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## Incidence of aflatoxin M<sub>1</sub> in fresh milk from small farms

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### Abstract

The objective of this work was to determine aflatoxin M<sub>1</sub> in fresh milk from fifty-two small farms in the city of Concórdia - SC, Brazil. Samples from the cooling tanks of each property were collected from November 2014 to January 2015. The QuEChERS method was used for the extraction of aflatoxin M<sub>1</sub>, and quantification was performed in UHPLC-FL. 40.4% of the analyzed samples (eg, 21 samples) showed contamination levels by aflatoxin M<sub>1</sub> above the maximum limit allowed by the Brazilian regulation, which is 0.5 µg L<sup>-1</sup>. These results suggest the importance of implementing Good Practices in obtaining feed for dairy cows, since the contamination of milk by aflatoxin M<sub>1</sub> occurs through the biotransformation of aflatoxin B<sub>1</sub>, after the ingestion of feed or silage contaminated by the animals, posing risk to the animals themselves, as well as to consumers of milk and dairy products.

**Keywords:** aflatoxins; QuEChERS; UHPLC-FL.

**Practical Application:** Contamination by aflatoxin M<sub>1</sub> of the evaluated fresh milk was high.

## 1 Introduction

Mycotoxins make up a diverse group of toxic substances produced by some types of fungi. The aflatoxin-producing species are *Aspergillus flavus*, *Aspergillus parasiticus* and others such as *A. nominus*, *A. tamarii*, *A. pseudotamarii*, *A. bombycis*, *A. ochraceoroseus* and *A. australis*. Exposure of animals to mycotoxins, through the ingestion of contaminated food, may result in harm to their health as well as that of humans, who are consumers of the products obtained from this raw material (Benkerroum, 2016; Gacem & Hadj-Khelil, 2016; Oliveira et al., 2016; Wilson et al., 2002).

Among aflatoxins, B<sub>1</sub> is the most toxic, followed by B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> (Bbosa et al., 2013). The aflatoxins are absorbed in the gastrointestinal tract of the animal and are biotransformed, primarily in the liver, by microsomal enzymes related to the cytochrome P450 (Joint FAO/WHO Expert Committee on Food Additives, 2002) in multiple forms, among which epoxidation and hydroxylation stand out. Hydroxylation is a process that can be reversible or irreversible, forming derivatives such as aflatoxin M<sub>1</sub>, which can be excreted through body fluids such as milk (Oliveira & Germano, 2003). Aflatoxin B<sub>1</sub> that has not been biotransformed can also be excreted (Scaglioni et al., 2014).

The maximum limit of aflatoxin M<sub>1</sub> in milk and dairy products, as stipulated by international regulations, ranges from 0 to 1.0 µg kg<sup>-1</sup>. The European Union and the United States of America, for example, set limits of 0.05 and 0.5 µg kg<sup>-1</sup>, respectively; Nigeria, 1.0 µg kg<sup>-1</sup> (Iqbal et al., 2015); and Brazil has a maximum limit of 0.5 µg kg<sup>-1</sup> for fluid milk (Brasil, 2011).

When it comes to the production of bovine milk, Brazil stands out in the international scenario. In 2015, it was the fifth largest producer in the world (35.00 billion liters), with the South Region being the largest Brazilian producer (12.32 billion liters). Among the Brazilian States, Santa Catarina is one of the major producers, with 8.7% of the volume of bovine milk produced in this country, with the Western mesoregion being responsible for 75.2% of the volume produced throughout the State in that year. These data show the importance of this region in the milk production chain at national and international levels (Instituto Brasileiro de Geografia e Estatística, 2016; United States Department of Agriculture, 2016).

Due to public health concerns and the consequent economic losses due to the presence of aflatoxins in foods, the objective of this study was to determine aflatoxin M<sub>1</sub> in fresh bovine milk produced in small farms in the city of Concórdia, located in the State of Santa Catarina, Brazil.

## 2 Materials and methods

### 2.1 Sampling

Samples of fresh bovine milk were collected between November 2014 and January 2015 in 52 small rural farms, with an average production of 120 L day<sup>-1</sup>, from Concórdia, Western region of Santa Catarina, in the South of Brazil.

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In each farm, a single direct collection was carried out in the morning, directly from the cooling tank, in duplicate, using Falcon tubes of 50 mL. These vials were placed in ice-cold isothermal boxes, sent to the laboratory and kept in a freezer at -12 °C until analyses.

## 2.2 Determination of aflatoxin M<sub>1</sub> in samples of fresh bovine milk

### Samples preparation

Samples of bovine milk, *in natura*, were thawed and centrifuged at 2600 g for 3 min at room temperature to remove the supernatant fat layer.

### Extraction of aflatoxin M<sub>1</sub>

The extraction of aflatoxin M<sub>1</sub> was performed in triplicate using the QuEChERS method (Sartori et al., 2015). 15 g of the defatted and homogenized milk were weighed, to which 10 mL of hexane (C<sub>6</sub>H<sub>14</sub>; Synth, Brazil) and 15 mL of acetonitrile (C<sub>2</sub>H<sub>3</sub>N; J.T. Baker, Brazil) with 1% acetic acid (C<sub>2</sub>H<sub>4</sub>O<sub>2</sub>; Dinâmica, Brazil) were added. This solution was homogenized for 1 min, manually and slowly. Thereafter, 6 g of magnesium sulfate (MgSO<sub>4</sub>; Synth, Brazil) and 1.5 g of sodium chloride (NaCl; Vetec, Brazil) were added, and the solution was homogenized manually and vigorously for 90 sec. Subsequently, the sample was centrifuged at 2600 g for 7 min at room temperature. The hexane phase was discarded and the acetonitrile phase was collected in amber flasks for further concentration of this fraction in a water bath at 60 °C. The obtained extract was conditioned and kept under freezing for further quantification of aflatoxin M<sub>1</sub>.

### Quantification of aflatoxin M<sub>1</sub>

For quantification of aflatoxin M<sub>1</sub> in the samples, a Shimadzu high-performance liquid chromatograph with fluorescence detector (UHPLC-FL) ( $\lambda$  excitation = 360 nm and  $\lambda$  emission = 450 nm) was used, equipped with a Kromasil C18 5 $\mu$  250 x 4.6 mm chromatographic column with an injection handle of 20  $\mu$ L and a flow of 1.0 mL min<sup>-1</sup>.

The method for quantification was validated by Scaglioni et al. (2014) and consisted in resuspending the dried extracts from the samples in 1 mL of the mobile phase (1% acetic acid: acetonitrile: methanol 40:35:25, by volume), shaking for 1 min in an ultrasonic bath. Later, the obtained solutions were transferred to a vial and centrifuged at 30186 g for 10 min at room temperature for later injection.

The concentration of aflatoxin M<sub>1</sub> in the samples was calculated using the equation of the calibration curve  $y = 2,246,144x - 6,973.83$ , with coefficient of determination ( $R^2$ ) 0.9983 and correlation coefficient of 0.9991 (Scaglioni et al., 2014). A sample of fresh milk, in triplicate, was fortified with 0.5  $\mu$ g L<sup>-1</sup> of aflatoxin M<sub>1</sub>, extracted by the QuEChERS method and quantified by the Scaglioni method (2014), to obtain the method recovery. A M<sub>1</sub> standard (Sigma-Aldrich, St Louis, MO, USA) was also injected at a concentration of 0.1  $\mu$ g L<sup>-1</sup>.

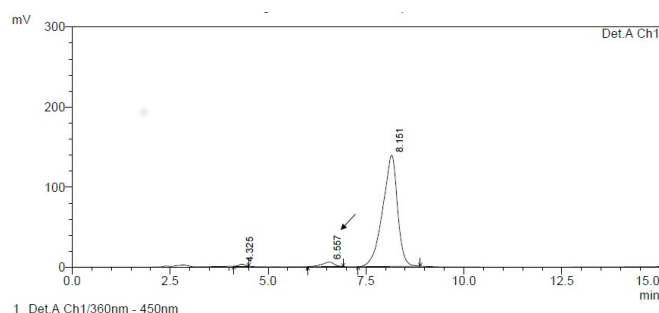
## 3 Results and discussion

The QuEChERS method was chosen to investigate aflatoxin M<sub>1</sub> in fresh milk samples, since it is a fast and simple method for extraction and purification prior to the determination of mycotoxins in this sample. The milk matrix contains high levels of proteins and fats, which would make it necessary to perform several cleaning steps to reduce the interferences for subsequent quantification, which can be avoided using the QuEChERS protocol. In addition to the use of this protocol, previous freezing of the sample was adopted, in order to facilitate the separation and removal of fat from the fresh milk.

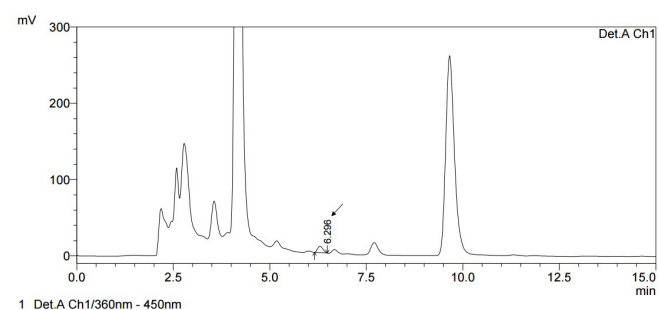
The chromatographic conditions previously established by Scaglioni et al. (2014) allowed adequate separation of aflatoxin M<sub>1</sub> (Figure 1). Figure 2 shows the chromatogram of the fortified sample, whose mean recovery value was 60% (0.3  $\mu$ g L<sup>-1</sup>). This chromatogram, when compared to the chromatogram of fresh milk samples (chromatogram illustrative in Figure 3), presented more interference peaks.

The presence of aflatoxin M<sub>1</sub> was detected in the 52 analyzed fresh milk samples, with values ranging from  $\leq$  LOD (0.09  $\mu$ g L<sup>-1</sup>) to 3.385  $\mu$ g L<sup>-1</sup> (Table 1). 40.4% of the analyzed samples (eg, 21 samples) were above the limit allowed by the Brazilian regulation, which is 0.5  $\mu$ g L<sup>-1</sup> (Brasil, 2011), and 59.6% (31 samples) showed levels higher than allowed by the European Union, which is 0.05  $\mu$ g L<sup>-1</sup> (Iqbal et al., 2015). It is important to notice that the recovery test performed with the fortified sample indicates that the values found may be even higher than those presented in Table 1.

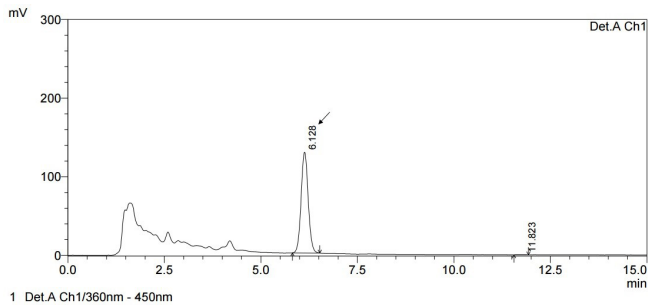
Several recent studies found aflatoxin M<sub>1</sub> contamination *in natura* or raw milk. Shuib et al. (2017) quantified this aflatoxin in fresh milk by UHPLC-FL after immunoaffinity column cleaning,



**Figure 1.** Chromatogram of aflatoxin M<sub>1</sub> standard (0.1  $\mu$ g L<sup>-1</sup>).



**Figure 2.** Chromatogram of milk sample previously fortified with aflatoxin M<sub>1</sub> (0.3  $\mu$ g L<sup>-1</sup>).



**Figure 3.** Chromatogram of fresh milk ( $0.73 \mu\text{g L}^{-1}$ ).

**Table 1.** Aflatoxin  $M_1$  content in fresh bovine milk.

| AFM1 contamination level<br>( $\mu\text{g L}^{-1}$ ) | Number of samples<br>(% of total analyzed samples) |
|--|--|
| $\leq \text{LOD}$ (0.09)                             | 24 (46.2%)   |
| $\leq \text{LOQ}$ (0.1 to 0.25)                      | 4 (7.7%)   |
| 0.26 to 0.49   | 3 (5.6%)   |
| $\geq 0.50$  | 19 (36.5%)   |
| $> 1.00$   | 2 (3.8%)   |
| TOTAL  | 52 (100%)  |

and 4% of the analyzed 102 samples showed contamination levels below  $0.5 \text{ g Kg}^{-1}$ , that is the maximum limit allowed by the Malaysian regulation. Michlig et al. (2016) quantified aflatoxin  $M_1$  in 160 samples of milk collected in cooling tanks of the largest producing region of Argentina, using immunoaffinity columns for cleaning and UHPLC-MS/MS for quantification. 38.8% of total analyzed samples were contaminated with levels lower than  $0.5 \text{ mg L}^{-1}$  that is set by the Common Market of the South (MERCOSUR) regulation. Hashemi (2016) analyzed 168 raw milk and 12 pasteurized milk samples in southern Iran using the ELISA method, and 55.6% showed a mean concentration of  $21.31 \text{ ng L}^{-1}$  aflatoxin  $M_1$ , all below the maximum limit established by Iran regulation ( $100 \text{ ng L}^{-1}$ ).

In Brazil, Pereira et al. (2005) verified that 52.8% of the 36 analyzed samples milk were contaminated with  $M_1$  in Minas Gerais (Brazil) and Oliveira et al. (2010) detected this aflatoxin in 36.7% of the 30 samples evaluated in São Paulo (SP, Brazil), but the values found in both studies were below that allowed by Brazilian regulation. Santili (2010) extracted and purified raw milk samples by immunoaffinity columns and quantified aflatoxin  $M_1$  by HPLC-FD, finding it in 49% of the 429 samples collected in São Paulo (SP, Brazil), with 0.7% being above the maximum limit allowed in Brazil. Picinin et al. (2013) collected 129 samples of bulk milk in the state of Minas Gerais, Brazil, and ELISA-tested and confirmed the presence of aflatoxin  $M_1$  by HPLC-FD in 18 samples ( $0.06$  to  $0.5 \mu\text{g L}^{-1}$ ).

It is known that aflatoxins are heat treatment stable (Assem et al., 2011; Prandini et al., 2009; Shundo et al., 2009). Studies carried out in Brazil (Scaglioni et al., 2014) and other countries (Farah Nadira et al., 2017; Armorini et al., 2016; Bellio et al., 2016; Zheng et al., 2013; Kabak & Özbey, 2012) prove that even in pasteurized or ultrapasteurized milk, aflatoxin  $M_1$  is found at levels often higher than the allowed maximum limit established by national regulations (Becker-Algeri et al., 2016).

Thus, the contamination level found in the fresh milk evaluated in this study is worrisome, since, of the 37.5% samples that showed contamination above the maximum limit allowed by Brazilian regulation ( $0.5 \mu\text{g L}^{-1}$ ), 9.5% showed levels at least twice higher than this maximum value. The contamination with mycotoxigenic species and the production of mycotoxins occurs under favorable environmental conditions (Gergiadou et al., 2012), which include specific climatic conditions,  $\text{CO}_2$  availability, temperature and water availability, as well as the interactions between them (Magan et al., 2011). Thus, ingestion of feeds and silages contaminated with aflatoxins will expose animals to these dangers (Mídio & Martins, 2000; Oliveira & Olivera, 2010), which may impair their reproductive efficiency, reduce feed conversion efficiency, increase mortality rates, reduce weight gain, cause anemia and jaundice, as well as have an impact on food safety (Feddern et al., 2013).

According to Schneider et al. (2016), the feed supplied to dairy herds in the region of Concordia, SC (Brazil) is predominantly silage and feed produced on the farm itself. The feed uses corn as the main raw material and sometimes concentrate, wheat bran, soybean meal and rice bran are added during the management, production and storage of feed and silages. There is a relation between food contaminated with mycotoxins, and that fed to animals, and metabolism of aflatoxin  $B_1$  in its hydroxylated metabolite as aflatoxin  $M_1$  in milk (Kang'Ethe & Lang'A, 2009). Therefore, the high percentage of samples containing aflatoxin  $M_1$  in the analyzed milk samples suggests this path of contamination. However, other factors such as the climate, the stage of lactation of the animals, productivity and health of the mammary gland of the animal, among others, may have contributed to the high incidence of aflatoxin  $M_1$  in this product. However, contamination may also occur in temperate zones, when meteorological conditions combine with environmental factors and agricultural practices that favor the growth of toxigenic molds and AF production (Gioviati et al., 2015).

## 4 Conclusion

40.4% of fresh milk samples showed contamination by aflatoxin  $M_1$  above the maximum limit allowed by Brazilian regulation ( $0.5 \mu\text{g L}^{-1}$ ). The contamination levels found in the evaluated samples were high and these results represent a food safety problem in the investigated small farms. This scenario indicates the importance of the adoption of measures to mitigate this problem, such as the implementation of Good Practices throughout the milk production chain.

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