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OCCURRENCE OF AFLATOXIN B1 IN CORN IN VOJVODINA, REPUBLIC OF SERBIA, OVER THE LAST FOURTEEN YEARS (2010-2023)

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SUMMARY

Mycotoxins, including aflatoxins, are pervasive contaminants in crops worldwide, with aflatoxin B1 being particularly significant due to its effects on agricultural production and food safety. Serbia has a well-developed crop production sector. The increasing risk of aflatoxin contamination in certain parts of Europe, particularly the Balkan Peninsula, became evident during the 2013 outbreak. This study presents the results of 14-year monitoring (2010–2023) of corn for the occurrence of aflatoxin B1 in the Republic of Serbia. A total of 1167 samples were analyzed, of which 422 samples (36%) exceeded the limit of quantification. Among these, 183 samples were above the EU regulatory limits (EC, 2002), while 150 samples were above the regulatory limits of the Republic of Serbia. The year 2012 was particularly prominent for the high occurrence and concentration of aflatoxin B1 (AFB1) in corn, when 128 out of 132 samples tested positive for AFB1. The average concentration in all positive samples was 55±57 µg kg^{-1}, with a maximum value of 367 µg kg^{-1}, which exceeded the Serbian legal limit by over ten times and the EU limit by more than fifteen times. High incidence of AFB1 in analyzed samples was also in 2022, with 61% of samples exceeding the AFB1 limit of quantification. The average concentration in 2022 was 47±81 µg kg^{-1}, with a maximum recorded value of 560 µg kg^{-1}. The results indicate that aflatoxin B1 will remain a persistent challenge in crop production, with certain years exhibiting exceptionally high levels of contamination.

Key words:
aflatoxin B1, corn, monitoring, Serbia

Abbreviations:
AFB1 - aflatoxin B1

INTRODUCTION

Mycotoxins are produced primarily by fungi of the genera *Aspergillus*, *Penicillium*, *Fusarim*, and *Alternaria* (Shabeer et al., 2022). Aflatoxins and other mycotoxins are prevalent in crops worldwide. Different estimations of mycotoxin contamination range from 25% to over 60% of all crops contaminated worldwide. Currently, approximately 25% of global crops exceed regulatory limits (EU and Codex), while detectable mycotoxin levels occur in 60–80% of samples (Eskola et al., 2020).

Mycotoxin can cause both acute and chronic toxicosis in humans and animals. Owing to the established legal limits on mycotoxin levels in food and feed, incidence of acute toxicosis in developed countries is rare.

The most notable case of acute aflatoxicosis, involving more than 100,000 turkey poults, is known as turkey x disease (Blount, 1961). Today, chronic exposure to aflatoxins at relatively low concentrations is more common. However, chronic exposure to aflatoxins is linked to numerous problems in animal production, including increased mortality, immune suppression, and overall reductions in animal productivity (Zain, 2011). Aflatoxin B1 (AFB1) can transfer from feed to milk as aflatoxin M1 (AFM1), posing health risk in the human food chain. This transfer ranges from 1% to 6%, depending on the animal health status, production, and other factors (Zentai et al., 2023). High levels of AFM1 in milk in the Republic of Serbia during 2013, with 86% of samples exceeding the permitted level (Kos et al., 2013), increased public concern over food safety and aflatoxin B1. According to Kos et al. (2013), the exposure data indicated that all categories of population were exposed to high risk related to the presence of AFM1. Following this outbreak and efforts to preserve domestic milk production in Serbia, several legislative changes concerning AFM1 were introduced over the following years (Jajić et al., 2019).

Crop production in Serbia is highly developed, covering approximately 3.38 million hectares. Depending on annual yield and climate conditions, Serbia ranks among the top 10 global corn exporters (USID, 2019). Until several decades ago, crops from Europe were generally not associated with high aflatoxin levels. A turning point was increased AFB1 in corn produced in the Po Valley, Italy, caused by high temperature, drought, and strong insect damage in 2003 (EFSA, 2004). Some other parts of Europe, including the Balkan peninsula, experienced the 2013 aflatoxin outbreak (de Rijk et al., 2015), when AFB1 concentrations in corn intended for animal feed reached 21-204 µg kg^{-1}, while the allowed concentration was at 20 µg kg^{-1} (EC, 2002). The current regulations in the Republic of Serbia permit AFB1 concentration of up to 30 µg kg-1 in feedstuff (Official Gazette No. 4/2010, 113/2012 and 27/2014). The undeniable climatic changes raise the global food safety issues. The most likely scenario predicts a 2°C temperature increase in Europe, which is expected to elevate the food safety risks of AFB1 contamination in corn (Battilani et al., 2016).

Animal production in Serbia heavily relies on locally produced corn and its quality. Apart from the problems in production caused by aflatoxin, it can pose health risk due to transfers from feed to milk. The 2012 outbreak of AFB1 in corn (Kos et al., 2014) significantly affected dairy production the following year (Horvatovic et al., 2018), as increased aflatoxin M1 level in milk deterred consumers from purchasing milk and dairy products, which subsequently led to a reduction in the number of dairy herds. This paper presents the results of a 14-year monitoring study (2010-2023) of aflatoxin AFB1, covering 1167 samples analyzed to assess AFB1 contamination.

MATERIAL AND METHODS

A total of 1167 whole-grain maize samples were collected from 2010 to 2023. The samples, provided by traders, were collected by official controllers in accordance with EU regulations to address uneven distribution of mycotoxins. Incremental samples were combined in aggregate samples weighing approximately 5-10 kg, which were subsequently homogenized and quartered to obtain 500 g of laboratory samples. These laboratory samples were refrigerated at -20 °C until the analysis.

Chemicals and Reagents

A mixed aflatoxin standard (in methanol) was purchased from SupelcoTM (Bellefonte, PA, USA), containing 1.026 ± 0.006 of AFB1. Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Iodine (p.a.) was purchased from Lach-Ner (Neratovice, Czech Republic). Deionized water (Millipore, Bedford, MA, USA) was used for the mobile phase in HPLC analysis.

Each representative 500 g sample was ground to a particle size of 1 mm using a laboratory mill (KnifetecTM 1095 mill, Foss, Hoganas, Sweden). The sample clean-up procedure was performed using MycoSep®224AflaZon SPE columns (RomerLab, USA) following the manufacturer´s instructions. Briefly, subsamples of 25.0 g were extracted with 100.0 mL of acetonitrile/distilled water (84:16, *v/v*) and shaken vigorously for thirty minutes in a laboratory shaker (Griffin and George, Wembley, England). The extracts were filtered through Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The filtrates were collected, and 5.0 mL was transferred to a glass tube. The MycoSep® clean-up column was inserted into a test tube with the sample extract, forcing the extract to filter upwards through the column packing material. Afterwards, 2.0 mL of the upper layers was transferred into another glass cuvette and evaporated under a stream of nitrogen (Reacti-Therm I#18821, Thermo Scientific, Bellefonte, PA, USA). The dry residue was dissolved in 0.40 mL of mobile phase and transferred to an HPLC vial through a regenerated cellulose (RC, 4 mm, 0.2 m) premium syringe filter (Agilent Technologies, UK).

HPLC analysis of Aflatoxins

Aflatoxin B1 concentrations were measured using an Agilent 1260 Infinity HPLC system (Agilent Technologies Inc., USA) consisting of a solvent degassing unit, a quaternary pump, an autosampler, a thermostated column and a spectrofluorometric detector (FLD). The FLD was set to excitation and emission wavelengths of 365 and 435 nm, respectively. Water/methanol/acetonitrile (60:20:20, *v/v/v*) at a flow rate of 0.90 mL min^{-1} under isocratic conditions were used as a mobile phase. The total run time was 10 min. Separation was achieved using a ZORBAX Eclipse Plus C18 column (4.6 x 100 mm, i.d. 3.5µm) (Agilent Technologies Inc., USA). The column temperature was set at 42 °C and 10.0 µL standards and samples were injected in the duplicate. Post-column derivatization was conducted with a

Pinnacle PCX instrument (Pickering Laboratories Inc., California, USA). The reagent used for derivatization was iodine (100 mg in 200 mL-1 of I2 in water according to European Standard CEN/EN 12955:1999). The volume of the reactor was 1.4 mL, the flow rate was 0.3 mL min^{-1} and the reactor temperature was 85 °C. The iodine solution was degassed using a nitrogen gas stream. The chromatograms were analyzed by Chemstation LC software (Agilent Technologies Inc., USA).

Statistical analysis

Statistical analysis was performed using the Statistica software version 13.5.0.17 (TIBCO Software, Palo Alto, CA, USA). Descriptive statistical data were calculated and presented as mean, maximum, minimum and median values.

RESULTS AND DISCUSSION

The results of the 14-year monitoring period are presented in the Table 1, Figure 1 and Figure 2. It should be noted that 2012 was a turning point in AFB1 monitoring in corn in Serbia, leading to generally more frequent testing. However, the annual number of samples collected depended primarily on suspicions of AFB1 presence in each harvest. Over the fourteen-year period, a total of 1167 samples were analyzed, of which 422 samples, or 36%, were above the limit of quantification. Among these, 183 samples were above the EU regulatory limits (EC, 2002), while 150 samples were above the regulatory limits of the Republic of Serbia ("Official Gazette No. 4/2010, 113/2012 i 27/2014) over the whole period of 14 years. The highest number of analyzed samples was in 2015 (247 samples). However, only 20 of the samples, or 8%, were above the limit of quantification. The highest incidence of AFB1 contamination in corn was in 2012, when 128 out of 132 samples were positive for the presence of AFB1. The average concentration in all positive samples in 2012 was 55±57 µg kg^{-1}, with a maximum level of 367 µg kg^{-1}, which exceeded the Serbian legal limit by over ten times and the EU limit by more than fifteen times. A high incidence of AFB1 in analyzed samples was also in 2022. In this year, 61% of the samples exceeding the AFB1 limit of quantification, while the average concentration was 47±81 µg kg^{-1}, with a maximum detected level of 560 µg kg^{-1}. In our study, the average concentration in 2012 was 55±57 µg kg^{-1}, with a maximum concentration 367 µg kg^{-1}. The 2012 outbreak of AFB1 in corn in Serbia is well documented. In the study by Kos et al. (2013), 68.5% of the samples were positive for AFB1, with an average value of 36.3 µg kg^{-1}, and 29.5% of the samples had concentrations in the range 50-90 µg kg^{-1}. In the years 2009-2011, AFB1 was not detected in corn samples in the same study. In another study (Kos et al., 2018), 72.3% of 600 corn samples from the 2012 harvest contained aflatoxin B1. The maximum concentration was 111.2 µg kg^{-1}, with the average value of 37.4 ± 26.2 µg kg^{-1} for all positive samples. Undoubtedly, 2012 was a critical year for AFB1 contamination in corn, which was further evidenced by high concentrations of aflatoxin M1 in milk and dairy products in 2012-2013 (Kos et al., 2014; Tomašević et al., 2015; Jajić et al., 2019).

Table 1. The results on the occurrence of AFB1 in corn in the period 2010-2023

Year	**Samples**	**>LOQ**	**>LOQ (%)**	**Average ±SD (µg kg^{-1})**	**Med**	**>EUR**	**>RS**	**Min**	**Max**
2010	15	1	7	20	2	0	0	2	2
2011	13	2	15	18± 19	18	1	1	4	31
2012	132	128	97	55±57	40	83	72	2	367
2013	40	38	95	36±31	19	19	17	2	93
2014	8	6	75	30±23	27	4	3	7	64
2015	247	20	8	31±46	14	9	7	3	210
2016	161	2	1	12±7	12	0	0	7	17
2017	47	12	26	79±72	71	7	7	1	178
2018	89	2	2	15±11	15	1	0	7	23
2019	50	13	26	3±2	2	0	0	2	9
2020	31	23	74	2±1	2	0	0	2	6
2021	93	31	33	14±34	6	3	1	1	196
2022	182	111	61	47±81	18	54	40	3	560
2023	59	33	56	11±16	5	2	2	3	84

Legend: ->LOQ above the limit of quantification; ->LOQ (%) – above the limit of quantification in percent; - >EUR above EU regulations (20 µg kg^{-1}); >RS above the Republic of Serbia regulations (30 µg kg^{-1})

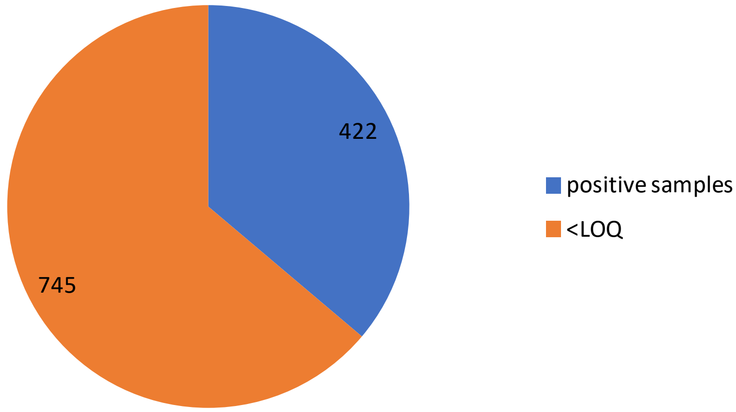

Figure 1. The occurrence of AFB1 in corn in the period 2010-2023

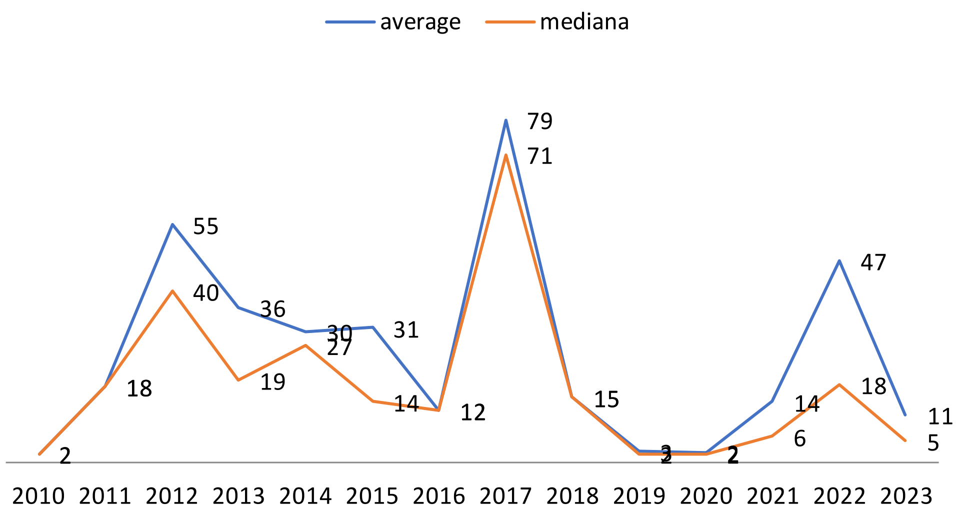

Figure 2. The occurrence of AFB1 (μg kg^{-1}) in the period 2010-2023

The conditions under which crops are grown worldwide are expected to change in the following decades, with a projected rise in global temperatures by +2 to +5 °C, and an increase in atmospheric CO_2 concentrations from 350 to 700 or 900–1000 ppm. These changes will inevitably affect the occurrence of aflatoxins at the global level (Medina et al., 2014; Battilani et al., 2016).

It is possible that, in previous decades, when aflatoxin B1 was not expected to be found in corn in this region, its occurrence may have gone undetected. After all, the first significant outbreak of aflatoxin in Europe was recorded in the Po River Valley, Italy, in 2003 (EFSA 2005). Serbia was not an isolated region, as the whole of Europe, especially the Balkan countries, were affected by aflatoxin B1 contamination in 2012 (de Rijk et al., 2015). Our results indicate that some years were relatively unaffected by aflatoxin, while some other years had high incidence of aflatoxin. In this 14-year monitoring study, there were two years with high incidence of aflatoxin in corn produced in Serbia: 2012 and 2022. These results stress the need for further control of aflatoxin B1contamination of corn in Serbia. There are different approaches for mitigating aflatoxin contamination in crops. Apart from the use of preventive measures such as irrigation and planting schedules, there are also other possibilities. A possible solution is application of biological control methods using non-toxigenic strains of Aspergilus flavus (Savić et al., 2020). The susceptibility of corn hybrids used in Serbia to Aspergillus and aflatoxin accumulation also varies, so using different hybrids may be another method for controlling contamination (Barošević et al., 2022).

Aflatoxin B1 poses health risk in the human food chain, particularly through consumption of corn and corn products and milk and dairy products. Several studies over recent decades have assessed the health risk associated with AFB1 exposure, showing that estimated daily intake varies depending on the investigated population: the highest value was determined for children, adolescents, females, and men, respectively. Based on mean values of the overall estimated daily intake, potential cases of hepatocellular carcinoma caused by exposure to AFB1 were in the range of 0.01–0.02

cases/year/105 individuals, depending on the population group (Udovicki et al., 2021). A comparative study of average aflatoxin concentrations in corn based on published studies covering a ten-year period and risk assessment in three Balkan countries, Serbia, Greece, and Croatia, found that the intake of aflatoxins through corn consumption was the highest in Serbia (Udovicki et al., 2019). The results from this study indicate significant exposure risks to this mycotoxin. Additionally, risk assessments on aflatoxin M1 occurrence in milk showed that all age groups, especially children, are exposed to high risk related to AFM1 presence in milk (Kos et al., 2014).

CONCLUSION

Climate change is undeniably progressing, and addressing its consequences is imperative, whether we are adequately prepared or not. The aflatoxin outbreaks were an unforeseen challenge with significant impacts on Serbian agriculture. Twelve years later, agricultural producers in Serbia should be more ready to mitigate these challenges through application of appropriate agrotechnical practices and selection of resistant corn hybrids. Regular monitoring remains one of the strategies for controlling and detecting problems that are expected to occur in the future.

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