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Aflatoxin M1 occurrence in Serbian milk and its impact on legislative

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ABSTRACT. Serbia is a country which has repeatedly changed aflatoxin M1 (AFM1) legislation in milk. As a country that clearly has aspiration toward the EU membership, Serbia implemented the EU legislation for this mycotoxin at level of 0.050 µg/kg. However, due to high occurrence of AFM1 in milk, legislation has been changed several times in the past few years as an effort to preserve domestic milk production.

This paper presents the results of four years monitoring of different milk types taken from Serbian market and from Serbian farmers. The samples were analyzed by liquid chromatography on ODS Hypersil column with fluorescence detector (FLD), after cleanup on immunoaffinity column. Limit of quantification was 0.005 µg/kg, while obtained mean value for trueness was 95.1%, respectively. Average AFM1 levels in 2013, 2014, 2015 and 2016 were 0.205, 0.127, 0.238. and 0.269 µg/kg, respectively. Overall occurrence of AFM1 was 80.9%, with the average content of 0.216 µg/kg (ranged from 0.005 to 5.078 µg/kg). According to this, 49.1% of samples were above the EU regulation.

In years to come, Serbia will have a challenge to produce the milk that is in compliance with the permitted level of AFM1. Especially, when it is known that in not so distant future, an increase in temperature as a result of the certain climate changes is expected.

Keywords: aflatoxin M1, high-performance liquid chromatography, legislation, milk, Serbia

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INTRODUCTION

Aflatoxins (AFs) in food and feed are recognized as a public health problem of considerable importance. Williams et al. (2004) estimated that 4.5 billion of the world's population is exposed to AFs. Because security blankets in crops at pre-harvest and post-harvest level are not as strict as in developed countries, populations of developing countries are the most susceptible to aflatoxicosis illness (Williams et al., 2004). The same problem occurs with milk derivatives in developing countries for the reason that they have not accepted and assumed amenities as quick as developed countries (Lizárraga-Paulín et al., 2011).

Aflatoxin losses to livestock and poultry producers from aflatoxin-contaminated feeds include death and the subtler effects of immune system suppression, reduced growth rates, and losses in feed efficiency. Other adverse economic effects of AFs include lower yields of food and fiber crops (Anon, 1989). Among all AFs, the aflatoxin B1 (AFB1) is genotoxic and is considered to be the most potent hepatocarcinogenic substance (Van Egmond and Jonker, 2004; Zein, 2011).

Aflatoxins M1 (AFM1) and M2 (AFM2) are thermo-resistant hydroxylated metabolites produced by lactating animals consuming aflatoxin contaminated feeds (Lizárraga-Paulín et al., 2011). In farm animals, AFB1 and aflatoxin B2 (AFB2) are converted into AFM1 and AFM2 metabolites with the ratio of 1–3% between AFB1 and AFM1 (Ali et al., 1999; Herzallah, 2009). Cows can convert AFB1 into AFM1 within 12–24 hours after ingestion of contaminated feed and the highest levels are reached after a few days (Ayar, 2007). After exclusion of contaminated feed from diets, the AFM1 concentration in the milk decreases to an undetectable level after 72 hours (Van Egmond, 1989; Gimeno, 2004; Özdemir, 2007). However, AFs carry-over from feed into milk is exponentially increased (Britz et al., 2013). These authors suggested that in the case of high yielding cows with the average milk production of 45 kg and daily intake of 25 kg dry matter (DM), aflatoxin B1 needed to be below 1.4 µg/kg to ensure milk production with AFM1 levels lower than 0.05 µg/kg (Britz et al., 2013).

To reduce the risk of exposure, many countries have regulated the maximum level (ML) of AFB1 in

feed (and have set or proposed ML of AFM1 in milk). Currently, the legal limits of AFB1 in feedstuffs are highly variable from the European Union (EU) countries to other countries (the EU has a limit of 5 µg/kg for dairy feed) (European Commission, 2003). In Serbia, proposed ML of AFB1 is harmonized with EU since the April 2014 (Serbian Regulation, 2014). Regarding the regulation of AFM1, European Union has established ML in raw milk of 0.05 µg/kg (European Commission, 2006). This level was also set in Serbia (Serbian Regulation, 2011) but since then, it has been changed several times.

The aim of this paper was to investigate the occurrence of AFM1 in different types of milk during 2013–2016, in Serbia and its impact on Serbian legislation. Such data would certainly be a contribution to food safety assessment of this very important foodstuff, and could surely assist to finally determine ML of AFM1 in milk, on a long-term period in Serbia.

MATERIALS AND METHODS

Samples

Milk samples were randomly collected in Serbia during four-year period (2013–2016). Samples were then divided into four groups, regarding year 2013 (raw milk, pasteurized milk, UHT milk, and samples of organic pasteurized milk), three groups in samples originating from year 2014 (raw milk, UHT milk, and organic pasteurized milk) while in samples from 2015, only raw milk samples were analyzed. In the last year of monitoring, samples of raw and pasteurized milk were tested. Samples of raw milk were collected from small dairy farms; pasteurized milk samples were collected from small and big milk producers while UHT and organic milk samples were collected from big milk producers. All milk samples were produced in Serbia. Immediately after collection samples were transported to the laboratory and analyzed.

Reagents

Acetonitrile was purchased from Sigma Aldrich (Buchs, Switzerland) while n-hexane was obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was purchased from Thermo Fisher Scientific (Cheshire, United Kingdom).

Sample preparation for high-performance liquid chromatography (HPLC) analysis was done using AflaStar™ M1 R-Immunoaffinity Columns (IAC) (Romer Labs Inc., Union, MO, USA). Deionized water (electric conductivity, $< 3.5 \mu\text{S/cm}$) from reverse osmosis filtration system DS – 83 (Amtast, USA), was used. Nitrogen gas was obtained from Messer (Belgrade, Serbia).

AFM1 standard with certified concentration of $10 \mu\text{g/ml}$ was purchased from Sigma Aldrich (Buchs, Switzerland). Standard stock solutions were prepared in acetonitrile and stored at -18°C . These solutions were used for solvent based calibration. The standard solutions were stored under refrigerator conditions (4°C).

Sample preparation

Fifty ml of warm milk ($35 - 37^\circ\text{C}$) was filtered through a quantitative filter paper for fast filtration (Filtros Anolia, Barcelona, Spain) and applied to the IAC. Flow rate of milk was approximately $1-3 \text{ ml/min}$. After the milk completely passed, IAC was rinsed with 20 ml of deionized water. The AFM1 was eluted with 4 ml of acetonitrile. Eluate was collected and evaporated to dryness at 50°C using gentle stream of nitrogen.

Since AFM1 in milk samples occur in small concentrations, post derivatization step for HPLC-FLD analysis is required to enhance its fluorescence (Chen et al., 2005). This was achieved by adding $200 \mu\text{l}$ of TFA and the same volume of n-hexane to the residue from the evaporated acetonitrile eluate or to the AFM1 working standards, vortexed for 30 s, and kept in the dark for 10 min at 40°C . Further, after evaporation $300 \mu\text{l}$ of water:acetonitrile (75:25, v/v) mixture was added to the vials and vortexed for 30 s.

HPLC determination

The HPLC instrument was an Agilent 1260 (Agilent Technologies Inc., USA) system equipped with a Chemstation Software (Agilent Instrument Utilities, ChemStation for LC 3D systems, Rev. B.04.03), fluorescence detector (FLD), a binary pump, a μ -degasser, an auto sampler and Agilent column (Hypersil ODS C18, $4.6 \times 100 \text{ mm}$, $5 \mu\text{m}$). The mobile phase consisted of an isocratic mixture of

water:acetonitrile (75:25, v/v) and flow rate was 1.0 ml/min . Twenty microliters of standards and samples were injected into the HPLC column. The fluorescence detector was set to an excitation and emission wavelengths of 360 and 423 nm, respectively. The retention time was around 2.1 min.

Analytical quality control

Calibration curves used for quantitative determination were constructed on the basis of the area under the AFM1 chromatographic peaks, using seven AFM1 working standard solutions. Analytical quality control was implemented according to the Commission Regulation (European Commission, 2002). The linearity of the method was assessed by standard ranging from $2.5-50 \text{ ng/ml}$. The correlation coefficient was 0.9999. The limit of quantification (LOQ) for liquid chromatography determination based on ten times the ratio of the standard deviation of intercept and slope of the calibration curve, was 0.25 ng/ml of AFM1, which is equivalent to $0.005 \mu\text{g/kg}$ of AFM1 in sample. Method accuracy was investigated by analyzing certified reference material MI1142-1/CM (Progetto Trieste, Padova, Italy) in six replicates and the mean value for trueness was 95.1%.

RESULTS

In this study, a total of 423 milk samples were analyzed to determine concentration of AFM1. Obtained results were summarized in Table 1. Overall occurrence of AFM1 was 80.9%, with the average content of $0.216 \mu\text{g/kg}$ ($0.005 - 5.078 \mu\text{g/kg}$). For easier interpretation, the results were classified, based on the contamination level, into five groups: $<0.005 \mu\text{g/kg}$, $0.005-0.05 \mu\text{g/kg}$, $0.05-0.25 \mu\text{g/kg}$, $>0.25 \mu\text{g/kg}$ and $>0.5 \mu\text{g/kg}$. According to this, 49.1% of samples were above the EU regulation (European Commission, 2006).

As can be seen, in all 4 groups of samples from 2013 a very high level of contamination was established, ranging from 80.0 to 100.0%, respectively. The highest contamination with AFM1 was found in pasteurized milk (100.0%), as well as the highest mean value of this mycotoxin ($0.270 \mu\text{g/kg}$). Similar average content of AFM1 was found in raw milk ($0.231 \mu\text{g/kg}$), and slightly lower in UHT milk (0.145

Table 1. Occurrence of AFM1 in Serbian milk during four-year period (2013–2016)

Year	Category	No.	Positive samples	Range of conc. (µg/kg)					Average±SD	Min-Max
				<0.005	0.005-0.05	0.05-0.25	>0.25	>0.5		
2013	Raw	64	55 (85.9)	9 (14.1)	29 (45.3)	18 (28.1)	8 (12.5)	4 (6.3)	0.231±0.719	0.005–5.078
	Pasteurized	22	22 (100.0)	0 (0.0)	1 (4.5)	14 (63.6)	7 (31.8)	2 (9.1)	0.270±0.268	0.037–1.215
	UHT	39	37 (94.9)	2 (5.1)	8 (20.5)	22 (56.4)	7 (17.9)	0 (0.0)	0.145±0.107	0.007–0.411
	Organic	5	4 (80.0)	1 (20.0)	3 (60.0)	1 (20.0)	0 (0.0)	0 (0.0)	0.044±0.039	0.016–0.101
	Total	130	118 (90.8)	12 (9.2)	41 (31.5)	55 (42.3)	22 (16.9)	6 (4.6)	0.205±0.508	0.005–5.078
2014	Raw	47	39 (83.0)	8 (17.0)	23 (48.9)	11 (23.4)	5 (10.6)	4 (8.5)	0.153±0.329	0.008–1.486
	UHT	16	14 (87.5)	2 (12.5)	8 (50.0)	6 (37.5)	0 (0.0)	0 (0.0)	0.051±0.038	0.010–0.114
	Organic	1	1 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.008	0.008
	Total	64	54 (84.4)	10 (15.6)	32 (50.0)	17 (26.6)	5 (7.8)	4 (6.3)	0.127±0.288	0.008–1.486
2015	Raw	126	98 (77.8)	28 (22.2)	25 (19.8)	46 (36.5)	27 (21.4)	17 (13.5)	0.238±0.346	0.006–2.613
2016	Raw	68	53 (77.9)	15 (22.1)	19 (27.9)	21 (30.9)	13 (19.1)	9 (13.2)	0.353±0.716	0.008–3.928
	Pasteurized	35	19 (54.3)	16 (45.7)	17 (48.6)	2 (7.7)	0 (0.0)	0 (0.0)	0.035±0.049	0.007–0.233
	Total	103	72 (69.9)	31 (30.1)	36 (35.0)	23 (22.3)	13 (12.6)	9 (8.7)	0.269±0.630	0.007–3.928
Total		423	342 (80.9)	81 (19.1)	134 (31.7)	141 (33.3)	67 (15.8)	36 (8.5)	0.216±0.470	0.005–5.078

No.: number of samples

Positive samples: number (percentage)

Range of concentrations: number (percentage)

Average ± SD: average concentration (µg/kg) ± standard deviation (µg/kg)

Min–Max: minimum and maximum concentrations (µg/kg)

µg/kg). Maximum concentration of AFM1 (5.078 µg/kg) was found in a raw milk sample. Particularly worrying fact was that even 95.5% of pasteurized milk and 74.4% of analyzed UHT milk samples established AFM1 concentration greater than ML defined by EU Regulation (European Commission, 2006) and Serbian Regulation (Serbian Regulation, 2011) that was in force at the time of analysis.

As for the samples obtained in 2014, the situation is considerably different. Although the presence of AFM1 remained very high (> 80%), the number of samples containing AFM1 in concentration higher than EU and Serbian MLs (European Commission, 2006; Serbian Regulation, 2011) was significantly smaller. Maximum concentration of AFM1 was again found in sample of raw milk, but it was significantly lower (1.486 µg/kg) compared to the previous year. An encouraging fact is the significantly lower average content of AFM1 in UHT milk (0.051 µg/kg), which is slightly above the ML defined by EU Regulation.

During 2015, a high percentage of raw milk samples containing AFM1 over the current regulations was established once more. This number is higher than in 2014 or even in relation to 2013, when it

comes to raw milk. However, these results cannot be associated with the entire 2015. To be precise, in the period from January to mid-September 2015, only one sample (4.3%) exceeded the current regulations, at a concentration of 0.073 µg/kg. During this period was found both, the lowest presence of AFM1 (17.4%) and its lowest mean value (0.034 µg/kg). In samples analyzed in period from mid-September 2015 to November 2015, we started establishing the above mentioned very high levels of AFM1. Differences in results between the two periods in 2015 are shown in Table 2.

In 2016, there was still a problem with AFM1 contamination since 69.9% of samples contained levels above LOQ. Also, a high proportion (34.9%) of samples containing AFM1 above European ML persisted. The average AFM1 levels were 0.716 µg/kg (raw milk) and 0.049 µg/kg (pasteurized milk). This is encouraging, since pasteurized milk is used for human consumption.

DISCUSSION

Presence of AFM1 in milk depends primarily on the presence of AFB1 in feed. Hot and dry weather

Table 2. Differences in occurrence of AFM1 in Serbian milk during 2015

Year	Period	No.	Positive samples	Range of conc. (µg/kg)					Average±SD	Min–Max
				<0.005	0.005-0.05	0.05-0.25	>0.25	>0.5		
2015	Jan – Sep 15	23	4 (17.4)	19 (82.6)	3 (13.8)	1 (4.3)	0 (0.0)	0 (0.0)	0.034±0.030	0.008–0.073
	Sep 15 – Nov	103	94 (91.3)	9 (87.4)	22 (21.4)	45 (43.7)	27 (26.2)	17 (16.5)	0.247±0.351	0.006–2.613
Total	126	98 (77.8)	28 (22.2)	25 (19.8)	46 (36.5)	27 (21.4)	17 (13.5)	0.238±0.346	0.006–2.613	

No.: number of samples

Number of positive samples: number (percentage)

Range of concentration: number (percentage)

Average ± SD: average concentration (µg/kg) ± standard deviation (µg/kg)

Min–Max: minimum and maximum concentrations (µg/kg)

conditions during maize growing season 2012 were favorable for *Aspergillus* molds growth and AFs productions. Many authors from Serbia confirmed the presence of AFs in maize from mentioned growing season. Jajić et al (2013) investigated the occurrence of aflatoxin in 44 samples of maize and established presence of 63.6% with the average value of 74.5 µg/kg. Levels of AFs exceeded the ML established by European Regulation (European Commission, 2006) in 45.5% analyzed samples. Kos et al. (2013) detected AFs in 68.5% of maize samples with the mean level of 36.3 µg/kg. High levels of AFs in maize (69.9%), as well as in maize silage (38.0%), which is primarily used for dairy cattle diets, were found by Lević et al. (2013). Since maize is mainly used as a component of animal feed, it is most likely the reason for the appearance of AFM1 in milk and milk products. Particularly interesting is the period September–November 2015 (Table 2), because the low presence of AFs in maize from 2015 harvest (unpublished results) could not indicate a potential occurrence of AFM1 in milk. Regardless, the AFM1 contamination of milk in Banat region was very important. According to unofficial data of Serbian Ministry of Agriculture, the cause of milk contamination was shortened period for preparation of silage intended for cows. This situation was particularly evident in Banat region, from where the contaminated samples originated. The authors of this study did not have the possibility to analyze the mentioned silage samples, because that was under the authority of the Ministry of Agriculture.

Presence of AFM1 in milk in an earlier period in Serbia was published in few reports. Janković et al.

(2009) analyzed 23 milk samples using ELISA method, and in 3 (13.0%) samples concentration of AFM1 was higher than 0.05 µg/kg. In 70 cow's milk samples, Polovinski-Horvatović et al. (2010) used TLC method after immunoaffinity column clean-up to determine AFM1 and have found that none were contaminated with AFM1 in concentration greater than 0.05 µg/kg. Lower occurrence of AFM1 contamination reported in these reports from Serbia, compared to the results obtained in this and subsequent studies, can be explained with the absence of AFs in maize and other feed material in Serbia in previous years (Kokić et al., 2009; Jakšić et al., 2011; Kos 2013). In all the reports, ELISA test kit was used for AFs determination. During this period in the neighboring Republic of Croatia, Bilandžić et al. (2010), by applying ELISA method, analyzed AFM1 in 61 raw milk samples and found that only 1.6% of samples was contaminated with AFM1 in concentration greater than 0.05 µg/kg.

A few years later, the situation in the Republic of Serbia and the whole region, when it comes to AFM1 in milk, appeared to be completely different. In period February–May 2013 in Republic of Serbia, Škrbić et al. (2014) analyzed 50 samples of sterilized, pasteurized and raw milk using UHPLC-MS/MS method. They found that 76.0% of analyzed samples were above the maximum allowed limit set by European legislation. The highest average level of AFM1 was found in raw (0.49 µg/kg), while the lowest average level was found in pasteurized milk (0.19 µg/kg). The authors also found that the average level of AFM1 was decreasing during the February–

April-May period. Kos et al. (2014) investigated the occurrence of AFM1 in larger number of samples of cow's milk (150) by applying ELISA method. AFM1 was detected in 98.7% of analyzed cow's milk samples in concentrations ranged from 0.01 to 1.2 µg/kg. Further, even 129 (86.0%) cow's milk samples contained AFM1 in concentration greater than ML of 0.05 µg/kg defined by European Union (EU) Regulation. Vuković et al. (2013) investigated 111 milk samples and detected AFM1 in 95 (85.6%) samples. However, 62 (55.9%) samples were contaminated at a level above the ML accepted by the European regulation and 4 (3.6%) above Serbian ML for AFM1, which was in force at the time. The authors applied HPLC-FLD method for the AFM1 determination. Torović (2015) determined AFM1 levels, using HPLC-FLD method, in 80 samples of milk and 21 samples of infant formulae. Samples were contaminated in the range 0.02–0.32 µg/kg, whereby AFM1 exceeded European ML in 75.0% of the samples.

Practically at the same time, in the immediate surroundings, in neighboring Croatia, Bilandžić et al. (2014) analyzed a very representative number of samples of raw milk (3736) and UHT milk (706) collected in the period from February to July 2013 in Croatia, using ELISA method. AFM1 levels exceeded the EU ML values in 27.8% of raw and 9.6% of UHT milk samples.

The results of our tests, and tests of researchers from the Western Balkans region certainly contributed to significant changes in the Serbian regulations in the last 4 years. Namely, with the first reports of high presence and content of aflatoxin in feed, and then in milk, in the Republic of Serbia was in force legislation which prescribed the aflatoxin ML of 50 µg/kg in maize and 10 µg/kg in complete mixtures for dairy cows (Serbian Regulation, 2010) as well as 0.050 µg/kg of AFM1 in milk (Serbian Regulation, 2011). As the Ministry of Agriculture, based on the initial results, found that most of the milk present in the Serbian market contained quantities of AFM1 greater than those prescribed in Serbian Regulation at that time (Serbian Regulation, 2011), it adopted a new regulation that prescribed ML of AFM1 in milk at 0.500 µg/kg (Serbian Regulation, 2013). In April 2014 the Ministry, to contribute to the reduc-

tion of AFM1 in milk, tightened regulation that prescribed the content of aflatoxin in feed: at 30 µg/kg in maize and 5 µg/kg in complete mixes for dairy cows (Serbian Regulation, 2014). At the same time, new regulation (Serbian Regulation, 2014a) returned the ML of AFM1 in milk to 0.050 µg/kg, which entered into force on July 1, 2014. However, the test results indicated that milk was still contaminated with AFM1 at levels higher than 0.050 µg/kg in high percentage of milk samples (> 30% in our tests). The Ministry reacted very quickly by adopting new regulations in which the maximum content of AFM1 in milk was raised to 0.250 µg/kg (Serbian Regulation, 2014b). This legal act placed specified ML of AFM1 in milk until the end of 2014, and as of 01.01.2015 ML returned to the European level of 0.050 µg/kg. During September, in samples of milk the concentration of AFM1 is significantly increased, which is indicated by our results. During this period, the analysis of 103 milk samples showed that 91.3% of samples contained AFM1 and 69.9% of samples exceeded the ML. The Ministry, probably under pressure from producers and milk processors, acted again, raising the ML of AFM1 in milk at 0.250 µg/kg (Serbian Regulation, 2015).

One of the first EU countries facing the problem of AFM1 in milk was Italy (EFSA, 2004). Extensive measures have been taken to prevent recurrence of the problem. Several studies showed how the implementation of aflatoxin M1 monitoring plan in milk reduced this problem (Nachtmann et al., 2007; Schirone, 2015). Unfortunately, even with the all actions taken to prevent the problem, AFM1 has been found in milk and milk products from Italy in the recent period, which is presented on the RASFF (six notifications in 2016).

CONCLUSION

It was predicted that a temperature increase of +2°C in Europe will probably cause a problem with aflatoxin B1 in the years to come (Battilani, 2016). Serbia and the surrounding countries should expect a struggle with AFB1 and AFM1 in the future. A high occurrence of AFM1 in 2016 may lead to a conclusion that Serbia, and probably the entire region, would still have a problem with this mycotoxin. Therefore, the high level of AFM1 in investigated

samples of milk confirmed that constant monitoring throughout the milk production chain is necessary to minimize health risks related to the presence of this toxin in milk.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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