Incidence of aflatoxin M₁ in milk used for the production of Istrian cheese

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Abstract

This study was undertaken to determine the presence and levels of aflatoxin M₁ (AFM₁) in ewe’s milk produced by different plants in the Istrian region of Croatia, and to compare the obtained results with maximum of AFM₁ tolerance limits in milk that are accepted by some of the countries such as Croatia. The occurrence of AFM₁ contamination in investigated milk samples was determined by ELISA (Enzyme Linked Immunosorbent Assay) technique. A total of 18 samples of commercial whole milk were analyzed. AFM₁ was found in all of the milk samples examined. The mean value was 0.028 μg L⁻¹. The range of contamination levels varied among different farms. None of investigated samples did not contain AFM₁ in concentrations that exceeded the maximum acceptable levels (0.05 μg L⁻¹) that are accepted by Croatia.

Key words: mycotoxins, aflatoxin B₁, aflatoxin M₁, ELISA, ewe’s milk

Introduction

Of all mycotoxins, aflatoxin B₁ (AFB₁) is considered to be the most toxic/carcinogenic compound (Škrinjar et al., 1992; Duraković et al., 2011). Mammals that ingest AFB₁-contaminated diets eliminate into milk amounts of the principal 4-hydroxilated metabolite known as “milk toxin” or aflatoxin M₁ (AFM₁) (Galvano et al., 1996). AFM₁, recently reported as hepatic and carcinogenic to humans (European Commission, 2006), is a metabolite found in the milk of lactating animals which have consumed feedstuffs contaminated by aflatoxin B₁ (AFB₁) (Škrinjar et al., 1992; Galvano et al., 1996; Kamkar, 2006). Because of the binding of AFM₁ to the
protein fraction, in particular the association with casein, this metabolite can be present also in dairy products with contaminated milk. When cheese making is carried using AFM₁ contaminated milk, this toxin is likely to have become enriched in the final curd compared to that found in milk (Kamkar, 2006). Therefore, it is necessary to note whether AFM₁ is present in final product like cheese, because its concentration in it has been reported to be around 2.1-4.5 times higher than in original milk used in the production of this cheese type (Kamkar, 2006). There is a general consensus that approximately 1-3% of the AFB₁ initially present in animal feeds appear as AFM₁ in milk, but this transmission rate was shown to vary from animal to animal, from day to day, and from one milking to the next (Škrinjar et al., 1992). Milk is a highly variable product that rapidly loses its homogeneity and spoils if untreated. Since milk may be processed in numerous ways, the effect of storage and processing on stability and distribution of AFM₁ are of a great concern. The incidence of AFM₁ is often higher in commercial milk than in raw milk, because of the dilution of uncontaminated bulk milk by only a few contaminated samples (Kamkar, 2006). On the other hand, a seasonal trend in milk contamination was noted that lower levels of AFM₁ occurring during the summer months when animals consumed more grass that concentrated feeds (Galvano et al., 1996). The toxicological concern with AFM₁ arises in principle from its close structure similarity to AFB₁ (Figure 1), which has been shown to be of the most potent carcinogens (Duraković et al., 2011).

AFM₁ is relatively stable in raw and processed milk and milk products, and is unaffected by pasteurization or processing into cheese. Thus, if raw milk contains AFM₁, cheese made from such milk also contains AFM₁ (Galvano et al., 1996). Traditional Istrian cheese is produced from raw ewe’s milk on small scale farms across Istria and it is left to ripen 90-120 days (Mrkonjić-Fuka et al., 2010). The production and consumption of cheese, especially Istrian cheese is widespread in the Istrian region of Croatia (Samaržija et al., 2003). For this purpose, this study was designed to determine the presence and levels of AFM₁ in Istrian milk, that is especially sold and consumed in Istria region, and to compare the obtained results with the maximum of AFM₁ tolerance limits in milk that are accepted by some of the countries such as Croatia.

**Material and methods**

**Sampling**

Investigated milk was collected from six farms located at different areas in the Istrian region of Croatia during the year 2007. Due to the unequal beginning of the production of Istrian cheese at different farms, the collection of samples started at two farms in May, two farms in June and two farms in July 2007. All samples were collected in triplicates, transported to the laboratory at 4 °C, and frozen at -20 °C. A total specimen contained 18 milk samples, which were used for AFM₁ analysis, and randomly obtained from six different milk producers who delivered their milk to the Department of Microbiology, Faculty of Agriculture, Zagreb, Croatia. The milk samples were taken six times at 15 day intervals.
Methods

In this study, we employed a reliable method for ELISA determination of aflatoxins in milk, which we consider to be a better method for controlling aflatoxins. Determination of AFM₁ was based on Enzyme Linked Immunoassay using RIDASCREEN IMMUNOLAB test kit (Immunolab GmbH, Kassel, Germany), which also contained AFM₁ standard. This method is quick, reliable, and costs effective for estimating AFM₁ and has been included in the official collection of test procedures by the German Federal Board for Health. Solvents used during the experiment were analytically pure, purchased from Merck (Darmstadt, Germany), and were prepared according to van Egmond (1989). Most of the reagents used were in the RIDASCREEN test kit, which included microtiter plate coated with capture antibodies, AFM₁ standard solutions (1.3 mL each, 0 ppt, 250 ppt, 500 ppt, 1000 ppt, 2000 ppt), peroxidase conjugated aflatoxin, anti-aflatoxin M₁ antibody, substrate/chromogen stained in red and stop solution that contains 1N sulphuric acid.

Preparation of the milk samples

Preparation of samples was conducted according to the instructions of the RIDASCREEN test kit. 10 mL of milk samples were chilled to 10 °C and then centrifuged for 10 min. at 3500 rpm. An aliquot (50 μL per well) of the milk was used directly in the test. The IMMUNOLAB aflatoxin M₁ quantitative test is based on the principle of an enzyme-linked immunosorbent assay. The AFM₁ was assayed quantitatively by ELISA, according to the standard calibration curve (Figure 2).

![Figure 2. Calibration curve of AFM₁.](image)

Evaluation of AFM₁

The absorbance values obtained for the standards and the samples were divided by the absorbance value of the first standard (0 standard) and multiplied by 100. Therefore, the zero standard is thus made equal to 100% and the absorbance values are quoted in percentages. The absorption is inversely proportional to the AFM₁. According to the test preparation record, the lower detection limit is 0.01 μg L⁻¹ for milk.

Calculation of extrapolated values of AFB₁ concentration in cattle feeding stuffs

Many researchers reported that there was a linear relationship between amount of AFM₁ in milk and AFB₁ in feed consumed by the animals (Bakirci, 2001). It has been suggested that only 1.6% of ingested AFB₁ is converted to AFM₁ by the dairy cattle. Hence, the values of AFB₁ contamination in feeding stuffs were back calculated by the formula given below (Rostogi et al., 2004):

\[
AFB₁ (μg kg⁻¹) = \frac{AFM₁ (ng L⁻¹) \times 100}{1.6 \times 1000}
\]
Results and discussion

In the present work it was intended to investigate quantitatively the detection and determination of AFM₁ whose presence in Istrian milk was of special interest. As shown in Table 1, all of the 18 milk samples were found to be contaminated with AFM₁ at < 0.025-0.037 μg L⁻¹. All concentrations were below the maximum tolerated levels in liquid milk (50 ng L⁻¹), in Croatia. AFB₁ concentration in ewe’s feedstuffs was shown in Table 2. All concentrations of AFB₁ in ewe’s feedstuffs also did not exceed maximum tolerated levels (5 μg kg⁻¹) (EU Commission, 2006). The percentages of absorbance obtained in the competitive ELISA with the calibration curve (Figure 2) allow to calculate the AFM₁ concentration in μg L⁻¹ in the samples (Table 1), for each kind of milk-dairy farm milk. All samples were below the advisory limit of 0.05 μg L⁻¹.

Table 1. AFM₁ contamination in ewe’s milk samples in Istria region during the year 2007.

<table>
<thead>
<tr>
<th>Sample category</th>
<th>Samples analyzed</th>
<th>Positive samples</th>
<th>AFM₁ range (μg L⁻¹)</th>
<th>Exceeding EC/Codex regulations (50 ng L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid milk</td>
<td>18</td>
<td>18 (18)</td>
<td>&lt;0.024-0.037</td>
<td>None</td>
</tr>
<tr>
<td>May</td>
<td>6</td>
<td>6 (6)</td>
<td>&lt;0.024-0.037</td>
<td>None</td>
</tr>
<tr>
<td>June</td>
<td>6</td>
<td>6 (6)</td>
<td>&lt;0.024</td>
<td>None</td>
</tr>
<tr>
<td>July</td>
<td>6</td>
<td>6 (6)</td>
<td>&lt;0.024-0.029</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 2. Extrapolated AFB₁ concentration in cattle feedstuffs based on AFM₁ contamination in milk samples.

<table>
<thead>
<tr>
<th>Sample category</th>
<th>Samples analyzed</th>
<th>Positive samples</th>
<th>AFB₁ range (μg kg⁻¹)</th>
<th>Exceeding EC/Codex regulations (5 μg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle feedstuffs</td>
<td>18</td>
<td>18 (18)</td>
<td>&lt;1.5-2.3</td>
<td>None</td>
</tr>
<tr>
<td>May</td>
<td>6</td>
<td>6 (6)</td>
<td>&lt;1.5-2.3</td>
<td>None</td>
</tr>
<tr>
<td>June</td>
<td>6</td>
<td>6 (6)</td>
<td>&lt;1.5</td>
<td>None</td>
</tr>
<tr>
<td>July</td>
<td>6</td>
<td>6 (6)</td>
<td>&lt;1.5-1.8</td>
<td>None</td>
</tr>
</tbody>
</table>

The AFM₁ concentrations encountered were within the tolerance level. The results indicate that the incidence of AFM₁ in ewe’s milk commercialized in Istria is not serious. However, with regard of this particular contamination in Croatia, the information is limited. The situation suggests that more samples will have to be analyzed and the survey conducted over a more extent period, in order to obtain data corresponding in various climate-humidity and temperature conditions. Since milk is one of the most important human foods and the main nutrient for growing young, who are notably vulnerable and potentially more sensitive than adults, it must therefore, be monitored for contaminants, including AFM₁.

Conclusions

Ewe’s milk samples were analyzed for the presence and concentration of aflatoxin M₁ using an enzyme-linked immunoassay. A total of 18 samples aforementioned materials were analyzed originating from plants of Istria region of Croatia. Levels of aflatoxin M₁ in ewe’s milk were found far below the tolerance level (highest value 0.037 μg L⁻¹). Thus, considering the current scientific information, the general human exposure to AFM₁ by the consumption of contaminated ewe’s milk is not significant in Istrian region of Croatia. However, considering the fact that small numbers of samples were analyzed and through a short time period, the levels of AFM₁ and AFB₁ in milk and milk products should be controlled and monitored continuously. Accordingly, it is important to maintain low levels of AFB₁ in dairy animals’ feeds.

References


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