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PRESENCE OF ZEARELENONE IN THE MOST COMMONLY GROWN WHEAT CULTIVARS IN SERBIA

ABSTRACT: A total of 45 samples of wheat from three different locations in Vojvodina were analyzed for the presence of zearalenone. Analytical methods based on clean-up by solid-phase extraction (SPE) columns and detection by liquid chromatography were used after validation. Limit of detection for ZEA in wheat was 18.6 µg/kg and the limit of quantification was 56.5 µg/kg. Recovery values ranged between 86% and 97%. The occurrence of ZEA in wheat was rather high with 53.3% of positive samples with the average value of 330 µg/kg. Incidences were found from 68 µg/kg to 1079 µg/kg. Contamination levels were above the established maximum limit for unprocessed cereals, other than maize, in as many as seventeen samples. These results were compared to the results of investigation of deoxynivalenol and fumonisin content, established in our previous work on the same samples. The results obtained were also compared to those of the neighboring countries where the relevant data existed and to the data of previous studies in our country.

KEY WORDS: HPLC, wheat, zearalenone

INTRODUCTION

Fusarium molds appear as the most common contaminants of grains in the mild climatic zone. As a result, mycotoxins are often found in such substrates in larger or smaller concentrations, depending on storage and microclimatic conditions (K l a r i ć, 2008). The main groups of Fusarium toxins commonly found in grains are trichothecenes (including deoxynivalenol, nivalenol, T-2

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and HT-2 toxins), zearalenone (ZEA) and fumonisins B1 and B2. *Fusarium* toxins pose safety concerns for grains intended for direct consumption due to their harmful impact on human health.

Fusarium, *Penicillium* and *Aspergillus* genera are the most frequently isolated fungi contaminating cereals, feedstuffs, vegetables and fruit in Serbia (L e v i ć et al., 2004). Fungi from the *Fusarium* genera are especially present, and with them *Fusarium* mycotoxins, first of all zearalenone, which presence is mainly associated with *F. graminearum* and it is the highest in years with abundant precipitation and lower temperatures at the end of summer and the beginning of autumn (L e v i ć et al., 2004).

In Serbia, grain production, especially wheat and maize, represents an important economic factor considering the fact that our country is mostly agricultural. Out of 3.099.000 ha of area sown with major crops, 493.000 ha was sown with wheat in 2010 (Statistical Office of the Republic of Serbia, 2012). In our country, main crops are wheat, barley and maize which are particularly important as food, feed and raw materials for breweries and distilleries. Grain-based products represent the main part of human diet and infants, in particular. Babies and young children are exposed to higher doses of toxins per body weight unit compared to adults because of their proportionally higher consumption of cereals. This poses concern for consumer health and requires continuous control of foodstuffs and foods for chemical and biological contaminants.

The Commission of the European Communities (Commission Regulation 1126/2007) established the following tolerance values for ZEA in cereals and cereal-based products: unprocessed cereals other than maize (100 µg/kg), unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling (350 µg/kg), cereals intended for direct human consumption, cereal flour, bran and germ as end products marketed for direct human consumption (75 µg/kg), refined maize oil (400 µg/kg), bread (including small bakery commodities), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereal (50 µg/kg), maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals (100 µg/kg), processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children (20 µg/kg) and processed maize-based foods for infants and young children (20 µg/kg). With respect to the aforementioned cereals and their products, Serbian regulation (Službeni glasnik, 2011) established the same tolerance levels as Commission Regulation did, and for feed (Službeni glasnik, 2010) the following tolerance levels were set: in complete and supplementary mixtures for piglets, young mails and gilts is 500 µg/kg, in complete and supplementary mixtures for other categories of pigs is 1000 µg/kg while in complete and supplementary mixtures for cattle, sheep and goats is 3000 µg/kg.

In Serbia, available data for the distribution of *Fusarium* mycotoxins in cereal production are still limited. There is no national data base established for the collection of this kind of data in order to use them for prediction and prognosis of annual mycotoxin risk exposure of the local population.

Considering the aforementioned facts, the primary aim of this study was to gain an insight into ZEA presence in Serbia on the basis of analysis of 45 wheat samples from the 2010 harvest and to compare the results of investigation of deoxynivalenol and fumonisin content, established in our previous work on the same samples. The samples were taken directly from the fields immediately after the harvest before entering further the food chain. The levels of ZEA were determined after SPE clean-up by liquid chromatography with DAD, allowing detection limits in the ppb (18.6 µg/kg) range.

MATERIALS AND METHODS

2.1. Materials

Acetonitrile and methanol (both HPLC grade) were purchased from Sigma (St. Louis, MO, USA). Water (HiPerSolvChromanorm, HPLC grade) was purchased from Sigma (St. Louis, MO, USA). Zearalenone mycotoxin was purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). MycoSep® 224 (AflaZon) clean-up columns were obtained from Romer (Romer Labs. Inc., Union, MO, USA) and paper filter (Whatman No. 4) was obtained from Whatman (Maidstone, UK).

Samples

A total of 45 samples of wheat, from three different locations in Vojvodina, were analyzed for the presence of zearalenone. They were collected from the most important agricultural area in the country – Vojvodina, with 3 different regions: north-western (Bačka), north-eastern (Banat) and south-western region (Srem). The samples of wheat represent the most common cultivars grown in Vojvodina.

Due to the irregular mycotoxin distribution among the crops and kernels, a proper sampling was ensured according to EU requirements (Commission Regulation 401/2006). Manual sampling was performed by trained inspectors with grain probes which are authorized for official control of contaminants. Depending on the weight of bulk lot, from 3 to 100 incremental samples of 100 g or more, were taken randomly and combined into a representative sample of 1–10 kg weight. Each sample was transported to the laboratory immediately and was stored at low temperature in a dark place. All samples were milled on a laboratory mill and a portion was taken for the analysis of contaminants.

Extraction and clean-up

Twenty-five grams of ground sample were extracted with 100 ml acetonitrile:water (84:16, v/v) by high speed blending for 3 min. The extract

was filtered through paper filter, acidified with 50 μ l acetic acid and 2 ml was cleaned-up by Mycosep® 224 (AflaZon) columns. The cleaned-up extract was evaporated to dryness.

Liquid chromatographic analysis

The equipment consisted of an Agilent 1260 Infinity Liquid Chromatography system, equipped with a μ -Degasser (G1379B), 1260 binary pump (G1312B), 1260 standard autosampler (G1329B), 1260 thermostated column compartment (G1316A), 1260 diode array and multiple wavelength detector (G1315C), and a column Hypersil ODS (100 x 4.6 mm i.d., particle size 5 μ m, Agilent Technologies, USA).

After evaporation, the residue was redissolved in 400 μ l methanol, and a 15 μ l aliquot of the solution was injected into the LC system. A mobile phase consisting of a mixture of methanol–water (70:30, v/v) was used at 0.5 ml/min with UV detection at 236 nm. The mobile phase was filtered through a 0.45 μ m pore size cellulose filter membrane (Agilent Technologies, USA).

RESULTS AND DISCUSSION

Validation parameters of method for ZEA determination were estimated using the recovery tests of samples of the studied wheat matrix. The accuracy of the method was determined with the recovery of fortified blank grain samples at three levels with three replicates for each level and three injections for each replicate (nine injections per level). Recoveries obtained for wheat were in the range 86 – 98%. These recoveries comply with the requirements of European Commission concerning analytical methods development (Commission Regulation 657/2002).

The limit of detection (LOD) and the limit of quantification (LOQ) are determined on the basis of standard deviation of the response and the slope of the linearity plot (Reason, 2003). The LOD was calculated as $3.3\alpha/b$ and LOQ as $10\alpha/b$, where α was the standard deviation of the response about the line of the best fit and b was the slope of the calibration curve. Detection limit (LOD) for wheat was below 18.6 μ g/kg while limit of quantification (LOQ) was close to 56.5 μ g/kg. The obtained results showed that the proposed analytical method fitted well for the purpose of control of ZEA in grain samples.

Zearalenone content was determined in fifteen cultivars of winter wheat which have been the most commonly grown cultivars in Serbia since 1955. The occurrence of ZEA in these samples originated from the trial fields of the Institute of Field and Vegetable Crops and it is summarized in Table 1. Banatka is a native population, San Pastore and Libellula are Italian cultivars, Zlatna Dolina is a Croatian cultivar. Serbian cultivars used in the study were created at the Institute of Field and Vegetable Crops, Novi Sad (Sava, Novosadska rana 2, Balkan, Lasta). All the mentioned cultivars are presently grown

in Serbia. Serbian cultivars that are most widespread and encompassed by this study are Novosadska rana 5, Renesansa, Pesma, Cipovka, Dragana, Simonida, and Zvezdana. Grain samples were obtained from 15 winter wheat cultivars grown in 2010 at the following Serbian locations: Novi Sad (Bačka), Sremska Mitrovica (Srem) and Pančevo (Banat). The locations are characterized by semiarid conditions, with dry, hot spring and summer, neutral autumn and moderately cold winter. At each location, the plots were rotated with soybean. Positive samples were contaminated with ZEA at levels from 68 – 1079 µg/kg. According to the data in Table 1, more than half of the analyzed wheat samples were ZEA-contaminated at mean level of 330 µg/kg. The contamination levels in as many as seventeen samples were above the maximum limit of 100 µg/kg, a level set in Europe (Commission Regulation 1126/2007). Contamination was found in samples with visually healthy (asymptomatic) kernels.

Tab. 1. – Occurrence of zearalenone in wheat samples in Serbia from the 2010 harvest

Cultivar	Location/Concentration of ZEA (µg/kg)			
	Pančevo (Banat)	Novi Sad (Bačka)	Sremska Mitrovica (Srem)	
Banatka	92	ND	231	
San Pastore	ND	ND	821	
Libellula	214	ND	ND	
Zlatna Dolina	ND	ND	351	
Sava	73	ND	327	
Novosadska rana 2	68	71	158	
Balkan	ND	ND	301	
Lasta	181	467	143	
Novosadska rana 5	ND	ND	944	
Renesansa	241	ND	356	
Pesma	88	ND	734	
Cipovka	ND	ND	293	
Dragana	71	ND	1079	
Simonida	ND	ND	139	
Zvezdana	ND	ND	469	Total
No. of positive samples (%)	8 (53.3)	2 (13.3)	14 (93.3)	24 (53.3)
Average (µg/kg)	129	269	453	330
Range (µg/kg)	68 – 241	71 – 467	193 – 1079	68 – 1079
Median (µg/kg)	90	269	339	236

The incidence and level of contamination of studied cereal samples with ZEA did not show comparable levels as it was reported for previous harvest years in the country. The frequency of contamination seems to be highly dependent of weather conditions. The analysis of wheat samples for human consumption in 1995 (P a r r y et al., 1995) showed a widespread contamination (70% incidence) with low to medium levels of ZEA (V r a b c h e v a, 1996;

N e n o v, 1997; V a l c h e v a et al., 2003; V r a b c h e v a et al., 2004). As regards the occurrence of ZEA in wheat in neighboring countries, V r a b c h e v a (1996) reported 69% of positive samples in Bulgarian wheat, while M l a d e n o v a and M a n o v a (2009) obtained quite lower number of positive wheat samples (1.9%). In Romanian wheat, during the period from 2008-2010, ZEA occurred in 10% of wheat samples (G a l b e n u et al., 2011). Š k r b i ć et al. (2011) found no ZEA in wheat samples collected during the harvest of 2007.

In our previous study on the same samples, the contents of deoxynivalenol (DON) and fumonisins (FUMs) were determined (J a k š i ć et al., 2012). We compared these results to those obtained for ZEA, in terms of better estimation of mycotoxin contamination (Table 2). As it can be seen, the number of contaminated samples was quite similar for all toxins found in the samples from Banat region, while the number of fumonisin contaminated samples from Bačka and Srem regions was lower than in the other two. The highest number of positive samples was established in Srem region where the highest concentration of all three toxins was obtained. Also, it can be said that the distribution of DON and ZEA contamination is well correlated unlike the distribution of fumonisin contamination.

Tab. 2. – Comparison of occurrence of deoxynivalenol, fumonisin and zearalenone in crops in Serbia from the 2010 harvest

Cultivar	Location/content µg/kg								
	Pančevo (Banat)			Novi Sad (Bačka)			Sremska Mitrovica (Srem)		
	FUMs	DON	ZEA	FUMs	DON	ZEA	FUMs	DON	ZEA
Banatka	ND	229	92	ND	ND	ND	28	392	231
San Pastore	ND	ND	ND	ND	ND	ND	ND	352	821
Libellula	81	312	214	ND	144	ND	27	136	ND
Zlatna Dolina	ND	ND	ND	38	ND	ND	ND	312	351
Sava	427	104	73	359	ND	ND	414	356	327
Novosadska rana 2	ND	132	68	ND	156	71	ND	364	158
Balkan	419	ND	ND	ND	ND	ND	ND	316	301
Lasta	ND	288	181	ND	384	467	263	164	143
Novosadska rana 5	68	ND	ND	55	ND	ND	37	864	944
Renesansa	351	196	241	417	ND	ND	525	660	356
Pesma	72	292	88	84	ND	ND	78	984	734
Cipovka	401	ND	ND	ND	ND	ND	ND	288	293
Dragana	36	232	71	ND	ND	ND	ND	308	1079
Simonida	ND	ND	ND	441	ND	ND	394	292	139
Zvezdana	ND	ND	ND	ND	ND	ND	52	692	469
No. of positive samples (%)	8 (53.3)	8 (53.3)	8 (53.3)	6 (40.0)	3 (20.0)	2 (13.3)	9 (60)	15 (100)	14 (93.3)
Average (mg/kg)	232	223	129	232	228	269	202	432	453
Range (mg/kg)	36-427	104-312	68-241	38-441	144-384	71-467	27-525	136-984	193-1079
Median (mg/kg)	216	230	90	221	156	269	78	352	339

The obtained results for wheat harvested in 2010 confirmed that this crop should be continuously controlled in order to protect the population against unallowable risk of mycotoxin contamination. The results of the present study are particularly important for the wheat production which should be regarded as a potential source of *Fusarium* mycotoxins. This requires increased control measures for agricultural production starting from the field, before foodstuffs enter the manufacturing process and food chain.

CONCLUSIONS

Samples of wheat grown in Serbia were tested for zearalenone, produced by common *Fusarium* species. The incidence of ZEA contamination of wheat (53.3%) was found to be quite high, especially in Srem region (93.3%). It should be noted that the average value (330 μ g/kg) in the examined wheat samples was very high, and in 17 out of 45 samples ZEA content exceeded the maximum tolerable level set by EU and Serbian regulations. By comparing these data to DON and FUMs content in the same samples, it can be said that ZEA distribution responds to DON distribution but not to distribution of FUMs. This study indicates the existence of a serious risk related to the occurrence of DON in the food chain in Serbia and importance of frequent monitoring of this mycotoxin. Since the new Serbian regulation for control of mycotoxins in food was adopted and harmonized with EU regulations in April 2011, it is expected that more frequent control of ZEA will be conducted and that more data on its occurrence in Serbia will be available. In the future, based on the obtained results, it will be necessary to form a national database of ZEA occurrence in the food chain in Serbia.

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ПРИСУСТВО ЗЕАРАЛЕНОНА У НАЈЧЕШЋЕ УЗГАЈАНИМ СОРТАМА ПШЕНИЦЕ У СРБИЈИ

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Резиме

На присуство зеараленона анализирано је укупно 45 узорака пшенице са три различите локације у Војводини. Коришћене су аналитичке методе засноване на пречишћавању екстракцијом на чврстој фази, те квантификација течном хроматографијом, након валидације методе. Граница детекције за зеараленон у пшеници је износила 18,6 µg/kg, а граница одређивања 56,5 µg/kg. Ефикасност методе је била у опсегу од 86% до 97%. Зеараленон је био присутан у 53,3% испитиваних узорака, са просечним садржајем од 330 µg/kg. Добијене вредности садржаја зеараленона су биле у опсегу од 68 µg/kg до 1079 µg/kg. У чак седамнаест узорака је пронађена концентрација овог токсина која превазилази максимални дозвољени садржај зеараленона у нетретираним житарицама. Ови резултати су упоређени са вредностима садржаја деоксиниваленола и фумонизина у истим узорцима добијеним у нашим претходним истраживањима. Резултати су такође упоређени са доступним резултатима добијеним у нашој и суседним земљама током претходних година.

КЉУЧНЕ РЕЧИ: HPLC, пшеница, зеараленон

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