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Assessment of aflatoxin M_1 levels in ewe's raw milk used for the production of Istrian cheese

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Summary

This study was undertaken to determine the presence and levels of aflatoxin M_1 (AFM_1) in ewe's raw milk produced by different farms in the Istrian region of Croatia, and to compare the obtained results with maximum of AFM_1 tolerance limits in ewe's milk that are accepted by some of the countries such as Croatia. All ewe's milk samples were collected in triplicates at two farms in May, two farms in June, and two farms in July during the year 2007. The occurrence of AFM_1 contamination in ewe's milk samples was investigated by ELISA (Enzyme Linked Immunosorbent Assay) technique. A total of 18 samples of raw ewe's milk were analyzed. AFM_1 was found in all of the ewe's milk samples examined. The mean value was 0.028 μg/L. None of the samples did not contain AFM_1 in concentration that exceeded the maximum acceptable levels (0.05 μg/L) that are accepted by Croatia.

Key words: moulds, aflatoxin B_1, aflatoxin M_1, ELISA, ewe's milk

Introduction

Fungi are a major cause of deterioration and spoilage in stored crops. They render unfit for human and animal consumption perhaps as much as 1 % of world's supply of grain and oilseeds (Johnson, 1948). Spoilage fungi attack food and feed crops after harvest, whenever environmental conditions become favorable for their proliferation.

Moisture content of the seed or grain, its viability and physical state, ambient temperature, length of storage, and the activity of stored product, insect and mites are the main factors that determine both the initiation and extent of mould growth. These factors also govern the metabolism of a given fungus strain to its capacity for toxin production.

The capacity of fungi to produce toxic metabolites has been known since the turn of century. As early as 1913, Alsberg and Black of the US Department of Agriculture conjectured that the products of mould growth might be involved in diseases. Certainly, the first described form of a mycotoxicosis, ergotism, has been known to man from such of this recorded history. In modern times, the ability of fungi to synthesize highly toxic compounds was noted during extensive screening of microorganisms for antibiotic production, but the significance of these findings was not fully appreciated. However, the eruption of "Turkey X" disease in England in 1960, followed by the discovery of aflatoxins, resulted in a drastic reappraisal of the mycotoxins problem. Survey of foods and feeds revealed that the problem was

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worldwide, rather than confined to any geographical area (Blount, 1961).

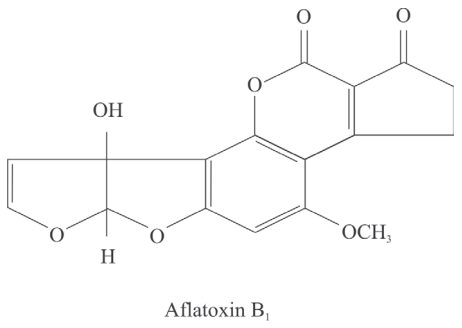
Aflatoxins are a group of highly toxic secondary metabolites produced by certain species of moulds from genera *Aspergillus*, *Penicillium* and *Rhizopus* during their growth on plants and its products (Goldblatt, 1969). As aflatoxins are carcinogenic, mutagenic, and teratogenic to animals and humans, contamination of feed and food is a current problem (Piva et al., 1995; Galvano et al., 1996; Duraković, 2007; Duraković et al., 2008, 2011b; Prado et al., 2011; Zafar et al., 2011). Of all mycotoxins, aflatoxin B_1 (AFB_1) is considered to be the most toxic/carcinogenic compound (Škrinjar et al., 1992; IARC, 1993b; Duraković et al., 2011a).

Mammals that ingest AFB_1-contained diets eliminate into milk amounts of the principal 4-hydroxilated metabolite known as "milk toxin" or aflatoxin M_1 (AFM_1) (Galvano et al., 1996). Recently Rothschild (1992) classified AFB_1 and AFM_1 as class 1 and 2B (or probable) human carcinogens, respectively. There is a general consensus that approximately 1-3 % of the AFB_1 initially present in animal feeds appear as AFM_1 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 (van Egmond and Paulsch, 1986; Fallah et al., 2011; Iha et al., 2011).

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 AFM_1 are of a great concern. Purchase et al. (1972) reported that the aflatoxin M content of freeze dried milk was reduced during processing. They found a reduction ranging from 32 % to 87 % for processes such as pasteurization, sterilization, preparation of evaporated milk, roller drying, and spray drying. Reduction in aflatoxin content was confirmed by duckling bioassay. Aflatoxin was not detected in cottage cheese prepared from the pasteurized milk, but was present in the whey.

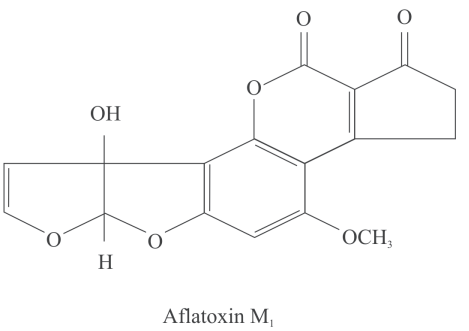
According to Stoloff (1977) and Kourousekos (2011), milk has the great demonstrated potential for introducing aflatoxin residues from edible animal tissue into the human diet. Moreover, as milk is the main nutrient for growing young, whose vulnerability is noteworthy and potentially more sensitive than that of adults, the occurrence of human breast milk, commercially available milk, milk products and protein concentrates based on milk such as whey protein concentrates are one of the most serious problems in food hygiene (Matijević et al., 2008; Brnčić et al., 2009). AFM_1 is hepatic carcinogenic metabolite found in milk of lactating animals that had ingested AFB_1-contaminated feed (van Egmond, 1989; Škrinjar et al., 1992; Galvano et al., 1996; Kamkar, 2006; Atasever et al., 2011).

There is a linear relationship between the AFM_1 content in milk and the consumption of AFB_1 via foodstuff (Sasshara et al., 2005; Fallah et al., 2011). It has been estimated that about 0.3-6.2 % of AFB_1 present in animal feed pass as AFM_1 in milk (Creppy, 2002; Rubio et al., 2011). Although the toxicity of AFM_1 is less than AFB_1, its cytotoxic, genotoxic and carcinogenic effects are well demonstrated. Hence the IARC of WHO initially categorized AFM_1 as a Group 2 human carcinogen (IARC,



Aflatoxin B_1

Figure 1. Chemical structure of aflatoxin B_1 (Duraković, 2007)



Aflatoxin M_1

Figure 2. Chemical structure of aflatoxin M_1 (Duraković et al., 2011a)

Table 1. Current and proposed legal limits (μg/L/kg) for aflatoxin M_1 in milk and dairy products for human consumption in various European countries and USA* (van Egmond and Wagstaffe, 1987; Creppy, 2002; Manetta et al., 2005; Kamkar, 2008)

Country	Raw milk	Dairy products
European Union[a]	0.05 (C)	0.05 (C)
Austria	0.05 (V) 0.01 (V) pasteurized infant milk	0.02 (P) butter 0.25 (P) cheese 0.4 (P) powdered milk
Belgium	0.05 (V)	0.10 (P) milk powder for infant foods
Bulgaria	0.50 (V)	0.10 (P) milk powder for infant foods
Croatia	0.05 (V)	0.025 (P) milk powder for infant foods
Czech Republic	0.05 (V) 0.10 (V) children's milk 0.50 (V) adult's milk	
France	0.05 (P)	0.03 (P) milk powder for infant foods 0.05 (P) adult's milk
Germany	0.05 (P)	0.10 (P) milk powder for infant foods
The Netherlands	0.05 (P)	0.05 (P) milk powder for infant foods 0.20 (P) cheese 0.02 (P) butter
Russia	0.05 (V)	0.10 (P) milk powder for infant foods
Sweden	0.05 (P)	0.10 (P) milk powder for infant foods
Switzerland	0.05 (C)	0.01 (C) milk powder for infant foods 0.25 (C) cheese 0.02 (C) butter
USA (FDA)[b]	0.50 (V)	

[a]Legislation common to all member countries: EU Regulation 466/2001
[b]Legislation was complied from the FAO publication: Worldwide regulations for mycotoxins 1995 - A compendium. FAO Food and Nutrition Papers, No. 64, Roma, 1997
*(C) = current limit ; (P) = proposed limit ; (V) = voluntary limit

1993a), but has transferred it to Group 1 according to recent investigations (IARC, 2002). Occurrence of AFM_1 in milk and derivatives is a worldwide concern since these products are frequently consumed in market and therefore could be important vehicles for introducing aflatoxin residues into the human diet (Ardic, 2009; Iha et al., 2011).

The incidence of AFM_1 is often higher in commercial milk than in raw milk, because of the dilution of uncontaminated bulk milk by only a few contaminated samples (Škrinjar et al., 1995; Kamkar, 2006). On the other hand, a seasonal trend in milk contamination showed that lower levels of AFM_1 occurred during the summer months, when animals consumed more grass than concentrated feeds (Galvano et al., 1996; Fallah et al., 2011).

The toxicological concern with AFM_1 arises in principle from its close structure similarity to AFB_1 (Figures 1 and 2), which has shown to be of the most potent carcinogens (Duraković, 2007; Duraković et al., 2011a).

To reduce this risk, most of the developed countries have regulated the maximum permissible levels of AFB_1 in foods and feeds as well as the levels of AFM_1 in milk and milk products, such as cheese and milk powder for infant foods (Table 1), until 1997, when the European Union has set the maximum residue limit (MRL) of 50 ng/L/kg for AFM_1

in milk (FAO Food and Nutrition Papers, 1997), and recently also MRL of 25 ng/L/kg in baby food (Commission Regulation EC No. 683/2004).

Currently the limits are highly variable, depending on the degree of development and economic involvement of the countries insetting regulatory limits: according to van Egmond (1989) and the Codex Alimentarius Commission (2001), regulatory limits seem to be a practical compromise between the need to have carcinogen-free commodities and the economic consequences of setting regulatory limits. Current and proposed limits for AFM_1 in selected European countries and USA are given in Table 1.

The European Commission (EC) has set a limit of 50 ng/L for AFM_1 in milk (EC, 2001), while the US Food and Drug Administration (US FDA, 1997) prescribed the maximum level for AFM_1 in milk 10-fold higher (500 ng/L) than the current level in the EC (Table 1).

AFM_1 is relatively stable in raw and processed milk and milk products, and is unaffected by pasteurization or processing into cheese (Sefidgar et al., 2011). Thus, if raw milk contains AFM_1, cheese made from such milk also contains AFM_1 (Galvano et al., 1996; Rubio et al., 2011). Traditional Istrian cheese is produced from raw ewe's milk on a 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 AFM_1 in Istrian ewe's milk, that is especially sold and consumed in Istria region, and to compare the obtained results with maximum of AFM_1 tolerance limits in milk that are accepted by some of the countries such as Croatia.

Materials and methods

Sampling

Investigated ewe's raw milk was collected from six farms located at different areas in the Istrian region of Croatia during the year 2007. Due to unequal beginning of the production of Istrian cheese at different farms, the collection of ewe's milk samples started at two farms in May, two farms in June and two farms in July 2007. All samples were collected in triplicates and transported to the laboratory inside a digital portable refrigerator stored at 4 °C, and frozen at -20 °C. A total specimen contained 18 ewe's raw milk samples, which were used for AFM_1 analysis, and randomly obtained from six different ewe's milk producers who delivered their milk to the Department of Microbiology, Faculty of Agriculture, Zagreb, Croatia. The ewe's milk samples were taken six times at 15 days intervals.

Methods

The most common analytical methods employed for AFM_1 determination are thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA). Among them, ELISA is often used for routine screening due to its several advantages, such as rapidity, simplicity and cost-effective (Rosi et al., 2007; Kursat et al., 2011). In this study, we employed a reliable method for ELISA determination of aflatoxins in raw ewe's milk, which we consider to be a better method for controlling aflatoxins (Rastogi et al., 2004; Radoi et al., 2008). Determination of AFM_1 was based on ELISA using RIDASCREEN IMMUNOLAB Aflatoxin M_1 test kit (Immunolab GmbH, Kassel, Germany). The test kit is sufficient for 96 determinations (including the calibration curve).

This method is quick, reliable, and costs effective for estimating AFM_1 and has been included in the official collection of test procedures by the German Federal Board of Health. Solvents used during the experiments were analytically pure and purchased from Merck (Darmstadt, Germany), and were prepared according to van Egmond (1989). AFM_1 standard solutions used for the construction of the calibration curve were at levels of 0, 5, 10, 20, 50, 100, 200, 500 and 1000 pg/mL and were all included in the ELISA test kit.

Preparation of raw ewe's milk samples

Preparation of samples was conducted according to the instructions of the IMMUNOLAB test kit. Briefly, raw ewe's milk samples (5 mL each) were chilled at 10 °C and then centrifuged for 10 min. at 3500 rpm. After centrifugation, the upper cream layer was completely removed by aspirating through a Pasteur pipette. An aliquot (100 μL per

well) of the skimmed ewe's milk was used directly in the test.

Test procedure

AFM_1 standards or the prepared raw ewe's milk sample solutions were added to microtiter wells in duplicate. During incubation for 60 min. at room temperature in the dark, the antibody binding sites are occupied proportionally to the AFM_1 concentration. The liquid was then removed completely from the wells, which were washed twice with distilled water. In the next step, any remaining free binding sites are occupied by the enzyme conjugate (enzyme labeled toxin), which was added (100 μL) and incubated for another 60 min. at room temperature in the dark. Any unbound enzyme conjugate was then removed in a washing step. Enzyme substrate (urea peroxide, 50 μL) and chromogen (tetramethylbenzidine, 50 μL) were added to each well and incubated for 30 min. at room temperature in the dark. Bound enzyme conjugate converts in colorless chromogen into a blue product.

Then, the addition of the stop reagent (100 μL per well) led to a color change from blue to yellow. The measurement was made spectrophotometrically at 450 nm (optional reference wavelength 600 nm). The mean values obtained of the absorbance values, obtained for the standards and the ewe's milk sample, were divided by the absorbance value of the first standard (zero standard), and multiplied by 100. Therefore, the zero standard is thus made equal to 100 % and the absorbance values are quoted in percentages (Lopez et al., 2003):

$$\% \text{ absorption} = \frac{\textit{Absorption standard (or sample)}}{\textit{Absorption zero standard}} \text{ x } 100\%$$

Evaluation of AFM_1

The standard calibration curve obtained in the present study for AFM_1 detection by using the ELISA test is depicted in Figure 3. The absorption is inversely proportional to the AFM_1 concentration in the raw ewe's milk sample. The AFM_1 concentration in μg/L corresponding to the absorption of each ewe's milk sample can be read from this curve. The mean lower detection limit was found to be 0.01 μg/L for ewe's milk. The results are consistent with Kaniou-Grigoriadou et al., 2005.

Calculation of extrapolated values of AFB_1 concentrations in ewe's feeding stuffs

Many researchers reported that there was a linear relationship between the amount of AFM_1 in milk and AFB_1 in feed consumed by the animals like cows and ewes (Bacher et al., 1998; Bakirci, 2001; Rastogi et al., 2004). It has been suggested that only 1.6 % of ingested AFB_1 is converted to AFM_1 by the dairy cattle. The values of AFB_1 in dairy cattle feeds are extrapolated from the back calculation of the values of AFM_1 obtained from the analysis of ewe's milk samples. Therefore, the values of AFB_1 contamination in dairy cattle feeding stuffs were back calculated by the formula given below (Price et al., 1985; Forbisch et al., 1986; Rastogi et al., 2004):

$$AFB_1(\mu g/kg) = \frac{AFM_1 \text{ (ng/L) x } 100}{1.6 \text{ x } 1000}$$

Results and discussion

Performance of analytical method

The values of AFM_1 calculated for the standards were entered in a system of coordinates on semilogarithmic paper against the AFM_1 concentration in ng/L (Figure 3). The resolution of the curve within the range 1-10 ng/L was insufficient to enable reliable interpolation of AFM_1 concentration based on their corresponding absorbance values, i.e. % maximum absorbance at 450 nm (Figure 3). Therefore, concentration values of AFM_1 obtained from the curve within this range were not considered reliable. However, the standard curve for the range 10-100 ng/L was reliable, and within this range, the inverse correlation between AFM_1 concentrations in the standard or in the samples, and their correspondent absorbance values at 450 nm was good (Figure 3). The percentages of absorbance obtained in the ELISA with the calibration curve (Figure 3) allow calculating the AFM_1 concentration in μg/L in the ewe's milk samples (Table 2), for each kind of milk-dairy farm milk.

In the present work, it was intended to investigate quantitatively the detection and determination of AFM_1, whose presence in Istrian raw ewe's milk was of special interest. As shown in Table 2, all of the 18 raw ewe's milk samples were found to be contaminated with AFM_1 at <0.025-0.037 μg/L. The mean value was 0.028 μg/L. All samples were

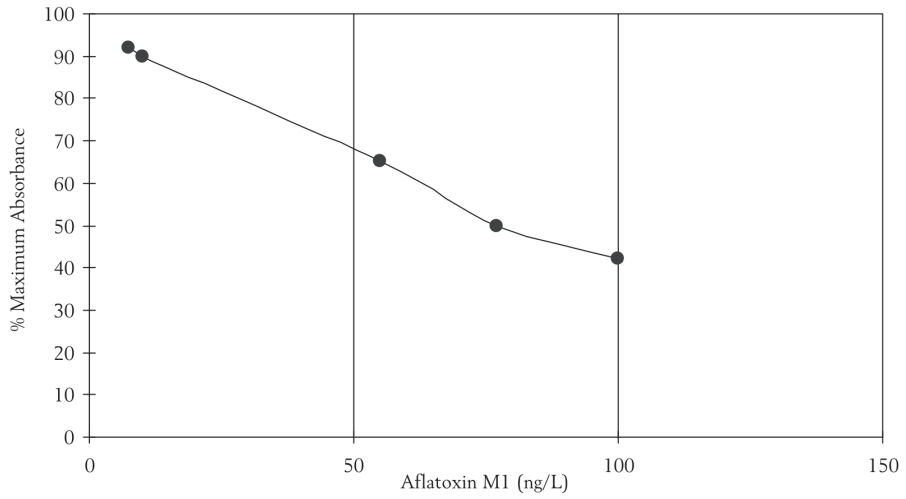


Figure 3. Calibration curve of aflatoxin M_1. The values on the ordinate represent absorbance at 450 nm for the ewe's milk sample or the standard divided by absorbance at the same wavelength for the control

below the advisory limit of EU regulations (0.05 μg/L).

It is known that contamination of ewe's milk is a result of exposure of AFB_1 to ewes through feedstuffs (Price et al., 1985; Forbisch et al., 1986; Rubio et al., 2011). Using the factor, the content of AFB_1 in the dairy cattle feed was extrapolated from AFM_1 contamination in ewe's milk samples (Table 3). It can be seen from the results that the feed contamination with AFB_1 in ewe's feed did not exceed 1.5-2.3 μg/kg, with an average of 1.9 μg/kg (Table 3). Moreover, the levels of AFB_1 in ewe's feed are very low, and in no case did not exceed the levels that are recommended by EC regulations (5 μg/kg) (European Commission, 2006).

Kamkar (2008) reported that in the spring and summer, samples mean concentrations of AFM_1 were significantly lower than that of samples measured in winter and autumn. The researcher showed that 40 % of the samples exceeded the EU limits, while none of the samples exceeded US limits (ten fold higher than EU limits) (Table 1). He pointed out the importance of seasonal factors in AFM_1 levels. Other researchers also reported a higher incidence of AFM_1 contamination during cool seasons, than hot ones (Blanco et al., 1988; Lopez et al., 2003). Due to the seasonal conditions in winter, dairy cows are fed with animal feed that exceed the allowed AFB_1 content.

Thus far, a relationship between AFM_1 occurrence level in milk and AFB_1 content in dairy cattle feed was reported (Wood, 1991; Guan et al., 2011). The most important factors on the amount of AFB_1 were temperature and moisture, since some moulds like *A. flavus* and *A. parasiticus* can easily grow in feeds having moisture between 13-18 %, and environmental moisture between 50-60 % (Jay, 1992). The mean concentrations of AFM_1 were de-

Table 2. AFM_1 contamination in ewe's milk samples in Istria region during the year 2007

Sample category	Samples analyzed	Positive samples	AFM_1 range (μg/L)	Exceeding EC/Codex regulations (50 ng/L)
Liquid ewe's milk	18	18 (18)	0.024-0.037	None
May	6	6 (6)	0.024-0.037	None
June	6	6 (6)	0.024	None
July	6	6 (6)	0.024-0.029	None

Table 3. Extrapolated AFB_1 concentration in dairy cattle feedstuffs based on AFM_1 contamination in ewe's milk samples

Sample category	Samples analyzed	Positive samples	AFB_1 range (μg/kg)	Exceeding EC/Codex regulations (5 μg/kg)
Dairy cattle feedstuffs	18	18 (18)	1.5-2.3	None
May	6	6 (6)	1.5-2.3	None
June	6	6 (6)	1.5	None
July	6	6 (6)	1.5-1.8	None

termined in spring and summer seasons as 0.024-0.037 and 0.024-0.029 μg/L, respectively. The mean concentrations of AFB_1 in dairy cattle feedstuffs were determined in spring and summer seasons as 1.5-2.3 and 1.5-1.8 μg/kg, respectively. Tables 2 and 3 show the obtained results.

Concentrations of AFM_1 were lower than the Maximum Residue Level (MRL) accepted by European Union, 50 ng/L/kg (European Commission, 2006). There is no statistically significant difference between AFM_1 and AFB_1 levels between spring and summer seasons. In this study, the occurrence of AFM_1 indicates that the levels of aflatoxins were higher in the spring. These results could be attributed to the effect of the type of feed intake to the residual levels of aflatoxins in raw ewe's milk in the spring and the first months of summer as a result of low feed quality. These results are in agreement with some other studies (Ghiasian et al., 2007; Herzallah, 2009; ElKhoury et al., 2011).

Conclusions

Raw ewe's milk samples were analyzed for the presence and concentration of aflatoxin M_1 using an enzyme-linked immunoassay. The contamination level of the ewe's milk samples with this mycotoxin observed in this study was usually lower than 50 ng/L. This food product provides no potential risk for human health. The initial approach to control the occurrence of AFM_1 in ewe's milk and milk products had been to control the AFB_1 contamination of animal feed. The contamination in the field, however, is very difficult to control, because it is influenced primarily by climate conditions, such as relative humidity and temperature. Soil moisture and insects damage are also important factors in the contamination of commodities. However, it is reported that the highest concentrations of aflatoxins are associated with the postharvest growth of *Aspergillus* moulds on poorly stored stuffs. For this reason, animal feeds should be checked regularly for AFB_1, and storage conditions of feeds must be under strict control. Milk and dairy products containing high levels of AFM_1 are prohibited for human consumption.

Beside this, it is important to have low levels of AFB_1 in the feeds of dairy animals, and in order to achieve this purpose, feeds of dairy cows and ewes should be kept away from contamination as much as possible. In recent past, it has been indicated that many countries of Europe showed relatively low levels of AFM_1 contamination in milk and milk products (Trucksess, 1997, 1998, 1999). The occurrence of AFM_1 at such low levels in European countries may be a result of stringent AFB_1 regulation in complementary foodstuffs for dairy cattle.

Procjena količine aflatoksina M_1 u sirovom ovčjem mlijeku za upotrebu u proizvodnji Istarskog sira

Sažetak

Istraživanje je provedeno radi određivanja prisutnosti i količine aflatoksina M_1 (AFM_1) u sirovom ovčjem mlijeku proizvedenom na različitim farmama u Istarskoj regiji Hrvatske i usporedbe dobivenih rezultata s maksimalnim dopuštenim vrijednostima AFM_1 u ovčjem mlijeku, koje su prihvaćene u pojedinim zemljama poput Hrvatske. Svi uzorci ovčjeg mlijeka sakupljeni su u triplikatu na dvije farme u svibnju, dvije farme u lipnju i dvije farme u srpnju tijekom 2007. godine. Učestalost kontaminacije uzoraka ovčjeg mlijeka s AFM_1 testirana je ELISA imunoafinitetnim postupkom. Analizirano je ukupno 18 uzoraka sirovog ovčjeg mlijeka.

AFM_1 nađen je u svim istraživanim uzorcima ovčjeg mlijeka. Srednja vrijednost iznosila je 0,028 μg/L. Niti u jednom uzorku nisu nađene koncentracije AFM_1 koje prelaze maksimalno dopuštenu razinu (0,05 μg/L) što je prihvaćena u Hrvatskoj.

Ključne riječi: plijesni, aflatoksin B_1, aflatoksin M_1, ELISA, ovčje mlijeko

Acknowledgements

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References

1. Alsberg, C.L., Black, O.F. (1913): Products of mould growth and disease. U.S. Department of Agriculture. *Bulletin of Plant Industry 270*, 1-12.
2. Ardic, M. (2009): Occurrence of Aflatoxin M_1 in Raw Ewe's Milk produced in Sanliurfa, Turkey. *Asian Journal of Chemistry, 21* (3), 1966-1970.
3. Atasever, M.A., Atasever, M., Ozturan, K. (2011): Aflatoxin M_1 levels in retail yoghurt and ayran in Erzurum in Turkey. *Turkish Journal of Veterinary and Animal Sciences 35* (1), 59-62.
4. Bacher, U., Martlbauer, E., Terblan, G. (1998): Detecting aflatoxin M_1 in milk from selected parts of Bavaria by using ELISA. German Federal Republic. *On Dairy Science Abstract 52*, 901-907.
5. Bakirci, I. (2001): A study on the occurrence of aflatoxin M_1 in milk and milk products produced in Van province of Turkey. *Food Control 12*, 47-51.
6. Blanco, J.L., Domingues, L., Gomez-Lucia, E., Garayzabal, J.E.E., Goyache, J., Suarez, G. (1988): Behavior of aflatoxin during the manufacture, repining and storage of Manchego-type cheese. *Journal of Food Science 77*, 1223-1231.
7. Blount, W.P. (1961): Turkey "X" disease. *Turkeys (Journal of British Turkey Federation) 9* (2), 52, 55-58, 61, 77.
8. Brnčić, M., Bosiljkov, T., Ukrainczyk, M., Tripalo, B., Rimac-Brnčić, S., Karlović, S., Karlović, D., Ježek, D., Vikić-Topić, D. (2009): Influence of Whey Protein Addition and Feed Moisture Content on Chosen Physicochemical Properties of Directly Expanded Corn Extrudates. *Food and Bioprocess Technology: an International Journal*, DOI10.1007/s11947-009-0273-0.
9. Codex Alimentarius Commission (2001): Comments submitted on the draft Maximum level for Aflatoxin M_1 in milk. *Codex committee on food additives and contaminants, 33rd session*, Hague, The Netherlands.
10. Creppy, E.E. (2002): Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters 127*, 19-28.
11. Duraković, L. (2007): *The influence of chosen parameters on the growth of the mold Aspergillus flavus ATCC 26949 in mixed culture and biosynthesis of aflatoxins B_1 and G_1*. PhD Thesis. Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia.
12. Duraković, S., Duraković, L., Vahčić, N., Skelin, A., Duraković, Z. (2008): Affect of moulds growth in mixed cultures on production of aflatoxins on maize hybrid. *Cereal Research Communications 36* (Part 3, Supplement 5), 1615-1618.
13. Duraković, L., Blažinkov, M., Skelin, A., Sikora, S., Delaš, F., Mrkonjić-Fuka, M., Huić-Babić, K., Redžepović, S. (2011a): Temperature-dose relationships with aflatoxin M_1 in milk on the brine shrimp (*Artemia salina*) larvae. *Mljekarstvo 61* (2), 145-153.
14. Duraković, L., Skelin, A., Sikora, S., Delaš, F., Mrkonjić-Fuka, M., Huić-Babić, K., Blažinkov, M. (2011b): Impact of new synthesized analogues of dehydroacetic acid on growth rate and vomitoxin accumulation by *Fusarium graminearum* under different temperatures in maize hybrid. *African Journal of Biotechnology 10* (52), 10798-10810.
15. ElKhoury, A., Atoui, A., Yaghi, J. (2011): Analysis of aflatoxin M_1 in milk and yoghurt and AFM_1 reduction by lactic acid bacteria used in Lebanese industry. *Food Control 22* (10), 1695-1699.
16. European Commission (2001): European Commission Regulation, Regulation (EC) No. 466/2001 of 8 March 2001, setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Commission L077*, 1-13.
17. European Commission (2004): European Commission Regulation, Regulation (EC) No. 683/2004 of 15 April 2004. *Official Journal of European Communities Legis. L 106*.
18. European Commission (2006): European Commission Regulation, Regulation (EC) No. 1881 of 19 December 2006, setting maximum levels for certain contaminations in foodstuffs. *Official Journal of the European Commission L364*, 5-24.
19. Fallah, A.A., Jafari, R.M., Yafari, T. (2011): Seasonal variation of aflatoxin M_1 contamination in industrial and traditional Iranian dairy products. *Food Control 22* (10), 1653-1656.
20. FAO - Food and Feed contaminants Publications (1997): Worldwide regulations for mycotoxins 1995. - A compendium. *FAO Food and Nutrition Papers (FAO)*, No. 64, FAO Rome (Italy), Food and Nutrition Div.; WHO, Geneva (Switzerland), 1997.
21. Forbisch, R.A., Bradley, B.D., Wagner, D.D., Long-Bradley, P.E., Hariston, H. (1986): Aflatoxin residues in milk of dairy cows after ingestion of naturally contaminated grain. *Journal of Food Protection 49*, 685-701.
22. Galvano, F., Galofaro, V., Galvano, G. (1996): Occurrence and stability of aflatoxin M_1 in milk and milk products: A worldwide review. *Journal of Food Protection 59* (10), 1079-1090.
23. Ghiasian, S.A., Maghsood, A.H., Neyestani, T.R., Mirhendi, S.H. (2007): Occurrence of aflatoxin M_1 in raw milk during the summer and winter seasons in Hamadan, Iran. *Journal of Food Safety 27*, 188-198.

24. Goldblatt, L.A. (1969): *Aflatoxin. Scientific Background, Control and Implications*. In: L.A. Goldblatt (Ed.). Academic Press, New York and London, pp: 1-54.
25. Guan, D., Pelwu, L., Zhang, Q. (2011): An ultra-sensitive monoclonal antibody-based competitive enzyme immunoassay for aflatoxin M_1 in milk and infant milk products. *Food Chemistry 125* (4), 1359-1364.
26. Herzallah, S.M. (2009): Determination of aflatoxins in eggs, milk, meat, and meat products using HPLC fluorescent, and UV detectors. *Food Chemistry 114*, 1141-1146.
27. IARC (1993a): Aflatoxins: naturally occurring aflatoxins (Group 1). Aflatoxin M_1 (Group 2B). *International Agency for Research on Cancer.* Lyon, France, *Vol. 56*, pp: 245-252.
28. IARC (1993b): Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. *International Agency for Research on Cancer*. Lyon, France, *Vol. 56*, pp: 345-395.
29. IARC (2002): Monograph on the evaluation of carcinogenic risk to humans. *World Health Organization, International Agency for Research on Cancer.* Lyon, France, *Vol. 82*, p: 171.
30. Iha, M.H., Barbosa, C.B., Okada, I.A. (2011): Occurrence of aflatoxin M_1 in dairy products in Brazil. *Food Control 22* (129), 1971-1974.
31. Jay, J.M. (1992): *Modern Food Microbiology, (4th Ed.)*. Chapman & Hall, New York.
32. Johnson, A.G. (1948): The role of fungi in the deterioration of grain in storage. *In: Preservations of Grains in Storage*. FAO Agricultural Studies, No. 2, pp: 88-91.
33. Kamkar, A. (2006): A study of the occurrence of aflatoxin M_1 in Iranian Feta cheese. *Food Control 17*, 768-775.
34. Kamkar, A. (2008): The study of aflatoxin M_1 in UHT milk samples by ELISA. *Journal of Veterinary Research 63*, 7-12.
35. Kaniou-Grigoriadou, I., Eleftheriadou, A., Mouratidou, T., Katikou, P. (2005): Determination of aflatoxin M_1 in ewe's milk samples and the produced curd and Feta cheese. *Food Control 16*, 257-261.
36. Kourousekos, G.D. (2011): Aflatoxin effects on animal health and milk composition of the ruminants. *Journal of the Hellenic Veterinary Medical Society 62* (1), 38-47.
37. Kursat, K., Ramazan, C., Tekinsan, K.K. (2011): Detection of aflatoxin M_1 levels by ELISA in white-brined Urfa cheese consumed in Turkey. *Food Control 22* (12), 1883-1888.
38. Lopez, C.E., Ramos, I.L., Ramadan, S.S., Bulacio, L.C. (2003): Presence of aflatoxin M_1 in milk for human consumption in Argentina. *Food Control 14* (1), 31-34.
39. Manetta, A.C., Di Giuseppe, L., Giammarco, M., Fusaro, J., Simonella, A., Gramenzi, A., Formigoni, A. (2005): High-performance liquid chromatography with post-column derivatisation and fluorescence detection for sensitive determination of aflatoxin M_1 in milk and cheese. *Journal of Chromatography, A. 1083*, 219-222.
40. Matijević, B., Božanić, R., Tratnik, Lj., Jeličić, I. (2008): The influence of whey protein concentrate on growth and survival of probiotic bacteria in whey. *Mljekarstvo 58* (3), 243-255.
41. Mrkonjić-Fuka, M., Engel, M., Skelin, A., Redžepović, S., Schloter, M. (2010): Bacterial communities associated with the production of artisanal Istrian cheese. *International Journal of Food Microbiology 142*, 19-24.
42. Piva, G., Galvano, F., Pietri, A., Piva, A. (1995): Detoxification methods of aflatoxins: a review. *Nutritional Research 15*, 689-714.
43. Prado, G., Madeira, J.E.G., Morais, V.A.D. (2011): Reduction of Aflatoxin B_1 in Stored Peanuts (*Arachis hypogaea*) Using *Saccharomyces cerevisiae*. *Journal of Food Protection 74* (6), 1003-1006.
44. Price, R.L., Paulson, J.H., Lough, O.G., Ginng, C., Kurtz, A.G. (1985): Aflatoxin conversion by dairy cattle consuming naturally contaminated whole cottonseed. *Journal of Food Protection 48*, 11-15.
45. Purchase, I.F.H., Steyn, M., Rinsma, R., Tustin, R.C. (1972): Reduction in the aflatoxin M content of milk by processing. *Food and Cosmetics Toxicology 10*, 383-387.
46. Radoi, A., Targa, M., Prieto-Simon, B., Marty, J.L. (2008): Enzyme-Linked Immunosorbent Assay (ELISA) based on superparamagnetic nanoparticles for aflatoxin M_1 detection. *Talanta 77*, 138-143.
47. Rastogi, S., Dwivedi, D.P., Khanna, K.S., Das, M. (2004): Detection of aflatoxin M_1 contamination in milk and infant milk products from Indian markets by ELISA. *Food Control 15*, 287-290.
48. Rosi, P., Borsari, A., Lasi, G., Lodi, S., Galanti, A., Fava, A., Girotti, S., Ferri, E. (2007): Aflatoxin M_1 in milk: reliability of immunoenzymatic assay. *International Dairy Journal 17*, 429-435.
49. Rothschild, L.J. (1992): IARC classes AFB_1 as class I human carcinogen. *Food Chemistry 34*, 62-66.
50. Rubio, R., Licon, C.C., Berruga, M. (2011): Occurrence of aflatoxin M_1 in the Manchego cheese supply chain. *Journal of Dairy Science 94* (6), 2775-2778.
51. Samaržija, D., Antunac, N., Pećina, M., Havranek, J. (2003): Quality of artisanal hard cheeses produced in the Mediterranean area of Croatia. *Milchwissenschaft 58* (1-2), 43-46.
52. Sassahara, M., Netto, D.P., Yanaka, E.K. (2005): Aflatoxins occurrence in foodstuff supplied to dairy cattle and aflatoxin M_1 in raw milk in the north of Parana state. *Food and Chemical Toxicology 43*, 981-984.
53. Sefidgar, S.A.A., Mirzae, M., Assmar, M. (2011): Aflatoxin M_1 in Pasteurized Milk in Babol city, Mazandaran Province, Iran. *Iranian Journal of Public Health 40* (1), 115-118.
54. Stoloff, L. (1977): Aflatoxins: an overview. *In: Proceedings of a conference on mycotoxins in human and animal health*. Pathotox Publishers Inc.: Park Forest South, pp: 7-28.
55. Škrinjar, M., Stubblefield, R.D., Vujičić, I.F., Stojanović, E. (1992): Distribution of aflatoxin-producing moulds and aflatoxins in Yugoslav dairy cattle feed and raw milk. *Acta Microbiologica Hungarica 39* (2), 175-179.

56. Škrinjar, M., Danev, M., Dimić, G. (1995): Investigations on the presence of toxigenic fungi and aflatoxins in raw milk. *Acta Alimentaria 24* (4), 395-402.

57. Trucksess, M.W. (1997): Mycotoxins. *Journal of AOAC International 80*, 119-126.

58. Trucksess, M.W. (1998): Mycotoxins. *Journal of AOAC International 81*, 128-137.

59. Trucksess, M.W. (1999): Mycotoxins. *Journal of AOAC International 82*, 488-495.

60. van Egmond, H.P., Paulsch, W.E. (1986): Mycotoxins in milk and milk products. *Netherlands Milk and Dairy Journal 40*, 175-188.

61. van Egmond, H.P., Wagstaffe, P.J. (1987): Development of Milk Powder Reference Materials Certified for Aflatoxin M_1 Content (Part I). *Journal of the Association of Official Analytical Chemists 70* (4), 605-610.

62. van Egmond, H.P. (1989): Aflatoxin M_1: occurrence, toxicity, regulation. *In: Mycotoxins in Dairy Products*, H.P., van Egmond (Ed.). Elsevier Applied Science, London, pp: 11-55.

63. Wood, G.E. (1991): Aflatoxin M_1. *In: Mycotoxins and Phytoalexins*, R.P. Sharma and D.K. Salunkhe (Eds.). CRC Press, Boca Raton, pp: 145-164.

64. Zafar, I.S., Bhati, I.A., Rafique, B.I.A. (2011): Aflatoxin Contamination in Chillies from Punjab Pakistan with Reference to Climate Change. *International Journal of Agriculture and Biology 13* (2), 261-265.