Romanian Journal for Plant Protection, Vol. XIII, 2020
ISSN 2248 – 129X; ISSN-L 2248 – 129X

A 2019 STUDY ON TOTAL AFLATOXINS IN ROMANIAN MAIZE (*ZEA MAYS* L.) SAMPLES

Irina Smeu[1*], *Elena Mirela Cucu*[1], *Alina Alexandra Dobre*[1], *Hellene Casian*[2]

[1] National R&D Institute for Food Bioresources IBA - Bucharest, Microbiology-ELISA Laboratory, Romania
[2] University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Agriculture, Bucharest, Romania

[*] Correspondence address:
National R&D Institute for Food Bioresources IBA – Bucharest
5th Băneasa Ancuța Street, 2nd District, 020323 Bucharest, Romania
Tel: 004 031 620 58 33/133
Fax: 004 031 620 58 34
E-mail: irina.smeu@bioresurse.ro

http://www.doi.org/10.54574/RJPP.13.01

Abstract: Mycotoxin contamination represents a clear public health concern. In this context, a maize survey was conducted in Romania, to monitor the occurrence of total aflatoxins in maize samples collected during the 2019 growing season from fields located in all counties. A total of 95 maize samples were collected along with information regarding the specific location of fields, the applied agronomic practices and cropping systems. ELISA method was used for the quantification of AFs. The results showed 88 contaminated samples. Only one sample registered aflatoxin levels higher than the limit of 10.00 µg/kg, settled by the Commission Regulation (EC) No 1881/2006 for maize to be subjected to soring or other physical treatment before human consumption or use as an ingredient in foodstuffs. The highest AFs level was 77.59 µg/kg, noted by a maize sample from Argeș County (the South-Muntenia development region, macro region 3). When referring to the analysed samples, the total aflatoxin contamination was independent of the type of hybrid, but strongly influenced by the pedo-climatic differences between counties. The southern counties proved to represent critical risk areas for aflatoxin contamination when referring to maize crops. These results highlight the importance of an effective and sustainable mycotoxin management along the food and feed chain, as well as the need of mapping the mycotoxin risk areas.

Keywords: *aflatoxins, maize, Romania*

INTRODUCTION

Cereals are very susceptible to fungal attacks, both in the field and during storage. Depending on environmental conditions, a fungal infection, mainly produced by species of *Aspergillus*, *Fusarium* and *Penicillium*, may result in a mycotoxin contamination of the crop (Smeu et al., 2017).

Mycotoxins are a group of toxic compounds produced by spore-forming fungi. A wide range of food products could be contaminated with mycotoxins, pre- and post-harvest (Zain, 2011). Consequently, a regular contamination can be expected for cereals and cereal-based commodities (Montes et al., 2012). Thus, there is an increasing awareness of the hazards to both human and animal welfare represented by mycotoxins present in food and feed.

Aflatoxins are a major class of toxic and carcinogenic mycotoxins produced primarily by fungi belonging to *Aspergillus* section Flavi, mainly *Aspergillus flavus* and *A. parasiticus*, which contaminate a wide range of agricultural products (Cotty et al., 1994; Wang et al., 2019; Sserumaga et al., 2020). *Aspergillus* fungi infect cereal crops in the field and may produce aflatoxins at pre- and/or post-harvest stages (Sserumaga et al., 2020). High or chronic exposure levels of aflatoxins can lead to acute hepatitis, liver cancer, lung cancer and stomach cancer (Wang et al., 2019). The toxin is classified by the International Agency for Research on Cancer as a Group 1 carcinogen (IARC, 1993). Given their highly toxigenic nature, at European level, the presence of aflatoxins is strictly regulated, being imposed maximum levels in various commodities (EC Commission Regulation No. 1881/2006).

Even though there is knowledge of the occurrence of aflatoxins in maize samples collected across Europe (Torović, 2018; Assunção et al., 2018; Udovicki et al., 2019), there is not so many information of the aflatoxin levels in maize samples from Romanian counties, even if maize cultivation is common in Romania (Battilani et al., 2016). In this context, the current study was undertaken to monitor the occurrence of total aflatoxins in maize samples collected during the 2019 harvest from fields located in all major maize-producing Romanian counties. It was necessary to investigate aflatoxin concentrations at the field level in as many areas as possible, in order to identify areas in more need of intervention. Thus, the information on aflatoxin levels in the Romanian counties will be essential to identify hotspot regions in Romania and will aid to design appropriate and cost-effective aflatoxin management strategies to prevent aflatoxin contamination right at the source.

MATERIALS AND METHODS

Maize samples. Ninety-five (N = 95; 1 kg/sample) samples of maize were randomly collected in 2019 from private cereal farmers from 33 Romanian counties, immediately after harvest. The sampling was done by inspectors of the County Agriculture Directorates of the Romanian Ministry of Agriculture and Rural Development, according to the European guidelines (EC Commission Regulation No. 401/2006). Upon arrival, all samples were transferred into paper bags and stored in the dark until their assessment. All samples were received along with information regarding the specific location of fields and the applied agronomic practices (hybrid type, previous crops, incorporation of crop residues, sowing date, fertilisation and fungicide information etc.), which were filled in by farmers into a structured questionnaire dedicated to this study.

Geographic Coordinates. The Northern latitude and Eastern longitude of the location of the field of each sample were determined using Google Maps (Google Maps, 2019), based on the information given by farmers in the questionnaires that accompanied the maize samples (Figure 1). Furthermore, in order to reference the origin of samples, the European nomenclature of territorial units for statistics (NUTS) was used, based on the European regulation (EU Commission Regulation 2016/2066).

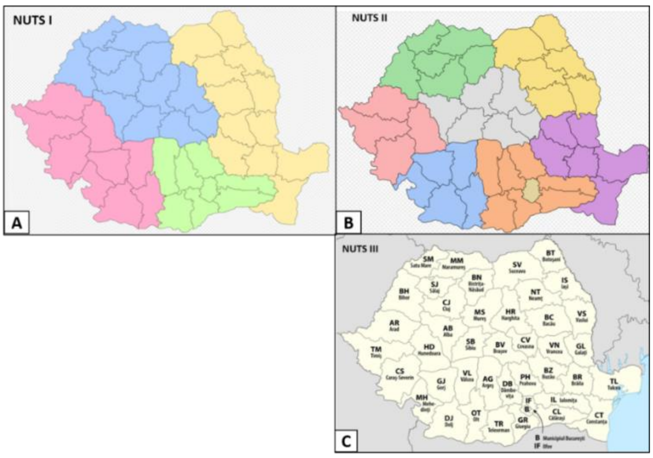

Figure 1. The NUTS (Nomenclature of Territorial Units for Statistics) regions of Romania: (A) NUTS I – Macroregions; (B) NUTS II – Regions; (C) NUTS III - Counties

Mycotoxin analysis. A competitive enzyme linked immunosorbent assay (ELISA) was selected for the quantitative analysis of total aflatoxins. The assessment was performed with commercially available test kits, according to the manufacturer's instructions (Ridascreen® Aflatoxin Total, R-Biopharm AG, Germany). Thus, all samples were first finely ground using a laboratory mill (MRC Ltd., Israel) and mixed thoroughly to achieve complete homogenization. Furthermore, 2 grams of grinded sample were homogenized in 10 mL methanol / distilled water (70/30; v/v) and mixed vigorously for 10 minutes at room temperature using an orbital shaker (GFL Gesellschaft für Labortechnik mbH, Germany). All extracts were then filtered using a grade 1 filter paper (Whatman™, UK) and the obtained filtrates were further diluted in 600 μL distilled water (100/600; v/v). There were employed 50 μL standard solutions and prepared samples to separate duplicate wells. A volume of 50 μL of the enzyme conjugate was added to each well, followed by 50 μL of the antibody solution. The plate was gently mixed by hand and incubated for 30 minutes at room temperature in the dark. After the incubation period, the liquid was poured out of the wells and the plate was vigorously taped upside down against absorbent paper to ensure complete removal of liquid from the wells. This was followed by the washing procedure (250 μL washing buffer, repeated three times). There were added 100 μL of substrate/chromogen to each well. The plate was again very well mixed by hand and incubated for 15 minutes at room temperature in the dark. After incubation, 100 μL of the stop solution were added to each well. The absorbance was measured at 450 nm using a Sunrise™ plate reader (Tecan Group Ltd., Switzerland). The RIDA®SOFT Win software was used for the evaluation of the immunoassays. For each sample, two replicates have been used. The average of these results has been employed in data analysis. A mycotoxin quality control material (Trilogy Reference Material, Naturally Contaminated Aflatoxin Corn, Trilogy Analytical Laboratory, Inc., USA) was used was used for each measurement, to ensure the quality of the analyses.

Data analysis. ELISA tests were run in duplicate for each sample. Results are reported as the mean ± standard deviation and include the recovery of the used quality control material. The uncertainty of the method was 0.34 μg/kg. The correlation between aflatoxin levels and agronomic and pedological parameters was evaluated by applying ANOVA single factor test. Statistical analysis was performed using IBM® SPSS® Statistics 20 (IBM Corp., USA). Significance was defined at $P < 0.05$.

RESULTS AND DISCUSSIONS

Prevalence of total aflatoxin contamination in maize samples in Romania. The current study documents total aflatoxin levels in 2019 maize samples across Romania. When using ELISA method, an accurate quantification is only possible within the range of the calibrators - values of the given standards provided by the kit multiplied by the corresponding dilution factor (e.g. 35 for cereals and feed), which results in a range of 1.75 - 20.25 μg/kg total aflatoxins. The calculation of the results was done using the cubic spline function for RIDA®SOFT Win software. The analysis of 95 maize samples revealed that most of the evaluated samples showed no contamination with aflatoxins beyond threshold set by the European regulations, which stipulates 10.00 μg/kg as the maximum level of aflatoxins for maize subjected to sorting or other physical treatment before human consumption (EC Commission Regulation No. 1881/2006). For 64 samples, the concentrations of total aflatoxins were lower than the limit of detection of the ELISA kit (1.75 μg/kg). For all samples outside the calibrator range, the mathematical function had to be extrapolated, which increased uncertainty (Weiss et al., 2016). As samples with negative test results still could contain a total aflatoxin contamination below the limit of detection of the assay, the 'out of range' function of the RIDA®SOFT Win software was applied for these samples, in order to receive a rough estimation of the concentrations of total aflatoxins for the assessed samples. Because samples with higher concentrations can be diluted to lie within the range of the calibrators, this step was applied to the only sample which was found having the total aflatoxin concentration higher than the given range. For the samples noted to have concentrations lower than the minimum value of the given range, there were no actions that could avoid the increased uncertainty obtained after applying the 'out of range' function of the software. For the ELISA assessments, the recoveries of the control material ranged from 92.42% up to 101.95%.

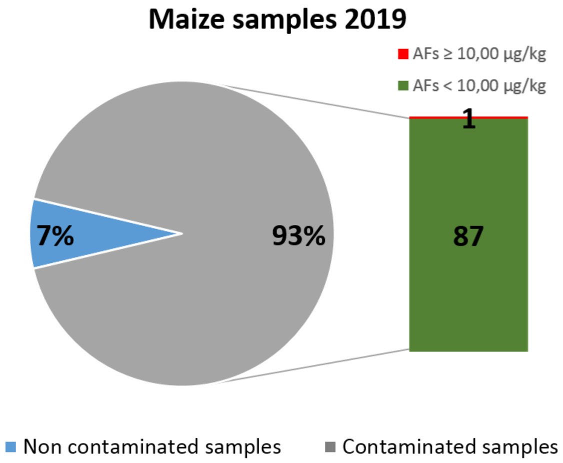

Figure 2. Contaminated maize samples, harvest 2019

Thus, only 7 samples (7.37%) were identified as having no detectable concentrations of total aflatoxins, while for 57 samples, the presence of concentrations of total aflatoxins was confirmed (< 1.75 µg/kg). Taken into account these results, there can be stated that a number of 88 maize samples (92.63%) showed total aflatoxin contamination (Figure 2). Out of the total number of assessed samples (95 maize samples), 31 noted concentrations of total aflatoxins over 1.75 µg/kg (32.63%). However, only one sample exceeded the total aflatoxin limit imposed by the European regulations and noted a concentration of 77.59 µg/kg (Table 1).

Table 1. Number (and percent) of 2019 maize samples from Romania with various levels of total aflatoxin concentrations

Sample category (based on the relevance of the total aflatoxin concentrations)	Number (and percent) of maize samples
Analysed samples	95 (100%)
< 1.75 µg/kg	64 (67.37%)
ND*	7 (7.37%)
0 – 1.75 µg/kg*	57 (60.00%)
1.76 – 5.00 µg/kg	29 (30.53%)
5.01 – 10.00 µg/kg	1 (1.05%)
> 10.00 µg/kg	1 (1.05%)

*Results for which the 'Out of range' function of the RIDA®SOFT Win software was applied; ND – not detected

We argue that we detected a wide range of aflatoxin levels. However, the concentrations found in most of the examined samples were relatively low. Only one sample noted an extremely high total aflatoxin level (77.59 μg/kg). Similar aflatoxin levels in maize samples have been reported by Kos et al. (2018) in neighbouring Serbia, where the maximum level was 111.2 μg/kg (72.3% contaminated samples) in 2012, a year which noted extreme drought conditions. The same authors noted for 2016, a year characterized by moderate weather conditions in Serbia, a maximum concentration of aflatoxins of 6.9 μg/kg (5.0% contaminated samples). These results highlight the impact of climate conditions in the occurrence of mycotoxins and furthermore, the obtained knowledge from our current study will aid in the development of risk maps and aflatoxin management strategies for the maize crop in Romania.

Distribution of total aflatoxins across Romania. Distribution of the assessed maize samples varied across Romania (Figure 3). There were received 14 maize samples (14.74%) from Macroregion 1 (North-West and Center development regions), 24 samples (25.26%) from Macroregion 2 (North-East and South-East development regions), 42 samples (44.21%) from Macroregion 3 (South-Muntenia and Bucharest-Ilfov development regions) and 15 maize samples (15.79%) from Macroregion 4 (South-West Oltenia and West development regions).

The majority of the samples were collected from the Macroregion 3 (42 samples, representing 44.21% of the total samples), where the South-Muntenia development region registered the highest number of maize samples (39 samples). While there were received an average number of 2 maize samples from each County, Ialomița County noted the highest number of collected samples (20 samples), followed by Argeș County, with 8 samples.

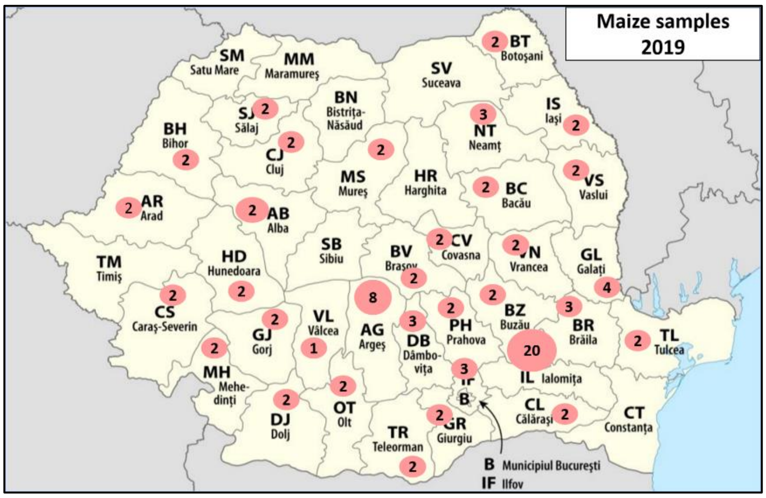

Figure 3. Distribution of the collected maize samples (NUTS III level), crop 2019.

When referring to the 2019 contaminated maize samples, results show that Macroregion 1 noted 2 maize samples with total aflatoxin concentrations below the limit of detection of the assay (<1.75 μg/kg, not detectable), 10 samples with aflatoxin levels in the range of 0 - 1.75 μg/kg and only 2 samples with total aflatoxin concentrations in the range of 1.76 - 5.00 μg/kg. No sample from this geographical area exceeded the maximum level of 4.00 μg/kg. The maximum level of total aflatoxins (3.62 μg/kg) noted in Macroregion 1 was noted by a maize sample from Sălaj County (North-West development region).

There were assessed 24 maize samples from Macroregion 2 and 3 samples recorded aflatoxin concentrations below the limit of detection of the assay (<1.75 μg/kg, not detectable), while 13 samples noted concentrations within the range of 0 - 1.75 µg/kg. A number of 8 samples registered concentrations of total aflatoxins between 1.76 - 5.00 µg/kg. No sample from this geographical area exceeded the maximum level of 10.00 µg/kg. The maximum concentration of total aflatoxins (4.28 µg/kg) from Macroregion 2 was noted by a maize sample from Brăila County (South-East development region).

From all the 42 maize samples from Macroregion 3, only 2 samples noted aflatoxin concentrations below the limit of detection of the assay (< 1.75 µg/kg, not detectable) and 25 samples recorded concentrations in the range of 0 - 1.75 µg/kg. A number of 14 samples registered concentrations of total aflatoxins between 1.76 - 5.00 µg/kg. There were no samples with total aflatoxin concentrations between 5.01 - 10.00 µg/kg. However, one sample exceeded the maximum level of 10.00 µg/kg. The highest concentration (77.59 µg/kg) of total aflatoxins was noted in Argeș County (South-Muntenia development region). This was also, the highest level of total aflatoxins noted for the 2019 maize crop in Romania.

Macroregion 4 of Romania was the most affected by the incidence of total aflatoxins (100% contaminated samples). However, none of these samples exceeded the total aflatoxin limit imposed by the European regulations (EC Commission Regulation No. 1881/2006). In this geographical area, there were noted 5 maize samples with total aflatoxin concentrations in the range of 1.76 - 5.00 µg/kg and one sample in the range of 5.01-10.00 µg/kg. The highest concentration of total aflatoxins (5.67 µg/kg) was noted by a sample from Olt County (South-West Oltenia development region).

In the current study across Romania, Argeș County (Macroregion 3, South-Muntenia development region) was identified as the region with the most number of contaminated samples (100%). There were assessed 8 maize samples from this County, which noted aflatoxin levels in the range of 2.75 - 77.59 µg/kg. Only one sample exceeded the total aflatoxin limit imposed by the European regulations and noted a concentration of 77.59 µg/kg. Other studies showed that 37% of maize samples (2002-2004) from the south-eastern region of Romania registered aflatoxin B1 contamination, where the highest contamination level was about 45 µg/kg (Tabuc et al., 2009). Also, for 2008-2010 maize samples from the same region, aflatoxin B1 contamination was observed in 38 % of maize samples. Mean level of contamination was about 3.2 µg/kg and the highest contamination levels observed being about 42.6 µg/kg. Aut of these samples, 4.76% exceeded EU regulation (Tabuc et al., 2011).

Thus, the southern areas of Romania acted as hotspot regions for aflatoxins, requiring implementation of aflatoxin management strategies to reduce mycotoxin contamination in the field, in order to result safe maize crops that will enhance trade and increase income and welfare of farmers and consumers.

Total aflatoxin contamination in correlation with the applied agronomic practices and cropping systems. When referring to the 2019 maize samples, the monitoring questionnaires indicated the use of Romanian hybrids only for 7.37% of the assessed samples. The main types of cultivated hybrids belonged to producers such as Corteva Agriscience™ (30.53%), DEKALB®, Monsanto Technology LLC (24.21%), KWS Seminţe SRL (12.63%) and Syngenta Agro SRL (6.32%). Hybrids belonging to other producers were also noted, but only in a limited number (1 to 3 maize samples). When referring to the analysed samples, the total aflatoxin contamination was independent of the type of hybrid. Also, no hybrid type showed statistically significant ($P > 0.05$) differences in total aflatoxin content between the different levels of used nitrogen fertilisation in the season of 2019 in Romania.

Variations in total aflatoxin levels in maize samples, even when they originate from the same geographical region, could be attributed to the type of farming systems. Along with differences in temperature, humidity, soil and hybrid type, these factors are thought to play an important role in the observed aflatoxin concentrations. The highest level of total aflatoxins was noted in Argeș County, an area of 682631 ha located in the central-south part of the country, Macroregion 3. Pedological researches revealed the existence of a great variety of soils in Argeș. The area from which the only maize sample that exceeded the maximum limit of total aflatoxins provided by European regulations comes from, is situated at the southern end of Argeș County and the northern end of Teleorman County. In this area, as forms of relief, plains and hills predominate. The climate of the locality is temperate-continental, with Eastern influences. Winters are harsh and with multiple precipitations, while summers are dry.

There were noted hybrids from 4 different producers in Argeș County. As in other regions, when referring to the analysed samples, the total aflatoxin contamination was independent of the type of hybrid, but strongly influenced by the differences of pedo-climatic conditions. Our results showed that the southern counties proved to represent critical risk areas for aflatoxin contamination on maize crops and taking into consideration the information provided by the monitoring questionnaires, the same counties noted poor agricultural practices, also. Thus, in order to prevent the occurrence of aflatoxins, farmers from hotspot regions for mycotoxin contamination could alternate maize with other crops like common beans and potatoes to support little to no growth of aflatoxin-producing fungi (Sserumaga et al., 2020).

CONCLUSIONS

The present study showed that maize is a potential source of aflatoxin exposure in certain regions of Romania. Our results indicate that high concentrations of aflatoxins are independent of the hybrid type as well as the different levels of nitrogen fertilisers. Hotsport regions for aflatoxins were identified in areas where environmental conditions are favourable for the occurrence of toxigenic fungi. Also, not correctly applied agronomic practices represent a favourable factor for aflatoxin contamination.

The results showed that only a small fraction of the analysed samples contained unsafe aflatoxin levels. However, the detected level of total aflatoxins which exceeded the threshold set by the European regulations (10.00 μg/kg) was 77.59 μg/kg (Argeș County, South-Muntenia development region, Macroregion 3). Thus, more research is needed for the hotspot regions for aflatoxin contamination of maize crops, in order to identify the aflatoxin-producing fungi and the correct selection of proper agronomic practices which favour reduced occurrence of total aflatoxins in regard to the specific environmental and climatic conditions of these areas.

ACKNOWLEDGEMENTS

The present study has received funding by the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No. 678781 (MycoKey project). The authors would like to thank the Romanian Ministry of Agriculture and Rural Development for sampling and Constanta Pîrvu and Veta Mertescu for preparing the maize samples for ELISA tests.

REFERENCES

ASSUNÇÃO, R., MARTINS, C., VIEGAS, S., VIEGAS, C., JAKOBSEN, L.S., PIRES, S., ALVITO, P. (2018). Climate change and the health impact of aflatoxins exposure in Portugal – an overview. *Food additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment. Foreword,* 35, 8, 1610-1621. Doi: 10.1080/19440049.2018.1447691.

BATTILANI, P., TOSCANO, P., VAN DER FELS-KLERX, H.J., MORETTI, A., CAMARDO LEGGIERI, M., BRERA, C., RORTAIS, A., GOUMPERIS, T., ROBINSON, T. (2016). Aflatoxin B1 contamination in maize in Europe increases due to climate change. *Scientific Reports*, 6, 24328, Doi: 10.1038/srep24328.

COTTY, P.J. (1994). Comparison of four media for isolation of *Aspergillus flavus* group fungi. *Mycopathologia*, 125, 157-162. Doi: 10.1007/bf01146521.

EUROPEAN COMMISSION (2006). Commission Regulation (EC) No. 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 European Union*, L70, 12-34.

EUROPEAN COMMISSION. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of European Union 2006*, L364, 5-24.

EUROPEAN COMMISSION (2006). Commission Regulation (EU) 2016/2066 of 21 November 2016 amending the annexes to Regulation (EC) No 1059/2003 of the European Parliament and of the Council on the establishment of a common classification of territorial units for statistics (NUTS). *Official Journal of European Union*, L322, 1-61.

Google Maps. (2019). Google Maps. [Online] Available at: https://www.google.com/maps/@46.0899012,25.0740693,6.83z [Accessed 31 Jan. 2020].

IARC [International Agency for Research on Cancer] (1993). IARC monograph on the evaluation of carcinogenic risk to humans. Volume 56: Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. ISBN 92 832 1256 8 (Lyon, France).

KOS, J., HAJNAL, E.J., ŠARIĆ, B., JOVANOV, P., MANDIĆ, A., ĐURAGIĆ, O., KOKIĆ, B. (2018). Aflatoxins in maize harvested in the Republic of Serbia over the period 2012-2016. *Food Additives and Contaminants: Part B Surveillance*, 11, 4, 246-255. Doi: 10.1080/19393210.2018.1499675.

MONTES, R., SEGARRA, R., CASTILLO, M.-Á. (2012). Trichothecenes in breakfast cereals from the Spanish retail market. *Journal of Food Composition and Analysis*, 27, 38-44. Doi: 10.1016/j.jfca.2012.05.004.

SMEU, I., CUCU, E.M., DOBRE, A.A., IORGA, E. (2017). Optimization and evaluation of ELISA immunoassay for mycotoxin detection of breakfast cereals. *Scientific Bulletin. Series F. Biotechnologies*, Vol. XXI, 265-269.

SSERUMAGA, J.P., ORTEGA-BELTRAN, A., WAGACHA, J.M., MUTEGI, C.K., BANDYOPADHYAY, R. (2020). Aflatoxin-producing fungi associated with pre-harvest maize contamination in Uganda. *International Journal of Food Microbiology*, 313, 108376. Doi: 10.1016/j.ijfoodmicro.2019.108376.

TABUC, C., MARIN, D., GUEVE,P., SESAN, T., BAILLY, J. D. (2009). Molds and mycotoxin content of cereals in Southeastern Romania. *Journal of Food Protection*. 72, 3, 662-665. Doi: 10.4315/0362-028X-72.3.662.

TABUC, C., ȚĂRANU, I., CĂLIN, I. (2011). Survey of moulds and mycotoxin contamination of cereals in South-Eastern Romania in 2008-2010. *Archiva Zootechnica*, 14, 4, 25-38.

TOROVIĆ, L. (2018). Aflatoxins and ochratoxin A in flour: a survey of the Serbian retail market. *Food Additives and Contaminants: Part B Surveillance*, 11, 1, 26-32. Doi: 10.1080/19393210.2017.1391335.

UDOVICKI, B., DJEKIC, I., GAJDOS KLJUSURIC, J., PAPAGEORGIOU, M., SKENDI, A., DJUGUM, J., RAJKOVIC, A. (2019). Exposure assessment and risk characterization of aflatoxins intake through consumption of maize products in the adult populations of Serbia, Croatia and Greece. *Food additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment Foreword*, 36, 6, 940-951. Doi: 10.1080/19440049.2019.1600748.

WANG, P., CHANG, P.-K., KONG, Q., SHAN, S., WEI, Q. (2019). Comparison of aflatoxin production of Aspergillus flavus at different temperatures and media: Proteome analysis based on TMT. *International Journal of Food Microbiology*, 310, 108313. Doi: 10.1016/j.ijfoodmicro.2019.108313.

ZAIN, M.E. (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society*, 15, 129-144. Doi: 10.1016/j.jscs.2010.06.006.