Agronomy Research **9** (Special Issue II), 461–468, 2011

Multi-mycotoxin Analysis by UHPLC-HESI-MS/MS: A Preliminary Survey of Serbian Wheat Flour

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Abstract. The aim of this paper is to present a high throughput method for the determination of eleven *Fusarium*, *Aspergillus* and *Penicillium* mycotoxins in wheat flour from Serbia. Mycotoxins were extracted from samples by one-step solvent extraction (acetonitrile-water (86:14, v/v)) without any cleanup and directly injected into an ultra-high pressure liquid chromatography/heated electrospray ionization-triple quadrupole mass spectrometry (UHPLC-HESI-MS/MS) system. The chromatographic separation was achieved in only 4 min. Quantification was performed by external calibration with matrix-matched standard solutions. The method recovery, linearity, limit of detection (LOD), and limit of quantification (LOQ) were determined. The developed method has been applied to the analysis of samples of wheat flour collected from Serbian local markets.

Key words: Multi-mycotoxin method, UHPLC-HESI-MS/MS, wheat flour, Serbia

INTRODUCTION

Mycotoxins are toxic natural secondary metabolites produced by several species of fungi on agricultural commodities in the field or during storage. The most predominant mycotoxins are the aflatoxins (AFs, among which AFB1, AFG1, AFB2 and AFG2 are the most analyzed ones) produced by *Aspergillus* species, ochratoxin A (OTA) produced by both *Aspergillus* and *Penicillium* species, and toxins from fungi belonging to the *Fusarium*, for example, deoxynivalenol (DON), zearalenone (ZON), T-2 and HT-2 toxins, and fumonisins (FB1 and FB2). Generally, mycotoxins are stable chemical compounds and can neither be completely removed from the food supply nor destroyed during processing and heat treatment, thus, monitoring of these contaminants in food and in feed are important issues associated with public health, agricultural production, food processing, and trade. The co-occurrence of different toxic compounds implies a potential risk of additional or even synergetic toxic effects after consumption of contaminated food/feed commodities. The Commission of the European Communities established the maximum level for several mycotoxins for cereals (Commission Regulation 1881/2006 (EC) amended by Commission Regulation 1126/2007 (EC)): 4 $\mu g\ kg^{-1}$ for total AFs, and 2 $\mu g\ kg^{-1}$ for AFB1 for all cereals and all products derived from cereals; 3 $\mu g\ kg^{-1}$ for OTA for all products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption; 750 μg

kg^{-1} of cereal flours for DON; 75 μg kg^{-1} for ZON in cereals intended for direct human consumption; and 1000 μg kg^{-1} for FBs in maize intended for direct human consumption. Statutory regulations do not exist for T-2 and HT-2, yet. In addition, it should be emphasized that the recent Serbian regulation (2011) has established the maximum level for the following mycotoxins AFs, OTA, DON, ZON, FB1 and FB2, and it is in line with mentioned EU regulations.

The analysis of mycotoxins is challenging as they are detected in very low concentrations in complex sample matrices. Since most commodities are contaminated with several mycotoxins, there is a need to develop multi-mycotoxin analysis methods for different feed/food matrices. In recent years, liquid chromatography coupled with tandem mass spectrometry (MS/MS) has become the most universal approach for mycotoxin analysis (Krska et al., 2008; Škrbić et al., 2011); the extensive review article of Zollner & Mayer-Helm (2006) gives an overview of the impact of modern LC/MS methodology in the field of mycotoxin research and analysis. Many routine laboratories use preparatory methods based on extraction/cleanup/pre-concentration steps regarding only single or small groups of similar mycotoxins (Manova & Mladenova, 2009; Sokolović & Šimpraga, 2006). Rarely, studies concern simultaneous determination of the mycotoxins produced by different fungal species; for instance, while analyzing *Fusarium* toxins, Biselli et al. (2004) included the analysis of OTA and AFs in food and feed matrices for additional verification purposes. Although the existing methods for mycotoxin determination are well established, and in some cases interlaboratory validated, the current trend is to introduce simple (one step-), broad scope procedures which, thanks to the use of modern separation/detection instrumental technologies, allow accurate determination of as many as possible major mycotoxins even at low levels in crude extracts, not applying the labor/cost-demanding clean-up step (Škrbić et al., 2011). Thus, in this paper a simple sample preparation technique with only one step of extraction was chosen to be used in order to allow fast analysis of eleven mycotoxins (*Fusarium*, *Aspergillus* and *Penicillium*) in crude extracts of wheat flour from Serbia. To our best knowledge, this is the first publication of a study on the coupling of UHPLC with HESI-MS/MS for determining simultaneously mycotoxins produced by *Aspergillus*, *Fusarium*, and *Penicillium* genera in wheat flour from the Serbian market.

MATERIALS AND METHODS

Reagents and chemicals

Individual standard stock solutions of aflatoxins B1 (2 μg ml^{-1}), B2 (0.5 μg ml^{-1}), G1 (2 μg ml^{-1}), and G2 (0.5 μg ml^{-1}), ochratoxin A (OTA, 1000 μg ml^{-1}), HT-2 toxin (100, μg ml^{-1}), T-2 toxin (100 μg ml^{-1}), deoxynivalenol (DON, 100 μg ml^{-1}), zearalenone (ZON, 100 μg ml^{-1}), fumonisins B1(FB1, 50 μg ml^{-1}) and B2 (FB2, 50 μg ml^{-1}) were purchased from Supelco Co. (Bellefonte, PA). All standards dissolved in acetonitrile were stored at −20°C in amber glass vials, and brought to room temperature before use. Two composite working standard solutions (solutions 1 and 2) were prepared by diluting the above-mentioned stock solutions in acetonitrile for all mycotoxins. The concentrations of each mycotoxin (ZON, HT-2, T-2, DON, FB1 and FB2) in the working solution 1 were 200 ng ml^{-1}. Working standard solution 2 contained other mycotoxins (AFB1, AFB2, AFG1, AFG2 and OTA) with concentration 2 ng ml^{-1}. These

composite solutions were used to prepare matrix-matched calibration standards by adding them in appropriate dilution to the extract of the uncontaminated sample to obtain concentration ranges that cover the maximum allowable concentrations and also the expected mycotoxin occurrence (in accordance with the available literature data). Ultra-pure water was produced by Milli-Q purification system (Millipore, Molsheim, France). Methanol, acetonitrile and ammonium acetate (all LC-MS gradient grade) were supplied from J.T. Baker (Deventer, The Netherlands); glacial acetic acid (p.a.) was obtained from LTG Promochem (Wesel, Germany).

Samples

In January 2011, fifteen wheat flour samples were collected randomly from different supermarkets within Novi Sad, the capitol of the northern Serbian province of Vojvodina, where the biggest producers of flour in Serbia are located. Five different brand names were selected in order to have a market-representative sampling. Packs of the samples were 1000 g containing different "types" of wheat flour. In Serbia, commercially available flours are classified into standards (called "types") based on the mineral (ash) content corresponding to different extraction rates during milling. The milling companies in Serbia use the national regulation 52/1995 for grading flour (Serbian regulation, 1995), with the ash content being analyzed by the producers according to the regulation 74/1988, (Serbian regulation, 1988). Flour samples belonged to type 400 (T 400; n=5) and type 500 (T 500; n=10). Type 400 is defined by an ash content of up to 450 mg per 100 g (dry basis matter) flour. Type 500 is defined by an ash content between 460 and 550 mg per 100 g (dry basis matter) of flour. They were stored in their original packets at 4–5^{o}C until analysis was carried out.

Sample preparation

The method used to prepare the crude extracts of the cereal flours was previously described by Škrbić et al. (2011) for preparation of wheat extracts for *Fusarium* mycotoxins analysis by HPLC-MS/MS. Only slight modifications were made with respect to amount of sample, volume of solvent used for extraction and dilution of the extract. Five grams of homogenized wheat flour samples were extracted by shaking with 20 mL of acetonitrile/water mixture (86:14, v/v) for an hour using an automatic shaker (Promax 2020, Heidolph Instruments, Germany). This solvent mixture has been used widely for extraction of mycotoxins, but the majority of the methods described in literature are based on the subsequent clean-up of the obtained extracts (Biselli et al., 2004; Biselli & Hummert, 2005; Zolner & Mayer-Helm, 2006), while “purification” of the extract was omitted in this study and compensated for by usage of modern UHPLC-MS/MS technique. The suspensions were filtered and an aliquot (1 mL) of filtered crude extracts was transferred into glass vials and diluted with 3 ml of the UHPLC mobile phase of the initial content (95% A and 5% B; explanation of the composition of the eluent A and B is given in the subsequent section). Thus, the final extracts contained 0.0625 g sample per mL. Before injection into the UHPLC-HESI-MS/MS system, the extracts were passed through the 0.2 μm nylon syringe filter.

Instrumentation

Ultra-high performance liquid chromatography performed by Accela™ (Thermo Fisher Scientific, USA) was used for separation of sample components. Hypersil GOLD™, 50 × 2.1 mm i.d., 1.9 μm column (Thermo Fisher Scientific, USA) was used with a flow rate of 0.5 ml/min, and the column temperature was maintained at 30^0C. The injection volume was 10 μL. The mobile phase consisted of eluent A containing water/acetic acid (99:1, v/v), and eluent B consisting of methanol/acetic acid (99:1, v/v). Both eluents contained 5 mM ammonium acetate. The gradient program started with 95% A and 5% B and was kept until 0.5 min; next, a linear gradient was applied, reaching 95% B after 3.04 min (holding time 2.1 min) and then switched back (6.20 min) to 95 % A (holding time 1.80 min), which was maintained till the end of the run at 8 min.

For analytes detection, triple quadrupole mass spectrometer TSQ Vantage (Thermo Fisher Scientific, USA) equipped with heated-electrospray ionization probe (HESI-II, Thermo Scientific, USA) was used. Heated-electrospray ionization (H-ESI) transforms ions in solution into ions in the gas phase by using electrospray ionization (ESI) in combination with heated auxiliary gas. In H-ESI, heated auxiliary gas aids solvent evaporation. For the analysis of mycotoxins in various matrices, H-ESI probe provides significant advantages (Huls et al., 2007). The dual desolvation zone design increases the ionization efficiency and helps to remove the clustering solvents, leading to higher signals (Huls et al., 2007). Parameters of the ion source were as follows: spray voltage - 3.4 kV, vaporizer temperature - 350^oC, sheath gas pressure - 40 arbitrary units, auxiliary gas pressure - 10 arbitrary units, capillary temperature - 270^oC. Argon was used as the collision gas and its pressure in the second quadrupole (Q2) was set to 1.5 mTorr. Other source parameters were automatically tuned for maximal intensity of a particular analyte in the respective time window. Instrument control and data collection were handled by a computer equipped with Xcalibur 2.1.0 (Thermo Fisher Scientific, USA).

RESULTS AND DISCUSSION

Acquisition parameters of the mass spectrometer were optimized by direct continuous pump infusion of 5 μg ml^{-1} standard solutions of each individual analyte standard (except for AFB1 and AFG1 (2 μg ml^{-1}) and AFB2 and AFG2 (0.5 μg ml^{-1})) dissolved in initial mobile phase into the mass spectrometer using a syringe pump at a flow rate of 10 μl min^{-1}. Data acquisition was performed initially in full scan to determine an abundant precursor ion. Next, the MS/MS fragmentation conditions were investigated and collision energies and S-lens voltages were optimized for each individual compound and/or transition. UHPLC-HESI-MS/MS parameters of mycotoxins separation and identification under optimized conditions on the Accela-TSQ Vantage system are shown in Table 1. All the analytes exhibited higher parent ion signal intensities in positive ion mode except for DON and ZON whose intensities were better in negative ion mode. Fragmentation reactions were done in selected reaction monitoring mode (SRM) by choosing the optimum voltage of collision energies for each compound. Two product ions were measured for all compounds: one was used as the quantifier ion and the other was used as the qualifier ion. In SRM mode, a mass resolution of 0.7 Da FWHM was set on the first (Q1) and the third (Q3) quadrupole and a scan width of 0.5 m/z was used.

Table 1. UHPLC-HESI-MS/MS parameters of mycotoxins under optimized conditions on TSQ Vantage.

Mycotoxins	t_R [a], min	Dwell time, s	Precursor ion, m/z	Product ions [b], m/z	CID [c], eV
AFB1	3.17	0.1	313.1 $[M+H]^+$	285.1/241.1	33/22
AFB2	3.07	0.1	315.1 $[M+H]^+$	287.2/259.2	28/25
AFG1	2.97	0.1	329.1 $[M+H]^+$	200.2/215.1	26/40
AFG2	2.87	0.1	331.0 $[M+H]^+$	245.0/189.1	40/28
OTA	3.91	0.1	404.1 $[M+H]^+$	239.0/221.0	35/23
DON	1.82	0.1	295.0 [M-H]-	265.1/205.2	-18/-24
HT-2	3.57	0.1	447.2 $[M+Na]^+$	345.5/285.5	19/20
T-2	3.74	0.1	489.0 $[M+Na]^+$	245.1/327.1	26/23
ZON	3.88	0.1	317.1 $[M-H]^-$	175.1/131.1	-26/-32
FB1	3.70	0.1	722.6 $[M+H]^+$	334.3/352.4	38/34
FB2	3.98	0.1	706.5 $[M+H]^+$	336.3/318.3	36/38

[a] Retention time
[b] Numerical values are given in the order quantifier/qualifier ion.
[c] Collision-induced dissociation energy for quantifier/qualifier ion.

Chromatographic separation was accomplished by choosing methanol (MeOH) as the organic phase and water as polar phase with the addition of 1% acetic acid to the mobile phases recommended for marked improvement of mycotoxin signals (Sulyok et al., 2006). Ammonium acetate was used to achieve better chromatography and to increase ionization efficiency of the analytes (Sulyok et al., 2006). Different mobile phase flow rates were also tested (0.3, 0.4, and 0.5 µl min^{-1}), selecting 0.5 ml/min as a compromise between sensitivity, resolution and analysis time. Lower flow rates did not allow an adequate sensitivity to be reached with the source design of the available MS/MS instrument. With the optimized conditions, the analysis time was 8 min, allowing high sample throughput and retention time ranges from 1.82 (DON) to 3.98 (FB2). Retention times of the corresponding compounds given in Table 1 were obtained for the optimal parameters of the SRM transitions. The chromatogram of the mixture of all analyzed mycotoxins spiked in crude extract of wheat flour at the lowest calibration levels are presented in Fig. 1.

The following parameters were evaluated in order to ensure the method quality: instrumental linearity, limits of detection (LOD), limit of quantification (LOQ), and recovery. Mycotoxins were quantified by external matrix-matched calibration procedure. Calibration solutions for matrix-matched calibration curves were prepared in uncontaminated flour extract. Calibration curves for all mycotoxins in wheat flour were linear with squared correlation coefficients (R^2) in the range of 0.9913-0.9990.

The detection and quantification limits for all mycotoxins were assessed at a signal to noise ratio of 3:1 and 10:1, respectively (Table 2). The LODs of the developed method were always lower than the maximum residue limits established by European Union (EC, 2007).

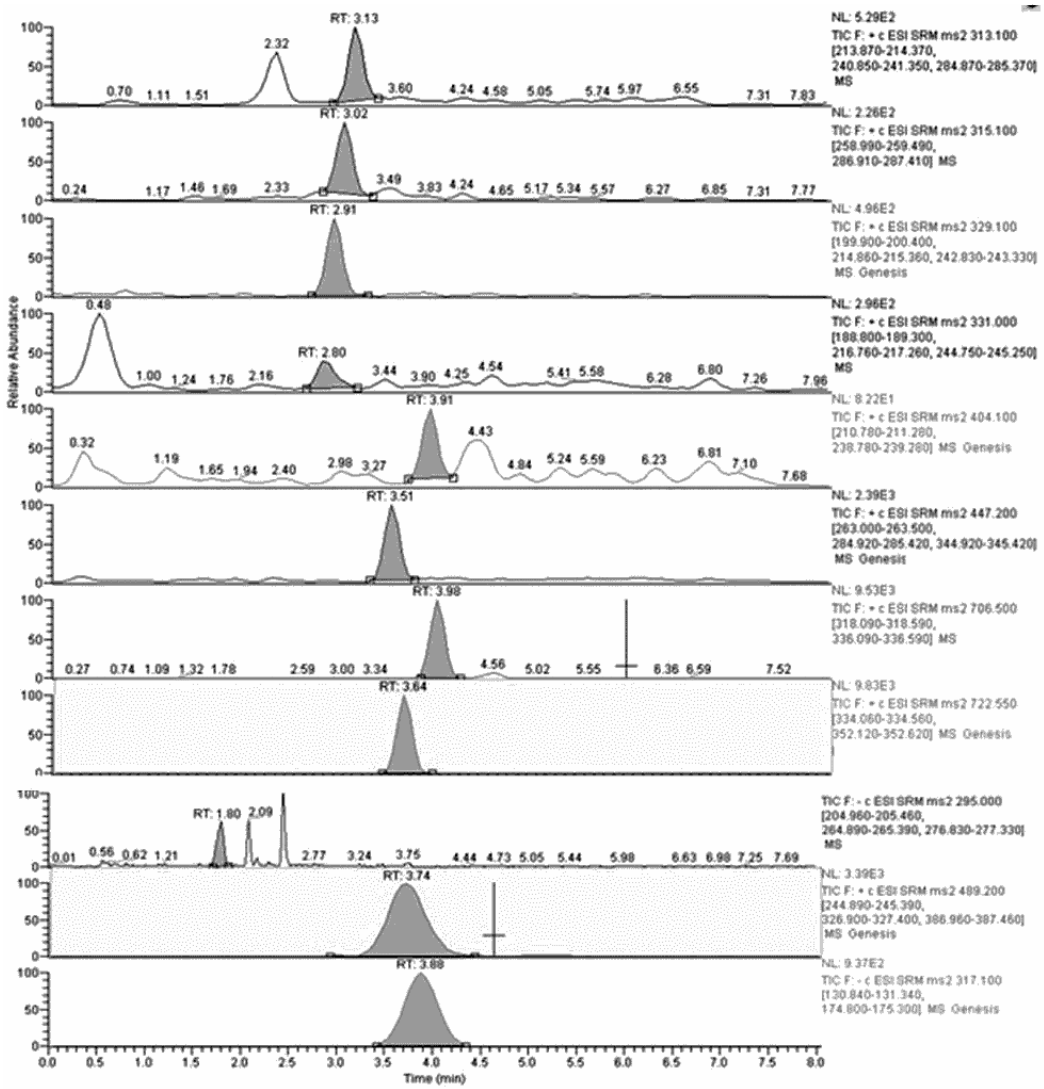

Figure 1. Chromatogram of the mixture of all analyzed mycotoxins spiked in crude extract of wheat flour at the lowest calibration levels.

Recoveries were determined by analyzing uncontaminated sample spiked (before extraction) at a level of 2 μg/kg for each of AFs, 2.5 μg kg^{-1} for OTA, 350 μg kg^{-1} for DON, 100 μg kg^{-1} for HT-2 and T-2, 35 μg kg^{-1} for ZON and 300 μg kg^{-1} for FBs. Recovery experiments were performed in duplicate. Spiked samples were left overnight at room temperature to allow solvent evaporation and equilibration between analytes and matrix. The recoveries were in the range defined by EU requirement (EC, 2006), except for FBs, for which recoveries were <60% (Table 2). Similarly, Sulyok et al. (2006) investigated analysis of 39 mycotoxins in crude extracts of wheat and maize with acetonitrile/water/acetic acid (79:20:1, v/v/v) and found also low (unsatisfactory)

recoveries of fumonisins (<60%). This implied that for FBs, a modified extraction approach should be used.

Table 2. Summary of validation data of U-HPLC/HESI-MS/MS method.

	AFB1	AFB2	AFG1	AFG2	DON	ZON	HT-2	T-2	OTA	FB1	FB2
LOD, μg kg^{-1}	0.7	0.2	0.5	0.9	0.3	0.4	0.9	1.4	2.1	0.05	0.01
LOQ, μg kg^{-1}	2.3	0.7	1.7	3.0	1.0	1.3	3.0	4.7	7.0	0.2	0.03
Recovery, %	121	110	112	113	72	74	130	107	87	<60	<60

Analysis of samples

The method was applied to determine mycotoxin levels in wheat flours collected from Serbian local markets. The most prevalent was DON followed by ZON and T-2, while AFs, OTA, HT-2, FB1, as well as FB2 were not detected in any of the samples. DON, ZON and T-2 were detected in 86.67%, 33.33% and 26.67% of the total number of samples, respectively. For calculation of average values, the samples with quantities below LOD were considered as LOD/2. The average (median) values obtained for DON, ZON and T-2 were 234 (210) μg kg^{-1}, 3.38 (<LOD) μg kg^{-1} and 4.42 (<LOD) μg kg^{-1}, respectively. All the samples were in compliance to the EU regulation, since non of them exceeded established limits.

CONCLUSION

An UHPLC/HESI-MS/MS method was developed for simultaneous analysis of mycotoxins included in EU Regulation for wheat flour. According to the obtained validation parameters, the method provides fast and simple screening multitoxin analysis of wheat crude extracts. The high sensitivity of the triple quadrupole analyzer allowed the minimization of sample treatment, facilitating the determination of the analytes without the need to apply clean-up of wheat extracts that might restrict the multiresiduality of the method. However, before recommendation of the method for large scale screening of mycotoxins in cereal products, there is a need for recovery trials for different matrices in order to determine its general applicability and robustness.

ACKNOWLEDGMENT: The results presented here are the part of project no. 172050 "Development and application of the advanced chromatographic and spectrometric methods in the analysis of xenobiotics and their degradation pathways in biotic and abiotic matrices", coordinated by Prof. B. Škrbić and supported by the Ministry of Education and Science of the Republic of Serbia and the EU FP7 CEFSER, Grant Agreement No. 229629.

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