Research Note

Incidence of Deoxynivalenol in Serbian Wheat and Barley

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ABSTRACT

One hundred thirty-nine small-grain cereal (wheat and barley) samples collected during the 2010 harvest in Serbia were tested for deoxynivalenol (DON) contamination. Samples were classified into four different groups and then analyzed by analytical methods based on cleanup by solid-phase extraction and detection by liquid chromatography after the validation. Limits of detection of DON were 18 and 22 µg/kg for wheat and barley, respectively, and limits of quantification were 60 and 73 µg/kg for wheat and barley, respectively. Obtained recovery values for wheat and barley samples ranged from 93.7 to 105.8% and from 84.7 to 89.2%, respectively. Analysis of 128 wheat samples showed that 100 (78.1%) of them were contaminated with DON at the levels ranging from 64 to 4,808 µg/kg. The contamination level of even 16 (12.5%) samples was above the established maximum tolerable limits adopted by the European Commission (EC) and Serbian regulation. In the 11 examined barley samples, DON was found in 3 (27.3%), with the levels ranging from 118 to 355 µg/kg, although none of the samples were contaminated above the limit for this cereal. The results obtained were analyzed as a function of climatic conditions and compared with the previous data on the presence of DON in Serbia.

The contamination of crops by fungi able to produce toxic metabolites is often unavoidable and represents a worldwide concern. Fungal invasions cause a reduction of crop yield and grain quality, which results in considerable financial losses, as well as impaired health in humans and animals (16, 22). The weather conditions during the plant growth, in particular, in the flowering stage, are a key factor that influences the mycotoxin formation and production (13, 14, 20, 25). The presence of Fusarium mycotoxins in small-grain cereals, both in fresh and stored grains, has been frequently reported worldwide (9, 18, 21). The main groups of these toxins that are commonly found in grains are trichothecenes (including deoxynivalenol [DON], nivalenol, and T-2 and HT-2 toxins), zearalenone, and fumonisins B1 and B2 (3). Fusarium toxins pose safety concerns for grains intended for direct consumption due to their harmful impact on human and animal health. Although DON is among the least toxic of the trichothecenes, it is the most frequently detected throughout the world, and its occurrence is considered to be an indicator of the possible presence of other, more toxic, trichothecenes (19).

Control and maximum tolerable levels of mycotoxins in food have been established in many countries. The Commission of the European Communities (8) established the following tolerance values for DON in cereals and cereal-based products: unprocessed cereals other than durum wheat, oats, and maize (1,250 µg/kg), unprocessed durum wheat and oats (1,750 µg/kg), unprocessed maize (1,750 µg/kg), cereal flour, including maize flour, maize grits, and maize meal (750 µg/kg), bread, pastries, biscuits, cereal snacks, and breakfast cereals (500 µg/kg), pasta (dry, 750 µg/kg), and processed cereal-based food for infants and young children and baby food (200 µg/kg). The Serbian regulation (29) for control of mycotoxins in food was adopted and harmonized with the European Union (EU) regulation in April 2011. Until then, control of DON in Serbia was obligatory only in complete feeding stuffs for pigs (28).

Serbia is an agricultural country, in which arable fields and gardens cover about 65.3% of used agricultural land. Grain production, especially wheat and maize, are an important economic factor (31). In 2010, the total arable land in Serbia amounted to 3,295,000 ha. Cereals were planted on 1,894,000 ha, of which 493,000 ha were planted with wheat. Overall wheat production in 2010 amounted to 1,630,546 tons, with an average yield of 3.4 tons/ha, which is 0.2 ton/ha less than in 2009 and 0.9 ton/ha less than in 2008 (32). In 2010, overall barley production amounted to 244,268 tons, with an average yield of 2.9 tons/ha (35). Furthermore, Serbia is a major regional producer of maize, ranking third in Central Europe, only behind Romania and Hungary (30), so in 2010, it was on the list of the top 10 corn exporters in the world (34).

Serbia is located in the continental climate belt, where the most frequently isolated fungi-contaminating cereals, feedstuffs, vegetables, and fruits are from the genera of Fusarium, Penicillium, and Aspergillus. Fungi from the Fusarium genera are especially prevalent, and among them Fusarium mycotoxins, with zearalenone being foremost.
The presence of zearalenone is mainly associated with *Fusarium graminearum* and weather conditions with abundant precipitation and lower temperatures at the end of summer and the beginning of autumn (17).

The availability of data concerning the distribution of *Fusarium* mycotoxins in cereals produced in Serbia is still limited. No national database for collecting this kind of data to use for prediction and prognosis of annual mycotoxin risk exposure for the local population exists. In the first reports of DON content in crops from Serbia (1, 10, 11), we analyzed a larger number of maize samples (226) and a smaller number of samples of wheat (59), soybean and soybean meal (42), sunflower (19), and barley (4), collected during 2004 to 2007.

Regarding the above, the primary aim of this study was to examine the impact of weather factors and the composition of land in different regions of Serbia for the presence of *Fusarium* head blight (FHB) in 21 varieties of wheat, which were mostly grown in Serbia in the period from 1955 to 2006. DON contamination of wheat and barley seeding material intended for 2010 to 2011 sowing was also examined. Moreover, the susceptibility of durum wheat (cultivar Durumko) to FHB was studied, as a variety with no available tolerance to FHB (4, 5).

### MATERIALS AND METHODS

**Chemicals.** Acetonitrile, methanol, and water (all high-performance liquid chromatography [HPLC] grade) were purchased from Sigma (St. Louis, MO). DON standard solution was also purchased from Sigma as an analytical standard. Calibrant solution was prepared in ethyl acetate–methanol (19:1, vol/vol) at the concentration of 0.1 mg/ml from a crystalline substance according to AOAC International method 986.17 (Association of Official Analytical Chemists, *J. AOAC Int.* 69:37, 1986). Stock solution was prepared by measuring 1.00 ml of calibrant solution of DON into a 10-ml volumetric flask and diluting to volume with ethyl acetate–methanol (19:1, vol/vol). Working calibrant solutions were prepared by evaporating the appropriate volume of the stock solution and diluting with 1.00 ml of methanol. Standard solutions were stored at 4°C.

**Samples and sample handling.** In total, 139 representative cereal samples (128 of wheat and 11 of barley) harvested in 2010 were analyzed. Samples were collected from three different regions of Vojvodina, the most important agricultural area in Serbia: northwestern (Bačka), northeastern (Banat), and southwestern (Srem) region. Samples of wheat and barley were classified into four groups: 22 samples that included 18 samples of wheat seed and 4 barley samples (intended for autumn 2010 seeding); 63 samples of wheat that were sown and harvested from the trial fields located in the three regions of Vojvodina (21 cultivars that have been most frequently sown in the recent decades); 22 samples of different wheat and barley cultivars (collected from the fields in Srem); and 32 samples of durum wheat (cultivar Durumko from the Institute of Field and Vegetable Crops, Novi Sad, Serbia). Improvements in sampling methods to detect mycotoxins and other quality attributes in food and feed products continue to be a high priority among regulatory agencies, international organizations, and commodity industries worldwide (26). Due to irregular distribution of mycotoxins among the crops and kernels, a proper sampling was ensured according to the EU requirements (7).

Manual sampling was performed by trained inspectors with grain probes that are authorized for official control of contaminants. Depending on the weight of bulk lot, 10 to 20 incremental samples of 100 g or more were taken randomly and combined in representative samples of 1- to 2-kg weights. Each sample was transported to the laboratory immediately and was stored in a freezer at −20°C until analysis. Prior to each analysis, the samples were allowed to reach room temperature. All samples were laboratory milled (UD Corporation, Boulder, CO) in such a way that >93% passed through a sieve with a pore diameter of 0.8 mm, and a portion was taken for analysis.

**Determination of DON in grains.** Grain samples (25 g) were extracted with 100 ml of acetonitrile-water (84:16, vol/vol) by high-speed blending on an Ultra-Turrax T18 (IKA, Staufen, Germany). The extract was filtered through no. 4 filter paper (Whatman, Maidstone, UK), and 3 ml was cleaned up on MycoSep 225 Trich columns (Romer Labs, Inc., Union, MO). The cleaned-up extract was evaporated just to dryness.

**HPLC analysis.** The equipment consisted of a 1200 Series HPLC System (Agilent Technologies, Santa Clara, CA) with a diode array detector (Agilent Technologies) and a column Hypersil ODS (100 by 4.6 mm inside diameter, particle size 5 μm; Agilent Technologies).

The DON analysis was performed after evaporation, the residue was redissolved in 300 μl of methanol, and a 15-μl aliquot of the solution was injected into the HPLC system. The mobile phase consisted of an isocratic mixture of water-acetonitrile (86:14, vol/vol), with a flow rate of 0.8 ml/min (2). The detection of DON was performed at 220 nm. The mobile phase was filtered through a 0.45-μm regenerated cellulose membrane filter (Agilent Technologies). Identification of DON was done by comparing the retention times and spectra of DON from samples with those of the standards.

**Validation performance and quality control.** Selectivity, linearity, recovery, limit of detection, and limit of quantification were determined to test the validity of the procedures used for DON determination. Selectivity of the procedure was evaluated by analyzing extracts of blank and the same spiked sample levels close to the regulated level fixed by the EU (7). To determine the recoveries of analyzed mycotoxin, blank samples of wheat and barley were spiked with low (80 μg/kg), medium (160 and 400 μg/kg), and high (800 μg/kg) levels for wheat. Three experiments were repeated on spiked samples for each matrix at all levels. The linearity of the method was estimated in the working range of 0.1 to 3.0 μg/ml, at eight calibration levels, each injected in duplicate. The correlation coefficient was 0.996. Statistical analysis (analysis of variance) was performed to check the goodness of fit and linearity, using Microsoft Excel software. The calculated limit of detection and limit of quantification were verified by the signal-to-noise ratio, which should be more than 3 and 10, respectively (Agilent Instrument Utilities, ChemStation for LC 3D systems, Rev. B.04.03, Agilent Technologies) (19).

**Weather factors.** Effects of monthly amounts rainfall and average air temperatures were studied in the period 2009 to 2010 (23) for the presence of DON.

### RESULTS AND DISCUSSION

The method performance for DON analysis was determined using recovery tests of samples of the two studied cereal matrices. The accuracy of the method was...
determined with the recovery of fortified blank grain samples at four (for wheat) and three (for barley) levels with three replicates for each level and three injections for each replicate. The obtained results are presented in Table 1. Recoveries obtained for wheat ranged from 93.7 to 105.8% and for barley from 84.7 to 89.2%. These recoveries comply with the requirements of the European Commission concerning development of analytical methods (7). Precision of the methods was calculated in terms of standard deviation and ranged between 4.5 and 12.6% for both matrices. Limits of detection for wheat and barley were 18 and 22 μg/kg, respectively, and the corresponding limits of quantification were 60 and 73 μg/kg. Obtained results show that the proposed analytical method fits well the purposes of assaying DON in grain samples. Results for the examined samples were not corrected for the recovery of the spike.

In this study, the presence of DON was determined in 128 wheat and 11 barley samples. Examined samples were classified into four groups, and obtained results are shown in Table 2.

The first analyzed group of samples contained 18 cultivars of winter wheat and 4 cultivars of winter barley intended for autumn sowing. From the obtained results, it can be seen that 15 (83.3%) wheat samples were contaminated with DON. The concentration range of 68 to 1,952 μg/kg and the percentage of positive samples (83.3%) can be considered extremely high. In two wheat samples, DON content was above the maximum tolerable level (1,250 μg/kg) adopted by the EC and Serbian regulation (29). None of the barley samples were contaminated with DON.

The second group of analyzed samples consisted of 63 cultivars of winter wheat collected from the trial fields (Srem, Banat, and Bačka) of the Institute of Field and Vegetable Crops. These samples represent the most common cultivars released in Serbia from 1955 to 2006. It is evident from Table 2 that of the 63 analyzed wheat samples from the second group, DON was present in 63.5% with the concentration range of 64 to 1,604 μg/kg. Only one sample was contaminated with DON in a concentration (1,604 μg/kg) above the maximum tolerable level (1,250 μg/kg) adopted by the mentioned regulations.

The third group represented the most common cultivars of wheat (Renesansa, Simonida, Evropa 90, and Zvezdana) and barley (Novosadski 525) that are presently grown in Vojvodina. These samples were collected from the Srem region. Among the 15 analyzed samples of wheat, 13 (86.7%) were contaminated. On average, the DON content in positive wheat samples was 1,318 μg/kg, which is above the maximum level (1,250 μg/kg) adopted by the EC (8) and Serbian regulation (29). The highest level in all examined samples was 4,808 μg/kg. It is particularly important to point out that the level of DON in 6 of 15 wheat samples was above the maximum tolerable level adopted by the mentioned regulations. The frequency of DON contamination of barley samples was 42.9%, with the average content in positive samples of 260 μg/kg, and all examined samples satisfied the corresponding regulations.

The occurrence of DON in durum wheat from the fourth group of samples is shown in Table 2. These samples included cultivar Durumko from the Institute of Field and Vegetable Crops. The Durumko cultivar is one of the three indigenous varieties of durum wheat produced by this recognized Serbian Institute (6). This variety is characterized by high-quality and high-yield potential, which is recognized by the pasta industry in Serbia. From the obtained results, all samples of durum wheat for human consumption were contaminated with DON at a very high average level of 1,161 μg/kg, with the highest content of 2,288 μg/kg. Content of DON in seven samples of durum wheat was above the maximum tolerable level (1,750 μg/kg) adopted by the EC (8) and Serbian regulation (29). These results are in agreement with those in the literature (4, 5).

Among the 128 analyzed wheat samples from 2010, 100 (78.1%) samples were contaminated with DON. DON concentrations in positive samples ranged from 64 to

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**TABLE 2. Presence of DON in all examined cereal samples**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cereal</th>
<th>No. of samples</th>
<th>No. (%) of positive samples</th>
<th>Avg ± SD</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wheat</td>
<td>18</td>
<td>15 (83.3)</td>
<td>537 ± 569</td>
<td>68–1,952</td>
<td>276</td>
</tr>
<tr>
<td></td>
<td>Barley</td>
<td>4</td>
<td>0</td>
<td>218 ± 120</td>
<td>64–512</td>
<td>226</td>
</tr>
<tr>
<td>2</td>
<td>Wheat—Banat</td>
<td>21</td>
<td>13 (61.9)</td>
<td>485 ± 575</td>
<td>80–1,604</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>Wheat—Bačka</td>
<td>21</td>
<td>6 (28.6)</td>
<td>469 ± 247</td>
<td>92–984</td>
<td>392</td>
</tr>
<tr>
<td></td>
<td>Wheat—Srem</td>
<td>21</td>
<td>21 (100.0)</td>
<td>1,318 ± 1,322</td>
<td>112–4,808</td>
<td>884</td>
</tr>
<tr>
<td>3</td>
<td>Wheat</td>
<td>15</td>
<td>13 (86.7)</td>
<td>260 ± 125</td>
<td>118–355</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>Barley</td>
<td>7</td>
<td>3 (42.9)</td>
<td>1,161 ± 566</td>
<td>304–2,288</td>
<td>1,198</td>
</tr>
<tr>
<td>4</td>
<td>Durum wheat</td>
<td>32</td>
<td>32 (100)</td>
<td>779 ± 742</td>
<td>64–4,808</td>
<td>546</td>
</tr>
<tr>
<td>Total</td>
<td>Wheat</td>
<td>128</td>
<td>100 (78.1)</td>
<td>260 ± 125</td>
<td>118–355</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>Barley</td>
<td>11</td>
<td>3 (27.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4,808 μg/kg, with mean level of 779 μg/kg. In 16 of the examined wheat samples, the content of DON was above the maximum tolerable level (1,250 μg/kg) adopted by the mentioned regulations. Of the 11 analyzed barley samples for DON analysis, 3 samples were positive with an average of 260 μg/kg.

It should be pointed out that the weather conditions in spring 2010 had a very strong influence on the production of DON in all examined groups of samples. Namely, the weather conditions during the plant growth, in particular in the flowering stage, have a major influence on the mycotoxin formation and production. According to the literature data, the optimal temperature for F. graminearum growth is 25°C, at a water activity above 0.88. In addition, it is known that the incidence of FHB is strongly associated with moisture at the time of flowering (anthesis) and the timing of rainfall, rather than its amount (13). According to a study on wheat (25), environmental effects accounted for 48% of the variation in DON across all fields, followed by variety (27%) and the previous crop (14 to 28%). Critical periods of weather were identified with DON concentrations in grain at maturity. Timing of these periods is relative to the average heading date (Zadoks 59) of the wheat crop. Three of the critical periods—from 4 to 7 days (first critical period) before heading and 3 to 10 days (second and third critical periods) after heading—represent the most important contributors to the variation in DON content. The period of 4 to 7 days before heading likely corresponds to inoculum production. In this period, rainfall amounts of >5 mm/day trigger an increase in DON potential, and daily minimum air temperatures less than 10°C limit the DON potential. Similarly, weather variables in critical periods 2 and 3 correspond to infection during flowering and fungal growth. The number of rainy days and days with relative humidity over 75% increase DON potential; in contrast, daily maximum temperatures over 32°C and average temperatures less than 12°C limit DON potential. During the fourth critical period (11 to 19 days after heading), daily maximum temperatures exceeding 32°C limit DON and rainfall events near maturity favor DON accumulation. For wheat, in the climatic region of Serbia, this is the middle of May and the beginning of June. Barley initially may not have been a main host for Fusarium, but it now appears as vulnerable as wheat (33). In comparison with wheat, in the climatic region of Serbia, barley flowers earlier at the beginning of May.

High contamination frequency of DON in wheat from the 2010 harvest can be explained by the weather conditions that were favorable for fungi growth and production of mycotoxins. Figure 1 shows the amount of average rainfall (Fig. 1A) and temperature (Fig. 1B) in Vojvodina and central Serbia for the period October 2009 to July 2010 compared with the average values for the long-term period of 1961 to 1990 (24), which defines the latest normal for Serbia, i.e., the arithmetic means of a climatological element computed over three consecutive decades in Serbia. The period 2009 to 2010 was considered because the investigated winter wheat samples were sown during October 2009, while their harvesting time was July 2010. Because all examined samples were from Vojvodina, we decided to show weather conditions also for this part of Serbia. As can be seen, the average temperatures in Vojvodina in the 2009 to 2010 period were slightly higher than the ones for the period 1961 to 1990 (Fig. 1B), while the amount of rainfall in certain months were significantly higher (Fig. 1A). The mean rainfall in May for several years in the entire region of Vojvodina was 56 ml/m², while in May 2010, it was 153 ml/m²; in the northwest part of Vojvodina, the average amount of rainfall was 195 ml/m². The weather conditions in May and June were very similar. The average temperatures were slightly higher in June, while the amount of rainfall was higher. In the northwest part of Vojvodina, the amount of rainfall was 240 ml/m², which is three times higher than the mean value for June (79 ml/m²) in the period 1961 to 1990. Therefore, precipitation in June in Vojvodina was significantly higher than the corresponding long-term mean (1961 to 1990), and the month of June can be characterized as extremely rainy (23). During 2010, there were two critical periods for infection of wheat by fungus of the Fusarium genus (12). The most critical period was from 14 to 18 May, and it coincided with the flowering stage of most varieties.

Based on these facts, it could be proposed that field conditions were favorable for the development of fungi and formation of mycotoxins, which is confirmed by the obtained results (Table 2). However, the incidence of DON contamination of all analyzed wheat samples was still unexpectedly high (78.1%), and it was the highest ever found in our practice of testing cereals on the presence of
DON. This especially holds for durum wheat, as all the samples were DON positive. Also, the average DON content in all positive wheat samples was very high (779 µg/kg), whereas for the samples of durum wheat, it was even higher, amounting to 1,161 µg/kg. With the second group of samples (Table 2; 21 wheat cultivars) the incidence of DON was somewhat lower (63.5%), with an average level of 390 µg/kg.

The incidence and the level of DON contamination of studied cereal samples are different from previous reported for the country in the previous harvest years. The analysis of wheat samples for human consumption in 2004 and 2005 showed a medium contamination (50.0 and 33.3% incidence) with different levels (1,230 and 182 µg/kg) of DON (10). In those years, rains were frequent in the period May to June, which is the critical period for development of fungi in spring wheat. During these 2 months, the amounts of rainfall were about 100 to 125% higher than the average value for 1961 to 1990, used for comparison. Furthermore, the number of analyzed samples was rather small (4 from 2004 and 12 from 2005) so that they cannot be considered as highly representative of the actual situation, and they represented just a preliminary evaluation of DON content in wheat cultivated in Serbia during 2004 and 2005. The analysis of 34 wheat samples from the 2006 harvest and only 9 samples from the 2007 harvest showed the contamination of 35.3 and 33.3%, respectively (11), whereas none of the samples contained DON above the EC tolerable value. The rainfalls during May and June of both years were at the level of long-term mean or somewhat lower. Also, the average temperature during 2006 was at the level of long-term mean, whereas during 2007, it was somewhat higher.

Similar results were obtained by Škrbić et al. (27) in the analysis of 54 samples of winter wheat from 2007, of which 27.8% were contaminated with DON at a level ranging from 41 to 309 µg/kg. In contrast to this, Km Jája et al. (15) found that all of the 20 analyzed samples of winter wheat from the 2009 harvest were contaminated with DON at a level ranging from 110 to 1,200 µg/kg, with an average in positive samples of 490 µg/kg. As for the weather conditions, the authors pointed out that the average temperatures during May of 2009 were within the long-term mean values and the rainfall was somewhat lower than the long-term mean, so it was possible to expect a lower contamination with DON. These authors also found an insignificant negative correlation between the DON level and the percentage of present Fusarium species. One of the reasons for such a high contamination with DON could be a consequence of the application of the enzyme-linked immunosorbent assay (ELISA) technique. Namely, it has been found that a significantly higher percentage of positive samples may arise as a consequence of the lower limit of determination of ELISA tests and that the results obtained by immunochromatographic method are higher than those measured by HPLC, which is in accordance with the literature (11). However, the differences in the results obtained by applying ELISA tests and HPLC are not drastically different from each other, and these results have considered with great reservation.

The analyzed samples from the 2010 harvest definitely confirmed that the frequency of contamination is highly dependent on weather conditions. High humidity during May and June of 2010 contributed to the development of Fusarium already in the field, and thus the production of DON. The obtained results for wheat confirmed that this crop should be continuously controlled to protect the population against the risk of mycotoxin contamination. The main outcome of the present study concerns the small-grain 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.

A number of economically important small-grain cereal lots grown in Serbia were tested for DON, produced by common Fusarium species. The incidence of DON contamination of wheat was found to be very high, and all examined samples of durum wheat were contaminated. It should be noted that in all examined wheat samples, the average value (779 µg/kg) was very high, and in 16 of 128 samples, DON content exceeded the maximum tolerable level given in the EU and Serbian regulations. Among 11 samples of barley, DON was found in 3 samples, with contents that were in accordance with the limits allowed by the EU and Serbian regulations. This study indicates the existence of a serious risk related to the occurrence of DON in the food chain of 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 estimated that in the future, there will be more frequent control of DON and more data about its occurrence in Serbia. In the future, based on the obtained results, it will be necessary to form a national database of DON occurrence in the food chain in Serbia.

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