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Monitoring of aflatoxin M1 in raw cow milk in Croatia during winter 2015

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Abstract

A total of 548 raw milk samples were collected in the western, central and eastern regions of Croatia during February and March 2015. Aflatoxin M1 (AFM1) concentrations were quantified by the enzyme immunoassay method. The method limits of detection (LOD) and quantification (LOQ) were 22.2 and 34.2 ng/kg, respectively. The mean AFM1 levels measured in the three regions were (ng/kg) as follows: western 3.69, central 3.11 and eastern 4.14. In total, the 548 samples analysed concentrations were below the LOD value and accordingly below the European Union maximum residue level (EU MRL) of 50 ng/kg. The results suggest an absence of use of contaminated with aflatoxin B1 supplementary feedstuff for lactating cows in winter 2015. Such results might be related to the improved storage conditions for feed as well as to the enhanced and more stringent feed control system for mycotoxins in Croatia.

Key words: aflatoxin M1, raw cow milk, ELISA, Croatian regions

Introduction

Milk and dairy products are among the most important dietary foods due to their content of fundamental nutrients such as proteins, lipids, macro- and microelements. However, industrial, agricultural and urban emissions cause environment pollution, which may result in the contamination of milk and dairy products with toxic contaminants such as heavy metals or mycotoxins (Bilandžić et al., 2011; Duarte et al., 2013). In recent years, the incidence of aflatoxin M1 (AFM1) presence in raw and commercially available milk and dairy products was reported in different countries, and a high intake of such products by consumers could have had negative health implications (Assem et al., 2011; Bilandžić et al., 2014; Fallah et al., 2011; Golge, 2014; Nemati et al., 2010; Rahimi et al., 2010; Škrbić et al., 2014).

Animals and humans can be exposed to aflatoxins after a direct ingestion of foods contaminated with the fungi *Aspergillus flavus* and *Aspergillus parasiticus* at some time during the growth, harvest or storage of foods (Bennet and Klich, 2003).

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Aflatoxin B1 (AFB1) is released in the stomach and small intestine after absorption and is then transported to the liver where it is primarily metabolized by microsomal mixed function oxidases (MFOs) to derivatives such as AFM1, aflatoxin P1 and aflatoxin Q1 (Sharma and Salunkhe, 1993). All metabolic products is released into the bloodstream and excreted in the urine or bile and some of these, particularly AFM1, are excreted in milk and eggs.

A comparative study of carcinogenicity of AFB1 and AFM1 in trouts and rats showed AFM1 to be significantly less potent hepatocarcinogen (Sharma and Salunkhe, 1993). Finally it was concluded that the carcinogenicity of AFM1 was about ten times lower in comparison to AFB1 (JECFA, 2001). According to these findings, the production of milk containing AFM1 in amounts up to 0.05 mg/kg (MRL value) would require the average food intake of about 40 mg/kg of AFB1 per day for dairy cows (FAO/WHO, 2002). After ingestion of contaminated foodstuffs, AFM1 appears in milk 2-3 days following ingestion and an additional 2-3 days are required for its excretion from milk and reduction to the zero level as when feed does not contain AFB1 (Prandini et al., 2009).

The variations of AFM1 concentrations in milk and dairy products in different countries are due to differences in geographic location, climatic factors, seasons and differences in the storage of animal feed and the application of good manufacturing practice in primary production (Duarte et al., 2013; Rahimi et al., 2010). An increased incidence of elevated concentrations is characteristic for tropical and subtropical regions such as Africa, South America (Brazil), Asia (Pakistan) and the Middle East, where high temperatures and drought, or conditions of warmth and humidity favour the growth of toxigenic fungi (Asi et al., 2012; Dutton et al., 2012; Elzupir and Elhussein, 2010; Fallah et al., 2011; Golge, 2014; Iqbal and Asi, 2013; Sadia et al., 2012; Picinin et al., 2013).

In 2013 and 2014, elevated concentrations of AFM1 were reported in cow's milk from the eastern regions of Croatia (Bilandzic et al., 2014, 2015) with elevated levels particularly during the autumn and winter months. Therefore, the aim of this study was to survey AFM1 concentrations in two spring months of 2015 in farms from western, central and eastern Croatia.

Materials and methods

Sample collection and preparation

A total of 381 raw cow's milk samples were collected at 2 farms from the central region and 8 farms from the western region during February and March 2015. In the same period, 167 samples were also taken from small dairy farms in eastern Croatia. Samples were cool stored at 2-8 °C or frozen at -20 °C until the analysis.

Prior to analysis, milk samples were centrifuged at 3500× g at 10 °C for 10 minutes. The upper cream layer was completely removed by aspirating through a Pasteur pipette. Skimmed milk (100 μL per well) was used directly in the test.

Chemicals and equipment

The Ridascreen enzyme immunoassay "Enzyme immunoassay for the quantitative analysis of aflatoxin M1" (R-Biopharm AG, Darmstadt, Germany) was used for the measurement AFM1 concentrations according to the manufacturer's instructions. Standard stock solution of AFM1 (1000 mg/L) was prepared by dissolving standard AFM1 (Sigma-Aldrich, St. Louis, USA) in methanol (analytical grade; Kemika, Zagreb, Croatia). Working solutions for the enrichment of milk samples were prepared by further dissolution of stock solution in methanol up to a concentration of 10 μg/L. Stock solution was stored at 4 °C for no longer than 6 months, and working solutions were prepared prior to each analysis.

Samples were prepared using the Vortex Genius 3 (IKA® -Werke GmbH & CO.KG, Germany) and centrifuge Rotanta 460R (Hettich GmbH & Co.KG, Tuttlingen, Germany). Microplate reader Sunrise Absorbance Reader (Tecan Austria GmbH, Salzburg, Austria) was used to measure the optical density at 450 nm.

Test procedure

The ELISA test procedure was performed according to the manufacturer's instructions. It has to be emphasized that in the case of AFM1 concentrations exceeding 80 ng/mL, samples have to be diluted with sample dilution buffer from the test kit and reanalyzed.

Method validation

The ELISA method was validated according the European Commission guidelines (European Commission, 2002) as previously described (Bilandžić et al., 2014). The validation parameters were (ng/kg): detection capacity (CCβ) 33.0, limit of detection (LOD) 22.2, and limit of quantification (LOQ) 34.2.

Statistical analyses

The Statistica 10 software package (StatSoft® Inc., Tulsa, USA) was used for statistical analyses. Concentrations of AFM1 were expressed as mean ± SD, minimum and maximum value.

Results and discussion

AFM1 is a stable molecule in raw and processed milk and is unaffected by the process of pasteurisation or treatments used during cheese production (Oruc et al., 2013). Therefore, it is important to set the effective control of raw milk and dairy products in accordance with the defined maximum residue levels set by the European Union (EU). The maximum residue level for AFM1 concentrations in milk, heat-treated milk and milk for dairy products approved by the EU (EU MRL) is 50 ng/kg, and 25 ng/kg for milk-based baby food (EC, 2006).

The AFM1 concentrations detected in raw milk in the period of February and March of 2015 in different Croatian regions are presented in Table 1. The mean AFM1 levels measured in the three regions were (ng/kg) as follows: western 3.69, central 3.11 and eastern 4.14. In total, the 548 analysed samples contained concentrations below the LOD value (22.2 ng/kg) and accordingly below the EU MRL of 50 ng/kg.

The incidence of elevated AFM1 concentrations is characteristic for countries with dry climate conditions or with seasons with long drought periods that favour the development of mould and elevated AFB1 levels in feed (Prandini et al., 2009). In recent years, studies have shown elevated AFM1 levels in the winter months in Iran, Pakistan, Turkey, China, Croatia and Serbia (Rahimi et al., 2010; Fallah et al., 2011; Iqbal and Asi, 2013; Golge, 2014; Xiong et al., 2013; Bilandžić et al., 2014; Škrbić et al., 2014).

Table 1. AFM1 concentrations in raw milk from western, central and eastern regions of Croatia collected during the period February-March 2015.

Region/ Farms	*N*	Mean (ng/kg)	SD	Min (ng/kg)	Max (ng/kg)
Western region					
F1	79	4.08	0.91	2.06	7.41
F2	55	3.77	0.84	2.23	5.69
F3	25	4.12	0.43	3.22	4.81
F4	22	3.36	0.74	2.08	4.55
F5	34	3.58	0.53	2.42	4.52
F6	65	3.32	0.47	1.74	4.16
F7	15	3.61	0.99	2.32	5.95
F8	22	3.32	0.43	2.64	4.13
Total	317	3.69	0.78	1.74	7.41
Central region					
F9	38	3.19	0.47	2.18	3.85
F10	26	2.98	0.42	2.23	4.01
Total	64	3.11	0.46	2.18	4.01
Eastern region					
Total	167	4.14	1.13	2.02	10.6

AFM1 concentrations reported in February 2013 in Serbia were in the range from 540 to 1440 ng/L (Škrbić et al., 2014). In studies from Pakistan, China and Turkey, 71 %, 72.2 % and 40.4 % milk samples, respectively, collected in winter concentrations exceeded the EU MRL value, with maximum concentrations of 845.5, 420 and 1101 ng/L (Iqbal and Asi, 2013; Xiong et al., 2013; Golge, 2014).

Also, elevated concentrations of AFM1 were reported in cow's milk from eastern Croatia in 2013 (Bilandžić et al., 2014). During the winter season (February and March) of 2013, 45.9 % and 35.4 % of raw milk samples contained AFM1 exceeding the EU MRL. The highest AFM1 level measured during those two months were 1105 and 1135.0 ng/L. A study showed seasonal variations of AFM1 concentrations, with an increase in concentrations during the winter and spring months, which was associated with the use of greater amounts of supplementary feedstuff, dry hay, corn and concentrated feed for lactating cows in winter than in summer months (Bilandžić et al., 2014). This was confirmed with elevated levels of AFB1 measured in 36.5 % of maize samples collected in three Croatian regions in 2013, with levels exceeding the maximal permitted level of 20 μg/kg (Pleadin et al., 2014).

Furthermore, in a study conducted in eastern, western and other regions of Croatia over four seasons in 2014, elevated levels were measured in winter (Bilandžić et al., 2015). During February and March, five milk samples from the eastern region and one sample from the western region of Croatia contained concentrations exceeding the EU MRL. However, in the present study, which is a part of the ongoing monitoring of AFM1 concentrations in milk in three regions of Croatia carried out in February and March of 2015, the mean AFM1 concentrations were below LOD values.

The results suggested an absence of the use of contaminated supplementary feedstuff for lactating cows in winter 2015, which may be the result of improved storage conditions for feed and enhanced and more stringent feed control system for mycotoxins in Croatia.

Praćenje aflatoksina M1 u sirovom kravljem mlijeku u Hrvatskoj tijekom zime 2015.

Sažetak

Ukupno 548 uzoraka sirovog mlijeka prikupljeno je u zapadnoj, središnjoj i istočnoj regiji Hrvatske tijekom veljače i ožujka 2015. Koncentracije aflatoksina M1 (AFM1) su određivane primjenom imunoenzimskog testa. Granice detekcije (LOD) i kvantifikacije (LOQ) testa su 22,2 i 34,2 ng/kg. Prosječne razine AFM1 izmjerene u tri regije su (ng/kg): zapadna 3,69, središnja 3,11 i istočna 4,14. U ukupno 548 uzoraka analizirane koncentracije su bile ispod LOD vrijednosti i sukladno tome ispod maksimalne dozvoljene koncentracije propisane u Europskoj uniji (EU MDK) od 50 ng/kg. Rezultati upućuju na odsutnost upotrebe dopunske hrane za krave muzare koja je kontaminirana s aflatoksinom B1 tijekom zime 2015. To može biti rezultat poboljšanih uvjeta držanja hrane te poboljšanog i postroženog sustava kontrole mikotoksina u hrani za životinje u Hrvatskoj.

Ključne riječi: aflatoksin M1, sirovo kravlje mlijeko, ELISA, regije Hrvatske

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