Bulgarian Journal of Agricultural Science, 25 (No 1) 2019, 124–128

Evaluation of aflatoxin M1 by ELISA in raw milk in Kosovo during 2016

Arieta Camaj[1*], Tahir Arbneshi[2], Bajram Berisha[1], Arben Haziri[2], Afërdita Camaj[2*]

[1]*University of Prishtina, Department of Food Technology and Biotechnology, Prishtina, Kosovo*
[2]*University of Prishtina, Department of Chemistry, Prishtina, Kosovo*
[*]*Corresponding authors:* arietacibrahimi@gmail.com, aferditacamaj@gmail.com

Abstract

Camaj, A., Arbneshi, T., Berisha, B., Haziri, A., & Camaj, A. (2019). Evaluation of aflatoxin M1 by ELISA in raw milk in Kosovo during 2016. *Bulgarian Journal of Agricultural Science*, *25*(1), 124–128

Aflatoxins are a class of mycotoxins that are found in agricultural crops produced mainly by fungi Aspergillus flavus and A. parasiticus. When aflatoxin B1 found in the food crops is consumed by cows, metabolizes to aflatoxin M1 (AFM1), which is excreted through milk. Aflatoxin M1 is a human toxin that causes several health problems. In 2016, during the winter and the summer season, 192 raw cow milk samples were collected from small farms in five major regions of Kosovo (Prishtina, Gjilan, Mitrovica, Peja, and Prizren), and analyzed for the aflatoxin M1 level, using Enzyme-Linked Immunosorbent Assay (ELISA). The number of positive samples was 80 (41.7%), from which 43 samples were from the winter season and 37 samples from the summer season. Prizren was the region with the highest number of positive samples for the two seasons examined. The region with the highest percentage of samples that exceeded the maximum tolerable limit for AFM1, according to EU Maximum Tolerable Limit, was Prishtina, with 15.4% of the samples. The difference of AFM1 level between the seasons was statistically significant ($P \leq 0.05$ to $P \leq 0.001$). The maximum mean concentrations of AFM1 recorded in the winter season were in the range of 0.004-0.109 ng/mL, and during the summer season they are in the range of 0.004-0.833 ng/mL, all coming from the region of Gjilan. The maximum range for this region resulted to be alarming, values that needed to be reevaluated by a confirming method, like HPLC, in another study. During 2016, the occurrence of AFM1 in raw cow's milk appears to be prevalent in Kosovo.

Keywords: aflatoxin M1; cow milk; ELISA; Kosovo

Introduction

The worldwide contamination of food and animal feed with mycotoxins is a significant problem. Mycotoxins are secondary metabolites of molds, which have adverse effects on humans, animals, and crops, that result in illnesses and economic losses (Hussein and Brasel, 2001). Aflatoxins are a group of mycotoxins produced by species Aspergillus flavus and A. parasiticus, with carcinogenicity, teratogenicity, and mutagenicity. Aflatoxins may be found in a wide range of agro-products, especially in grains, oilseeds, corns, and peanuts (Chen et al., 2014). Aflatoxins can be present in several forms in feedstuff, but most known are aflatoxin B1, B2, G1, and G2 (Akiyama et al., 2001). Aflatoxin B1 (AFB1) is a highly toxic metabolite that contaminates animal feed, and is known as a potent hepatocarcinogen in a variety of mammalians, including humans (IARC, 1993; Richard et al., 1993; Verma, 2004; Liu et al., 2012). Cows that consume AFB1 contaminated feed excrete aflatoxin M1 (AFM1) in their milk (Unusan, 2006). AFM1 is classified as a Group 1B agent by International Agency for Research on Cancer in 2002 (IARC, 2002). Due to the serious health problems AFM1 can cause, Kosovo also has set maximum tolerable limit for AFM1 in milk and milk products. According to the EU regulations the maximum tolerable limit of AFM1 should not exceed 0.05 μg/kg (European Commission, 2006b).

Some researches done in Kosovo and in the neighboring countries, do not indicate high incidence of AFM1 in milk, like data from 2009-2010 in Kosovo (Rama et al., 2016), Serbia (Polovinski-Horvatović et al., 2009), data from 2013 in Macedonia (Dimitrieska-Stojkovic et al., 2016), or in the countries of the region, like Republic of Croatia (Bilandžić et al., 2010). But, there are some studies that indicate contamination of milk with AFM1 in Serbia (Kos et al., 2014; Škrbić et al., 2014), and Croatia (Bilandžić et al., 2014a, 2014b). During 2013, in the region of Prishtina, Kosovo, there was a high incidence of AFM1 (81.0%) in pasteurized and UHT milk samples (Rama et al., 2015). Considering the high health risk potential that AFM1 presence in milk may cause, regular monitoring of this milk toxin should be carried.

Materials and Methods

Sampling and supplies

In 2016, during the winter and the summer season, 192 of raw cow milk samples were collected from small milking farms from five major regions of Kosovo: Prishtina, Gjilan, Mitrovica, Peja, and Prizren. From the total, 96 samples were collected during the winter season (February and March), and 96 samples were collected during the summer season (July and August) from the same farms. The samples were collected in small containers of 50 mL, based on the milk sampling method according to EU requirements (European Commission, 2006a). Milk samples were stored at -20°C until analyzed.

Pre analysis, each sample was centrifuged and filtered. To determine AFM1 using ELISA, the Max Signal "Aflatoxin M1 ELISA Test Kit" was used, purchased from Bioo Scientific, Bio Food and Safety (2013). The unique features of the Kit are: no need to extraction for milk samples; high sensitivity (0.004 ng/g) and low detection limit for milk (0.004 ppb); high reproducibility.

Procedure overview

Aflatoxin M1 antibody has been coated in the plate wells. During the analysis, sample is added to the wells for incubation. After washing the plate, the aflatoxin M1 – horseradish peroxidase (AFM1-HRP) conjugate is added to the wells for incubation. If the AFM1 residue is present in the sample, it will compete for AFM1 antibody, thereby preventing the AFM1-HRP from binding to the antibody attached to the well. The resulting color intensity, after addition of the HRP substrate (TMB), has an inverse relationship with the aflatoxin M1 residue concentration in the sample. Max Signal Aflatoxin M1 ELISA Test Kit has the capacity for 96 determinations or testing of 42 samples in duplicate (assuming 12 wells for standards).

The procedure of AFM1 analysis in the samples was performed by the competitive colorimetric ELISA assay. First, 200 µL of each AFM1 standard were added into different wells. Then, in each well 200 µL of each sample were added in duplicate. The plate was incubated for 45 minutes at room temperature. Then, the solution was decanted from wells and discarded the whole liquid. The plate was washed 3 times with 250 µL of 1X Wash Solution. After the last wash, the plate was left to dry. 100 µL of AFM1-HRP conjugate were added to each well. The plate was incubated for 15 minutes at room temperature. Then the liquid was discarded and washed 3 times with 250 µL of 1X Wash Solution and left to dry. 100 µL of TMB substrate was added to each plate. After incubating 15 minutes at room temperature, 100 µL of Stop Buffer was added to stop the enzyme reaction. The plate was put in the plate reader with 450 nm wavelength - "Microplate Reader, Sunrise, Tecan" (Max Signal "Aflatoxin M1 ELISA Test Kit" purchased from Bioo Scientific, Bio Food and Safety, 2013).

Results and Discussion

A total of 192 samples were analyzed for the presence of AFM1. The number of samples collected per season was 96, all collected in the same farms from five different regions of Kosovo. The distribution of concentration of AFM1 by the region and by the season is presented in Table 1 and Table 2, respectively.

From 192 samples examined, 80 samples (41.7%) were with positive result in AFM1; 43 (53.8%) positive samples

Table 1. AFM1 concentration in raw milk during the winter season, 2016

Region	No. of samples	No. of farms	No. of positive samples	AFM1 higher than 0.05 µg/L	Contamination level
Prishtina	13	13	4 (30.8%)	0 (0%)	0.006 – 0.014
Gjilan	13	13	5 (38.5%)	1 (7.7%)	0.004 – 0.109
Mitrovica	15	15	5 (33.3%)	1 (6.7%)	0.005 – 0.068
Peja	25	25	13 (52%)	1 (4%)	0.006 – 0.085
Prizren	30	30	16 (53.3%)	4 (13.3%)	0.007 – 0.073
Total	96	96	43 (44.8%)	7 (7.3%)	0.004 – 0.109

Table 2. AFM1 concentration in raw milk during the summer season, 2016

Region	No. of samples	No. of farms	No. of positive samples	AFM1 higher than 0.05 µg/L	Contamination level
Prishtina	13	13	4 (30.8%)	4 (30.8%)	0.312 – 0.637
Gjilan	13	13	3 (23.1%)	2 (15.4%)	0.009 – 0.833
Mitrovica	15	15	2 (13.3%)	0 (0%)	0.005 – 0.028
Peja	25	25	12 (48%)	0 (0%)	0.004 – 0.043
Prizren	30	30	16 (53.3%)	0 (0%)	0.004 – 0.040
Total	96	96	37 (38.5%)	6 (6.2%)	0.004 – 0.833

were from the winter season and 37 (46.2%) from the summer season. As a result, in the winter season there was a higher contamination than in the summer season for 7.6%. 13 (6.8%) of the contaminated samples exceeded the maximum tolerable limit of the EC according to the European Union maximum limit for AFM1, i.e. 0.05 µg/L. The maximum mean concentrations of AFM1 recorded in the winter season were in range of 0.004 – 0.109 µg/L and in the summer season they were in the range of 0.004 – 0.833 µg/L. As noticed in the Table 1, during the winter season, the region of Gjilan appears to have one sample (7.7%) with much higher maximum range than the tolerable limit, value that needed to be reevaluated with a confirmative method in the next study. Also, the region of Peja and Mitrovica resulted to have one sample each, with a higher range than the maximum tolerable limit. These were also the samples that needed to be confirmed with one another method. While, in the region of Prishtina none of the samples exceeded the maximum tolerable limit.

During the summer season, as noticed in the Table 2, in the region of Prishtina, four (30.8%) samples resulted to be positive in AFM1, all of them with greater values than the tolerable limit, values that needed to be confirmed in the next study; the region of Gjilan appears with two (15.4%) samples with greater range than the maximum tolerable limit. While, in the regions of Mitrovica, Peja, and Prizren none of the samples exceeded the maximum tolerable limit.

In terms of regional distribution of positive samples during winter and summer of 2016, Prizren was the region with the largest number of positive samples 32 or (53%), followed by the region of Peja with 25 (50%) positive samples.

The highest level of contamination was detected in the winter season in all regions, except for the regions of Prishtina and Gjilan, where contamination resulted greater during the summer season, as seen in Fig. 1 and Table 3. The results of these two regions where surprising, especially during the summer season, for which all the positive samples needed to be re-evaluated with another, confirmative, method in another study.

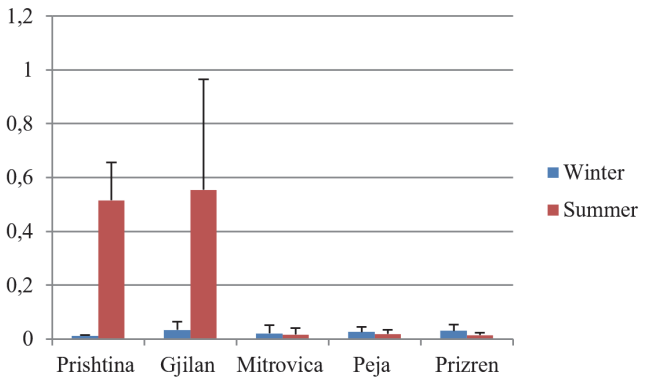

Fig. 1. The mean concentration of AFM1 in raw milk samples between the regions during the winter and the summer season 2016

In this study, significant differences are observed between the regions of Kosovo, in the winter and the summer season, as are shown in the Table 4. In the summer milk, the highest significant differences between the samples were observed between the regions of Prizren and Prishtina ($P \leq 0.001$). A significant difference of $P \leq 0.01$ was announced between the regions of Prishtina and Peja in the winter season samples, Mitrovica and Peja in the summer season samples, Prizren and Mitrovica in the summer season samples, and significant differences from $P \leq 0.05$ were appearing between the regions of Mitrovica and Prizren during the winter season samples. There is an absence of the statistical differences between the other regions ($P \leq 0.05$).

Table 3. Mean concentration and standard deviation values of AFM1 between the regions and the seasons

Region/Season	Prishtina	Gjilan	Mitrovica	Peja	Prizren
Summer	0.5155±0.140	0.5537±0.412	0.0165±0.023	0.0187±0.015	0.0140±0.010
Winter	0.0112±0.003	0.0334±0.031	0.0206±0.030	0.0256±0.019	0.0299±0.024

Table 4. Significant difference between the regions and the seasons

Region / Season	Prishtina/ Gjilan	Prishtina/ Mitrovica	Prishtina/ Peja	Prishtina/ Prizren	Gjilan/ Mitrovica	Gjilan/ Peja	Gjilan/ Prizren	Mitrovica/ Peja	Mitrovica/ Prizren	Peja/ Prizren
Winter	0.268	0.3721	0.0035*	0.00023***	0.6178	0.4778	0.2904	0.0990	0.0320*	0.5986
Summer	0.7794	0.04668*	0.0623	0.0571	0.1730	0.2132	0.2001	0.0064**	0.0092**	0.3474

* Significant difference in $P \leq 0.05$
** Significant difference in $P \leq 0.01$
*** Significant difference in $P \leq 0.001$

The major possible reason for some samples of Gjilan and Prishtina exceeding many times the maximum tolerable limit could be the human error, as a result, a further study must be done, using another analytical confirmative method, and a greater number of samples from the regions mentioned above. It was expected to have higher contamination during the winter season, because during the winter season, the climate conditions are more favorable for the moulds to develop, as well as there is more compound feeding of animals in the winter season than in the summer season.

Conclusions

From 192 samples been examined, 80 (41.7%) samples where positive in AFM1. Among them, 13 samples (6.8%) exceeded the European Union maximum Limit for AFM1 i.e. 0.05 μg/l. The maximum mean concentration of AFM1 recorded in the winter season was in the range of 0.014 – 0.109 μg/L, and during the summer season, it was in the range of 0.028 – 0.833 μg/L. In terms of regional distribution of positive samples during the winter and the summer season in 2016, Prizren was the region with the highest number of positive samples, 32 or (53%), followed by the region of Peja with 25 (50%) positive samples. The highest level of contamination was detected in the winter season for all the regions, except for the regions of Prishtina and Gjilan, where the summer milk was more contaminated with AFM1. It can be concluded that AFM1 contamination in the present investigation of the raw milk is relatively high and prevalent, which may be a potential public health problem in Kosovo, notably in the region of Prizren and the region of Peja. Also, the regions of Prishtina and Gjilan recorded alarmingly high maximum range which is a must for using another method to confirm those results. Farmers must be educated on proper animal feed storage, the potential health consequences of AFM1, and the importance of its control. Regular raw milk control must be done by government authorities to prevent any potential human and animal health risk. Further studies are needed to be done, with a greater number of samples per regions, and another confirmative method should be used for sample analysis. Based on these results, and considering the general public health situation in Kosovo, we strongly recommend the Food and Veterinary Agency of Kosovo to take concrete steps for establishing the continuous monitoring systems for AFM1 in raw cow milk.

References

Akiyama, H., Goda, Y., Tanaka, T., & Toyoda, M. (2001). Determination of aflatoxins B1, B2, G1 and G2 in spices using a multifunctional column clean-up. *Journal of Chromatography A*, *932*(1-2), 153-157.

Bilandžić, N., Varenina, I., & Solomun, B. (2010). Aflatoxin M1 in raw milk in Croatia. *Food Control*, *21*(9), 1279-1281.

Bilandžić, N., Božić, Đ., Đokić, M., Sedak, M., Kolanović, B. S., Varenina, I., & Cvetnić, Ž. (2014a). Assessment of aflatoxin M1 contamination in the milk of four dairy species in Croatia. *Food Control*, *43*, 18-21.

Bilandžić, N., Božić, Đ., Đokić, M., Sedak, M., Kolanović, B. S., Varenina, I., Tanković, S. & Cvetnić, Ž. (2014b). Seasonal effect on aflatoxin M1 contamination in raw and UHT milk from Croatia. *Food control*, *40*, 260-264.

Chen, R., Ma, F., Li, P. W., Zhang, W., Ding, X. X., Zhang, Q. I., Li, M., Wang, Y.R. & Xu, B. C. (2014). Effect of ozone on aflatoxins detoxification and nutritional quality of peanuts. *Food Chemistry*, *146*, 284-288.

Dimitrieska-Stojković, E., Stojanovska-Dimzoska, B., Ilievska, G., Uzunov, R., Stojković, G., Hajrulai-Musliu, Z., & Jankuloski, D. (2016). Assessment of aflatoxin contamination in raw milk and feed in Macedonia during 2013. *Food Control*, *59*, 201-206.

Hussein, H. S., & Brasel, J. M. (2001). Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology*, *167*(2), 101-134.

Kos, J., Lević, J., Đuragić, O., Kokić, B., & Miladinović, I. (2014). Occurrence and estimation of aflatoxin M1 exposure in milk in Serbia. *Food Control*, *38*, 41-46.

Liu, Y., Chang, C. C. H., Marsh, G. M., & Wu, F. (2012). Population attributable risk of aflatoxin-related liver cancer: systematic review and meta-analysis. *European Journal of Cancer*, *48*(14), 2125-2136.

Polovinski-Horvatović, M., Jurić, V., & Glamočić, D. (2009). Two year study of incidence of aflatoxin M1 in milk in the region of Serbia. *Biotechnology in Animal Husbandry*, *25*(5-6-2),

713-718.

Rama, A., Latifi, F., Bajraktari, D., & Ramadani, N. (2015). Assessment of aflatoxin M1 levels in pasteurized and UHT milk consumed in Prishtina, Kosovo. *Food Control*, *57*, 351-354.

Rama, A., Montesissa, C., Lucatello, L., Galina, G., Benetti, C., & Bajraktari, D. (2016). A study on the occurrence of aflatoxin M1 in milk consumed in Kosovo during 2009–2010. *Food Control*, *62*, 52-55.

Richard, J. L., Bennett, G. A., Ross, P. F., & Nelson, P. E. (1993). Analysis of naturally occurring mycotoxins in feedstuffs and food. *Journal of Animal Science*, *71*(9), 2563-2574.

Škrbić, B., Živančev, J., Antić, I., & Godula, M. (2014). Levels of aflatoxin M1 in different types of milk collected in Serbia: Assessment of human and animal exposure. *Food Control*, *40*, 113-119.

Unusan, N. (2006). Occurrence of aflatoxin M1 in UHT milk in Turkey. *Food and Chemical Toxicology*, *44*(11), 1897-1900.

Verma, R. J. (2004). Aflatoxin cause DNA damage. *International Journal of Human Genetics*, *4*(4), 231-236.

Received: 18.03.2018; ***Accepted:*** 15.05.2018

Bioo Scientific, Bio Food and Safety (2013). Max Signal "Aflatoxin M1 ELISA Test Kit"

European Commission (2006a). Commission regulation 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union, L 70, 12-34.

European Commission (2006b). Commission regulation 1881/2006 of 19 December2006 setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products. Official Journal of the European Union, L 364, 5-18.

IARC, International Agency for Research on Cancer (1993). Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. In IARC monographs on the evaluation of carcinogenic risk to humans (Vol. 56).

IARC, International Agency for Research on Cancer (2002). Monograph on the evaluation of carcinogenic risk to humans (Vol. 82). Lyon: IARC Press.