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Aflatoxins contamination of maize in Serbia: The impact of weather conditions in 2015

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ABSTRACT

In recent years climate changes recorded in temperate regions of Europe led to aflatoxins (AFs) contamination of maize. Thus, the aim of this study was to investigate the influence of weather conditions on aflatoxins (AFB1, AFB2, AFG1 and AFG2) content in 180 maize samples collected from the main maize growing regions (Western Bačka, North Banat, South Banat and Central Serbia) in Serbia after harvest in 2015. Aflatoxins (AFs) concentration was determined by validated high performance liquid chromatography with post column derivatization and fluorescence detection (HPLC-FLD). Presence of AFB1, AFB2, AFG1 and AFG2 were detected in 57.2%, 13.9%, 5.6% and 2.8% of maize samples with the concentration range of 1.3 – 88.8 µg kg^{-1}, 0.60 – 2.8 µg kg^{-1}, 1.8 -28.5 µg kg^{-1} and 2.1 – 7.5 µg kg^{-1}, respectively. The recorded smaller amount of precipitation and especially higher air temperatures during the summer of 2015 were favourable for AFs production, which resulted in 32.2% and 21.1% of samples being unsuitable for human consumption, since AFB1 and the sum of AFs concentrations were above 5.0 µg kg^{-1} and 10,0 µg kg^{-1}, respectively. Furthermore, the findings in this study indicate that the micro-climate conditions in the investigated regions had a great influence on the contamination frequency of maize with AFs. The highest percentage of samples unsuitable for human consumption, considering AFB1 and the sum of AFs content of 72.5% and 51.5%, respectively, were detected in Central Serbia, while the lowest percentage was detected in Western Bačka, 15.6% and 6.2%, respectively. These findings confirmed that maize should be continuously monitored in order to protect human and animal health from the harmful effects caused by AFs contamination.

Keywords: maize, aflatoxins, HPLC-FLD, Serbia, weather conditions

Introduction

Aflatoxins B1, B2, G1 and G2 (AFB1, AFB2, AFG1 and AFG2, respectively) are toxic secondary fungal metabolites produced mostly by *Aspergillus flavus* and *Aspergillus parasiticus* (Yabe et al. 1999). Hot and humid climates represent favourable conditions for the growth of *Aspergillus* species and production of the toxins (Santin 2005; Karami-Osboo et al., 2012). Therefore, aflatoxins (AFs) are very often found as contaminants in a wide variety of important agricultural products (particularly maize, wheat, rice, spices, dried fruits, and nuts) from tropical and subtropical areas of the world (Castells et al. 2008; Cotty and Jaime-Garcia 2007; Rustom 1997). The optimal temperature interval for *A. flavus* is from 25 °C to 42 °C, while it can grow under dry conditions with a water activity value of 0.78 (Santin 2005). The carcinogenic, mutagenic and teratogenic effects of AFs have been fully documented and International Agency for Research on Cancer classified AFs in primary group 1 carcinogenic compounds (IARC 1993, 2002, 2012). Since the presence of AFs may affect human and animal health, maximum levels (ML) as well as obligatory control of AFs have been established in numerous countries over the world. According to the European Union (EU) (European Commission 2006a) and Serbian Regulations (Serbian Regulation 2014a) MLs of AFB1 and total AFs in maize intended for human consumption is 5.0 and 10.0 μg kg^{-1}, respectively. If maize is intended for animal feed, concentration of AFB1 cannot be greater than 20.0 μg kg^{-1} and 30.0 μg kg^{-1} in the European Union (European Commission 2003) and Serbia (Serbian Regulation 2014b), respectively. Several recently published studies indicated that extreme weather conditions as a result of climate change and global warming are increasingly affecting the mycotoxin map in Europe and also world-wide (Battilani et al. 2016; Gilbert et al. 2016; Paterson and Lima 2010; Tiradoet al. 2010; Wu and Mitchell 2016). Based on these studies and predicted climate change, aflatoxin producing fungi and consequently AFs are expected to become more prevalent in temperate regions of Europe which have not faced with this problem before. Furthermore, RASFF reports indicate that in the recent years (2012, 2013, 2014, 2015) AFs contamination were observed in maize originating from Serbia, Croatia, Hungary, Bulgaria, Romania, Slovakia, Greece, Italy and Poland (https://webgate.ec.europa.eu/rasff-window/portal). Moreover, registered severe drought in the summer of 2012 influenced AFs contamination of maize and milk from this part of Europe, which was supported by the published data from Serbia (Kos et al. 2013; Kos et al. 2014; Škrbić et al. 2014; Stefanovic et al. 2015; Tomašević et al. 2015), Croatia (Pleadin et al. 2014, 2015), and Macedonia (Dimitrieska-Stojković et al. 2016). The consequence of severe drought in 2012 in Serbia led to the fact that maize yield was approximately 40.0% lower than in 2011. Furthermore, in 2012/2013 Serbia had significantly lower income due to the approximately 7 times lower amount of maize intended for export in comparison to the previous year (IndexMundi www.indexmundi.com/ agriculture).

Since quite similar weather conditions were recorded during maize growing season in 2015 and considering that the Republic of Serbia represents a leader in terms of maize production and exports in Europe and is among the top ten exporters in the world (Maslac, 2015; 2016), the aim of this study was to investigate AFs levels in maize originating from the four different maize growing areas in Serbia. The presence of AFs was investigated in terms of weather conditions recorded during maize growing season in 2015.

Materials and method

Samples

A total of one hundred and eighty (n=180) whole grain maize samples was collected after harvest in 2015. The samples were collected from Northern (Western Bačka, North Banat, and South Banat regions in the Autonomous Province of Vojvodina) and Central Serbia, which represent the most important maize growing areas in Serbia. Depends of the maize growing seasons, around 70% of total maize production in Serbia are produced in Northern and Central Serbia Statistical Office of the Republic of Serbia, http://webrzs.stat.gov.rs/WebSite). Maize production in Serbia is mainly based on small family as well as commercial production, both private and state owned, and most of them do not have their own silos for storage. Therefore, official traders after the harvest collect and transport maize samples to the silos or directly to river ports (due to the limited storage space for maize). Examined maize samples in this study were provided from traders. Sampling was performed by official controllers according to the EU requirements (European Commission 2006b) in order to overcome irregular mycotoxins distribution. Incremental samples were combined in aggregate samples of approximately 5-10 kg. Aggregate samples were homogenized and quartered to obtain a 500 g of laboratory samples which were refrigerated at -20 °C until the analysis.

Chemicals and reagents

A mixed aflatoxin standard (in methanol) was purchased from Supelco™ (Bellefonte, PA, USA), containing 1.026 ± 0.006, 0.311 ± 0.001, 1.046 ± 0.001 and 0.322 ± 0.004 μg mL^{-1} of AFB1, AFB2, AFG1 and AFG2, respectively. Acetonitrile and methanol (all of 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 HPLC analysis.

Sample preparation

500 g of each representative sample was ground to a 1 mm particle size using laboratory mill (Knifetec^{TM} 1095 mill, Foss, Hoganas, Sweden). The sample clean-up procedure was performed using MycoSep®224AflaZon SPE columns (RomerLab, USA) according to the manufacturer´s procedure. 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 Griffin flask shaker (Griffin and George, Wembley, England). Extracts were filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The obtained filtrates were collected, and 5.0 mL was transferred to the glass tube. The MycoSep® clean-up column was pushed into a test tube with the sample extract, forced the extract to filter upwards through the packing material of the column. Thereafter, 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 μm) premium syringe filter (Agilent Technologies, UK).

HPLC analysis of aflatoxins

The aflatoxin concentrations were determined by 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 an excitation and emission wavelengths of 365 and 435 nm, respectively. Water/methanol/acetronitrile (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. The 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 to 42 °C and 10.0 µL standards and samples were injected into the duplicate. The samples were analysed after post-column derivatization with a Pinnacle PCX instrument (Pickering Laboratories Inc., California, USA). The reagent used was iodine (100 mg 200 mL^{-1} of I_2 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 solution of iodine was degassed using a nitrogen gas stream. AFG2 was eluted first, followed by AFG1, AFB2 and AFB1 with retention times of 4.1, 4.8, 5.3 and 6.4 min, respectively. The chromatograms were analysed by Chemstation LC software (Agilent Technologies Inc., USA).

Method performance

The validation parameters for the applied HPLC-FLD method were determined, calculated and expressed according to the European Official Decision procedure for confirmatory chromatographic methods (European Commission 2002) as well as Technical Report CEN/TR 16059:2010 from European Committee for Standardization. The proposed method was validated with the respect to linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability and reproducibility. Standard curves were obtained daily by duplicate injection of ten concentrations in the range of 0.50–100.0 ng mL^{-1} for AFB1 and AFG1 and in the range of 0.15–30.0 ng mL^{-1} for AFB2 and AFG2. The squared correlation coefficients (R^2) were above 0.998 for all curves. Limits of detection and quantification were calculated according to the following equations (Miller and Miller, 2010): LOD=3.3 s_a b^{-1} and LOQ=10 s_a b^{-1}, where s_a is the standard deviation of the intercept and b is the slope of the regression line, obtained from the calibration curve. After carrying out the theoretical LOD and LOQ calculation, the values were verified by injecting a blank sample fortified at the LOD and LOQ level in 6 replicates and the levels of precision were determined. LODs and LOQs were 0.4 and 1.3 μg kg^{-1} for AFB1, 0.20 and 0.60 μg kg^{-1} for AFB2, 0.40 and 1.4 μg kg^{-1} for AFG1 and 0.60 and 1.8 μg kg^{-1} for AFG2. The level of precision for LODs and LOQs was 65.0% and 70.8% for AFB1, 53.9% and 77.1% for AFB2, 59.9% and 71.5% for AFG1, and 75.3% and 88.8% for AFG2. The analytical quality of the applied method in terms of recovery, repeatability and reproducibility was assured by the analysis of the certified reference material (CRM) as well as spiked uncontaminated maize samples. Naturally contaminated maize sample with a certified sum of AFs content (5.7±0.90 μg kg^{-1}) was used as CRM (8092/8096/8076, Neogen corporation, Lansing, USA). This CRM contained 5.0, 0.50 and 0.20 μg kg^{-1} of AFB1, AFB2 and AFG1, respectively. The precision of the method was expressed in terms of repeatability i.e. as the relative standard deviation (%RSDr) of 6 replicates at two concentration levels of 13.0 and 52.0 μg kg^{-1} for the sum of AFs (5.0 and 20.0 μg kg^{-1} of AFB1 and AFG1, and 1.5 and 6.0 μg kg^{-1} of AFB2 and AFG2) of spiked uncontaminated maize samples. The spiked samples were left overnight in the refrigerator prior to analysis. Furthermore, repeatability was also checked by analysis of CRM in 4 replicates. The within-laboratory reproducibility (%RSD_R) was determined by preparing and analising the fortified maize samples at the same concentration levels as for the repeatability, over the course of three days, using the same instrument and by the same operators. Relative standard deviations under reproducibility conditions (RSD_R) were not determined after CRM analysis due to the small amount of available CRM. The method validation data are present in Table 1. Based on the obtained validation data, the developed method was successfully validated according to the criteria specified in the European Official Decision procedure for confirmatory methods (European Commission 2002) as well as Technical Report CEN/TR 16059:2010 from the European Committee for Standardization.

Statistical analysis

Descriptive statistics for all data were expressed as the mean ± standard deviation (SD) using STATISTICA software version 12.5 (StatSoft Inc. 2015, USA). The significant differences between samples were calculated according to post-hoc Tukey's HSD ("honestly significant differences") test, for unequal sample sizes, at a $p < 0.05$ significance level, 95% confidence interval.

Results and Discussion

The distribution of the contaminated maize samples between four different regions of Serbia and the concentration ranges of the investigated AFs is shown in Table 2. Maize samples from Central Serbia were most frequently contaminated with a sum of AFs (90.9%), AFB1 (90.9%), and AFB2 (39.4%), and had the highest mean concentration of the sum of AFs (18.5 ± 19.4 µg kg^{-1}), AFB1 (16.7± 15.4 µg kg^{-1}), and AFG1 (16.2 ± 17.5 µg kg^{-1}). Contrary to this, the lowest mean concentration of AFB1 (5.3 ± 3.8 µg kg^{-1}) and the sum of AFs (6.7 ±7.6 µg kg^{-1}) were determined in the maize samples from Western Bačka. Furthermore, in 65.6% of maize samples from the same region, presence of AFs was not detected. The confidence intervals of aflatoxins concentration, shown in Table 2, were evaluated using the equation given in the literature (Czaban et al., 2015), by using the numbers of contaminated samples, and the total number of examined samples.

$$\frac{2\cdot Y+u_a^2-K}{2\cdot(n-u_a^2)}<p<\frac{2\cdot Y+u_a^2+K}{2\cdot(n-u_a^2)} \qquad (1)$$

where: $K = u_a \cdot \sqrt{x}$ and $x = u_a^2 + 4 \cdot Y \cdot (1 - Y/n)$, where Y is the number of infected maize kernels; n is the total number of maize kernels tested; and u_α is the Student's t-value obtained from tables. This calculation showed that there were statistically significant differences in aflatoxins content (95% confidence level) in the observed regions (AFB1, AFB2 and AFs), while there was no differences in AG1 and AFG2 content between observed regions (Table 2).

The contribution of the maize samples contaminated with AFB1 and the sum of AFs concentrations higher than prescribed MLs for human and animal consumption are presented in Table 3. Among 180 analysed maize samples 58 (32.2%) and 38 (21.1%) samples were contaminated with AFB1 and the sum of AFs in concentrations above MLs of 5.0 and 10.0 μg kg^{-1}, respectively (Serbian Regulation 2014a; European Commission 2006b).

The highest percentage of samples with AFB1 (72.7%) and sum of AFs (51.5%) concentrations higher than MLs was observed in Central Serbia, while the lowest percentage of contaminated maize samples (higher than MLs) were observed in Western Bačka (15.6% for AFB1 and 6.2% for the sum of AFs).

On the other hand, among 180 analysed maize samples intended for animal consumption, 13 (7.2%) and 9 (5.0%) samples were contaminated with AFB1 above MLs (Table 3) according to EU and Serbian Regulation, respectively (European Commission 2006b; Serbian Regulation 2014b). None of the maize samples from Western Bačka contained AFB1 above ML for animal feed (European Commission 2006b; Serbian Regulation 2014b), while 44 (24.2%) and 16 (9.1%) of maize samples from Central Serbia was not suitable for animal feed according to EU and Serbian Regulation, respectively.

The confidence intervals of aflatoxin concentration above the ML in maize samples from 2015: AFB1> 5.0 (μg kg^{-1}), AFs> 10.0 (μg kg^{-1}), AFB> 20.0 (μg kg^{-1}) and AFB14> 30.0 (μg kg^{-}

^{1}) shown in Table 3, were evaluated using the equation given in the literature (Czaban et al., 2015), by using the numbers of contaminated samples, and the total number of examined samples. This calculation showed that there were statistically significant differences in aflatoxins content (95% confidence level) in the observed regions. Moreover, the highest confidence intervals of aflatoxin concentration above the ML in maize samples were observed from Central Serbia.

The regression models for aflatoxin contaminations in grains, according to the average temperatures and sum of precipitation in July and August 2015 are presented in Table 4. The coefficients of determination for AFB1 and AFs equations according to average temperatures are very high (0.959 and 0.964, respectively), and these regression models are statistically significant at $p<0.01$ level. The sum of precipitation in July and August are less effective to AFB1 and AFs content, according to Table 4 (coefficients of determination are 0.771 and 0.829, respectively, statistically significant at $p<0.05$ level). The temperatures in July and August, in Serbia in 2015 were the strongest factor of maize grain contamination by aflatoxins, especially AFB1. Regression models statistics for AFB1>5.0 μg kg^{-1}, AFs>10.0 μg kg^{-1}, AFB1>20.0 μg kg^{-1} and AFB1>30.0 μg kg^{-1}, according to average temperature and sum of precipitation in July and August, 2015 is presented in Table 5. The r^2 coefficients for AFB1 and AFs equations according to average temperatures are very high (between 0.946 and 0.994), statistically significant at $p<0.01$ or $p<0.05$ level.

The first evidence of high AFs contamination of maize from Serbia was reported by Kos et al. (2013). In maize samples referred to the genus 2012, AFs were detected in 68.5% of the samples, while 53.5% of the samples were unsuitable for human consumption (> 10.0 μg kg^{-1} of the sum of AFs). Furthermore, 27.0% of the analysed maize samples were unsuitable for animal consumption since the concentration of AFs was higher than 50.0 μg kg^{-1} according to Serbian Regulation which was valid at that time of investigation (Serbian Regulation 2010). Beside study reported by Kos et al. (2013) there are only a few published studies related to the AFs in maize from Serbia. A study conducted on 53 maize samples collected during 2002 in the region of Vojvodina (Northern Province of Serbia) indicated that 16.9% of the samples were contaminated with AFs in concentrations lower than 10.0 μg kg^{-1} (Mašić et al.,2003). In maize samples from 2009 year Jakšić et al. (2011) determined AFs in 7 out of 20 examined maize samples. In contaminated samples, AFs concentrations were detected in the range from 2.0 to 4.3 μg kg^{-1}. Furthermore, two other studies indicated that AFs rarely occurred in maize as well as in agricultural products from Serbia (Matić et al, 2009; 2010).

Also, authors from neighboring countries Croatia (Pleadin et al. 2014) and Macedonia (Dimitrieska-Stojković et al. 2016) reported the presence of AFs in 38.1% and 29.2% of maize samples (referred to the genus 2012), respectively. As the authors from the above mentioned countries have emphasized, weather condition changes during previous years, particularly during 2012 maize growing season (high temperatures and lack of rainfall) could be the reason for the AFs contamination of maize, since such weather conditions were favourable for fungi formation and AFs synthesis.

AFs levels obtained in this study could also be explained by recorded weather conditions, best described by meteorological data provided by the Republic Hydrometeorological Service of Serbia. The summer in 2015 was one of the hottest summers in the last ten years in Serbia. Weather condition parameters for studied regions during maize growing season 2015 (April – September) are shown in Table6. Nonetheless, the data in Figure 1 and Figure 2 shows monthly average values of air temperatures and sum of precipitation in the four investigated regions in comparison to average values of these parameters from long-term period (1981-2010), respectively. As can be seen (Figure 1), the monthly average values of air temperatures in the period from April to September in 2015 were considerably higher than the values for the same months in the long-term period (1981-2010). Higher air temperatures were especially pronounced during the period of three months (July-September), in which monthly average air temperatures were higher in all investigated regions (from 1.4 to 3.8 °C), than the average temperatures in the long-term period (1981-2010) (Tabela 6). Regarding the average sum of precipitation, it can be seen (Figure 2) that a lower amount of precipitation was recorded during April, June and July, in all investigated maize growing regions. Furthermore, August was characterized by higher sum of precipitation in all regions with the exception of Central Serbia (Figure 2). It could also be noted from Table 6 that, during the observed period of six months, daily temperatures very often exceed 25 °C (96 - 110 days) and 35 °C (12 - 26 days).

Furthermore, in Western Bačka, North Banat, South Banat and Central Serbia recorded the sum of precipitation during the whole maize growing season (April-September, 2015) was lowered for 12.0%, 13.0%, 5.0% and 12.0% than the average sum of precipitation in the long-term (1981-2010), respectively.

It should be noted that July (Figure 1 and 2) was a month with the highest mean of air temperatures and with the lowest sum of precipitations in all investigated maize growing regions. In Western Bačka, North Banat, South Banat and Central Serbia sum of precipitation in July was lowered for 60.0%, 77.0%, 95.0% and 83.0% than the average sum of precipitation in long-term period (1981-2010), respectively. Moreover, monthly average air temperatures were higher in all investigated regions from 2.2 to 3.8 °C than the average temperatures in the long-term period (1981-2010) (Table 6). Therefore, drought conditions characterized by high air temperatures and lower amount of precipitation during June and July in maize growing season 2015 were favourable for *A. flavus* growth and AFs synthesis (as seen in Fig. 2). Furthermore, the findings in this study indicate that the micro-climate conditions in investigated regions had a great influence on the contamination frequency of AFs in maize samples. As a consequence of the drought conditions in Central Serbia (June – August), the maize samples from this region were characterized by the highest percentage of contaminated samples (90.9%), by highest percentage of samples unsuitable for human and animal consumption as well as by highest confidence intervals of aflatoxin concentration above ML for human and animal consumption (Table 3).On the other hand, as a result of weather conditions (Figure 1, 2; Table 6), during the maize growing season in 2015, the lowest share of contaminated samples (34.4%) with a lowest mean concentration of AFs was observed in Western Bačka (Table 2). Furthermore, mostly due to the extreme summer weather, Serbia's maize production in 2015 was lower for 31.4% than maize production in 2014 (Statistical Office of the Republic of Serbia, http://webrzs.stat.gov.rs/WebSite). Beside Serbia, the maize production in 2015 was reduced in the whole Balkan region (Maslac, 2016).

The obtained results in this study were not compared with the other results from this part of Europe, since, to the best of the author's knowledge, this study represent the first report of AFs contamination of maize referred to the genus 2015.

In order to get a better understanding of changes in weather conditions in the regions of Serbia, where the test samples were encouraged, we analysed the mean of the air temperatures and the sum of precipitation in the summer months (July and August) for each year in the period from 1981 to 2015 (Figures 3 and 4). The significantly higher temperatures are observed in 2012 and 2015, as seen from Fig. 3. Furthermore, the lowest amount of precipitation during the summer months were recorded during 2000 (in all regions), and during 2012 and 2015 (in certain regions). Moreover, these data confirm one of the reason for the occurrence of AFs in maize from 2012 and 2015 production years. It should be noted that they are not published papers related to the phenomena of AFs occurrence in maize in 2000, although the weather conditions in that year were favorable for the growth of *Aspergillus* species and the production of AFs.

In summary, the obtained results indicate the necessity for continuous monitoring of AFs occurrence in maize from Serbia, as well as in this part of Europe due to the observed weather conditions changes in the recent years. Furthermore, these results could be useful for developing a climate prediction model which could be of a great importance for further improvement in agriculture in Serbia as well as in the rest part of Europe. Such prediction model may contribute to the human health impact prevention of different diseases which human health impact. From all the above, there is a need to develop effective disease management strategies such as use of biocontrol products with atoxigenic *A. flavus* active ingredients (Umesha et al. 2016; Wambacq et al. 2016; Weaver et al. 2016) in order to minimize negative impacts on health, trade, and income.

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Figure Caption

Figure 1. Monthly mean of air temperature in 2015 (April – September) for investigated regions in comparison to the long-term annual values (1981–2010).

Figure 2. Monthly sum of precipitations in 2015 (April – September) for investigated regions in comparison to the long-term annual values (1981–2010).

Figure 3. Avarage values of air temperatures recorded in each investigated regions for summer months (Jul – August) for the period 1981- 2015. Data for period from 1986 to1990 are not available on the website of Rebublic Hydrometeorological Service of Serbia

.**Figure 4.** Sum of precipitation recorded in each investigated regions for summer months (Jul – August) for the period 1981- 2015. Data for period from 1986 to1990 are not available on the website of Rebublic Hydrometeorological Service of Serbia.

Table 1. Method performances for determination of AFB1, AFB2, AFG1, AFG2 and the sum of AFs.

Mycotoxins	Recovery (%)			RSD_r			RSD
	CL (µg kg^{-1})				CL (µg kg^{-1})		CL (µg
	CRM	5.0*/1.5**	20.0*/6.0**	CRM	5.0*/1.5**	20.0*/6.0**	5.0*/1.5**
AFB1*	97.0	94.7	104.2	3.9	3.9	5.0	5.1
AFB2**	75.5	98.0	100.9	7.9-	2.4	2. 9	3.0
AFG1*	-	85.0	109.4	-	4.4	4.8	7.1
AFG2**	-	84.1	87.3	-	6.0	6.2	6.6
AFs***	91.2	90.1	104.5	3.2-	3.7	3.2	4.0

CL: concentration level (µg kg^{-1}).
CRM: certified reference material with a certified content of 5.0, 0.50, 0.20 and 5.7±0.90 µg kg^{-1} of AFB1, A
AFs, respectively (8092/8096/8076, Neogen corporation, Lansing, USA).
LOD: limit of detection (µg kg^{-1}).
*Spiked levels of blank samples with AFB1 and AFG1 (5.0 and 20.0 µg kg^{-1}).
** Spiked levels of blank samples with AFB2 and AFG2 (1.5 and 6.0 µg kg^{-1}).
*** Spiked levels of blank samples with sum of AFs (13.0 and 52.0 µg kg^{-1}).
RSD_r: relative standard deviation calculated under repeatability conditions (%).
RSD_R: relative standard deviation calculated under reproducibility conditions (%).

Table 2. Observed levels of AFB1, AFB2, AFG1, AFG2 and the sum of AFs in the maize samples from the f
during 2015.

Region	Mycotoxins	N/N_{total}	Contamination frequency (%)	Interval of concentration (μg kg^{-1})	Mean ± SD* (μg kg^{-1})	C
Western Bačka	AFB1	11/32	34.4	1.3– 13.1	5.3 ± 3.8	2
	AFB2*	1/32	3.0	1.1	1.1	
	AFG1*	1/32	3.0	13.9	13.9	
	AFG2*	0/32	0.0	< LOQ	–	
	AFs	11/32	34.4	1.3 – 28.1	6.7 ±7.6	2
North Banat	AFB1	16/25	64.0	1.4 – 32.3	8.8 ± 8.6	6
	AFB2	5/25	20.0	0.61 -1.5	1.0± 0.45	1
	AFG1	1/25	4.0	4.4	4.4	
	AFG2	0/25	0.0	< LOQ	–	
	AFs	16/25	64.0	1.4 – 33.8	9.4 ± 9.3	6
South Banat	AFB1	46/90	51.1	1.3 – 88.8	10.3 ± 16.3	4
	AFB2	6/90	6. 7	0.66 – 2.8	1.5 ± 0.94	
	AFG1	6/90	6. 7	1.8 – 14.4	5.9 ± 5.5	
	AFG2	4/90	4.4	2.2 – 7.5	3.8 ± 2.5	
	AFs	46/90	64.0	1.3 – 91.4	11.6 ± 19.0	4
Central Serbia	AFB1	30/33	90.9	1.4 – 63.5	16.7 ± 15.4	9
	AFB2	13/33	39.4	0.60 – 2.4	1.3 ± 0.49	3
	AFG1	2/33	6.1	3.8– 28.5	16.1 ± 17.5	
	AFG2	1/33	3.0	4.1	4.1	
	AFs	30/33	90.9	1.4 – 86.3	18.5 ± 19.4	9
All regions	AFB1	103/180	57.2	1.3 – 88.8	11.4 ± 14.5	
	AFB2	25/180	13.9	0.60 – 2.8	1.3 ± 0.61	
	AFG1	10/180	5. 6	1.8 -28.5	8.6 ± 8.6	
	AFG2	5/180	2. 8	2.2 – 7.5	3.8 ± 2.2	
	AFs	103/180	57.2	1.3 – 91.4	12.7 ± 17.3	

Letters (a-b) indicate the statistically significant differences ($p < 0.05$) between aflatoxins content (AFB1,
AFs) according to the post-hoc Tukey's HSD test ("honestly significant differences"). *The means of all pos
in this column.

Table 3. Percentage of non-compliant maize samples intended for human and animal consumption according
Regulations.

	Human consumption				Ani	
	AFB1[1,2] > 5.0 (μg kg^{-1})		AFs[1,2] > 10.0 (μg kg^{-1})		AFB1[3] > 20.0 (μg kg^{-1})	
Region	Percentage (%)	95% confidence intervals	Percentage (%)	95% confidence intervals	Percentage (%)	95% confidence intervals
Western Bačka	15.6	8.6-42.3[a]	6.2	2.1-27.2[a]	0.0	0.0-15.0[a]
North Banat	28.0	19.4-67.8[a]	24.4	15.6-62.0[ab]	4.0	0.90-28.7[ab]
South Banat	24.4	18.1-38.1[a]	15.6	10.2-27.3[a]	4.4	1.8-12.3[a]
Central Serbia	72.7	71.0-100.0[b]	51.5	44.6-87.8[b]	24.2	16.1-53.9[b]
All region	32.2		21.1		7.2	

[1] European Commission (2006a)
[2] Serbian Regulation (2014a)
[3] European Commission (2003)
[4] Serbian Regulation (2014b)

Table 4. The regression models statistics for the prediction of AFB1, AFB2, AFG1, AFG2 and sum of AFs according to the average temperatures and sum of precipitation in July and August 2015.

	AFB1			AFB2			AFG1			AFG2		
Temp.	Est.	SD	p	Est.	SD	p	Est.	SD	p	Est.	SD	p
a	-91.6	14.9	0.025	-0.7	3.6	0.860	-51.5	78.3	0.578	-33.5	24.5	0.3
b	4.1	0.6	0.021	0.1	0.1	0.638	2.5	3.2	0.514	1.4	1.0	0.2
Effect	MS	F	p	MS	F	p	MS	F	p	MS	F	p
Regr.	244.4	176.6	0.006	3.1	39.0	0.025	215.1	5.6	0.151	11.6	3.1	0.2
r^2		0.959			0.131			0.236			0.511	
Perc.	Est.	SD	p	Est.	SD	p	Est.	SD	p	Est.	SD	p
a	23.7	5.3	0.047	1.8	0.4	0.037	19.5	11.7	0.236	8.8	1.7	0.0
b	-0.3	0.1	0.122	0.0	0.0	0.227	-0.2	0.2	0.490	-0.1	0.0	0.0
Effect	MS	F	p	MS	F	p	MS	F	p	MS	F	p
Regr.	238.1	30.7	0.032	3.1	85.4	0.012	216.3	5.8	0.146	14.5	17.5	0.0
r^2		0.771			0.597			0.260			0.892	

MS - mean square; Est. - estimation; Regr. - regression model; p - p - level;

F - F value; SD - standard deviation; Temp. - average temperature in July and August, 2015;

Perc. - sum of precipitation in July and August, 2015; a, b - regression coefficients for model: AFy=a+b·x; A AFG1, AFG2 or AFs; x - Temp. or Perc.

Table 5. Regression models statistics for the prediction of: AFB1>5 μg kg^{-1}, AFs>10 μg kg^{-1}, AFB1>20 μg $^{-1}$ contaminants in maize, according to average temperature and sum of precipitation in July and August, 2015

	AFB1>5 μg kg^{-1}			AFs>10 μg kg^{-1}			AFB1>20 μg kg^{-1}			
Temp.	Est.	SD	p	Est.	SD	p	Est.	SD	p	Est.
a	-521.5	39.8	0.006	-392.5	65.6	0.027	-229.3	13.4	0.003	-75.
b	22.5	1.6	0.005	16.8	2.6	0.024	9.6	0.5	0.003	3.2
Effect	MS	F	p	MS	F	p	MS	F	p	MS
Regr.	6888.2	348.369	0.003	3476.3	64.652	0.015	619.1	275.268	0.004	107.
r^2		0.990			0.953			0.994		
Perc.	Est.	SD	p	Est.	SD	p	Est.	SD	p	Est.
a	101.3	35.3	0.103	69.3	31.9	0.162	37.8	13.5	0.107	13.3
b	-1.4	0.7	0.192	-0.9	0.6	0.283	-0.6	0.3	0.151	-0.2
Effect	MS	F	p	MS	F	p	MS	F	p	MS
Regr.	6228.1	9.161	0.098	2976.2	5.374	0.157	522.4	5.282	0.159	92.2
r^2		0.653			0.515			0.721		

MS - mean square; Est. - estimation; Regr. - regression model; p - p - level;

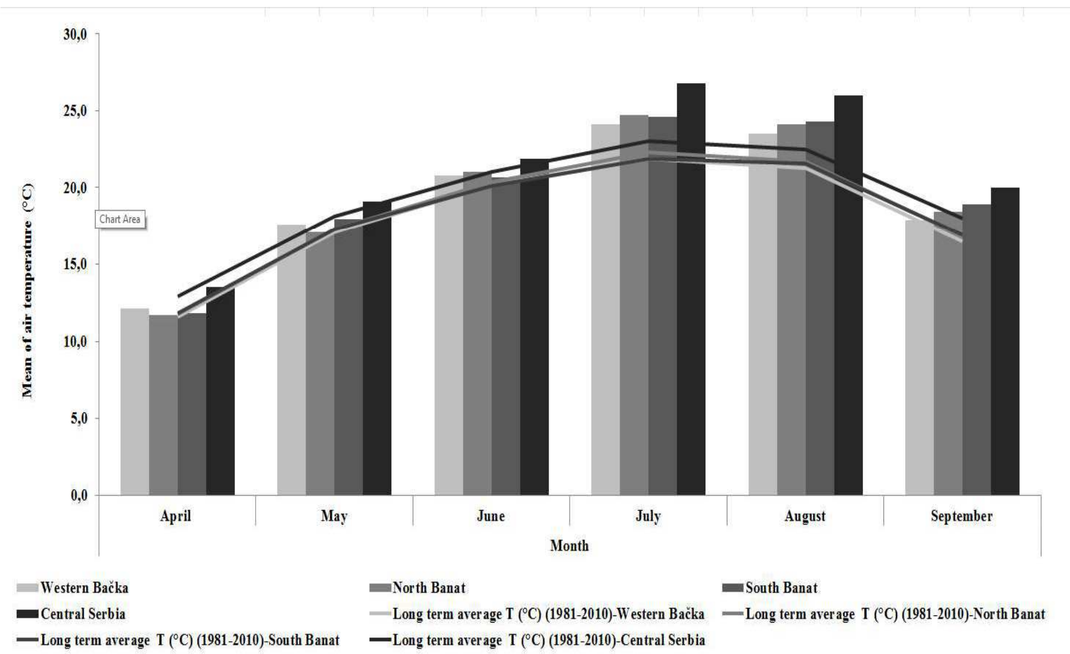
F - F value; SD - standard deviation; Temp. – average temperature in July and August, 2015;

Perc. – sum of precipitation in July and August, 2015; a, b - regression coefficients for model: AFy=a+b·x; A AFs>10, μg kg^{-1}, AFB1>20 μg kg^{-1}, AFB1>30 μg kg^{-1}; x - Temp. or Perc.

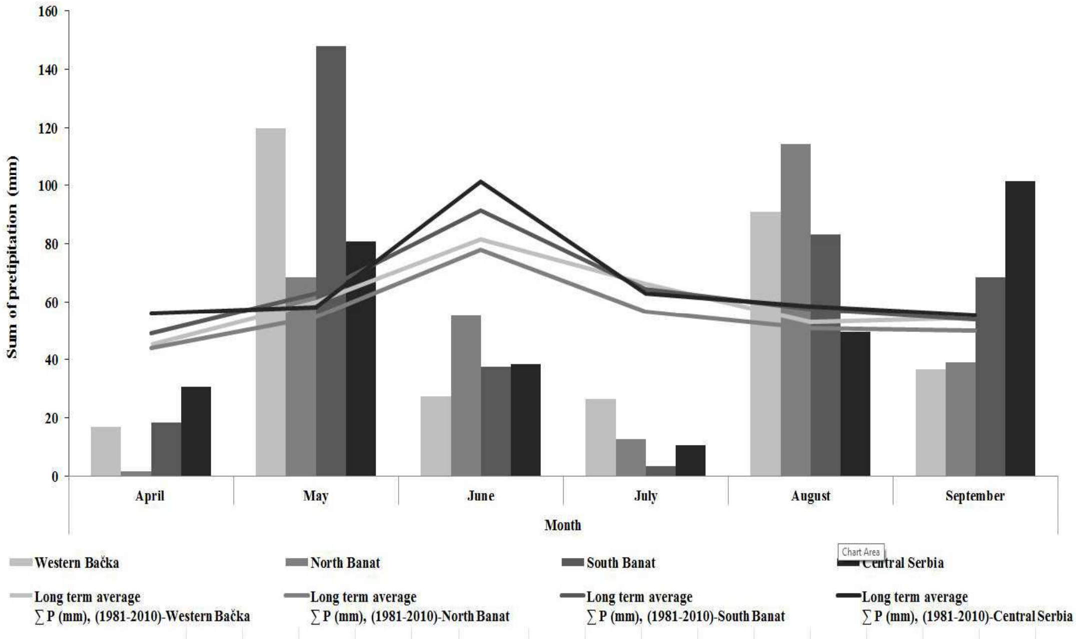
Table 6. Temperature and precipitation parameters for Western Bačka, North Banat, South Banat and Central Serbia regions in Re
September, 2015)

Region	Deviation of T (° C)**						Deviation T average (° C)	N Tmax> 25 ° C	N Tmax> 35 ° C	p
	April	May	June	July	August	September				
Western Bačka	0.50	0.50	0.60	2.2	2.2	1.4	1.2	96	12	
North Banat	-0.05	-0.20	0.65	2.4	2.4	1.6	1.1	104	23	
South Banat	0.05	0.65	0.55	2.7	2.7	2.0	1.4	105	26	
Central Serbia	0.60	1.0	0.90	3.8	3.5	2.0	2.0	110	25	

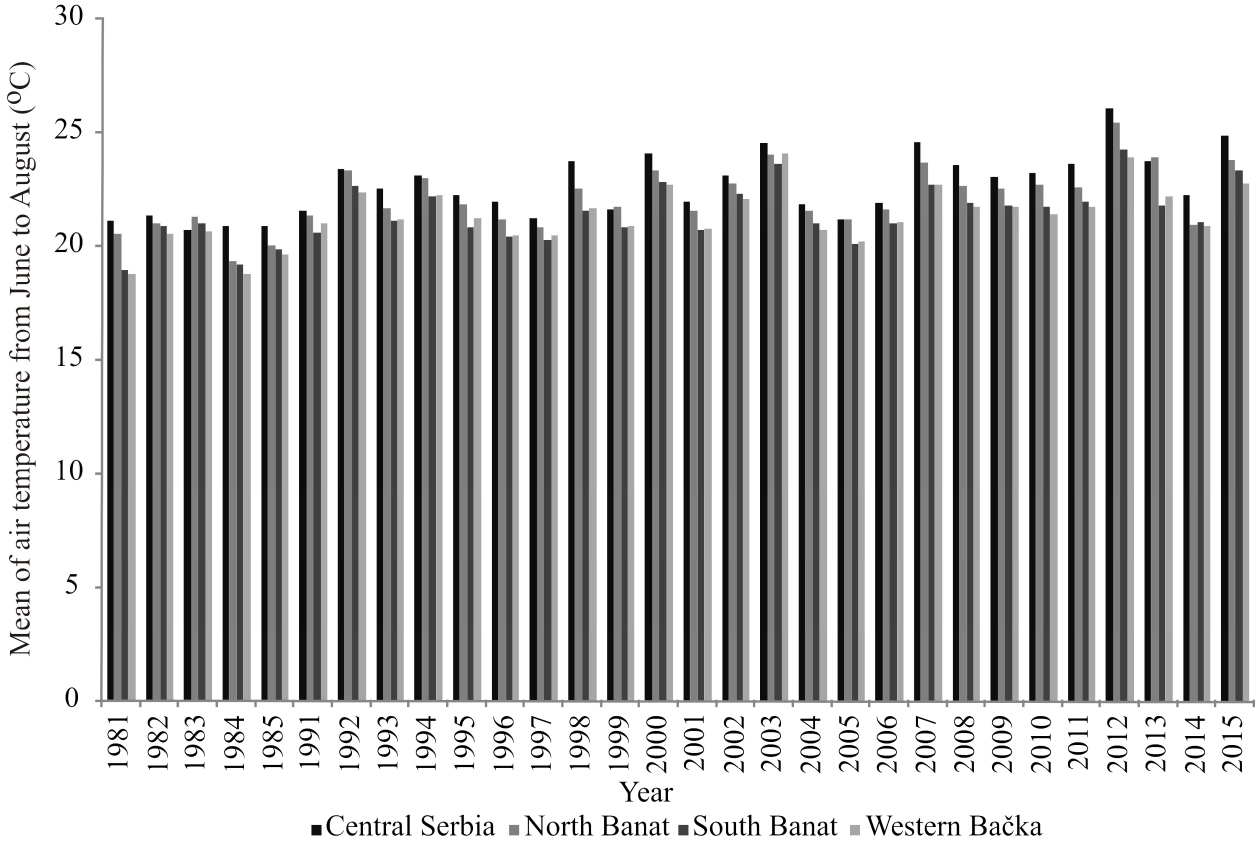
T: temperature.
N: numbers of days.
P: precipitation.
∑: sum.
*:percentage of the sum of precipitation for period April-September during 2015 in comparation with long-term average sum of pr
**:deviation of temperature (°C) for period April-September during 2015 in comparation with long-term average temperatures (19



30,0
25,0
20,0
15,0
10,0
5,0
0,0
Mean of air temperature (°C)
Chart Area
April
May
June
July
August
September
Month
Western Bačka
North Banat
South Banat
Central Serbia
Long term average T (°C) (1981-2010)-Western Bačka
Long term average T (°C) (1981-2010)-North Banat
Long term average T (°C) (1981-2010)-South Banat
Long term average T (°C) (1981-2010)-Central Serbia



160
140
120
100
80
60
40
20
0
Sum of pretipitation (mm)
April
May
June
July
August
September
Month
Chart Area
Western Bačka
North Banat
South Banat
Central Serbia
Long term average ∑ P (mm), (1981-2010)-Western Bačka
Long term average ∑ P (mm), (1981-2010)-North Banat
Long term average ∑ P (mm), (1981-2010)-South Banat
Long term average ∑ P (mm), (1981-2010)-Central Serbia



Mean of air temperature from June to August (°C)
30
25
20
15
10
5
0
1981
1982
1983
1984
1985
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
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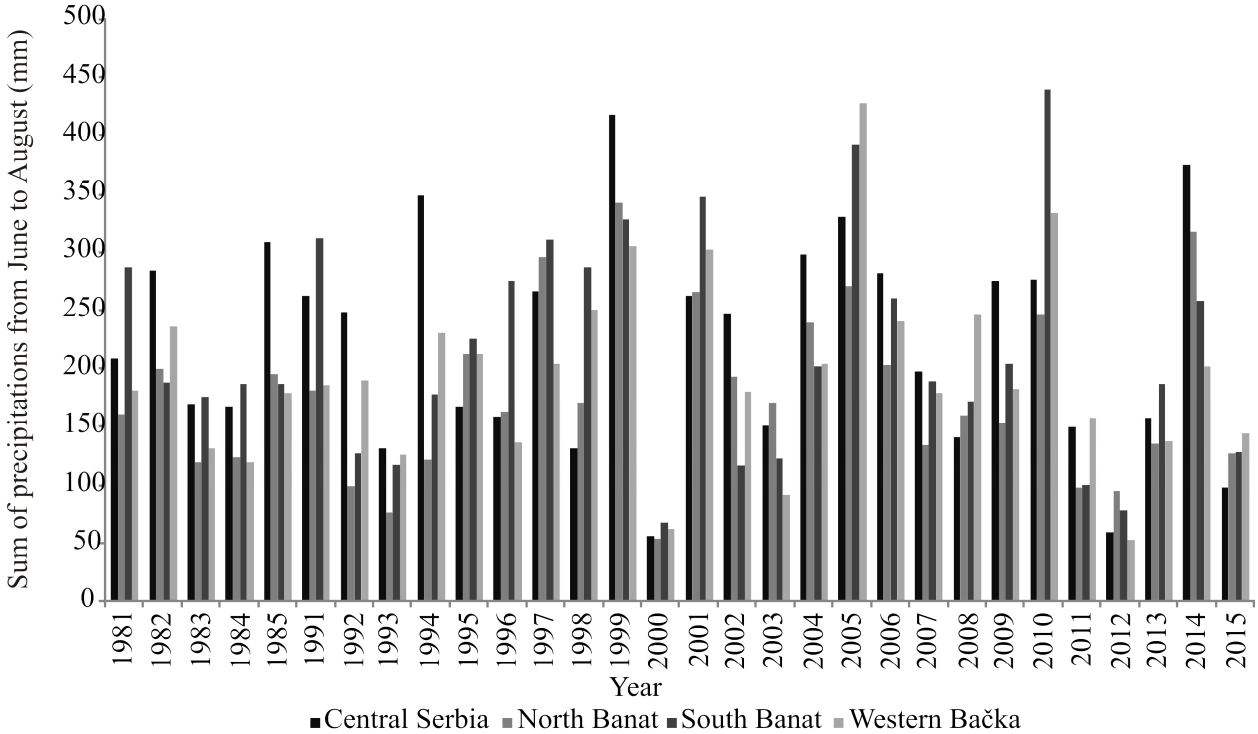
2010
2011
2012
2013
2014
2015
Year
Central Serbia
North Banat
South Banat
Western Bačka


Sum of precipitations from June to August (mm)
500
450
400
350
300
250
200
150
100
50
0
1981
1982
1983
1984
1985
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
Year
Central Serbia
North Banat
South Banat
Western Bačka