer months when larger amounts of grass were consumed or in regions where grazing periods were longer (Brown, 1982). Galvano and Galofaro (1996) also observed a seasonal trend in milk contamination by AFM1. They
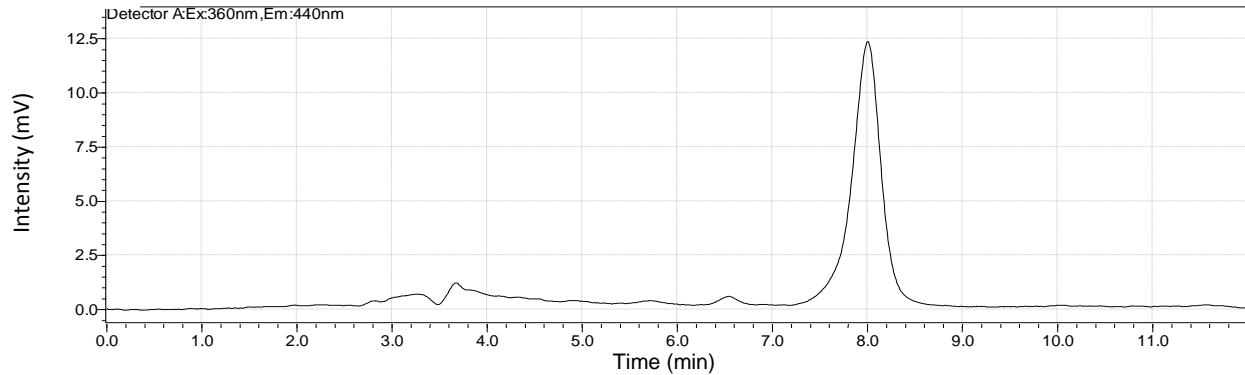
Figure 3. Chromatogram of the AFM1 standard at the concentration of 2.5 µg/mL.
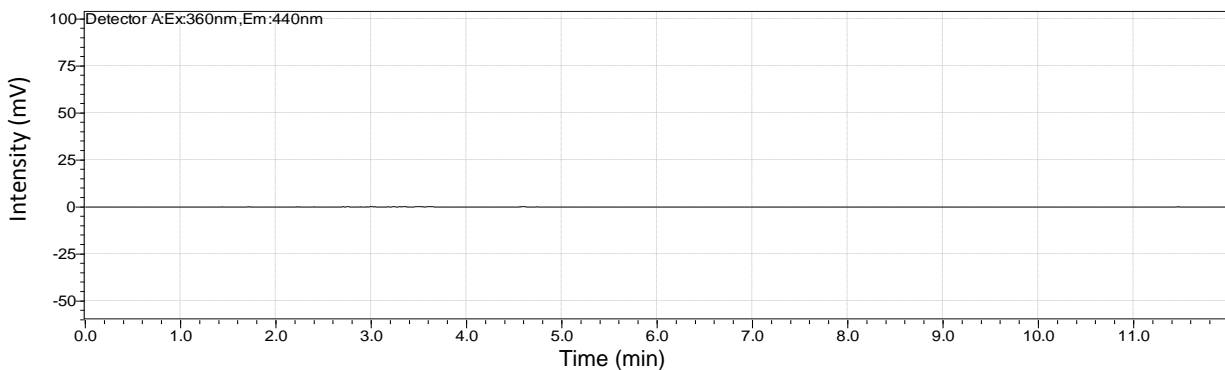

Figure 4. Chromatogram of the NI 18 sample with negative result.

found that lower rates occur during the summer months, when animals are commonly fed on pasture, unlike in winter when animals are fed by rations. Studies carried out by Embrapa (2017) show that in the north, in the rainy season, well managed pastures are capable of providing good nutrition conditions to herds conditioned to a pasture diet, requiring only mineral replacement. The reality of the Amazonian herd differs from other Brazilian states, such as the southern and southeastern regions of the country, where the animals receive rations during winter periods and the levels of contamination by AFM1 are more expressive, as evidenced in the work of Picinin et al. (2013) that evaluated AFM1 levels in raw milk samples from Minas Gerais in three different periods of the year: dry in which the rainfall is 7.9 mm and the average temperature is 19.3°C, the transition with precipitation of 100.3 mm and temperature of 20.3°C and rainfall with precipitation of 187.6 mm and temperature of 22.3°C. The authors observed higher AFM1 levels in the dry period (0.036 µg/L), followed by the transition period (0.017 µg/L) and rainy season (0.006 µg/L). The authors justified the differences due to the feeding provided to the

animals in the different periods. During the dry season, supplemental feedings were provided to the livestock in feedlot. During the transition period, the cattle were kept in confinement and under pasture. However, during the rainy season, when animals are generally free for grazing, the risk of contamination has decreased (Picinin et al., 2013). It should be borne in mind that the comparison between the results of this study and those carried out by other authors is complex since the contamination of the products may vary according to locality, climatic conditions, agricultural practices adopted with the herd, among others.

Conclusion

All samples of curd cheese (AI and NI) were found to be consistent with other authors' work on *mc* and *aw*. However, there is a need for specific legislation with reference values for these variables in this type of cheese. None of the 25 samples of cheese from independent producers in the rural area of the state and

sold in supermarkets, fairs and other food establishments in the city of Manaus, showed contamination by AFM1. This is probably due to the fact that in the Amazon region, cows and buffaloes are fed exclusively on pasture all year round, not receiving feed or supplements contaminated by aflatoxins. However, it would be important to continue sampling and analysis in the following years to understand the risks related to the conditions of the different producing regions in the state of Amazonas. The absence of AFM1 contamination in the cheeses under the conditions analyzed does not rule out the continuous monitoring of this mycotoxin in the region, since the favorable conditions for the production of aflatoxins can vary according to several factors specific to our locality as the type of food given to dairy cattle. The storage conditions of the feed being offered to the milk-producing herd and the hygienic-sanitary conditions at the place of sale of the finished product must be observed in order to avoid causing other contamination.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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