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Aflatoxin M_1 in raw milk in the region of Vojvodina

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Abstract

Aflatoxin M_1 was in the spotlight of public attention in Serbia and in the region in 2013 due to high level of this mycotoxin found in milk and milk products. Maximum allowed concentration of aflatoxin M_1 in milk by EU regulation is 50 ng/kg, while in the Republic of Serbia, allowed concentration by the current regulation is 250 ng/kg. During seven months period, from May to November, samples of raw milk were being taken from six farms, with 60 to 330 cows, from the region of Autonomous Province of Vojvodina, the Republic of Serbia, for the purpose of monitoring the occurrence of aflatoxin M_1 in milk and its variation during this period. The highest level of 39.8 ng/kg was found in November, while the mean value for the whole period was 7.9 ng/kg. None of the samples had higher level of this mycotoxin than allowed by EU regulation or by current legislation of the Republic of Serbia. The second group of samples included a total of 38 samples of raw milk, from the period of October and November, were taken from different producers from the region of Vojvodina and analyzed on the occurrence of aflatoxin M_1. In this group of samples, the occurrence of aflatoxin M_1 was much higher, with the average value of 230 ng/kg. The highest found value was 864 ng/kg. In 13 samples, the aflatoxin M_1 content was higher than the allowed by the legislation of the Republic of Serbia, while in 24 (63.2 %) samples determined concentration exceeded the value allowed by EU regulation. Data from this work suggest huge differences in the occurrence of aflatoxin M_1 in milk between the producers in this region. Regular monitoring is necessary to avoid situation with the elevated level of aflatoxin M_1 in milk. This measure will protect both the consumers and the producers of milk and milk product.

Key words: raw milk, aflatoxin M_1, Vojvodina

Introduction

Milk and milk products are significantly present in human nutrition. Estimated consumption of milk and milk products in Europe is 340 g/day per person (WHO, 2003) while estimation of consumption in Serbia is a 177.5 g/day (Škrbić et al., 2014). The younger population is main consumer of milk and milk products hence special attention is permanently present of both the consumers and the governments for the quality of those products. Aflatoxin M_1 can be found in milk and in the recent few years it has been in the center of public attention in Republic of Serbia. Europe is not traditionally associated with the high level of aflatoxins. However, depending on the year and climatic condition during the year, contamination can occasionally occur with fungi and consequently with aflatoxins. Aflatoxin M_1 contamination arises from the aflatoxin B_1, which can be found in broad spectrum of feed and food (EFSA, 2004). Aflatoxin B_1 is in the first

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group of carcinogens and stronger carcinogen than aflatoxin M_1 (IARC, 2012). First aflatoxin M_1 was put in the second B group as a possible carcinogen (IARC, 1993) while in some of the following classifications, due to the proven properties, it was moved in the first group and it contained 2-10 % of carcinogens of aflatoxin B_1 (IARC, 2002). Aflatoxins are very stable compounds. Heat treatment of milk or the production of the yoghurt does not influence the occurrence of this mycotoxins. Moreover, the technological process of production of cheese leads to the accumulation of aflatoxin M_1 due to the binding of the aflatoxin M_1 for the casein fraction of milk (Cavallarin et al., 2014). Predominately, in dairy cattle nutrition, the corn is considered to be a source of contamination of the aflatoxin B_1 and consequently the aflatoxin M_1. However, silage can be the source of mycotoxin contamination and therefore the source of the increase of aflatoxins in nutrition of dairy cows (Gonzalez Pereyra et al., 2008). Moreover, accumulations of aflatoxins in silage can happen due to air exposure during conservation or during feed-out phase (Cavallarin et al., 2011). Occasionally, the source of high contamination of aflatoxins can be a by-product of industry which is used in cattle nutrition. That was the case in Sweden when a by-product of Basmati rice included in less than 10 % of compound cattle feed was the source of aflatoxin contamination (Nordkvist et al, 2009).

Only 60 countries worldwide had regulation for aflatoxin M_1 in milk at the end of 2003. However, only 34 countries established limit on 50 ng/kg which is one of the lowest permitted levels. Most of these countries (28 countries) are members of European Union while some of them are from Africa, Asia and Latin America (FAO, 2004). In many other countries like a USA (FDA, 1996) or Brasil (Iha et al., 2011) legal regulation for this mycotoxin is ten times higher (500 ng/kg aflatoxin M_1). Until the 2011 in the Republic of Serbia the allowed concentration of aflatoxin M_1 in milk was 500 ng/kg when as aspiration toward European standard a legal limit was reduced to the 50 ng/kg of milk (Serbian Regulation, 2011). Due to high contamination of corn in Vojvodina, and hence increased values of aflatoxin M_1 in milk, legal permitted level of aflatoxin M_1 in milk was returned in 2013 to the previous level of 500 ng/kg milk (Serbian Regulation, 2013), while during 2014 it was once again returned to the 50 ng/kg. By the current law regulation (Serbian Regulation, 2015) adopted in 2015, permitted level of aflatoxin M_1 in milk is 250 ng/kg. In some countries of the European Union, the dairy established internal system for a preventive or corrective action because of their consumers' protection or due to problems related with the increased level of aflatoxin M_1 in the past. In an Italian dairy, when value of aflatoxin M_1 in milk from suppliers exceeds 10 ng/kg the dairy reacts on preventive level and contacts the supplier, while in the situation when aflatoxin M_1 exceeds 20 ng/kg the supplier is suspended until concentration in milk is not returned to internal permitted level (Visciano et al., 2015). Swedish Dairy Association established the action limit for aflatoxin M_1 in milk for start of the investigation and trace back studies at 8 ng/kg (Swedish Dairy Association, 2007).

Until recently, increased level of aflatoxin M_1 in countries of EU in milk was very rare according to the data from EFSA. Out of 11.831 analyzed samples only 0.06 % contained levels of aflatoxin M_1 above legal limit. Incidence of aflatoxin M_1 in samples from the individual farms was 1.8 % with the higher occurrence, compared to all samples (EFSA, 2004). However, this does not necessarily mean there is no risk for the occurrence of aflatoxin M_1 in milk in the countries of Europe. Contamination of corn with the aflatoxin B_1 occurred in Italy due to the combination of climatic factors and insects. The result of this contamination was the increased occurrence of aflatoxin M_1 in milk. Namely, this toxin was found in 6 % of samples at the beginning of 2003, and furthermore increased to 7.8 % of samples, by the end of the year (EFSA, 2004). Study from Slovenia, in the period from 2004 to 2005 showed that out of 40 samples of milk products made in small dairy plants, in 10 % of samples aflatoxin M_1 exceeded 50 ng/kg (Torkar and Vengušt, 2008). Milk samples collected during 2007 in Hungary, indicated much higher concentration of aflatoxin M_1 (up to 0.45 μg/kg), than maximum levels adopted by EU regulations (RASFF, 2007). High temperature and drought caused high aflatoxin B_1 contamination of corn, in 2012. As a consequence, the occurrence of aflatoxin M_1 in milk in Serbia was very high in the following year.

The aim of the present study is to investigate the occurrence of the aflatoxin M_1 in milk in the region of Autonomous Province of Vojvodina, the Republic of Serbia. Production of milk in Serbia is very fragmented with lots of small producers. Therefore, the milk samples from two different types of producers were analyzed: milk from six farms with 50-100 cows and milk produced by small individual producers with a few cows per farm.

Material and method

Samples

A total of 80 raw milk samples were collected and divided into two groups. First group of 42 samples in total, were taken during the period of seven months (from May to November 2015) from six farms in Vojvodina (farms I, II, III, IV, V and VI) containing 60 to 330 cows (170, 100, 60, 70, 130 and 330 cows). Once per month, samples were analyzed for aflatoxin M_1 content using the ELISA method. The second group consisted of 38 samples which were taken from individual producers during October and November of 2015, also from the region of Vojvodina. The samples were analyzed for the presence of aflatoxin M_1 using the HPLC method.

Reagents and standards

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 HPLC analysis was done using AflaStar™ M1 R-Immunoaffinity Columns (IAC) (Romer Labs Inc., Union, MO, USA). Deionized water (electric conductivity, <3.5 μS/cm) from reverse osmosis filtration system using a reverse osmosis system DS - 83 (Amtast, USA). Nitrogen gas was obtained from Messer (Belgrade, Serbia). Aflatoxin M_1 standard with certified concentration of 10 μg/mL was purchased from Sigma Aldrich (Buchs, Switzerland). Standard stock solutions were prepared in acetonitrile and stored at -18 °C. Those solutions were used for solvent based calibration. The standard solutions were stored under refrigerator conditions (4 °C).

Sample preparation and analysis by ELISA

Before analysis the samples were centrifuged at 3500 rpm for 10 min to separate a fat layer. The supernatant (skimmed milked) were used directly in the test (100 μL per well). The immunochemical analysis was performed using Veratox® for Aflatoxin M_1 Test Kit (Neogen, Lansing, MI, USA) with five calibration standard solutions (0, 5, 15, 30, 60 ng/kg). Analytical procedure was carried out according to manufacturer's instructions. Regarding ELISA method, precision and accuracy were determinated. This was carried out the same way as in case of the LC method, using the same certified reference material. Method was accurate with trueness of 104.6 %, and precise with obtained HORRAT value of 0.98.

Sample preparation and analysis by HPLC

Fifty mL of warm milk (35-37 °C) was filtered through a quantitative filter paper for fast filtration (Filtros Anoia, Barcelona, Spain) and applied to the IAC. After the milk completely passed, IAC was rinsed with 20 mL of ultra-pure water. The aflatoxin M_1 was eluted with 4 mL of acetonitrile. Eluate was collected and evaporated to dryness at 50 °C using gentle stream of nitrogen. The derivatization step was done by adding 200 μL of TFA and the same volume of n-hexane to the residue from the evaporated acetonitrile eluate or to the aflatoxin M_1 working standards, vortexed for 30 s, and incubated for 10 min at 40 °C. Further, after evaporation 300 μL of mobile phase was added to the vials and vortexed for 30 s.

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 x 100 mm, 5 μm). 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 of the LC method was carried out by determining linearity, precision, LOQ and method accuracy. The linearity of the method was assessed by standard ranging from 2.5-50 ng/mL.

The correlation coefficient was 0.9999. Precision was evaluated in terms of interlaboratory reproductivity by analyzing certified reference material MI1142-1/CM (Progetto Trieste, Padova, Italy) on two different days in four replicates (eight replicates in total) and the obtained HORRAT value was 0.46, which was lower than the maximum value of 2 for precise methods. The limit of quantification (LOQ), based on ten times the ratio of the standard deviation of intercept and slope of the calibration curve, was 0.25 ng/mL of aflatoxin M_1, which is equivalent to 0.005 μg/kg of aflatoxin M_1 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 and discussion

The monthly occurrence of aflatoxin, for the period of seven months, on six farms from Vojvodina is shown in Table 1. The mean value of aflatoxin M_1, in the entire period, was 7.94 ng/kg. The maximum concentration (38.9 ng/kg) was detected in November. Furthermore, the monthly mean value 23.4 ng/kg was the highest in this month. All six samples from June were below the limit of detection. During October and November there was an increase in the mean value of aflatoxin.

Table 1. Occurrence of aflatoxin M_1 in raw milk during seven-month period in Vojvodina

	Concentration of aflatoxin M_1 (ng/kg)			
Month	Mean	Minimum	Maximum	SD
May	2.90	4.50	7.32	3.51
Jun	0	0	0	0
July	12.1	9.80	16.8	2.66
August	9.62	5.20	13.01	2.91
September	3.06	4.34	4.34	1.63
October	4.46	5.09	21.67	8.67
November	23.42	4.50	39.8	12.3
Total	7.94	4.50	39.8	9.36

The occurrence of aflatoxin M_1 on farms in Vojvodina during period of seven months is shown in Table 2. Out of six farms, which participated in this research, the highest value of aflatoxin M_1 (38.9 ng/kg) was detected in November on the farm number one, while the highest mean value (10.6 ng/kg), was established on the farm number five. The lowest mean vale for the period of seven months (3.39 ng/kg) was found on farm number two where the lowest maximum value (10.0 ng/kg) was established, too. None of the samples from the six farms during seven months exceeded a maximum level of aflatoxin M_1 adopted by the EU regulation or by the Serbian regulation.

Results on the occurrence of the aflatoxin M_1 in milk samples, taken from the individual producers, is shown in Table 3. In 38 samples of raw milk, mean value for the aflatoxin was 230 ng/kg and the maximum value was 864 ng/kg. Fourteen samples complied with the permitted level of aflatoxin M_1 adopted by the EU regulation, while in 24 samples, the aflatoxin M_1 content exceeded that level. In 13 samples, the aflatoxin M_1 content was above the legal limit permitted by the Serbian regulation from 2015.

In the recent period aflatoxin M_1 was in the spotlight of public attention in Republic of Serbia due to the high contamination of milk with this mycotoxin during 2013 (Kos et al., 2014; Škrbić et al., 2014). Increased level of aflatoxin M_1 was most probably the consequence of the contamination of corn with fungi which consequently led to the contamination with aflatoxin B_1 in 2013 in Serbia. Some of the corn exported in EU had high concentration

Table 2. Occurrence of aflatoxin in raw milk on six farms during a seven-month period in Vojvodina

	Concentration of aflatoxin M_1 (ng/kg)			
Farm	Mean	Minimum	Maximum	SD
I	10.3	4.32	39.8	13.8
II	3.39	4.30	10.0	3.72
III	7.96	5.09	24.0	9.46
IV	7.17	4.30	18.9	7.55
V	10.6	4.30	21.7	8.08
VI	8.22	4.34	32.9	11.9

of aflatoxin B_1 (RASFF, 2013a). Moreover, during 2013, corn from the entire region had high level of aflatoxin B_1 during 2013. Therefore, the notification was posted on RASFF portal about corn from Hungary (RASFF, 2013b), Romania (RASFF, 2013c) and Bulgaria (RASFF, 2013d). Consequences were the increased interest in aflatoxin M_1 in milk as the food which is quite present in human diet.

Some of the previous research from this region did not indicate the possibility of the high presence of aflatoxin M_1 in milk in Serbia (Polovinski-Horvatović et al., 2009), Kosovo and Metohija (data from 2009-2010) (Rama et al., 2016) or in the countries of the region, like Republic of Croatia (Bilandžić et al., 2010). However, during 2013 in Serbia was detected high level of aflatoxin M_1 in milk, up to 1440 ng/kg (Škrbić et al., 2014). Also, high levels of aflatoxin M_1 (up to 1135 ng/kg) were detected in Croatia (Bilandžić et al., 2014).

Results of the first group of samples indicate that aflatoxin M_1 content in milk was within permitted levels according to both, Serbian and more strict, EU regulations. During October and November, increased content of this mycotoxin was established in samples of raw milk from farms. These results are consistent with some previous studies conducted by other researchers who reported an increase in concentration during autumn and winter months (Panariti, 2001; Visciano et al., 2015). However, samples from the individual producers in significant number of samples had concentrations of aflatoxin above EU regulation (EU, 2006) and even above Serbian regulation (Serbian Regulation, 2015). The maximum found value was 864 ng/kg. Higher concentration of aflatoxin M_1 in this study was found in milk samples from the area of Banat (Vojvodina) in October and November. As a response to this situation, during October 2015, the government of Republic of Serbia increases maximum level of aflatoxin M_1 in milk to 250 ng/kg (Serbian Regulation, 2015).

In Serbia, milk production is very fragmented, and the average Serbian farm has 2.8 dairy cows; almost half of primary producers have only one cow, and only one fifth have 5 or more cows (Lončar and Ristić, 2011). According the Chamber of Commerce of Vojvodina (2013), only 4 % of all producers have 50-100 dairy cows. Huge differences could be observed between the first group of samples taken from the farms and the second group originating from the individual producers, which is relatively hard to explain. Milk, as a final product, is an aggregate of many milk producers, and the concentration in this milk represents the average of multiple producers. In Sweden, during the routine control of milk, it was found that they consume milk with 12 and 13 ng/kg of aflatoxin M_1. Therefore, the trace back study was done in order to find the reason for the increases. Some producers had milk contaminated with aflatoxin up to 257 ng/kg (Nordkvist et al, 2009). According to some previous researches in Serbia, the amount of aflatoxin in milk from small producers was extremely high (Škrbić et al., 2014), up to 1440 ng/kg in one sample.

Throughout this year the value of this mycotoxin was high in all types of milk due to high contamination of corn with aflatoxin B_1. From the point of view of the quality of milk it is important to know the amount of aflatoxin in the final products as a pasteurized milk, UHT milk or cheese. However, in order to avoid the possibility of increased level of aflatoxin in milk, it is important to trace all producers. It seems, on the basis of the results from this work, that individual producers have problems with the achieving and the obtaining good quality milk.

Table 3. Occurrence of aflatoxin M1 in raw milk from the individual producers

The range of concentration of aflatoxin M_1 in milk (ng/kg)							
Total	<5 ng/kg	5-50 ng/kg	51-250 ng/kg	>250 ng/kg	Total sample mean ng/kg±SD	Positive mean ng/kg ±SD	Range
38	6 (15.8 %)	8 (21 %)	11 (28.9 %)	13 (34.2 %)	230±257	273±258	6-864

Conclusion

Results from this work showed a large difference between the average concentrations of aflatoxin M_1 in milk which have been taken from bigger farms and from the milk from individual producers. It can be explained by the fact that most producers have learned from previous experience in 2013 and are now more cautious and have a better quality control system of feeds included in the diet of cows and consequently in average lower level of aflatoxin M_1 in milk. Individual producers, however have less economic ability to control feed ingredients involved in feeding dairy cows and probably poorer agricultural practices. Further more extensive research would be needed for a more accurate conclusion.

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Aflatoksin M_1 u sirovom mlijeku u regiji Vojvodine

Sažetak

Aflatoksin M_1 je bio u središtu pozornosti javnosti u Srbiji ali i u regiji u 2013. godini, zbog visoke razine u mlijeku i mliječnim proizvodima. Najveća je dopuštena koncentracija aflatoksina M_1 u mlijeku po propisu EU 50 ng/kg, dok je u Republici Srbiji dopuštena koncentracija prema važećem propisu 250 ng/kg. Uzorci sirovog mlijeka prikupljani su sa šest gospodarstava, sa 60 do 330 krava, s područja Autonomne Pokrajine Vojvodine, Republike Srbije, kontinuirano tijekom sedam mjeseci razdoblja (od svibnja do studenog), u svrhu praćenja pojave aflatoksina M_1 u mlijeku i njegova promjena u tom razdoblju. Najviša razina 39,82 ng/kg pronađena je u studenome, dok je srednja vrijednost za cijelo razdoblje bila 7,94 ng/kg. Nijedan od svih uzoraka nije imao višu razinu ovog mikotoksina od razine dopuštene propisom EU ili važećem propisu Republike Srbije. Druga skupina uzoraka, ukupno 38 uzoraka sirovog mlijeka, iz razdoblja listopad/studeni, uzeto je od različitih proizvođača iz regije Vojvodine i analizirano na pojavu aflatoksina M_1. U ovoj skupini uzoraka pojava aflatoksina M_1 bila je znatno veća, s prosječnom vrijednošću od 230 ng/kg i najvišom utvrđenom od 864 ng/kg. U 13 (34,27 %) uzoraka utvrđena je koncentracija aflatoksina M_1 veća od dopuštene po zakonodavstvu Republike Srbije, dok su u 24 (63,16%) uzorka utvrđene koncentracije prelazile vrijednosti dopuštene propisom EU. Podaci ovog rada ukazuju na velike razlike u pojavi aflatoksina M_1 u mlijeku između proizvođača u ovoj regiji. Redovito praćenje je potrebno da bi se izbjegle situacije s povišenom razinom aflatoksina M_1 u mlijeku, te da se zaštite i potrošači i proizvođači mlijeka i mliječnih proizvoda.

Ključne riječi: sirovo mlijeko, aflatoksin M_1, Vojvodina

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