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SEASONAL VARIATION OF AFLATOXIN M1 IN DAIRY PRODUCTS DURING 2015 IN SERBIA

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ABSTRACT

During the last two years, official regulations about aflatoxin M1 (AFM1) level in milk are periodically changed. The cause of the variation in the concentration levels of AFM1 in milk during different seasons. The Serbian Government does not have the required regulations for the level of AFM1 in dairy products. The official regulations exist only for raw and heat treated milk.
The aim of this study was to monitor the level of AFM1 concentration in two groups of commercial dairy products (fermented dairy products and milk drinks) during different seasons (autumn, spring, winter and summer) in 2015 year. The level of AFM1 varied during the season and the higher level of AFM1 recorded during the autumn, while during the summer period AFM1 level is much lower. However, level of AFM1 in different seasons was not significantly different and generally is much less than in previous years.
The study indicates that current level of AFM1 in fermented dairy products and milk drinks is satisfactory. As it known, changes in the concentration of AFM1 in dairy products are a direct result of changes in the concentration of AFM1 in raw milk. However, based on literature data and results of this study it could be concluded that raw milk could be contaminated due to improper feed of cows, but is not accepted by dairy processors. These results indicate that situation regarding safety of dairy products has been improved, but still is essential to introduce monitoring programs for primary milk producers in order to avoid their losses.

Key words: Aflatoxin M1, dairy products, season, Serbia

Introduction

Milk is a highly nutritious food, and it is a source of necessary macro and micronutrients for the growth, development and maintenance of human health. However, it may also be a source of natural food contaminants that may cause disease. Milk and dairy products contamination with aflatoxin M1 (AFM1) is important problem worldwide especially for developing countries in Mediterranean and Middle East region for the last ten to twenty years (Prandini et al., 2009). The presence of this mycotoxin in dairy products is important issue, especially for children and infants, who are more susceptible than adults.
Because aflatoxins are carcinogenic to animals and humans, they are monitored closely in the food supply. Milk that is sold commercially is checked for aflatoxin M1. When aflatoxin M1 is found at concentrations of 0.05 parts per billion (ppb) or greater, the milk is discarded because it cannot be used for products that go into the human food supply. The AFM1 concentration in milk and dairy products within EU is usually at very low level and below MRL (Prandini et al., 2009) indicating a strong control and continuous monitoring of feedstuff.
Opposite to that the Serbian Government because of poor supervision and monitoring of aflatoxin in milk during different seasons, finds a solution in frequent changing regulations and limits for the presence of AFM1 in milk. In order to harmonize regulations with the EU, the Serbian government established MRL of AFM1 in raw milks and heat treated at 0.05 μg/kg (Serbian Regulation 2011, 2014). However, in 2013 a big crisis in terms of the AFM1 contamination in milk (Tomašević et al., 2015) led to the fact that the Serbian Government increased the maximum tolerance level from the

EU level of 0.05 to 0.5 μg/kg (Serbian Regulation 2013). 2014 as a result of the step taken to reduce the level of AFM1 in milk and dairy products, there have been new changes in legislation and the limit is again restored at the 0.05 μg/kg (Serbian Regulation 2014). The latest legislative change occurred in the end of 2015(Serbian Regulation 2015), which is the limit for the level of AFM1 milk was set to 0.25 μg/kg.
The aim of this study was to investigate the occurrence of AFM1 in dairy products and its seasonal variations during 2015 years. In a matter of fact, we wanted to check how moving the level of aflatoxin M1 in dairy products, as a direct result of the quality of raw milk during different seasons, regardless of the prescribed permissible level of AFM1 in raw milk by the Serbian Government. We hope that this research will improve the monitoring and control of AFM1 raw milk and as a consequence to mark consistently and better quality of dairy products in Serbia.

Material and methods

Sampling and preparation
The chemicals and supplies used in the study were: AFM1 commercial kit (r-biopharm, RIDASCREEN, Aflatoxin M1, Germany); methanol and hexane, GLC grade-pesticide residue grade, (Fisher Chemical Scientific, UK).
A total of 660 samples including fermented dairy products (yoghurt and sour cream) and milk drinks (pasteurized, UHT and chocolate milks) were stored at 2-8○C until further analysis of AFM1. Duplicate analyzes were performed for each test sample.

Milk drinks
The liquid samples were cooled to 10°C and shaken manually to ensure sample homogeneity before being opened. Centrifuge milk drinks samples for 10 min, and after centrifugation, remove upper cream layer completely.

Fermented dairy products - yoghurt and cream samples
Bottles of yoghurt were shaken manually for 1 min before being opened to ensure that the mixtures were homogeneous. Preparation of yoghurt samples is based on the dilution of the 5g sample with 5ml water, after cooling at 10°C and shaking, the mixture was centrifuged for 10 min. The upper layer was aspirated and discarded. A 200 mL supernatant was diluted with 200 mL sampler buffered.
Cream samples (2.5g) were mixed with 10 mL methanol and incubated in water bath for 30 min at temperature 50°C. Then, samples were cooled to 10°C and centrifuged for 10 min. 2 ml of the supernatant placed in a test tube where 2 ml of hexane were added, stirred and cooled to 10°C. After centrifugation of about 10 min upper layer was removed completely. A 50 mL supernatant was diluted with 200 mL sampler buffered.

Aflatoxin analysis by ELISA (procedure and method validation)
Determination of AFM1 was done by Enzyme Linked Immuno-sorbent Assay (ELISA) method using standard validated commercial kit (r-Biopharm, RIDASCREEN, Aflatoxin M1, Germany). The test kit is sufficient for 96 determinations (including calibration curve). Kit is designed for the detection of AFM1 in milk, milk powder and cheese. Because of the advantages of the method, Elisa test is recommended for aflatoxin M1 in milk control, where screening method is necessary.
The analytical quality of the ELISA method was assured by the use of certified reference material (CRM). Milk powder with certified AFM1 content of 36.1±6.8 ng/kg (RealCheck AFLAM1 MilkHigh-Level MI211UK) was used as reference material. The validation parameters (Table 1) were calculated and expressed according to the requirements of British standard BS EN ISO 14675: 2003 and their values were in accordance with this standard. The quality of the results was tested through participation in a proficiency test organized by TESTVERITAS (Italy) of lyophilized milk.

The proficiency test results were satisfactory according to the calculated z-score values of -0.40 and 0.30, respectively (acceptable range $-2 \leq z \leq 2$).

Table 1. Validation parameters for ELISA test

Validation parameters	Milk	Milk powder	Cheese
LOD (µg/kg)	0.006	0.006	0.021
LOQ (µg/kg)	0.020	0.020	0.070
RSDr (%)	0.88	0.31	1.45
Recovery (%)	96.00	98.00	101.00

LOD: limit of detection (µg/kg); LOQ: limit of quantification (µg/kg); RSDr: relative standard deviation calculated under repeatability conditions;

Results and discussion

Seasonal variations and the presence of AFM1 in dairy products

Seasonal variations and the presence of AFM1 in dairy products during the different seasons of 2015 year (autumn, spring, winter and summer) are shown in Table 2.

Table 2. The overview of level AFM1 in dairy products during different seasons of 2015 in Serbia

Year	Seasons	Total number of sample	Range of AFM1 concentration (µg/kg) number of samples (frequency distribution. %)				Total % exceed EU (>0.05 µg/kg)	Mean±SD	Min.	Max
			≤0.025	0.026-0.05	0.051-0.5	> 0.5				
Milk drinks	Winter	105	91 (86.67%)	4 (3.81%)	10 (9.52%)	0 (0%)	10 (9.52%)	0.018±0.047[a]	0.005	0.310
	Spring	32	27 (84.38%)	2 (6.25%)	3 (9.37%)	0 (0%)	3 (9.37%)	0.015±0.037[a]	0.005	0.187
	Summer	24	21 (87.50%)	3 (12.50%)	0 (0%)	0 (0%)	0 (0%)	0.007±0.006[a]	0.005	0.048
	Autumn	197	138 (70.05%)	51 (25.89%)	8 (4.01%)	0 (0%)	8 (4.01%)	0.018±0.013[a]	0.005	0.149
	Total	**358**	**277 (77.37%)**	**60 (16.76%)**	**21 (5.86%)**	**0 (0%)**	**21 (5.86%)**	**0.018±0.021[A]**	**0.005**	**0.310**
Fermented dairy products	Winter	73	68 (93.15%)	1 (1.37%)	4 (5.48%)	0 (0%)	4 (5.48%)	0.017±0.047[a]	0.005	0.320
	Spring	18	16 (88.89%)	1 (5.55%)	1 (5.55%)	0 (0%)	1 (5.55%)	0.012±0.032[a]	0.005	0.185
	Summer	19	17 (89.47%)	2 (10.52%)	0 (0%)	0 (0%)	0 (0%)	0.007±0.006[a]	0.005	0.048
	Autumn	192	138 (71.87%)	51 (26.56%)	3 (1.56%)	0 (0%)	3 (1.56%)	0.022±0.015[a]	0.005	0.149
	Total	**302**	**239 (79.14%)**	**55 (18.21%)**	**8 (2.64%)**	**0 (0%)**	**8 (2.64%)**	**0.019±0.024[A]**	**0.005**	**0.320**

[a,b] values denoted with the same small letter are not significantly different at the level of 5% ($P > 0.05$); [A, B] values denoted with the same capital letter are not significantly different at the level of 5% ($P > 0.05$)

The average AFM1 concentration in dairy products in 2015 year for milk drinks and fermented dairy products were 0.018 and 0.019 µg/kg and 5.86 and 2.64% of samples exceeded EU MRL. In 2013 and 2014 year, about 38% of dairy products were above EU MRL. The high AFM1 contamination of dairy products was a consequence of high raw milk contamination due to bed climate conditions in 2012 year and improper feeding of dairy cows in 2013 year (Škrbić et al.

2014). Big crisis in 2013 was followed by great public attention and as mentioned above Government solved it by regulation change.
Our unpublished data indicated that incidence of contamination of raw milk samples with AFM1 at the end of 2014 year was high (29.95% samples was above EU MRL) indicating that problem reappeared. However, compared to 2013 year, current situation is quite different. From results present in this study it could be seen that safety of dairy products were significantly improved but contamination of raw milk still exist and strongly depend of season (unpublished data). Hence, it can be concluded that dairy processors responded to the pressure aroused from the previous crisis (in 2013) and took more responsibility by refusing the reception of contaminated raw milk from farmers. Due to that we could say that the consumers are more protected in term of AFM1 intoxication, but the primary milk producers are still dealing with financial losses. A high occurrence of AFM1 in milk and dairy products were presented in numerous studies (Fallah, 2010; Golge, 2014; Iqbal et al. 2013; Kos et al., 2014; Tavakoli et al., 2012), but usually in countries where both climatic and technological conditions stimulate their formation.
Results of this study also indicate that seasonal variations of AFM1 concentration in dairy products were not found. However, the highest level of AFM1 concentration was found during winter and autumn seasons in both dairy products group. Seasonal variation of AFM1 concentration was presented by many authors. The most of them reported higher concentration of AFM1 in cold seasons as compared to hot seasons (Bilandžić et al., 2015; Hussain and Anwar, 2008; Xiong et al., 2013). Elevated concentrations of AFM1 determined in milk in different countries in dry periods or during winter are influenced by the use of greater amounts of mixed supplementary feedstuff, dry hay and corn contaminated with high levels of AFB1 (Asi et al., 2012; Fallah, 2010). Tomašević et al. (2015) reported that seasonal variation of AFM1 concentrations in heat treated milk samples followed the trend observed in raw milk analysing a big set of data in 2013 and 2014 year. They found that in these years the significant higher AFM1 concentration was during winter and spring as consequence of bed quality feed produced in previous year.

Conclusion

Results of this study showed quite satisfactory situation regarding AFM1 contamination in dairy products. Despite the raw milk contamination that appears in some seasons this data indicate that dairy processors introduced much more control and refuse to accept contaminated raw milk.
However, primary milk producers still need to improve conditions and introduce more control in order to reduce AFM1 in raw milk and avoid financial losses.
Strict regulations and continuous feed control in Serbia would contribute to AFM1 level reduction to the EU level. The current advancements in analytical techniques could help to continuous implementation of strict regulation. Also, increasing the awareness and education level of primary milk producers can contribute to minimize of AFM1 occurrence in raw milk and dairy products.

References

Asi, M. R., Iqbal, S. Z., Ariño, A., & Hussain, A.: Effect of seasonal variations and lactation times on aflatoxin M1 contamination in milk of different species from Punjab, Pakistan. *Food Control*, 25, 34-38, 2012.
Bilandžić, N., Varenina, I., Kolanović, B. S., Božić, Đ., Đokić, M., Sedak, M., Tanković, S., Potočnjak, D., Cvetnić, Ž.: Monitoring of aflatoxin M1 in raw milk during four seasons in Croatia. *Food Control*, 54, 331-337, 2015.
Fallah, A. A.: Aflatoxin M1 contamination in dairy products marketed in Iran during winter and summer. *Food Control*, 21, 1478-1481, 2010.
Golge, O. : A survey on the occurrence of aflatoxin M1 in raw milk produced in Adana province of Turkey. *Food Control*, 45, 150-155, 2014.

Hussain, I., Anwar, J.: A study on contamination of aflatoxin M1 in raw milk in the Punjab province of Pakistan. *Food Control*, 19, 393-395, 2008.
Iqbal, S. Z., Asi, M. R., Jinap, S.: Variation of aflatoxin M1 contamination in milk and milk products collected during winter and summer seasons. *Food Control*, 34, 714-718, 2013.
Kos, J., Lević, J., Đuragić, O., Kokić, B., Miladinović, I.: Occurrence and estimation of aflatoxin M1 exposure in milk in Serbia. *Food Control*, 38, 41-46, 2014.
Prandini, A., Tansini, G., Sigolo, S., Filippi, L., Laporta, M., & Piva, G.: On the occurrence of aflatoxin M1 in milk and dairy products. *Food and Chemical Toxicology*, 47, 984-991, 2009.
Serbian Regulation. (2011). Maximum allowed contents of contaminants in food and feed. In 28/11 (pp. 2-7): Official Bulettin of the Republic Serbia.
Serbian Regulation. (2013). Amendment on Serbian regulation 2011 "Maximum allowed contents of contaminants in food and feed". In 20/13. Belgrade: Official Bulettin of the Republic Serbia.
Serbian Regulation. (2014). Amendment on Serbian regulation 2011 "Maximum allowed contents of contaminants in food and feed". In 29/14: Official Bulettin of the Republic Serbia.
Serbian Regulation. (2015). Amendment on Serbian regulation 2011 "Maximum allowed contents of contaminants in food and feed". In 84/15: Official Bulletin of the Republic of Serbia
Serbian Regulation. (2016). Amendment on Serbian regulation 2011 "Maximum allowed contents of contaminants in food and feed". In 35/16: Official Bulletin of the Republic of Serbia
Škrbić, B., Živančev, J., Antić, I., Godula, M.: Levels of aflatoxin M1 in different types of milk collected in Serbia: Assessment of human and animal exposure. *Food Control*, 40, 113-119, 2014.
Tavakoli, H. R., Riazipour, M., Kamkar, A., Shaldehi, H. R., & Mozaffari Nejad, A. S.: Occurrence of aflatoxin M1 in white cheese samples from Tehran, Iran. *Food Control*, 23, 293-295, 2012.
Tomašević, I., Petrović, J., Jovetić, M., Raičević, S., Milojević, M., Miočinović, J.: Two year survey on the occurrence and seasonal variation of aflatoxin M1 in milk and milk products in Serbia. *Food Control*, 56, 64-70, 2015.
Xiong, J. L., Wang, Y. M., Ma, M. R., & Liu, J. X.: Seasonal variation of aflatoxin M1 in raw milk from the Yangtze River Delta region of China. *Food Control*, 34, 703-706, 2013.