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A COMPARATIVE STUDY OF THREE METHODS OF EXTRACTION OF MYCOTOXINS FROM BEER

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ABSTRACT. Three sample preparation methods: solvent extraction, solid-phase extraction (SPE) and stir bar sorptive extraction (SBSE) to assess the occurrence of 11 mycotoxins in beer (pale, dark and non-alchoolic) samples were compared. In order to select the best extraction procedure, the sample matrix effects and the effect of the dilution of the sample were investigated by addition of the analytes before and after the extraction procedure was carried out. The study revealed that SPE (with Oasis HLB cartridge) procedure offered the best results compared with the other two extraction methods: relative standard errors under 16% and recovery of the analytes better than 85%. An Ultrahigh Performance Liquid Chromatography coupled with Mass Spectrometry (UPLC-MS/MS) method was used to identify and confirm the mycotoxins.

Keywords: *mycotoxins, extraction method, SPE, matrix effects, sample dilution effect*

INTRODUCTION

Mycotoxins are toxic secondary metabolites formed by certain *Aspergillus* spp., in particular *A. flavus* and *A. parasiticus*, which produce them on many plant products [1]. They have been detected as natural contaminants of barley, maize and sorghum malts [2]. Mycotoxins can survive the technological steps of beer production to the extent of 18–20% of the amount initially found in malt or corn grits; most of the losses occurred in the malt mash, boiled wort and final fermentation steps [3].

Maximum levels for mycotoxins in beer have been established by European Commission [4, 5] and classified by IARC [6].

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Beer is a complex matrix and for this reason, extraction procedures for mycotoxins from beer has to be carefully studied [7].

UPLC-MS/MS is a powerful technique used to analyze many types of chemical residues in food and feed products [8]. The chromatographic separation has to be preceded by an efficient sample treatment technique in order to reduce, as much as possible, the sample matrix effects on the separation, detection and quantification steps. The most common techniques for preparation of the samples for UPLC-MS/MS procedures are solvent addition [9], solid phase extraction [10], liquid phase microextraction [11] and accelerated solvent extraction [12]. Used more rarely, but with very good results, is the stir bar sorptive extraction method [13]. The main drawback of these techniques is the fact that these procedures have to be optimized for each compound of interest, the results not being able to be transferred to other analytes. To compensate for the sample matrix effects the use of internal standards will be the first option, but the cost of this approach as well as their commercial availability for every analyte prevent their application in multi-residue extraction procedures.

In this context, the main objective of this work was to compare the performances of three sample preparation methods (directly solvent addition to the beer, solid-phase extraction (SPE) with Oasis HLB SPE cartridge and SBSE (stir bar sorptive extraction)) used for the confirmation and quantization of 11 microtoxins by a UPLC-MS/MS method.

Representative mycotoxins (Table 4) were selected based on the published reports and the frequency of appearance of these compounds in beer samples [14].

RESULTS AND DISCUSSION

The present work focus on the optimization of the sample extraction method of 11 mycotoxins from beer. The optimization of chromatographic separation and MS detection were presented in another report [15], where, beside the information presented in experimental section and especially in Table 5, there were determined the linear range (0.15 – 10 ppb for aflatoxins G1, G2, B1, B2 and OTA, 1,5 – 100 ppb for FB1, FB2, T-2 and ZEA and 15 – 1000 ppb for DON and HT-2), the repeatability and intermediary precision (with relative standard deviations smaller than 13%), accuracy, limit of detection (smaller than 1.2 ppb) and limit of quantification (smaller than 3.5 ppb).

As it can be seen in Table 4 the logP values for the 11 mycotoxins considered in this study differ from -1.41 for DON until +4.39 from FB2 that make a difficult task to find the optimal extraction conditions for all the analytes.

Due to the complexity of composition of the beer, the sample matrix effects must be evaluated in order to obtain a correct quantification of mycotoxins. It was also taken into account that the influence of the matrix upon the estimation of the concentration of the analytes can be reduced by dilution of the raw samples [16]. Sample matrix effects may include any change in the analyte ionization process due to co-elution of the analyte with contaminants from the sample. Matrix-matched calibration curves are used for compensation of the sample matrix effects, considering that all the analytes will be equally affected [17]. Sample matrix may induce changes in the MS/MS signal, changes that can be constant and independent of the quantity of the analyte from the sample, variable and proportional with the quantity of the analyte, or a combination between the two [18].

To extract and concentrate the studied mycotoxins from beer three methods of sample preparation were used: (a) addition of solvent; (b) SPE and (c) SBSE and a comparison regarding the yield of extraction and sample matrix effect were made. The effect of dilution of the sample was also studied. To simplify the graphs only 3 of the 11 studied mycotoxins were presented: DON (logP = -1.41), AFB1 (logP = 0.45) and FB2 (logP = 4.39).

As it can be seen from Figure 1 when the method with solvent addition for sample preparation is applied to a mixture of analytes made in purified water, the percent of yield of recovery of the analytes is between 85 and 95%. When the same procedure is applied to a sample of beer fortified with the same concentration of analytes, the yield of recovery decrease until 45% in case of DON when no dilution of sample was applied. In case of dilution of the sample the percent of recovery is constantly better for all the analytes.

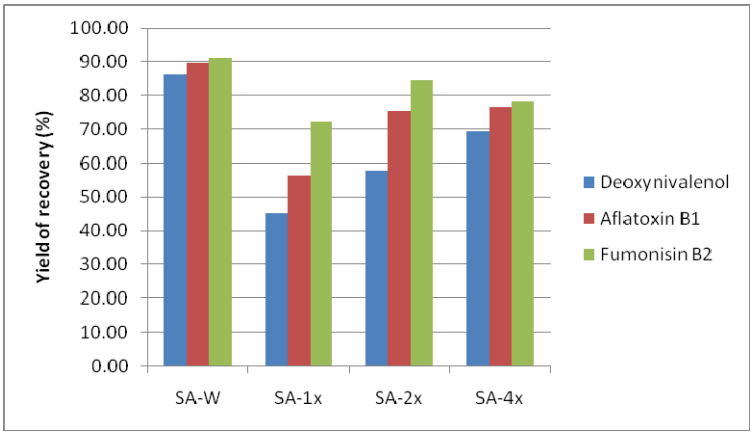

Figure 1. The effect of dilution of the sample on the yield of recovery of the analytes when the method with solvent addition (SA) was used for sample preparation (W – water instead beer, 1x, 2x and 4x – degree of sample dilution

Figure 2. The effect of dilution of the sample on the yield of recovery of the analytes when the SPE method was used for sample preparation (W – water instead beer, 1x, 2x and 4x – degree of sample dilution)

As similar results were obtained when the other two methods of sample preparation were used (i.e. SPE in Figure 2 and SBSE in Figure 3), explicitly when the extraction method is applied to beer fortified to the analytes the degree of recovery is lower in case when the analytes were added to purified water and because the yield of recovery of the analytes increase with the dilution of beer sample, one can conclude that the sample matrix has a major effect on the extraction procedure.

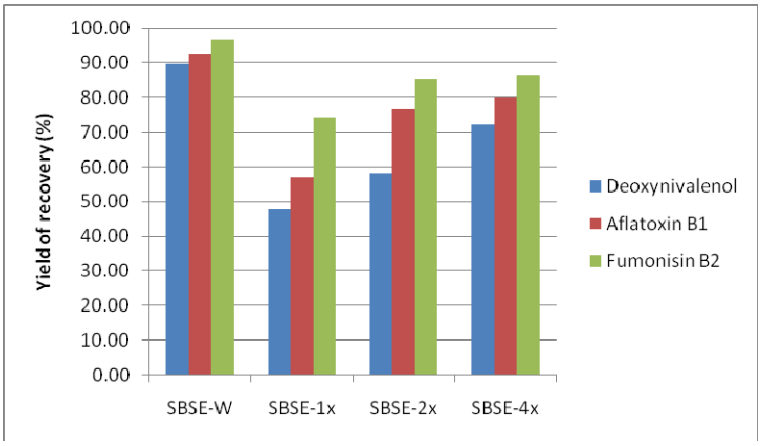

Figure 3. The effect of dilution of the sample on the yield of recovery of the analytes when the SBSE method was used for sample preparation (W – water instead beer, 1x, 2x and 4x – degree of sample dilution

In order to find out which of the three methods of sample preparation is more efficient in the recovery of the analytes, the degree of the recovery of the analytes added to purified water and to beer 4x diluted was graphically presented (Figure 4). Based on the results one may conclude that the method of choice for extraction of the analytes from beer sample is SPE, but a 4x dilution of sample mast be performed before loading the beer in the SPE cartridge.

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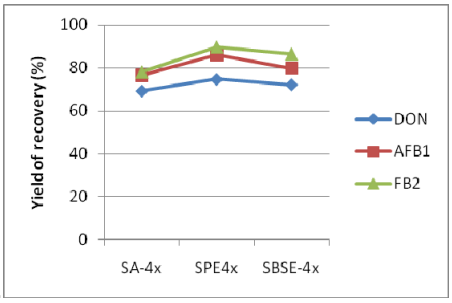
b. 

Figure 4. Comparison between the three extraction methods (SA – solvent addition, SPE – solid phase extraction, SBSE – stir bar sorptive extraction). **a**. Extraction methods applied to analytes dissolved in purified water (W) and **b**. Extraction methods applied to beer sample diluted 4x and fortified with known concentration of analytes.

The dilution experiments presented above, although reveal the fact that the beer matrix interfere with the quantization of the mycotoxins, cannot explain if the reduced yield of recovery of the analytes from the fortified samples is due to the interaction of the contaminants from beer during the sample preparation procedure with the analytes or these contaminants influence the analytes ionization process in MS detector. In order to explain which of the two phenomena have a bigger influence, there were realized series of experiments when the samples were fortified with the analytes at the beginning of the sample preparation method and at the end of this procedure. Practically, for each sample preparation method 3 results were obtained: the analytes were added to purified water (w), the analytes were added to beer before the extraction procedure (bex) and after the extraction procedure (aex) was applied to beer. Matrix effect (ME), recovery (RE) and overall process efficiency (PE) were assessed as described by Matuszewski et al. [19]: ME(%) = (aex/w)*100; RE(%) = (bex/aex)*100; PE(%) = (bex/w)*100.

Values of ME(%) around 100% indicate the absence of matrix effects, values lower than 100% point out a suppression of the ionization of the analytes (adsorption of the analytes or a interference with the ionization or detection of the analytes in MS instrument), while values higher than

100% reveal a fake enhancement process (interferences in the ionization interface or other non-normal phenomena leading the artificial increase of the signal in MS detector) [20]. The sample matrix effect was estimated for pale, dark and non-alcoholic beer.

The results presented in Table 1 reveals that in the case of pale beer, the sample matrix interfere with the correct evaluation of the analytes as all the values are lower than 100%. The smallest effect is registered when the sample is prepared by SPE procedure. Similar results were obtained for dark and non-alcoholic beers.

The influence of contaminants from the beer sample on the correct evaluation of the concentration of the analytes, during the sample preparation procedures are revealed by the PE (%) values. Smaller values than 100 indicate the fact that beer contains compounds that contribute to the reduction of the concentration of the analytes in the solution obtained after sample preparation. In this case the best method of extraction was also SPE.

Finally, RE (%) indicates which of the two possible interferences with the signal assigned to the analytes has a greater influence - sample preparation procedure or the ionization and detection in MS instrument. If the obtained values are smaller than 100, the influence upon the sample preparation method prevails (the reduction of the actual concentration of the analytes take place). When the RE value is larger than 100 the chromatographic separation procedure is the one that is influenced by the presence of the contaminants that were not eliminated from the processed sample during sample preparation method. As it can be seen from Table 1 RE do not show a clear tendency of values to be smaller or bigger than 100, to reveals which of the three studied extraction methods is better, as it was the case with the values of ME and PE, when SPE method has presented better results than the other two extraction methods. This means that in the case of some of the studied mycotoxins, depending on their chemical structure, the interactions with the contaminants take place during sample separation procedure and in the case of other analytes this interaction take place during the chromatographic separation process.

From the results (Tables 1 – 3) one may conclude that for extraction of the 11 mycotoxins, the smaller interferences with the quantification of the analytes are obtained when beer samples are prepared by SPE using Oasis HLB cartridges. For all types of beer studied (pale, dark and non-alcoholic) with SPE sample preparation method the percent of recovery of the analytes was better than 85%, which is comparable with other published studies [21-23]. Therefore, this method was used to assess the presence of the 11 micotoxins in real beer samples.

Table 1. Evaluation of the sample matrix effect for pale beer in case of application for the extraction of the 11 mycotoxins of a sample preparation method based on solvent addition (SA), solid phase extraction (SPE) and stir bar sorptive extraction (SBSE) procedure

Analyte	SA			SPE			SBSE		
	ME	RE	PE	ME	RE	PE	ME	RE	PE
DON	86.30	96.42	83.21	93.41	96.95	90.56	76.51	104.59	80.03
AFG2	84.32	93.96	79.23	99.82	91.16	91.00	81.92	87.76	71.89
AFG1	83.64	96.13	80.40	97.35	92.18	89.73	80.84	100.20	81.00
AFB2	84.74	94.47	80.06	87.86	100.86	88.61	84.67	93.30	79.00
AFB1	83.94	93.87	78.79	95.96	89.91	86.28	82.43	93.78	77.30
FB1	83.69	95.55	79.97	96.73	89.15	86.23	84.63	95.69	80.98
T-2	87.99	92.12	81.05	88.94	104.09	92.58	84.78	102.53	86.92
HT-2	85.07	97.48	82.93	93.72	93.38	87.52	86.11	95.03	81.84
ZEA	89.93	94.82	85.27	97.37	95.63	93.12	88.27	94.43	83.36
OTA	81.21	102.30	83.07	94.82	93.26	88.43	90.70	94.94	86.12
FB2	87.56	95.56	83.67	96.61	90.6	87.53	90.47	100.07	90.53

ME – matrix effect (in %), RE – recovery (in %) and PE – process efficiency (in %).

Table 2. Evaluation of the sample matrix effect for dark beer in case of application for the extraction of the 11 mycotoxins of a sample preparation method based on solvent addition (SA), solid phase extraction (SPE) and stir bar sorptive extraction (SBSE) procedure

Analyte	SA			SPE			SBSE		
	ME	RE	PE	ME	RE	PE	ME	RE	PE
DON	81.01	105.84	85.74	99.68	90.68	90.39	64.12	101.84	65.29
AFG2	76.63	90.27	69.17	99.48	84.41	83.97	76.63	101.26	65.20
AFG1	79.97	96.83	77.43	97.00	78.20	75.85	79.97	98.30	81.81
AFB2	86.63	100.53	87.09	110.21	70.32	77.50	86.63	88.66	70.94
AFB1	84.28	91.57	77.18	110.11	71.99	79.26	84.28	99.72	78.50
FB1	78.52	105.79	83.07	91.07	110.37	100.52	78.52	94.10	70.80
T-2	82.33	91.79	75.57	79.62	115.05	91.61	82.33	90.44	69.34
HT-2	81.66	95.36	77.87	88.04	100.71	88.66	81.66	81.34	67.26
ZEA	90.82	116.15	105.48	85.47	104.45	89.27	90.82	78.10	69.68
OTA	85.90	92.32	79.30	82.38	94.77	78.07	85.90	97.90	82.81
FB2	69.06	112.72	77.85	105.46	76.14	80.30	69.06	100.29	83.17

ME – matrix effect (in %), RE – recovery (in %) and PE – process efficiency (in %).

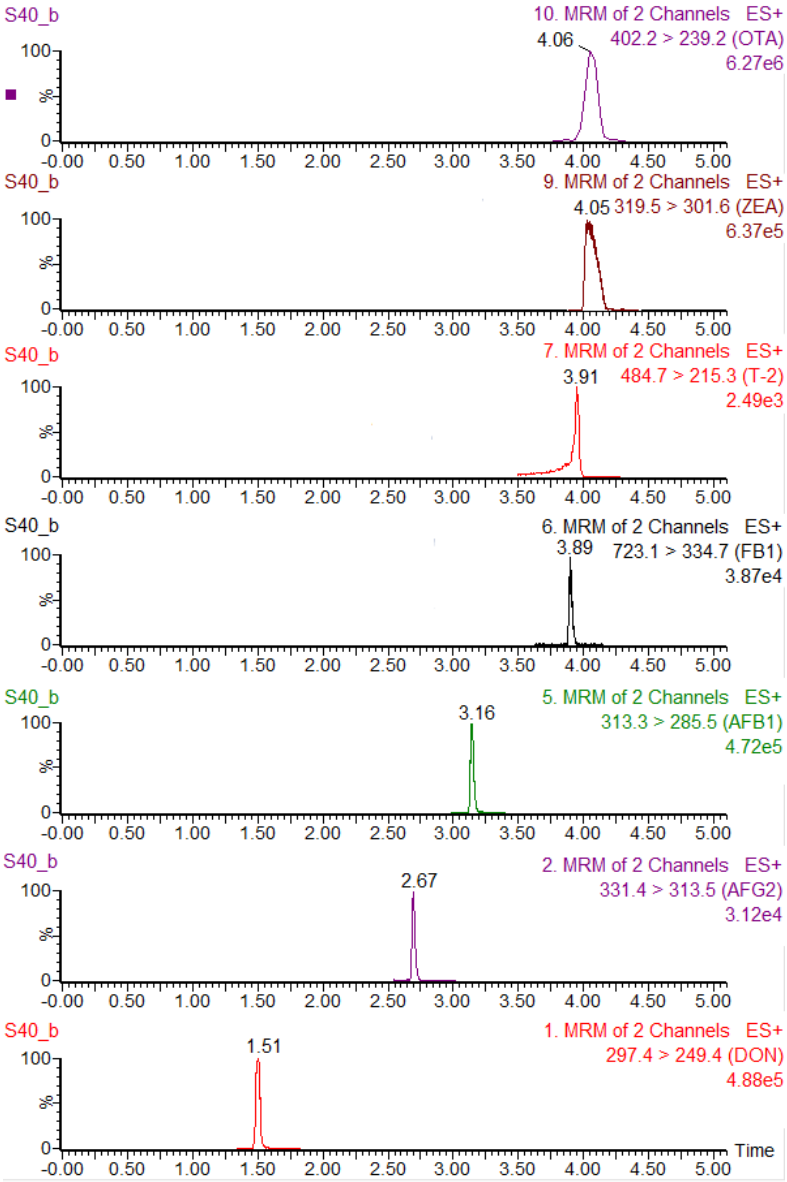


Figure 5. MRM chromatograms of a sample of pale beer (produced in UE and sold in a supermarket from Romania. There are presented only the chromatograms for the transitions used for quantification of the mycotoxins found in concentrations larger than the limit of the quantification.

Table 3. Evaluation of the sample matrix effect for non-alcoholic beer in case of application for the extraction of the 11 mycotoxins of a sample preparation method based on solvent addition (SA), solid phase extraction (SPE) and stir bar sorptive extraction (SBSE) procedure

Analyte	SA			SPE			SBSE		
	ME	RE	PE	ME	RE	PE	ME	RE	PE
DON	95.66	86.92	83.15	102.99	97.90	100.83	86.63	68.81	59.61
AFG2	84.96	96.04	81.60	93.91	99.30	93.25	84.96	77.59	52.52
AFG1	90.57	97.70	88.48	80.43	102.49	82.43	90.57	114.12	73.10
AFB2	100.31	88.51	88.79	87.05	93.54	81.43	100.31	101.50	80.83
AFB1	69.74	121.41	84.67	84.00	89.73	75.37	69.74	107.95	72.98
FB1	92.07	88.16	81.17	84.56	103.28	87.34	92.07	89.46	60.68
T-2	64.79	119.97	77.73	79.05	102.68	81.17	64.79	89.31	81.39
HT-2	72.41	114.60	82.99	97.05	80.70	78.32	72.41	131.74	91.57
ZEA	92.73	94.12	87.27	86.99	101.94	88.67	92.73	98.64	75.51
OTA	84.00	95.29	80.04	91.77	123.90	113.70	84.00	78.51	59.20
FB2	53.43	96.04	51.32	102.37	84.41	86.41	53.43	93.40	77.44

ME – matrix effect (in %), RE – recovery (in %) and PE – process efficiency (in %).

Applications to samples

The optimized extraction procedure (SPE with Oasis HLB cartridges) was applied for the identification and quantification of the 11 mycotoxins in commercial beers sold in Romania. Although the results and discussion of these study are presented elsewhere [15], it is worth to mention that from all the 54 analyzed samples only 2 have contained mycotoxins above the legal limit. In Figure 5 an example of the results obtained in case of a pale beer produced in EU but commercialized in Romania is presented. In this particular sample there were found 7 mycotoxins (with concentration above the quantification limit but below the legal limit).

CONCLUSIONS

Comparing the solvent addition, SPE and SBSE preparation sample methods, the most efficient regarding the relative standard error (under 16%) and yield of recovery of the added analytes (with a median value of 97%) was proved to be the SPE with Oasis HLB cartridges. For this extraction method the best results regarding the matrix effects and process efficiency were also obtained.

With SPE extraction method (with Oasis HLB cartridges), the selected compounds can be determined with acceptable precision and accuracy at lower concentration than the limit established by EU Commission Decision 2002/657/EC guidelines [24].

EXPERIMENTAL SECTION

Chemicals, Reagents and Materials

The mycotoxins used as standards were purchased from Sigma-Aldrich (via Redox, Bucharest): Aflatoxins B1, B2, G1, G2 (#40139-U Supelco; 25 µg/mL each component in acetonitrile), Fumonisins B1 (#34139 Fluka, 50 µg/mL in acetonitrile: water, 50:50) and B2 (#34142 Fluka, 50 µg/mL in acetonitrile: water, 50:50), Ochratoxin A (#34037 Fluka, 10 µg/mL in acetonitrile), HT-2 toxin (#34136 Fluka, 100 µg/mL in acetonitrile), T-2 toxin (#34071 Fluka, 100 µg/mL in acetonitrile), Deoxynivalenol (#34124 Fluka, 100 µg/mL in acetonitrile) and Zearalenone (#34126 Fluka, 100 µg/mL in acetonitrile). All other chemicals were of analytical grade. Ultrapure water was prepared with SG Ultra Clear 2001-B Water Deionization System (Cole-Parmer, via Nitech, Bucharest). Millex-GN nylon filters (0.20 µm, Millipore, Carrightwohill, Ireland) were used for filtration of any solutions before injection in UPLC system. For sample preparation / concentration by solid phase extraction (SPE) Oasis HLB cartridges of 200 mg (Waters, Mildford, USA) and by stir bar sorptive extraction (SBSE) glass bars with magnetic core, coated with silicone film with C18 arms (film thickness 1.0 mm, 10 mm length) (Gerstel, Mülheim an der Ruhr, Germany) were used.

In Table 4 there are presented the analytes used in this study and some related data.

Table 4. List of compounds included in the analyses

Nr.	Name of analytes	Abbreviation	CAS No.	Molecular mass (Da)	logP values
1	Deoxynivalenol	DON	51481-10-8	296,3	-1,41
2	Aflatoxin G2	AFG2	7241-98-7	330,2	-0,25
3	Aflatoxin G1	AFG1	1165-39-5	328,2	-0,17
4	Aflatoxin B2	AFB2	7220-81-7	314,2	0,37
5	Aflatoxin B1	AFB1	1162-65-8	312,2	0,45
6	Fumonisin B1	FB1	116355-83-0	721,8	2,2
7	T-2 toxin	T-2	21259-20-1	466,5	2,25
8	HT-2 toxin	HT-2	26934-87-2	424,2	2,27
9	Zearalenone	ZEA	17924-92-4	318,3	3,83
10	Ochratoxin A	OTA	303-47-9	403,8	4,31
11	Fumonisin B2	FB2	116355-84-1	705,8	4,39

log P predicted values from ACD/Labs' ACD/PhysChem Suite (http://www.acdlabs.com/products/pc_admet/physchem/physchemsuite/)

A stock solution containing 1000 ppb DON and HT-2, 100 ppb FB1, FB2, ZEA and T2 and 10 ppb OTA, AFB1, AFB2, AFG1 and AFG2 were prepared in a solution of 0.1% ammonium formate in 30% methanol (MeOH). The stock solution was used to make 7 serial dilutions (dilution factor 2) that were injected in the UPLC system to realize the standard curves. Similar standard curves were prepared using as dilution solution pale beer checked to be free of detectable traces of the analytes (matrix-matched calibration curves) [15].

Instrumentation

Chromatographic analyses were performed using an AcquityUPLC™ system (Waters, Milford, MA, USA), and separations were carried out using an AcquityUPLC™ BEH C18 column (100× 2.1 mm, 1.7 µm particle size) from Waters. The C18 column was equilibrated at 30 °C. The analytes were separated with a gradient elution profile realized with a mobile phase consisting of 0.1% ammonium formate in 100% methanol (mobile phase A) and an aqueous solution of 0.1% ammonium formate in 10% methanol (mobile phase B). The analysis started with 10% of mobile phase A at a flow rate of 0.35 mL/min, for 0.3 minute. Then the percentage of mobile phase A was increased linearly up to 30% in 1.2 minutes and further to 100% in 2.0 minutes; this composition was hold for 1.0 minute before being returned to 10% of mobile phase A, in 0.1 min, followed by a re-equilibration time of 0.4 minutes (total run time 5 minutes). The injection volume was always 10 µL (full sample loop). The UPLC system was coupled to a XevoTQD triple-quadrupole mass spectrometer with an orthogonal Z-spray–electrospray interface (Micromass, Manchester, UK). For the purpose of optimizing the MS parameters, the selected mycotoxins were dissolved in 0.1 ammonium formate in 30% methanol, at a concentration of 62.5 ppb DON and HT-2, 6.25 ppb FB1, FB2, ZEA and T2 and 0.625 ppb OTA, AFB1, AFB2, AFG1 and AFG2 and infused at 10 µL/min. The MS was operated in the positive electrospray (ESI+) mode with a capillary voltage 3.5 kV. The source and desolvation temperatures used were 140 and 400ºC, respectively. Nitrogen was used as the desolvation and cone gas at the flow rates of 650 and 50 L/h, respectively. Collision-induced dissociation was performed using argon (99.995%, Linde, Timisoara, Romania) as the collision gas at a pressure of 0.3 mbar in the collision cell. The selected precursor ions of the analytes were fragmented to their product ions in the collision cell and the two most intensive product ions per analyte were chosen for quantitative and confirmation purposes (see Table 5). The ions were monitored for a dwell time ranging from 0.01 to 0.04 s [15].

A vortex mixer (model Reax 2000), a rotary agitator (model Reax-2, end-over-end) from Heidolph (Schwabach, Germany), and an analytical AB204-S balance (Mettler Toledo, Greinfesee, Switzerland) were also used. An extraction manifold from Waters connected to a BüchiVac V-500 (Flawil, Switzerland) vacuum system was used for SPE experiments.

Table 5. MS/MS optimized conditions for studied mycotoxins (Rt – retention time; MRM – multiple reaction monitoring, CV – cone voltage, CE – collision energy)

Abbrev.	R_t (min)	Quantification transition			Confirmation transition		
		CV (V)	CE (V)	MRM transition	CE (V)	CV (V)	MRM transition
DON	1,51	25	10	297.4 > 249.4	25	15	297.4 > 231.3
AFG2	2,67	60	25	331.4 > 313.5	60	30	331.4 > 245.3
AFG1	2,83	40	25	329.2 > 243.1	45	25	329.2 > 311.4
AFB2	3,03	50	30	315.2 > 259.2	50	35	315.2 > 243.3
AFB1	3,16	30	25	313.3 > 285.5	30	30	313.3 > 241.3
FB1	3,89	45	40	723.1 > 334.7	40	35	723.1 > 352.8
T-2	3,91	25	20	484.7 > 215.3	25	15	484.7 > 245.4
HT-2	3,92	25	15	442.6 > 263.4	25	15	442.6 > 215.3
ZEA	4,05	30	10	319.5 > 301.6	30	12	319.5 > 283.6
OTA	4,06	25	20	404.2 > 239.2	25	15	404.2 > 358.2
FB2	4,07	55	30	707.1 > 336.7	50	30	707.1 > 354.7

Extraction Methods

The sample matrix effects on quantification of analytes was estimated for three extraction (sample preparation / sample concentration) methods.

Solvent extraction

In 10 mL of degassed beer (tested to be free of analytes) was added 0.4 mL diluted standard solution (62.5 ppb DON and HT-2, 6.25 ppb FB1, FB2, ZEA and T2 and 0.625 ppb OTA, AFB1, AFB2, AFG1 and AFG2 made in 0.1% ammonium formate in 30% MeOH). The most part of the proteins, polysaccharides and other contaminants were precipitated by addition of 40 mL of acetonitrile 100%. After 10 minute of gentle homogenization on rotary agitator, the precipitate was centrifuged at 4000 rpm for 10 minutes. From the supernatant 36 mL solution was recovered and further evaporated to dryness at 35 °C with a gentle stream of nitrogen. The residue was reconstituted to a final volume of 0.4 mL with 0.1% ammonium formate in 30% MeOH, filtered through a 0.20 µm filter and injected to UPLC system.

The extraction procedure was repeated, but 10 mL of purified water was used instead of beer.

To estimate the sample matrix effects on the extraction method another series of experiments was realized but the addition of the standard solution was carried out by adding 0.4 mL of diluted standard solution to the residue resulted after the evaporation of the solvent.

Another way to estimate the effects of the sample matrix was to dilute the sample. In a series of experiments, after the centrifugation step, the recovered supernatant (36 mL) was diluted with purified water in a ratio 1:1 and 1:3, respectively.

Solid phase extraction (SPE)

The Oasis HLB cartridge was conditioned with 5 mL of acetonitrile / methanol (50:50, v/v) and further with 5 mL purified water. To 10 mL degassed beer (tested to be free of analytes), 0.5 mL of diluted standard solution (same as above) was added. The homogenized mixture (10 seconds at 200 rpm on vortex) was percolated at 1 mL/min on a Oasis HLB cartridge. The non-bounded compounds were washed out with 5 mL of 5% acetonitrile. The mycotoxins were eluted by percolating the cartridge with 5 mL of 0.1% formic acid in 100% acetonitrile. The eluate was evaporated to dryness at 35 °C with a gentle stream of nitrogen. The residue was reconstituted to a final volume of 0.5 mL with 0.1% ammonium formate in 30% MeOH. After filtration through a 0.20 µm filter the solution was ready to be injected in UPLC system.

The extraction procedure was repeated, but 10 mL of purified water was used instead of beer.

To assess the influence of the sample matrix, two approaches were considered: addition of standard before chromatographic separation step and dilution of the sample at the earliest possible step. For this, in a series of experiments the extraction procedure was repeated but the diluted standard solution (0.5 mL) was added to the residue obtain after the evaporation of the solvent. Finally, a series of experiments was realized, but the beer sample (10 mL) was diluted 2x and 4x, respectively, before passing the beer through the SPE cartridge.

Stir bar sorptive extraction (SBSE)

Glass bar with magnetic core having C18 coating layer was used as a specific adsorbent and as a magnetic stirrer. Similarly as in SPE procedure, 10 mL of degassed beer were mixed with 0.5 mL of diluted standard solution and homogenized with SBSE for 10 minutes at 200 rpm. The glass bar was introduced for 10 minutes (200 rpm on a magnetic stirrer) in 5 mL of 5% acetonitrile in order to eliminate the non-bonded contaminants. The mycotoxins were eluted from the SBSE mixing the glass bar at 200 rpm, 10 minute in 5 mL 0.1% formic acid in 100% acetonitrile. The glass bar was removed and reconditioned (mixed successively with 10 mL 0.1% formic acid in 100% acetonitrile, 10 mL of dichloromethane, 10 mL 0.1% formic acid in 100% acetonitrile and 10 mL of 0.1% formic acid in 5% acetonitrile). The eluate was evaporated to dryness at 35 °C with a gentle stream of nitrogen. The residue

was reconstituted to a final volume of 0.5 mL with 0.1% ammonium formate in 30% MeOH. Then extraction procedure was repeated, but 10 mL of purified water was used instead of beer.

As in the previous described sample extraction method, two other series of experiments were carried out in order to estimate the sample matrix effects on the quantification of the analytes. In one series of the experiments the diluted standard solution (0.5 mL) was added to re-dissolve the residue obtained after the evaporation of the solvent. In another series of experiments, the beer sample (10 mL) was diluted 2x and 4x, respectively, before the interaction with the SBSE.

MATRIX EFFECTS

As it was described in the previous sub-section, the sample matrix effects were studied in beer samples checked to be free of traces of analytes, realizing several series of experiments. Series 1 represented the neat standard solution in water, series 2 and 3 were prepared similarly, but with beer, adding the standards either pre- or post- application of the entire procedure of the extraction methods described above. All series of experiments were realized in six replicates. Sample matrix effects (ME), recovery (RE) and overall process efficiency (PE) were calculated according to Matuszewski et al. [19]. In all these experiments, the analytes were quantified based of standard curves realized by dilutions of the analytes made in 0.1% ammonium formate in 30% methanol.

The optimized method was applied to assess the 11 mycotoxins in beers commercialized in Romania (Timisoara). Once bought, the beer samples were analyzed in the same day. The open containers were kept at 4 °C until the results were processed (no longer than a week).

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