Annual variations of *Fusarium* mycotoxins in unprocessed maize, wheat and barley from Bosnia and Herzegovina

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Summary

In this study, the occurrence of *Fusarium* mycotoxins deoxynivalenol (DON), zearalenone (ZEA) and fumonisins (FUM) was investigated in a total of 257 samples of unprocessed cereals (maize, wheat and barley), sampled annually in Bosnia and Herzegovina throughout the 2013-2015 harvesting period. The levels of mycotoxins were determined using a validated ELISA method. The results showed maize to be the most contaminated cereal among the three, with DON detected in 85%, ZEA in 73% and FUM in 67% of samples, the mean concentrations of the toxins observed during the study period thereby being 984±957 μg/kg, 326±314 μg/kg, and 1,259±1,161 μg/kg, respectively. Twenty samples (7.8%) were proven inadmissible for consumption as foodstuffs (Commission Regulation 1881/2006), and 3 samples (1.2%) were proven inadmissible even as feedstuffs (Commission Recommendation 2006/576/EC). Significantly higher ($p < 0.05$) mycotoxin levels determined in samples harvested in 2014 as compared to those harvested in 2013 and 2015 could be associated with heavy rainfall periods witnessed in 2014 that could favour the formation of moulds, and consequently also the increased production of *Fusarium* mycotoxins.

Keywords: cereals, deoxynivalenol, zearalenone, fumonisins, ELISA

Introduction

Mycotoxins are products of toxicogenic moulds that are frequently encountered as food or feed contaminants. A prominent group of mycotoxins are *Fusarium* mycotoxins synthesised by moulds belonging to the *Fusarium* genus (Tanaka et al., 1988; Creppy, 2002; Geraldo et al., 2006). Cereals represent a substrate that facilitates mould growth and consequently also the contamination with their metabolic products. The most commonly contaminated cereal is maize, followed by small grain cereals (wheat, sorghum, oat, barley and rice) and oilseeds (peanut and cotton seed). Contamination may occur as early as in the cultivation period, but also during the crop storage period (Schothorst and van Egmond, 2004; Glenn, 2007). *Fusarium* mycotoxins of relevance for the contamination of cereals and cereal-based products are zearalenone (ZEA), mostly found in maize and wheat, deoxynivalenol (DON), found in wheat, maize, barley, oat and rye, and fumonisins (FUM) which represent a group of mycotoxins (B_1, B_2 and B_3) mostly found in maize, and also not rarely concurrently present in combinations of two or more (Marin et al., 1998; SCF, 2002; Pleadin et al., 2015).

Research has shown that consumption of food or animal feeding on feeds and feed mixtures contaminated with *Fusarium* mycotoxins may jeopardise both human and animal health (IARC, 1993; Kabak et al., 2006; Pleadin et al., 2015). The end-impact of mycotoxins on human and animal health strongly depends on synergistic effects of various mycotoxins concurrently present in a biological substrate that may have serious health consequences despite of fairly low concentrations of an individual toxin in a given substrate (CAST, 2003; Erber and Binder, 2004). In case of cereal contamination with mycotoxins in concentrations surpassing the maximal permitted levels (MPLs) or guidance values (GVs) stipulated under applicable laws and regulations (Commission Regulation 1881/2006; Commission Recommendation 2006/576/EC), the cereal should not be released to the market or used as a food or feed ingredient. Besides health risks, financial risks should also be taken into account, given that the presence of *Fusarium* mycotoxins may substantially lower the crop yield and crop quality, thereby jeopardising the financial standing of agricultural producers with a consequential rise in prices of both raw materials and finalised food products.

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It has been well established that factors of relevance for the nascence of *Fusarium* mycotoxins are the presence of *Fusarium* mycotoxin-producing moulds, the level of humidity, environmental temperature, the degree of aeration, the presence of insects and mechanical grain damage, the level of contamination thereby most commonly depending on climate conditions witnessed during the cultivation period (Mateo et al., 2002; CAST, 2003; Pleadin et al., 2013). *Fusarium* mycotoxin contamination becomes an issue of special concern during rainy seasons characterised by substantial temperature variations, since these conditions favour mould contamination and therefore also an increased mycotoxin production.

Literature data have provided evidence substantiating the need for systematic monitoring of, and control over, cereals and cereal-based products, and/or the use of decontamination techniques, so as to prevent an unfavourable impact on human and animal health as well as to cut down economic losses suffered by the food and livestock industries (Pepeljnjak et al., 2008; Pleadin et al., 2012a; Pleadin et al., 2012b; Pleadin et al., 2013). In absence of universal physical, chemical or biological technique capable of removing most mycotoxins from cereals with no unfavourable effect on their nutritional value, prevention of mycotoxin contamination, control over cereals and clear definition of mycotoxin MPLs in foodstuffs and feedstuffs are of the outmost importance (Pleadin et al., 2013).

Given that data on natural occurrence of *Fusarium* mycotoxins in the last decades in Bosnia and Herzegovina are generally scarce and rare, particularly compared to those gathered by other European countries, this study aimed at gathering data on the occurrence of these mycotoyins in various types of unprocessed cereals harvested from fields across the country in a 3-year period, and to relate these data to climate conditions witnessed during the cultivation period.

Materials and methods

Samples

In the 2013-2015 timeframe, a total of 257 samples of unprocessed cereals, of which 115 maize samples, 84 wheat samples, and 58 barley samples, had been harvested from fields across Bosnia and Herzegovina. For the sole purpose of this study, cereals were sampled randomly during or immediately after harvesting (in a matter of days) from agricultural fields located in different areas seated in the northern, central, eastern and western part of the county. For the purpose of this study, cereals were divided in the laboratory into several groups based on the cultivation/harvesting year (2013, 2014 and 2015), and continuously analysed during the whole sampling period.

Sampling and sample preparation was conducted in accordance with the requirements of ISO 6497:2002 and ISO 6498:1998. Prior to the determination of mycotoxin concentration that made use of the ELISA method, cereals were ground to a fine powder using an analytical mill equipped with a 1.0 mm-diameter sieve (Cylotec 1093 Tecator, Sweden), and then stored at 4 °C pending analysis.

Determination of mycotoxins

Determination of DON, ZEA, and FUM was performed using the competitive ELISA test kits as instructed by the kit manufacturer (R-Biopharm, Darmstadt, Germany). Each kit contains a micro-titre plate with 96 wells coated with antibodies, standard solutions containing different concentrations of mycotoxins, an enzyme conjugate, an anti-antibody, a substrate, a chromogen solution (urea peroxide/tetramethylbenzidine), a stop solution, and washing and dilution buffers. Standards employed with the validation of analytical methods were provided by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals used for analyses were of an analytical grade.

ELISA tests were performed using a ChemWell auto-analyzer (Awareness Technology Inc. 2910, USA), the absorbance thereby being measured at 450 nm. In order to determine mycotoxin concentrations in the sampled material, a standard curve was plotted for each mycotoxin analysed. When determining final mycotoxin concentrations in a given sample, the dilution factor and the mean recovery rate determined for each mycotoxin were taken into account.

Statistical analysis

Statistical analysis was performed using Statistica Ver. 10.0 Software (StatSoft Inc. 1984-2011, USA), with a statistical significance set at 95% ($p= 0.05$). The Shapiro Wilks test was conducted so as to determine whether the results of the analysed parameters follow the normal distribution pattern ($p > 0.05$). For determining the differences in concentrations of the studied mycotoxins found in various cereals during various sampling years, parametric tests like the t-test, and one-way and two-way ANOVA were used, with the statistical significance being set at $p < 0.05$.

Validation of the ELISA method

The limit of detection (LOD) was calculated from the average of ten toxin-negative cereal mixture samples

(containing maize, wheat and barley in equal proportions; earlier analysed for the presence of *Fusarium* mycotoxins and used for validation as a blank material), plus tripled standard deviation (LOD = mean ± 3SD). To determine the limit of quantification (LOQ), the mean concentration determined with ten toxin-negative cereal mixture samples was summed up with six-fold standard deviation (LOQ = mean ± 6SD). The trueness was established using the maize-appropriate certified reference material (CRM) (n = 6) manufactured by Fapas (T04209QC, York, England) to which mean values and ranges of DON (mean value: 1.779 µg/kg; acceptable range: 1.257 to 2.301 µg/kg) and ZEA (mean value: 344 µg/kg; acceptable range: 214 to 473 µg/kg) were assigned. For each mycotoxin, the recovery rate was determined at three different levels (50, 100 and 200 µg/kg) by virtue of fortifying a toxin-negative cereal mixture standard working solution of the given mycotoxin (300 µg/L), followed by the analysis of three replicates at each "spiking" level.

Results and discussion

Numerous studies have confirmed a wide representation of mycotoxins in various cereals, strongly dependent on weather conditions seen during the cultivation period and the method of storage (Placinta et al., 1999; Pleadin et al., 2013). Due to its high adaptability, the *Fusarium* gender is particularly widespread, the cooler European regions thereby being especially convenient for the *Fusarium* mycotoxins' nascence, and therefore witnessing frequent contaminations with these toxins (Binder et al., 2007). These mycotoxins are present in numerous cultivated plant species, among which cereals, in particular maize and wheat are of special importance (EC, 2003; FAO, 2015). Given that these cereals are most commonly consumed around the world, the prevention of contamination and the implementation of systematic controls at all stages of food and feed production are very important.

In this study, the levels of *Fusarium* mycotoxins DON, ZEA, and FUM were investigated in samples of unprocessed cereals (maize, wheat and barley), sampled annually from fields in various areas of Bosnia and Herzegovina during the 2013-2015 timeframe. The levels of mycotoxins were compared based on the type of cereal in which they were found and the cultivation/harvesting year.

The analysis of mycotoxins was conducted using the validated quantitative ELISA method. Validation results are shown in Table 1.

The limit of detection (LOD) and the limit of quantification (LOQ) were the lowest for ZEA and the highest for FUM. According to the CRM-assigned values of these mycotoxins, DON and ZEA concentrations obtained with the determination of trueness were acceptable. Validation of the employed methodology resulted in the mean recovery rates of 95.7, 92.4 and 77.8% for DON, ZEA and FUM, respectively, as well as with the coefficients of variation (CV) ranging from 4.3 to 9.4%. Based on the obtained validation results and the validation criterion given under the Commission Decision 2002/657/EC, the applied quantitative ELISA method can be considered suitable for the determination of the investigated mycotoxins in cereals, as already concluded in many earlier studies (Krska et al., 2008; Pleadin et al., 2012b; Bryden, 2012; Pleadin et al., 2013).

The determined number (No) and the percentage of positive samples, the average (mean), the minimum (min) and the maximum (max) concentrations, together with the accompanying standard deviations (SDs), displayed for each investigated mycotoxin and each type of the studied cereal across the entire sampling period (2013-2015) are shown in Table 2.

Table 1. Results of validation of the ELISA method employed for determination of mycotoxins in various cereals[a]

Mycotoxin	LOD (µg/kg)	LOQ (µg/kg)	Trueness[b] (µg/kg)	Spiked level (µg/kg)	Recovery (%)	CV (%)
DON	23.2	30.1	1478	50	92.8	5.2
				100	94.6	6.1
				200	99.8	7.9
ZEA	3.5	4.2	367	50	87.6	4.3
				100	94.3	5.8
				200	95.4	8.6
FUM	26.8	32.7	-	50	75.6	6.1
				100	77.3	8.2
				200	80.5	9.4

LOD – limit of detection; LOQ – limit of quantification

[a]A mixture containing maize, wheat and barley in equal proportions was first analysed for mycotoxins under study as a blank (toxin-negative) material; [b]The CRM-assigned value (FAPAS T04209QC): DON = 1,779 µg/kg (1,257-2,301 µg/kg); ZEA = 344 µg/kg (214-473 µg/kg)

Table 2. The presence of *Fusarium* mycotoxins in unprocessed cereals sampled from fields in Bosnia and Herzegovina, reported for the entire sampling period (2013-2015)

Mycotoxin	Cereal	Positive/Total No of samples[a]	Positives (%)	Mean[b] (μg/kg)	SD (μg/kg)	Min (μg/kg)	Max (μg/kg)
DON	Maize	98/115	85	984	957	44	8,529
	Wheat	54/84	64	690	604	38	2,123
	Barley	22/58	38	365	177	32	578
ZEA	Maize	84/115	73	326	314	10	2,113
	Wheat	49/84	58	127	140	8	189
	Barley	20/58	34	92	80	11	84
FUM	Maize	77/115	67	1,259	1,161	42	3,275
	Wheat	42/84	50	414	316	35	328
	Barley	15/58	26	145	105	38	232

[a] Samples in which mycotoxin was detected (> LOD); [b] mean value of positives (> LOD)

Table 3. Comparison of the levels of each mycotoxin determined in an individual cereal sample against the maximum permitted levels (MPLs), and guidance values (GVs) defined for *Fusarium* mycotoxins in unprocessed cereals intended to be used as foodstuffs and feedstuffs

Mycotoxin	Unprocessed cereal	MPL (μg/kg)	> MPL[a] (No/%)	GV (μg/kg)	> GV[b] (No/%)
DON	maize	1,750	7/6.1	8,000	2/1.7
	wheat	1,750	4/4.8	8,000	0/0
	barley	1,250	0/0	8,000	0/0
ZEA	maize	350	4/3.5	2,000	1/0.9
	wheat	100	5/5.9	2,000	0/0
	barley	100	0/0	2,000	0/0
FUM	maize	4,000	0/0	60,000	0/0

[a]The number/percentage of samples in which mycotoxin concentration higher than the MPL defined for foodstuffs (Commission Regulation 1881/2006) was determined; [b]The number/percentage of samples in which mycotoxin concentration higher than the GVs for feedstuffs (Commission Recommendation 2006/576/EC) was determined

The MPLs for foodstuffs and the GVs of *Fusarium* mycotoxins in unprocessed cereals intended for use as feedstuffs, as well as the number/percentage of study samples in which these levels were exceeded, are shown in Table 3.

In maize, DON was detected in 85%, ZEA in 73% and FUM in 67% of samples. Their average concentrations (±SD) in maize were 984±957 μg/kg, 326±314 μg/kg, and 1,259±1,161 μg/kg, respectively. Given the MPLs of these mycotoxins in unprocessed maize (Commission Regulation 1881/2006), which equals to 1,750 μg/kg for DON, 350 μg/kg for ZEA and 4,000 μg/kg for FUM, DON levels higher than MPL were observed in 7 analyzed maize samples and those higher than ZEA MPL in 4 maize samples, while FUM concentrations higher than MPL were not determined.

Statistical analysis revealed significant ($p < 0.05$) mutual differences in concentrations of *Fusarium* mycotoxins found in individual maize samples, as well as significantly higher concentrations of all three investigated *Fusarium* mycotoxins in maize in comparison to other types of cereals (wheat and barley). Also, significantly higher concentrations of all three *Fusarium* mycotoxins were determined in maize sampled during 2014 in comparison to those sampled during 2013 and 2015. As for wheat and barley, significant mutual differences in mycotoxin concentrations or significant differences in concentrations of mycotoxins found in samples retrieved in various sampling years failed to be observed ($p > 0.05$).

In wheat, the highest mean concentration was observed for DON (690±604 μg/kg), and the lowest for ZEA (127±140 μg/kg); in barley, the highest contamination was also linked to DON (365±177 μg/kg), and the lowest to ZEA (92±80 μg/kg). Given the MPLs of these mycotoxins in unprocessed wheat and barley (Commission Regulation 1881/2006), which equals to 1,750 μg/kg and 1,250 μg/kg for DON, respectively, and 100 μg/kg for ZEA, DON levels higher than MPL were observed in 4 samples, while ZEA levels surpassing the MPL were established in 5 wheat samples. Concentrations higher than MPLs defined for these mycotoxins in barley were not determined.

In some of the samples, mycotoxin levels higher than permitted for foodstuffs and/or higher than

guidance value for feedstuffs were observed, of note, all samples in which mycotoxin levels were significantly increased (non-compliant samples) were sampled in the year 2014. Increased DON and ZEA levels were found in maize and wheat. Among 257 samples taken during a three-year period, 20 samples (7.8%) were not eligible for use as raw materials intended for food production, among which three samples (1.2%) were also non-compliant as feedstuffs. Other samples non-compliant for use as foodstuffs (17 additional samples) were acceptable for use as feedstuffs.

In many European countries, data have also shown substantial variations in *Fusarium* mycotoxin concentrations across various cereal types, various regions and various investigation periods (Placinta et al., 1999). In 11 European countries, 57% of cereal samples were positive for DON, among which most were either maize (89%) or wheat samples (61%): the above is consistently comparable to the results of this study, obtained throughout the investigated period (98% of DON-positive maize and 64% of DON-positive wheat samples). In the study by JECFA (2001), DON was detected in 59% of barley, 57% of wheat and 41% of maize samples in the maximal concentrations of up to 3,700 μg/kg in maize, 5,700 μg/kg in wheat, and 9,000 μg/kg in barley. Should the above-quoted JEFCA 2001 results be compared to the results of this study, it can be seen that the maximal DON concentrations established in maize within our study frame were significantly higher (8,529 μg/kg), while, at the same time, the concentrations of this mycotoxin determined in wheat (2,123 μg/kg) and barley (578 μg/kg) were significantly lower. In the German study performed by Müller and Schwadorf (1993), 79 out of 84 analysed wheat samples contained two to six *Fusarium* mycotoxins. DON and ZEA were detected in levels of up to 20,500 μg/kg and 8,040 μg/kg, respectively, whereas in Bulgaria, *F. graminearum* has been determined to be the major wheat pathogen, with levels of DON and ZEA rising up to 1,800 μg/kg and 120 μg/kg, respectively (Vrabcheva et al., 1996). The mean (127±140 μg/kg) and the maximal ZEA concentration (189 μg/kg) determined in wheat samples under this study are comparable to those established in the study by Vrabcheva et al. (1996).

In Croatia, the study by Domijan et al. (2005) revealed maize to be at constant risk of fungal development due to its nutrient composition. FUM was found in all analyzed maize samples in concentrations of 459.3±310.7 μg/kg, while ZEA (positive in 84% of the analysed samples) was present in concentrations of 3.84±6.68 μg/kg. The results obtained for maize sampled in 2010 showed the presence of DON in 85% of samples, with the maximum concentration of 17,920 μg/kg, and the presence of ZEA in 87.5% of samples, with the maximum concentration of 5,110 μg/kg (Pleadin et al., 2012b). The highest detected concentration of FUM was 25,200 μg/kg, with the mean value of 4,509 μg/kg; such a study outcome pointed towards mycotoxin-induced maize contamination that occurred after a heavy rainfall period. In this study, mean and maximum concentrations of all three *Fusarium* mycotoxins determined in maize were significantly lower (maximal DON and ZEA concentrations roughly two times lower and maximal FUM concentrations roughly seven times lower) than those determined in the above cited study by Pleadin et al. (2012b). These differences in mycotoxin concentrations obtained in this study as compared to previous studies conducted in Croatia might be attributed to facts given in conclusions of earlier studies conducted in Croatia, coming down to the dependence of mycotoxin occurrence and concentrations on climate conditions witnessed in a particular period (Domijan et al., 2005; Pepeljnjak et al., 2008; Pleadin et al., 2012a; Pleadin et al., 2012b), with significantly higher concentrations of *Fusarium* mycotoxins in cold and rainy years (Pleadin et al., 2012a; Pleadin et al., 2012b). It has been acknowledged that such a contamination is, to a significant degree, linked to specific cereal diseases caused by *Fusarium* pathogens, which could lead to multiple mycotoxin contaminations (Placinta et al., 1999).

In the study by Pleadin et al. (2013) performed in Croatia, as well as in this study, maize was shown to be the most contaminated cereal of them all, with DON as the most represented *Fusarium* mycotoxin (52.5%), followed by ZEA (40.5%) and FUM (37.5%). Mycotoxin concentrations higher than permitted were observed in 4 maize samples, and a single wheat sample. Authors concluded that, given that the study period was warm and dry, such a contamination might be associated with some factors other than climate conditions, which could cause *Fusarium* mycotoxin formation.

In this study, annual variations of DON, ZEA, and FUM concentrations during the 2013-2015 timeframe, given *per* the harvesting year and the type of cereal are shown in Fig. 1-3.

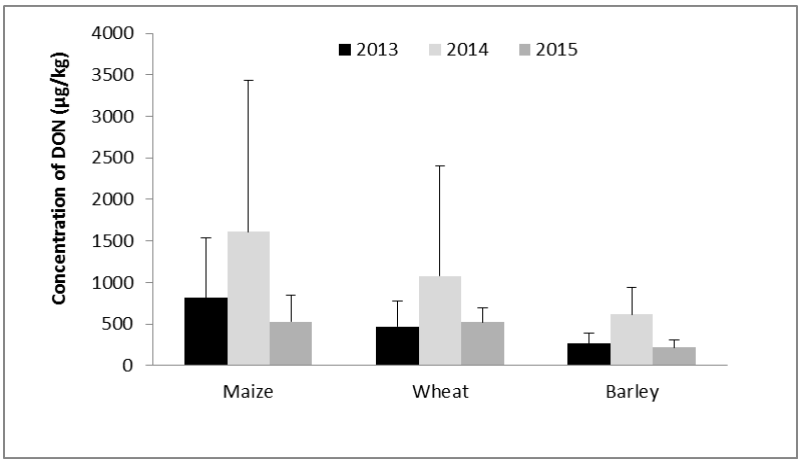

Fig. 1. Concentration of deoxynivalenol (DON) established in various types of cereals in various harvesting years

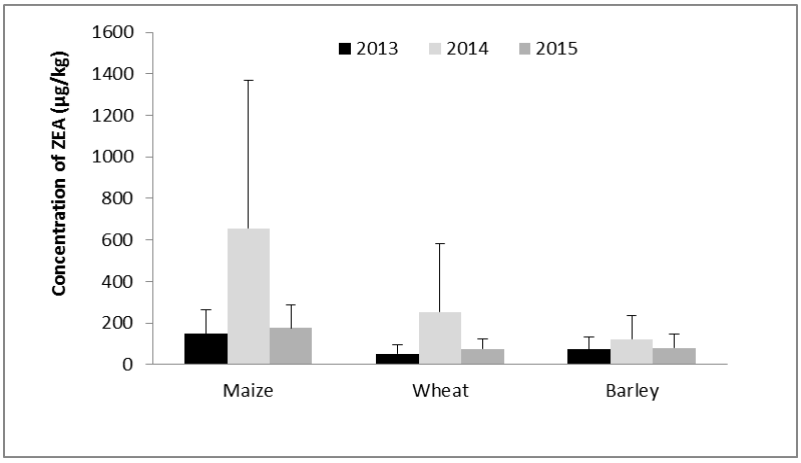

Fig. 2. Concentration of zearalenone (ZEA) established in various types of cereals in various harvesting years

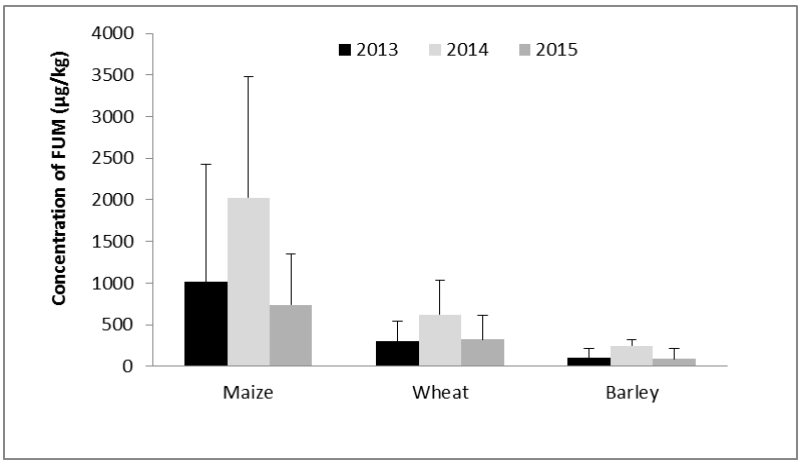

Fig. 3. Concentration of fumonisins (FUM) established in various types of cereals in various harvesting years

The highest mean concentrations of mycotoxins were determined in all three cereals in the year 2014. The results of ANOVA revealed statistically significant differences ($p < 0.05$) in DON, ZEA, and FUM levels between various types of cereals (i.e. maize in comparison to wheat and barley) and various cultivation/harvesting years (i.e. 2014 in comparison to 2013 and 2015). The maximum mean concentrations of mycotoxins were observed in maize sampled in 2014, with mean values of DON of 1,611±1,825 μg/kg, that of ZEA of 655±715 μg/kg, and that of FUM of 2,025±1,456 μg/kg. All samples, in which mycotoxin concentrations surpassed those stipulated for food and feed under the applicable legislation, were observed in cereals sampled during 2014.

Given the fact that high mycotoxin levels are usually associated with climate conditions, in particular humidity and temperature as the factors most critical for mould formation, and thus also mycotoxin production, in the studies of *Fusarium* mycotoxins occurrence dependent on weather conditions observed during the investigated period should be taken into account (Pleadin et al., 2013). Official weather reports for 2013 and 2014 show that in the period of cereal growth and harvesting (May-August) the investigated parts of Bosnia and Herzegovina (http://www.fhmzbih.gov.ba/latinica/KLIMATOLOGIJA/ANALIZA/K-sezona.php) were warm (75-91%) to very warm (91-98%). As for humidity, the year 2013 was either normal or dry to very dry (< 25%), whereas the year 2014 was highly (91-98%) to extremely humid (> 98%). In 2015, the period of concern was very warm (91-98%) and normal or dry to very dry (< 25%). Therefore, higher mean concentrations of *Fusarium* mycotoxins determined in cereals sampled in 2014 in comparison to those sampled in 2013 and 2015, could be linked to high or to extreme humidity seen during the cereal growth and harvesting period, which could enhance mould growth, and consequently also the production of *Fusarium* mycotoxins.

Conclusions

The observed average mycotoxin concentrations revealed maize to be the most contaminated among cereals, with DON as generally the most common *Fusarium* mycotoxin encountered, followed by ZEA and FUM. Significantly higher mean concentrations of all mycotoxins in all types of cereals under study, observed in 2014, can be associated to high or to extreme humidity witnessed during the cereal growth and harvesting period that could enhance mould growth and consequently also the production of *Fusarium* mycotoxins. Since generally high rates of contamination with *Fusarium* mycotoxins were detected, in order to ensure safety of cereals and cereal-based products used as foodstuffs and feedstuffs, it is necessary to establish consistent control over these contaminants and to annually determine and monitor their presence, taking into account climatic conditions during cereal growth period.

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