A rapid and sensitive method for the control of selected regulated and emerging mycotoxins in beer

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Abstract

A fast method using automated online solid-phase extraction (SPE) coupled to liquid-chromatography/high resolution mass spectrometric (LC-HRMS) detection for the determination of 12 mycotoxins in beer was developed and fully validated according to the performance criteria set in the European Union (EU) guidelines. Mycotoxin detection was achieved by full-scan HRMS with electrospray ionisation in positive ion mode within 13 min. No significant matrix interferences were observed. The lowest limits of detection (LODs) were achieved for emerging mycotoxins (beauvericin and enniatins) and ochratoxin A at levels between 0.01 and 0.02 µg/l, while the overall LODs were in the range of 0.01-0.30 µg/l. The elaborated method showed good performance in the analysis of beer samples with good linearity (R^2>0.999), intra-day and inter-day precision at the levels of <12% and <13%, respectively, and recoveries in the range of 80-119%. Finally, this fully automated, sensitive, and reliable online SPE-HPLC-HRMS method was successfully applied for the analysis of 25 Latvian beers and the obtained results were comparable or even better than those obtained using the well-established Quick, Easy, Cheap, Effective, Rugged, and Safe, the QuEChERS, methodology.

Keywords: *Fusarium* toxins, *Aspergillus* toxins, multi-mycotoxin, method development, high resolution mass spectrometry

1. Introduction

Fungi are natural biodeterioration agents of carbohydrate-rich agricultural commodities, while mycotoxins are secondary metabolites produced by toxigenic fungi and are known to cause severe effects in both humans and animals through their carcinogenic, teratogenic, nephrotoxic, and/or hepatotoxic properties (Tamura *et al.*, 2011; Wang and Li, 2015). The toxicity of mycotoxins has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the International Agency for Research on Cancer (IARC) (Table 1). According to latter, for example, aflatoxins are classified as group 1 carcinogens (sufficient evidence that they can cause cancer in humans), ochratoxin A (OTA) as group 2B carcinogen (some evidence that it can cause cancer in humans), while deoxynivalenol (DON) is a group 3 compound (insufficient evidence suggesting carcinogenicity) (IARC, 2017; WHO, 2002).

Cereals and other raw materials for beer (e.g. maize, wheat, barley, rice, hops, malt, etc.) are easily contaminated by various types of mycotoxins as long as the environmental conditions (humidity, temperature) are appropriate; thus mycotoxins can be occasionally found in beer and related drinks (Lhotská *et al.*, 2016; Pascari *et al.*, 2018; Tamura *et al.*, 2011; Wang and Li, 2015). For instance, detectable levels of OTA were found in beers originating from Spain, Italy, Belgium, Germany, Hungary, Turkey, and Czech Republic (Prelle *et al.*, 2013; Rahmani *et al.*, 2009; Soto *et al.*, 2014; Tangni *et al.*, 2002). However, OTA is not the sole mycotoxin that has been widely detected in beers, as researchers worldwide have reported the occurrence of aflatoxins, fumonisins, zearalenone (ZEA), DON, T-2 and HT-2 toxins in quantifiable levels (Bauer *et al.*, 2016; Habler *et al.*, 2017; Piacentini *et al.*, 2017; Rodríguez-Carrasco *et al.*, 2015; Rubert *et al.*, 2013). Therefore, the development of an effective, fast and accurate analytical method to monitor the occurrence of various toxins in beer is necessary for

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Table 1. Main characteristics of the selected mycotoxins.

Mycotoxin	Origin	Group of toxicity[1]	Molecular formula	pKa[2]	logP[2]
Aflatoxin B_1	*Aspergillus* spp.	1	$C_{17}H_{12}O_6$	17.79	1.73
Beauvericin	*Fusarium* spp.	–	$C_{45}H_{57}N_3O_9$	18.80	5.25
Dexynivalenol	*Fusarium* spp.	3	$C_{15}H_{20}O_6$	12.68	-0.76
Enniatin A	*Fusarium* spp.	–	$C_{36}H_{63}N_3O_9$	18.80	4.79
Enniatin A_1	*Fusarium* spp.	–	$C_{35}H_{61}N_3O_9$	18.80	4.39
Enniatin B	*Fusarium* spp.	–	$C_{33}H_{57}N_3O_9$	18.80	3.81
Enniatin B_1	*Fusarium* spp.	–	$C_{34}H_{59}N_3O_9$	18.80	4.06
HT-2 toxin	*Fusarium* spp.	–	$C_{22}H_{32}O_8$	12.98	1.32
Ochratoxin A	*Aspergillus* spp., *Penicillium* spp.	2B	$C_{20}H_{18}ClNO_6$	3.26	3.66
Sterigmatocystin	*Aspergillus spp.*	2B	$C_{18}H_{12}O_6$	8.38	2.73
T-2 toxin	*Fusarium* spp.	3	$C_{24}H_{34}O_9$	13.07	1.95
Zearalenone	*Fusarium* spp.	3	$C_{18}H_{22}O_5$	8.54	3.04

[1] Group of toxicity of mycotoxins evaluated by International Agency for Research on Cancer (IARC, 2017).
[2] pKa and logP values were predicted using interactive online software ALOGPS 2.1 according to Tetko *et al.* (2005).

ensuring the safety of consumers. Additionally, in an effort to further broaden the knowledge about mycotoxin occurrence, besides the mycotoxins typically regulated in plant-derived products, also some emerging mycotoxins like beauvericin (BEA) and enniatins are of great concern.

In recent years, the determination of organic microcontaminants, such as mycotoxins, has been mainly performed by high performance liquid chromatography coupled to mass spectrometry (LC-MS), now regarded as the standard reference method for this purpose (Rahmani *et al.*, 2009; Turner *et al.*, 2015). Although the use of MS, and especially MS/MS, provides high sensitivity, an analyte pre-concentration procedure is almost always necessary to achieve sufficiently low limits of detection for determining fungal contaminants at their typical concentrations (normally in µg/kg (µg/l) range). The most commonly used sample pretreatment methods for the determination of mycotoxins in beer include the traditional liquid-liquid extraction (LLE) (Hu *et al.*, 2014), QuEChERS extraction (Rodríguez-Carrasco *et al.*, 2015; Tamura *et al.*, 2011; Vaclavikova *et al.*, 2013), evaporate & shoot approach (Malachova *et al.*, 2012; Varga *et al.*, 2013), and offline solid-phase extraction (SPE) (Piacentini *et al.*, 2017; Rodriguez-Mozaz *et al.*, 2007; Rubert *et al.*, 2013). However, all of these methods are time-consuming and labour-intensive, thus several other sample pretreatment procedures like liquid-liquid micro-extraction (LLME) (Zhao *et al.*, 2017), micro-extraction by packed sorbents (MEPS) (Saito *et al.*, 2012; Savastano *et al.*, 2016), dilute & shoot approach (Abia *et al.*, 2013; Al-Taher *et al.*, 2013; Mastanjević *et al.*, 2018), and online SPE have been described (Lhotská *et al.*, 2016), although not for multi-mycotoxin analysis of beer (Campone *et al.*, 2017; Nistor *et al.*, 2017; Wang and Li, 2015).

The online coupling of the SPE technique with a chromatographic system enables a multidimensional separation with advantages over the traditional offline methods. The main advantage of the online SPE methodology is that the method can be performed faster with full automation. The other clear advantages of the online SPE technique are the reduced solvent consumption and less need for sample handling (Fumes *et al.*, 2017; Lhotská *et al.*, 2016). One additional crucial advantage of the online SPE-HPLC combination is the elimination of analyte losses by evaporation or by degradation during the sample preconcentration step, usually providing improved precision and accuracy (Watabe *et al.*, 2006). Higher sensitivity is also achieved in online configurations due to the complete transfer and analysis of the extracted species in the analytical system. The analysis of the whole sample leads to lower limits of detection or, alternatively, smaller sample volumes can be used to obtain sufficient sensitivity for a large variety of compounds (Lhotská *et al.*, 2016; Rodriguez-Mozaz *et al.*, 2007).

Due to the improvements in robustness and sensitivity of high resolution mass spectrometry (HRMS) in recent years, there has been a growing interest in switching from using triple quadrupole mass spectrometers to high resolution mass spectrometers (Kwok *et al.*, 2017). So far, the applicability of time-of-flight (TOF) and hybrid quadrupole TOF (qTOF) mass selective detectors in the

field of contaminant analysis has been limited due to their low sensitivity compared to triple quadrupole instruments (Kaufmann, 2014). However, by using an online SPE system to improve sensitivity, this approach could provide an accurate, reliable, and robust method for multi-analyte determination in complex matrices, with clear applications in the determination of mycotoxins. Moreover, the full-scan spectra obtained by this type of instrumentation offer an opportunity to identify untargeted compounds with similar chemical properties in the analysed samples anytime without reinjection.

As to our knowledge, no coupling of online SPE techniques with HPLC-TOF detection has been previously reported for the determination of mycotoxins in food and beverages. Thus, the purpose of this work was to show a simple and modern analytical tool for reliable control of mycotoxins at trace levels in beer, with a minimum of additional clean-up steps and maximum accuracy, simplicity, and selectivity.

2. Materials and methods

Samples

25 randomly selected beer samples with alcohol content ranging from 2.8 to 9.0% were analysed during this study. The sampled beers were produced in Latvia and were classified according to the producer label as: dark beer varieties (n=7), light beer varieties (n=11), and special or mixed varieties (n=7). All of the samples were obtained from local supermarkets. In order to avoid potential chemical composition changes, unopened samples were stored up to 3 weeks in amber glass bottles at 4 °C before the analysis. Visibly cloudy beers containing particles were first filtered through a paper filter (the other beers were used untreated) and a portion of 100 ml of each beer sample was degassed before the analysis by ultrasonication for at least 15 min.

Materials and reagents

The solvents used (acetonitrile and methanol) were of pesticide grade and were purchased from Sigma-Aldrich (Steinheim, Germany). Dimethylformamide (DMFA) of pesticide grade (≥99.8%) was purchased from Merck (Darmstadt, Germany). Ammonium acetate of reagent grade was purchased from Scharlab (Barcelona, Spain), while sodium chloride, magnesium sulphate, sodium sulphate, and formic acid of analytical grade were purchased from Merck Millipore (Darmstadt, Germany). The primary-secondary amine (PSA) and C18 sorbents were obtained from Phenomenex (Torrance, CA, USA). Deionised water was produced with a Millipore Milli-Q system (Darmstadt, Germany).

The standards of BEA, enniatin A (ENNA), enniatin A1 ($ENNA_1$), enniatin B (ENNB), and enniatin B1 ($ENNB_1$) were purchased from Cayman Chemical (Ann Arbor, MI, USA), while DON, aflatoxin B_1, HT-2, T-2, OTA, sterigmatocystin (STE), and ZEA were acquired from Romer Labs (Tulln, Austria). Standard stock solutions of all mycotoxins were prepared in acetonitrile, except BEA and enniatins that were kept in DMFA. The spiking solutions and calibration standards were prepared by serial dilution of stock solutions in acetonitrile and were stored in UV-protected glassware at 4 °C.

Online solid phase extraction and LC parameters

Online SPE and chromatographic analysis were performed with an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) comprised of a degasser, a dual pump, a column compartment, an autosampler fitted with a 500 µl injection loop, and a programmable Rheodyne® six-port switching valve at the side of the column compartment.

Automated sample pretreatment was performed using an online SPE methodology, with a HyperSep Retain PEP cartridge (2.1×10 mm, 30 µm) from Thermo Fisher Scientific. LC was performed with a Phenomenex Kinetex C18 (1.7 µm, 50 mm ×3 mm) analytical column used for the separation of target compounds at 40 °C. The separation process consisted of three steps involving the isolation of target analytes from endogenous interfering components (sample clean-up), elution of the target analytes from the cartridge for LC-HRMS analysis, and re-equilibration of the chromatographic system (Table 2).

The mobile phase A consisted of 0.5 mM aqueous ammonium acetate containing 0.1% of formic acid and the mobile phase B consisted of 0.5 mM ammonium acetate in acetonitrile containing 0.1% of formic acid. The following mobile phase composition programme was used: the fraction of eluent B was held at 30% for 3 min, then it was increased to 98% B within 0.5 min; followed by maintaining 98% B for 3 min. Thereafter, the mobile phase composition was again switched to 30% B over 3.5 min and the column was re-equilibrated for 3 min. The mobile phase flow rate was 0.35 ml/min. The injection volume was 500 µl.

Sample clean-up

In the loading step, the process was initiated by direct injection of degassed beer sample (500 µl) using the autosampler. The interfering matrix components were washed to waste with 70:30 (v/v) mixture of mobile phases A and B at the flow rate of 0.7 ml/min for 2 min, while mycotoxins were retained on the HyperSep Retain PEP cartridge. A second pump during the loading process conditioned the analytical column with the initial 70:30 (v/v) mixture of mobile phases A and B at the flow rate of 0.35 ml/min.

Table 2. Online solid-phase extraction (SPE) and high performance liquid chromatography conditions.[1]

Time (min)	Loading pump			Elution pump			Valve positions
	Flow rate (ml/min)	A (%)	B (%)	Flow rate (ml/min)	A (%)	B (%)	
0	0.7	70	30	0.35	70	30	Loading pump – SPE Elution pump – Analytical column
2	0.7	70	30	0.35	70	30	Loading pump – waste Elution pump – SPE – Analytical column
3	0.7	70	30	0.35	70	30	Loading pump – waste Elution pump – SPE – Analytical column
3.5	0.7	70	30	0.35	2	98	Loading pump – waste Elution pump – SPE – Analytical column
6.5	0.7	70	30	0.35	2	98	Loading pump – waste Elution pump – SPE – Analytical column
10	0.7	70	30	0.35	70	30	Loading pump – waste Elution pump – SPE – Analytical column
12	0.7	70	30	0.35	70	30	Loading pump – SPE Elution pump – Analytical column
13	0.7	70	30	0.35	70	30	Loading pump – SPE Elution pump – Analytical column

[1] A: 0.5 mM aqueous ammonium acetate containing 0.1% of formic acid; B: 0.5 mM ammonium acetate in acetonitrile containing 0.1% of formic acid.

Elution of mycotoxins

In the transfer step, the six-port valve was switched from the loading to transfer position and the HyperSep Retain PEP cartridge was thus connected to the analytical column. The mycotoxins were then transferred from the SPE cartridge to the analytical column by gradient elution at the flow rate of 0.35 ml/min. The ratio of mobile phases A and B was sharply increased to 2:98 and kept constant over 3 min, then the mobile phase composition was switched to 30% B again.

Re-equilibration step

At the end of the run, the six-port valve was switched to the initial position and the SPE cartridge was washed and re-equilibrated for 3 min in order to prepare the analytical system for the next injection. The total run time for each sample was 13 min.

Modified QuEChERS method

Beer samples were prepared using a previously published modified QuEChERS method (Bogdanova *et al.*, 2018). A portion of degassed beer sample (5 ml) was transferred in a 50 ml polypropylene (PP) tube. Acetonitrile (10 ml) and formic acid (20 µl) were then added and the mixture was vigorously shaken by hand for 30 s prior the addition of anhydrous $MgSO_4$ (4 g), NaCl (1 g), sodium citrate tribasic dihydrate (1 g), and sodium citrate dibasic sesquihydrate (0.5 g). The mixture was immediately shaken on Biosan Multi RS-60 programmable rotator (Riga, Latvia) at 60 rpm for 10 min, followed by centrifugation at 5,204×*g* on an Eppendorf 5810R centrifuge (Hamburg, Germany) for 10 min at 15 °C. The supernatant (10 ml) was then transferred to a new 15 ml PP tube for freezing out at -80 °C. After the sample was completely frozen (≈20 min), it was subjected to centrifugation at 5,204×*g* for 10 min at 15 °C. The supernatant (6 ml) was then transferred to a dispersive solid phase extraction (d-SPE) tube containing $MgSO_4$ (900 mg), PSA (450 mg), and C18 sorbent (150 mg), vortexed for approximately 30 s and centrifuged at 5,204×*g* for 10 min at 15 °C. The acetonitrile extract (3.75 ml) was then transferred to a new 15 ml PP tube and evaporated to dryness under a gentle stream of nitrogen at 50 °C. The dry residue was reconstituted with 150 µl of 0.1% aqueous formic acid/methanol mixture (60:40, v/v), resulting in a concentration factor of twenty five, and vortexed for 4 min. Finally, the solution was filtered through a Ultrafree-MC 0.22 µm hydrophilic PVDF centrifugal filter from Merck (Darmstadt, Germany) and transferred to an autosampler vial for HPLC-TOF-MS analysis (injection volume 15 µl).

HRMS parameters

The HRMS system was comprised of an Apollo II electrospray ionization (ESI) source and a compact qTOF mass analyser (Bruker Daltonik GmbH, Bremen, Germany). The MS system was controlled by otofControl 4.0 software

(Bruker Daltonik GmbH) and operated in positive mode for all mycotoxins. The source parameters were as follows: the applied voltages at the capillary cap and the spray shield were 4.5 kV and 0.5 kV, respectively. Laboratory grade nitrogen produced by Peak Scientific® generator (Billerica, MA, USA) was used as pressurised nebulizer gas and heated drying gas at the flow rate of 10 l/min. The drying gas heater temperature was adjusted to 200 °C. Nebulizer gas pressure was set at 200 kPa. The detection was carried out in the full scan mode at the *m/z* range of 50-1000 and 2 Hz scan rate. The measured masses with the respective ions are shown in Table 3. The mass extraction window applied for quantitative purposes was set to ±5 ppm. The mass accuracy throughout the analyses was better than 4 ppm at a resolution of 10,000 Full Width Half Maximum (FWHM). External instrument calibration for accurate mass measurements was performed before each batch of samples according to the instrument manufacturer's guidelines. Data acquisition was controlled by HyStar 3.2 software (Bruker Daltonik GmbH) and data analysis – by QuantAnalysis 4.3 (Bruker Daltonik GmbH), respectively, while further data evaluation was carried out using Microsoft Excel 2010 (Redmond, WI, USA).

Data analysis and the validation of analytical method

The proposed online SPE-HPLC-TOF-MS method was validated according to the performance criteria set in Commission Regulations (EU) No. 882/2004 (EC, 2004), 401/2006 (EC, 2006), 519/2014 (EC, 2014), the 'Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food' (Wenzl *et al.*, 2016), and ISO 5725 (ISO,1994). The limit of detection (LOD) and limit of quantification (LOQ) values, as well as the recovery and precision (intra-day and inter-day precision) of the analytical procedure were estimated in this study.

The validation of the method was performed using light beer sample previously analysed for the mycotoxins content and found to contain no detectable traces of selected substances. Recovery experiments were performed by spiking beer samples with standard solutions containing mycotoxins at three different concentrations. An overview of the spiking concentrations and the levels for each individual mycotoxin is presented in Table 4. The analysis at each spiking level was independently repeated, resulting in 3×6 individual results. A linear calibration function was used, including a forced intercept at zero and 1/x weighting. The linearity of the method was assessed from nine-point matrix-matched calibration by plotting the peak areas of each of the analytes against their concentrations and a linear least squares regression model was applied. Only the lower calibration levels (the first five calibration points) were used for the estimation of LOD/LOQ values. The recoveries were calculated as differences in the mycotoxin content in spiked and non-spiked samples relative to the spiking level. For these recovery analyses, the repeatability of the method was expressed as the relative standard deviation (RSD) in %. The combined standard uncertainties for selected mycotoxins were calculated for the lowest validation level with the refined level of confidence of 95% (k=2)

Table 3. High resolution mass spectrometry scan parameters for the selected mycotoxins with the retention times (RT) obtained using the online solid-phase extraction method.

Mycotoxin[1]	RT	Quantification ion	Theoretical mass, *m/z*	Measured mass, *m/z*	Confirmation ion	Theoretical mass, *m/z*	Measured mass, *m/z*
AFB_1	7.4	$[M+H]^+$	313.0707	313.0704	$[M+Na]^+$	335.0526	335.0529
BEA	7.6	$[M+NH_4]^+$	801.4433	801.4420	$[M+Na]^+$	806.3987	806.3971
DON	6.4	$[M+H]^+$	297.1333	297.1338	$[M+Na]^+$	319.1152	319.1163
ENNA	7.9	$[M+NH_4]^+$	699.4903	699.4904	$[M+Na]^+$	704.4457	704.4450
$ENNA_1$	7.8	$[M+NH_4]^+$	685.4746	685.4768	$[M+Na]^+$	690.4300	690.4314
ENNB	7.5	$[M+NH_4]^+$	657.4433	657.4454	$[M+Na]^+$	662.3987	662.4008
$ENNB_1$	7.6	$[M+NH_4]^+$	671.4590	671.4609	$[M+Na]^+$	676.4144	676.4153
HT-2	7.7	$[M+NH_4]^+$	442.2435	442.2421	$[M+Na]^+$	447.1989	447.1973
OTA	6.9	$[M+H]^+$	404.0895	404.0883	$[M+Na]^+$	426.0715	426.0710
STE	7.1	$[M+H]^+$	325.0707	325.0709	$[M+Na]^+$	347.0526	347.0531
T-2	6.8	$[M+NH_4]^+$	484.2541	484.2532	$[M+Na]^+$	489.2095	489.2087
ZEA	7.0	$[M+H]^+$	319.1540	319.1548	$[M+Na]^+$	341.1359	341.1368

[1] AFB_1 = aflatoxin B_1; BEA = beauvericin; DON = deoxynivalenol; ENNA = enniatin A; $ENNA_1$ = enniatin A_1; ENNB = enniatin B; $ENNB_1$ = enniatin B_1; HT-2 = HT-2 toxin; OTA = ochratoxin A; STE = sterigmatocystin; T-2 = T-2 toxin; ZEA = zearalenone.

Table 4. Recoveries and precision (RSD) of the method for determination of mycotoxins at different spiking levels.

Mycotoxin[1]	1st spiking level				2nd spiking level				3rd spiking level			
	Concentration, µg/l	Recovery (n=6), %	Intra-day RSD, (n=6), %	Inter-day RSD, (n=12), %	Concentration, µg/l	Recovery (n=6), %	Intra-day RSD, (n=6), %	Inter-day RSD, (n=12), %	Concentration, µg/l	Recovery (n=6), %	Intra-day RSD, (n=6), %	Inter-day RSD, (n=12), %
AFB_1	0.15	97	9	10	0.5	98	3	6	5.0	92	7	8
BEA	0.15	86	7	11	0.5	81	10	9	5.0	93	7	7
DON	1.5	112	10	11	5.0	111	9	9	50	103	9	9
ENNA	0.15	105	4	4	0.5	104	2	11	5.0	93	8	10
$ENNA_1$	0.15	80	12	12	0.5	92	2	13	5.0	99	4	11
ENNB	0.15	85	3	4	0.5	97	3	8	5.0	87	4	11
$ENNB_1$	0.15	102	6	6	0.5	82	5	9	5.0	85	6	6
HT-2	1.5	93	7	7	5.0	104	5	5	50	105	6	6
OTA	0.15	96	8	8	0.5	98	5	5	5.0	104	5	10
STE	0.15	85	8	10	0.5	103	9	9	5.0	89	3	8
T-2	1.5	119	8	8	5.0	105	7	7	50	100	5	5
ZEA	1.5	107	6	6	5.0	113	4	5	50	104	4	4

[1] AFB_1 = aflatoxin B_1; BEA = beauvericin; DON = deoxynivalenol; ENNA = enniatin A; $ENNA_1$ = enniatin A_1; ENNB = enniatin B; $ENNB_1$ = enniatin B_1; HT-2 = HT-2 toxin; OTA = ochratoxin A; STE = sterigmatocystin; T-2 = T-2 toxin; ZEA = zearalenone.

3. Results and discussion

Optimisation of the chromatographic separation

The effectiveness of C18 stationary phase as analytical column sorbent has been demonstrated in many other studies (Habler *et al.*, 2017; Lhotská *et al.*, 2016; Liu *et al.*, 2017; Turner *et al.*, 2015), therefore we selected this sorbent for exploring the possibility of liquid chromatography separation of the most commonly detected and emerging mycotoxins in beer.

Two different C18 analytical columns were tested: Luna® Omega Polar C18 (3 µm, 30×2.1 mm) packed with fully porous silica particles and Kinetex® C18 (1.7 µm, 50×3 mm) packed with core-shell silica particles. The parameter of resolution was almost identical on both columns and mycotoxins were efficiently separated under our chromatography conditions (Supplementary Figure S1). Methanol:water and acetonitrile:water gradient systems were applied for the separation of twelve mycotoxins, and it was concluded that methanol:water caused more pronounced peak broadening for lower mass mycotoxins on both columns. Thus, in order to perform an efficient separation over a wide molecular mass range, an acetonitrile:water gradient was used in our final version of the procedure in combination with solvent strength control.

Both columns showed no significant difference in separation efficiency, peak shape, or the column pressure even after a hundred injections, but the slightly higher back pressure of Luna® Omega Polar C18 column (about 10,000 kPa) turned our final selection in favour of Kinetex C18 analytical column (about 8,000 kPa).

In order to optimise the retention and elution conditions of the online SPE system, the gradient elution was optimised for efficiency and the initial gradient conditions were set with respect to the conditions of the online SPE step. The time of the valve switch was set to 2 min. The gradient elution program started at that moment from 30% acetonitrile, and the composition was kept constant for 1.5 min. The lower content of acetonitrile at the beginning of the separation enabled gradual washing of interfering residual compounds at the beginning of the chromatogram after the flow switch from the extraction column to the analytical column. Thus, the peak of DON was eluted over a slightly longer time and was separated from the very large peak of matrix components.

Several authors have reported the co-occurrence of DON and DON-3-glucoside in beer samples (Kostelanska *et al.*, 2009; Lancova *et al.*, 2008; Zachariasova *et al.*, 2008). Thus, in order to check whether DON-3-glucoside co-elutes with DON in our method and to guard against overestimating the concentration of DON in beer samples, several additional experiments were performed. It was concluded that our experimental conditions including the online SPE step allowed to retain DON on an online SPE cartridge, however it was not possible to retain DON-3-glucoside which was eluted already during the sample loading step (within the dead volume corresponding to the retention time of 5.2 min). This provided us an opportunity to distinguish and separate DON from DON-3-glucoside and made the method more specific for DON, but not suitable for the determination of DON-3-glucoside.

Between 1.0 and 1.5 min of the chromatographic run, the percentage of solvent B was linearly increased to 98% and held for 3 min. Thereafter, the percentage of solvent B was decreased to 30% over 3.5 min and kept constant for additional 2 min, then the valve was switched back to the online SPE position and the analytical column was equilibrated for 1 min more to the initial conditions for the next sample SPE step (Table 2). The separation of mycotoxins after the online SPE step is depicted on the spiked beer chromatogram in Figure 1. Under the optimal adjusted conditions, all mycotoxins were eluted selectively over 13 min time (including the pretreatment of sample) without matrix interference. It is important to mention that the mycotoxin eluting sequence during online SPE has changed compared to that observed for QuEChERS method and a single Kinetex C18 analytical column. In particular, during the online SPE the HT-2 toxin eluted after T-2 and AFB_1 eluted later than ZEA.

Optimisation of the HRMS parameters

Following the chromatographic separation, quantification of mycotoxins was performed using the TOF mass analyser. The positive-negative polarity switching of TOF instruments is not as fast as in the case of conventional quadrupole (QqQ) instruments or Orbitrap systems and, thus may result in excessive mass drift, providing poor mass accuracies (Kaufmann, 2014). Regarding this issue, all 12 mycotoxins were analysed in positive ionisation mode, forming protonated molecular ions and/or ammonium/sodium adducts. The detection was performed in a full scan mode (50-1000 Da) that was expected to provide an opportunity for identification of untargeted compounds without reinjection of the samples. Moreover, it was proved in various in-house experiments that the mass and spectral stability of our TOF system operating in multi-compound detection mode was higher when performing a full scan analysis rather than MS^2.

The general requirements for the quantification and confirmation of an analyte by the means of high resolution mass spectrometry in a full scan mode are to monitor the retention time of an analyte, to record chromatographic peaks by detecting the mass signal of 2 respective ions with a mass accuracy better than 5 ppm (preferably

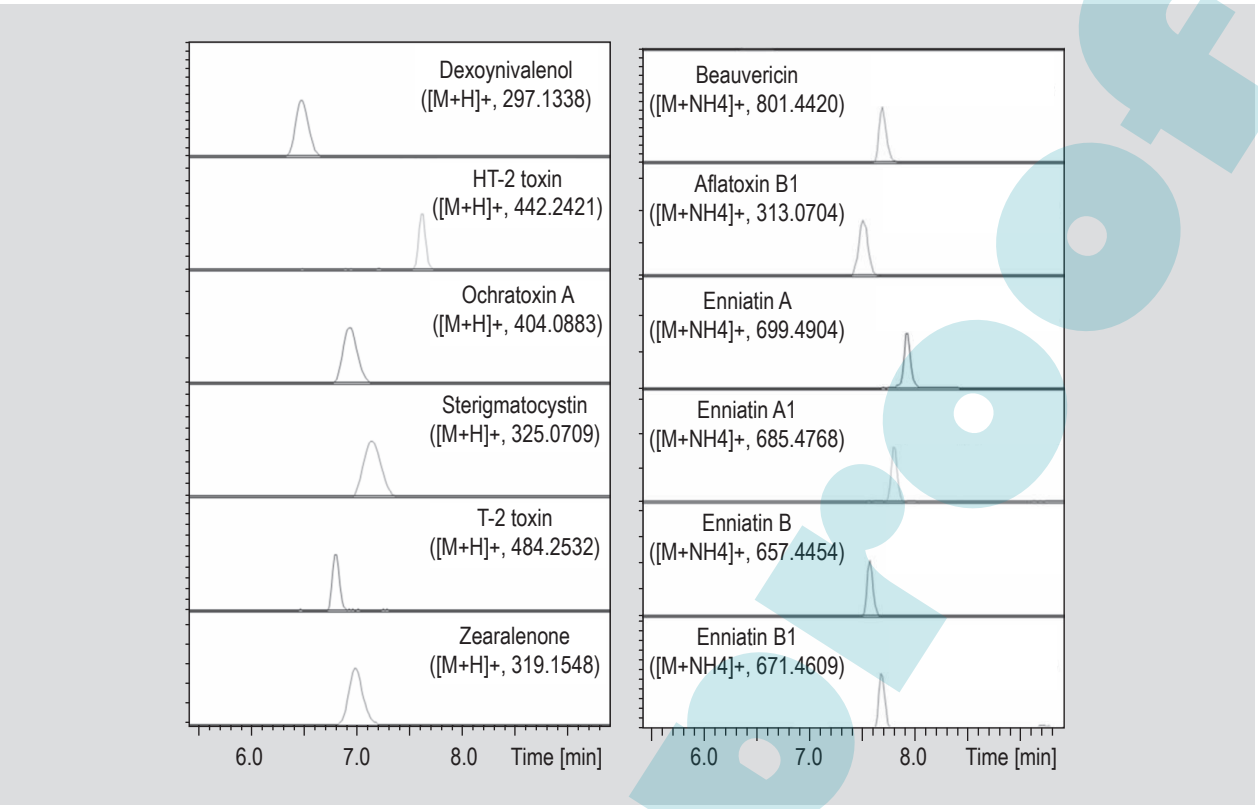

Figure 1. The chromatograms of fortified beer sample with the mycotoxins at the levels corresponding to the method limit of quantification (mass extraction window ±5 ppm).

including the molecular ion, protonated molecule or an adduct ion, and including at least one fragment ion), and to ensure ion ratio within ±30% of the average value for calibration standards from the same sequence (EC, 2016). The elaborated method showed high stability during all experiments, if all of the method conditions were met and the mobile phases were prepared daily. We did not observe variations in retention time larger than 0.1 min, thus the retention time of the compound can also be used as an identification point of the quantification method. The details of our optimised instrumental parameters, as well as the average experimental masses of quantification and confirmation ions of all analysed mycotoxins are summarised in Table 3.

As mentioned previously, the stability of the mass accuracy is a crucial parameter for the proper functioning of a TOF mass analyser. Thermal expansion of the flight tube or slight voltage fluctuations of the ion acceleration voltage directly affect the flight times, and thus the accuracy of mass measurements. For this reason, we performed mass-axis calibration before each batch of samples and added mass calibrant analysis after every 10 injections.

Optimisation of the automated solid phase extraction

The mycotoxins analysed in the present study have a rather broad range of physicochemical properties that pose a challenge for the optimisation of simultaneous online sample pretreatment. The key parameters of analytes that are relevant for retention on an SPE cartridge and further extraction are the pKa and logP values. For instance, STE (logP=2.73), OTA (logP=3.66) and, obviously, DON (logP=-0.76) are more polar compounds compared to enniatins that have mean logP values of 4.26. Also, the pKa values of these mycotoxins are significantly different: 18.8 for enniatins, 12.7 for DON, 8.38 for STE, and 3.26 for OTA. The pKa and logP values are shown in Table 1.

Selection of the extraction sorbent

Three different commercial SPE cartridges were evaluated for the retention of the analysed mycotoxins, including HyperSep Hypercarb (2.1×10 mm, 30 µm), HyperSep Retain-AX (2.1×10 mm, 30 µm), and HyperSep Retain-PEP (2.1×10 mm, 30 µm). In order to achieve the retention of the analytes without any matrix effects, all of the initial experiments were carried out using pure standards of mycotoxins. The results were obtained from at least 10-fold injections of the mycotoxin standard solution with the mycotoxins concentrations at the level corresponding

to the second spiking level of the validation study (Table 4). The results are shown in Table 5. Only the HyperSep Retain-PEP cartridge was able to retain all of the selected mycotoxins. The HyperSep Hypercarb cartridge packed with porous graphitic carbon, suitable for highly polar compounds and was not able to retain the less polar ZEA and STE or the highly acidic OTA. Important to mention, that besides the polarity or acidicity, also lipophilicity, dissociation of weak acids or bases, and also distribution coefficient affects the retention of compounds on different sorbents, and this could also be a reason of weak retention of AFB_1, BEA, STE, and ZEA. The HyperSep Retain-AX cartridge has been designed for the retention of acidic and non-polar analytes, thus showing extremely poor retention of the polar mycotoxins HT-2, AFB_1, and DON.

The elution of the target compounds from the HyperSep Retain-PEP cartridge was further evaluated. The SPE column loading, washing, and reconditioning parameters were optimised for the extraction recovery, sample matrix elimination, and prevention of carryover of analytes from contaminated to uncontaminated samples.

Optimisation of loading parameters

The effects of different loading parameters on the retention and elution of mycotoxins were evaluated by a direct injection of 2 µg/l standard solution containing 12 mycotoxins into the HPLC-TOF-MS system with and without the online SPE procedure. The percent recoveries were calculated by using averaged peak areas obtained from at least three online SPE runs and three direct injection runs. The direct injection method had the same analytical gradient as the online SPE method, and the observed retention times had a 3 min offset due to the duration of online SPE step, ensuring the same MS source conditions at the elution in both injection modes, thus enabling a direct comparison of peak areas. It was observed that an increase of the acetonitrile content in loading solvent resulted in peak broadening for STE and OTA, whereas the non-polar enniatins and BEA were much less affected by changes in acetonitrile concentration. A mixture of acetonitrile:water (30:70, v/v) was finally chosen as a compromise for the loading solvent.

Table 5. The retention effects of different solid-phase extraction (SPE) cartridges (n=10), %.

Mycotoxin[1]	Online SPE cartridge		
	HyperSep Hypercarb	HyperSep Retain AX	HyperSep Retain PEP
AFB_1	<80	<80	98±3
BEA	<80	95±2	97±3
DON	88±6	<80	79±7
ENNA	80±5	102±4	96±5
$ENNA_1$	81±4	99±2	95±5
ENNB	84±4	98±3	98±3
$ENNB_1$	84±3	101±4	98±3
HT-2	90±5	<80	81±4
OTA	<80	89±4	91±3
STE	<80	90±2	94±3
T-2	87±7	<80	87±5
ZEA	<80	94±3	92±4

[1] AFB_1 = aflatoxin B_1; BEA = beauvericin; DON = deoxynivalenol; ENNA = enniatin A; $ENNA_1$ = enniatin A_1; ENNB = enniatin B; $ENNB_1$ = enniatin B_1; HT-2 = HT-2 toxin; OTA = ochratoxin A; STE = sterigmatocystin; T-2 = T-2 toxin; ZEA = zearalenone.

The flow rate effect of the loading solvent was also optimised. The recoveries of mycotoxins decreased at higher flow rates of the loading solvent (>0.70 ml/min). Thus, in order to optimise the duration of analysis, the flow rate was selected at 0.7 ml/min with a constant loading time of 2 min. This moderate loading process (approximately 4 µl/s injected over 2 min) provided adequate time for effective adsorption of mycotoxins on the SPE sorbent and sufficient removal of most matrix components.

Increasing the injected sample volume provides an effective way to improve the sensitivity of the online SPE-HPLC method. However, this volume should be carefully adjusted to prevent the saturation of the detector that can not only lead to underestimation of the analyte abundance, but also to a shift of the observed *m/z* values. The injection volumes of 100-500 µl were tested by using a 500 µl stainless steel loop. No significant difference in SPE recovery was observed, while the linearity of the analyte peak area was still good. Thus, the large injection volumes of 500 µl were adopted for enhanced sensitivity, since no negative influence on the detector efficiency was observed.

Optimisation of washing solvent and washing time

A mixture of acetonitrile and water was used as the washing solvent. Since the samples were analysed without filtration and transferred from the sample loop to the SPE column with the washing solvent, the content of acetonitrile in the washing solvent had to be as low as possible to prevent the precipitation of proteins or mineral salts. The fraction of acetonitrile in the washing solvent tested in this study varied from 0 to 50% (v/v) at a flow rate of 0.7 ml/min. The optimal amount of acetonitrile in the washing solvent was found to be 30%, providing an adequate amount of water to completely wash off matrix components from the analytical system while avoiding any adverse effects on the determination of mycotoxins.

The washing time examined in this study ranged from 1 to 5 min at the solvent flow rate of 0.7 ml/min. The minimum volume of washing solvent after the SPE step was 2 ml, sufficient for preventing the transfer of salt residues to the ion source, for removing any clogging particles and for restoring analytical column performance. Since longer washing step times did not improve the clean-up efficiency, the valve switch was performed at the second minute to reduce the duration of the analyses.

Matrix effects

The matrix effects were evaluated by injecting standard solutions and fortified light beer samples containing 12 mycotoxins at nine different concentrations into the online SPE-HPLC-TOF-MS system. The matrix effect was investigated by calculating the percentage (ME%) of signal enhancement or suppression, according to Equation 1.

$$ME(\%)=100\times\left(\frac{a_m}{a_s}-1\right) \quad (1)$$

a_m: slope of the matrix-matched calibration;

a_s: slope of the calibration curve obtained by pure calibration standards.

None of the analysed mycotoxins showed ion enhancement in beer matrix, while ion suppression occurred to various extent (Table 6). Enniatins, BEA, and ZEA – the mycotoxins that were better retained on the SPE cartridge showed less or no matrix effects (less than 5%), compared to the more polar mycotoxins that were eluted faster – DON and T-2. Among these, DON showed the strongest ion suppression (-49%) as it was the most difficult to separate from the matrix components and also showed the lowest recovery in our method (Figure 1). These results were in a good agreement with those observed by Habler *et al.* (2017) and Zachariasova *et al.* (2010), where the authors reported an absence of matrix effects for non-polar enniatins and ZEA, along with strong ion suppression of polar analytes like DON-3-glucoside and DON. AFB_1 also showed rather strong ion suppression (-25%) comparable to that observed for T-2 toxin (-33%). The rest of the mycotoxins analysed in our study (HT-2, OTA, STE) were only slightly affected by ion suppression, at the levels about 10%. However, since strong adverse matrix effect was observed for some compounds, the matrix-matched calibration (MMC) approach was applied to the further mycotoxin analysis.

Analytical performance

The elaborated analytical method was validated for rapid and sensitive detection and quantification of twelve different mycotoxins in beer. The method provided good linearity, repeatability, accuracy, and high stability to meet the performance criteria set in EU legislation and guidelines.

Table 6. Main validation parameters of the elaborated mycotoxin determination method.[1]

Mycotoxin[2]	Linear range, µg/l	Linearity, r^2	Matrix effect, %	*i*-LOD, pg on-column	*i*-LOQ, pg on-column	*m*-LOD, µg/l	*m*-LOQ, µg/l	Uncertainty, %
AFB_1	0.02-10	0.99937	-25	10	34	0.021	0.068	9
BEA	0.02-10	0.99998	-0.8	8.8	29	0.018	0.058	16
DON	0.30-100	0.99904	-49	150	495	0.30	0.99	12
ENNA	0.02-10	0.99934	-1.3	9.4	31	0.019	0.062	8
$ENNA_1$	0.02-10	0.99995	-1.8	7.5	25	0.015	0.050	15
ENNB	0.02-10	0.99996	-2.2	6.5	22	0.013	0.043	13
$ENNB_1$	0.02-10	0.99987	-1.4	7.9	26	0.016	0.052	12
HT-2	0.30-100	0.99977	-6.6	125	413	0.25	0.83	6
OTA	0.02-10	0.99979	-9.2	7.5	25	0.015	0.050	8
STE	0.02-10	0.99988	-8.4	14	45	0.027	0.090	12
T-2	0.30-100	0.99945	-33	138	457	0.28	0.91	10
ZEA	0.30-100	0.99941	-4.8	144	475	0.29	0.95	9

[1] *i*-LOD = the instrumental limit of detection, assessed from the analysis of pure standard solutions; *i*-LOQ = the instrumental limit of quantification, assessed from the analysis of pure standard solutions; *m*-LOD = the method limit of detection, assessed from the analysis of fortified beer samples; *m*-LOQ = the method limit of quantification, assessed from the analysis of fortified beer samples.

[2] AFB_1 = aflatoxin B_1; BEA = beauvericin; DON = deoxynivalenol; ENNA = enniatin A; $ENNA_1$ = enniatin A_1; ENNB = enniatin B; $ENNB_1$ = enniatin B_1; HT-2 = HT-2 toxin; OTA = ochratoxin A; STE = sterigmatocystin; T-2 = T-2 toxin; ZEA = zearalenone.

Matrix-matched calibration and accuracy of the method

As explained previously, various extent of ion suppression was observed for the analysed mycotoxins in beer matrix, and different slopes of calibration curves were obtained as a result (Figure 1). The linearity of the method was assessed from nine-point MMC curves, obtained by fortifying blank beer samples with standard solutions of mycotoxins at the concentrations showed in Table 4. The peak areas of each of the analytes were plotted against their concentrations and a linear least squares regression model was applied, showing a good linearity (R^2>0.999). The linear ranges and the respective correlation coefficients are shown in Table 6.

The recoveries of mycotoxins calculated from MMC indicate that, contrary to the standard solution calibration, MMC may effectively compensate for the matrix effects of beer. The recoveries of mycotoxins from beer samples at low, moderate, and high spiking concentrations were in an acceptable range of 80-119%, as demonstrated in Table 4.

Precision of the method

The precision of the method was tested by analysing blank samples spiked with all twelve analytes at three different concentrations (Table 4). In particular, 18 spiked samples were processed per day, resulting in a total of 36 spiked samples analysed over two different days. The precision was expressed as the relative standard deviation (RSD) for both the intra-day and inter-day precisions. The results of the precision experiments confirmed the good performance of the elaborated method and are shown in Table 4.

The obtained mean RSD values for the between-days validation ranged from 3 to 13%, while the mean RSD values for the within-day validation were in the range of 2-12%. Compared to the previously reported methods using offline sample preparation or stable isotope dilution methodologies, the precision obtained in the present study was similar or even superior (Habler *et al.*, 2017; Hu *et al.*, 2014; Zachariasova *et al.*, 2010; Zhao *et al.*, 2017).

Method limit of detection and limit of quantification

The calculation of method LOD (*m*-LOD) was based on a single analysis on the test sample, ten independent analyses of the blank, and equal probabilities (α=β=0.05) for false positive and false negative detections (Wenzl *et al.*, 2016). The blank samples were processed by applying the whole analytical procedure. Homoscedasticity was assumed for the concentration range between *m*-LOD and the spiking level. The *m*-LOD and *m*-LOQ values of each analyte were calculated based on the Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food (Wenzl *et al.*, 2016), according to Equations 2 and 3, respectively.

$$x_{LOD}=3.86\times \frac{S_{y,b}}{b} \quad (2)$$

x_{LOD}: the limit of detection for the method;

The factor 3.86 reflects the number of experiments and the chosen error probabilities (α=β=0.05);

$S_{y,b}$: the standard deviation of the pseudo-blank signals;

b: the slope of the calibration curve close to LOD;

$$x_{LOQ}=3.3\times x_{LOD} \quad (3)$$

x_{LOQ}: the limit of quantification for the method.

Since practically no background noise was observed within 5 ppm of the theoretical *m/z* values for most of the target compounds in the full scan raw HRMS data, the evaluation of instrument sensitivity via S/N ratio was not feasible, as it would lead to overestimated results (Wenzl *et al.*, 2016). Thus, the instrumental LOD (*i*-LOD) and instrumental LOQ (*i*-LOQ) values were assessed from the analyses of pure standard solutions with analyte concentrations from 0.01 to 4 µg/l (0.005-2.0 ng injected on-column). Taking into account the dynamic range of the detector, only signals with intensities above 1×10^4 were used for reliable detection. The assessed *i*-LOD values for the analysed mycotoxins were in the range of 6.5-150 pg injected on-column, while *i*-LOQs were in the range of 22-495 pg injected on-column.

The obtained *m*-LODs and *m*-LOQs (Table 6) were comparable to those observed in traditional mycotoxin determination methods based on QuEChERS or offline SPE methodology followed by detection by the means of HPLC-MS/MS, HPLC-FLD or other techniques. (Habler *et al.*, 2017; Prelle *et al.*, 2013; Rubert *et al.*, 2013; Skarkova *et al.*, 2013; Tamura *et al.*, 2011; Zhao *et al.*, 2017). For example, the *m*-LOQs obtained in our study for emerging mycotoxins were in the range of 0.043-0.062 µg/l, demonstrating significantly improved method sensitivity compared to methods previously reported for the analysis of beer with *m*-LOQs in the range of 1.2-3.5 µg/kg (Hu *et al.*, 2014) and 2.0-5.0 µg/l (Vaclavikova *et al.*, 2013). In contrast to emerging mycotoxins, the *m*-LODs and *m*-LOQs for *Fusarium* toxins like DON, ZEA, and T-2 were not as remarkable. For example, Rodrigues-Carrasco *et al.* (2015) reported a GC-MS/MS method for the determination of mycotoxins based on a modified QuEChERS sample preparation, when the *m*-LOD was 0.05 µg/l for DON, 8 µg/l for ZEA, as well as 4 and 2 µg/l for T-2 and HT-2, respectively. Importantly, only the LOD for DON was better compared to our observed value of 0.3 µg/l. Meanwhile, Bauer *et al.* (2016) reported an enzyme immunoassay method for mycotoxin determination with LOD for ZEA

at the level of 0.14 µg/l, which was half of our achieved value of 0.29 µg/l. Lhotská *et al.* (2016) reported an online SPE method with an LOD for OTA at the level of 0.003 µg/l, which was five times lower than our observed value (0.015 µg/l). However, it should be mentioned that the two latter methods focussed on the determination of only two mycotoxins, instead of 12 according to our methodology. With regard to the *Aspergillus* toxins AFB_1 and STE, our obtained *m*-LODs (0.021 µg/l and 0.027 µg/l, respectively) and *m*-LOQs were lower than those reported by Rubert *et al.* (2013) and were comparable to the results achieved by Zhao *et al.* (2017).

Finally, it should be noted that the analysis of one sample according to the proposed methodology can be completed in only 15 min, and the consumed volume of acetonitrile is less than 10 ml (including chromatographic separation), whereas the rest of the analytical parameters are comparable and meet the current and foreseeable requirements.

Analysis of real samples

The applicability of the elaborated method was assessed through the analysis of 12 mycotoxins in Latvian beers. In order to prove the long term stability of the developed method and to avoid false positive findings, quality assurance and quality control (QA/QC) checks were performed by the addition of different spiked beers at two different levels (corresponding to the first and the third calibration levels) in every sample batch. The observed concentrations of mycotoxins in beer samples (Supplementary Table S1) varied from undetectable levels to 115 µg/l for DON, thus representing a broad range of mycotoxin contamination. Similar results were obtained in a studies by Peters *et al.* (2017) and Varga *et al.* (2013) when more than 70% of analysed beers were contaminated with DON with the concentrations up to 475 µg/l (Peters *et al.*, 2017; Varga *et al.*, 2013). STE, ZEA, BEA, ENNA, and $ENNA_1$ were not detected during the study, whereas DON was detected in 60% of the analysed beer samples. ENNB and $ENNB_1$ were detected in 20% of the analysed beer samples at the concentration range of 0.05-1.56 µg/l. In addition, ENNB was always detected in beers at about five times higher concentrations than those of $ENNB_1$. This was in line with the findings that enniatins occasionally are present in beer samples obtained from retail stores in the European countries (Habler *et al.*, 2017; Hu *et al.*, 2014; Rubert *et al.*, 2013; Vaclavikova *et al.*, 2013). However, the incidence and concentration of enniatins varied considerably between the samples in the published studies, as did the ratio between ENNAs and ENNBs. Only 4 beers (16%) were contaminated with OTA and the detected levels ranged from 0.02 to 0.10

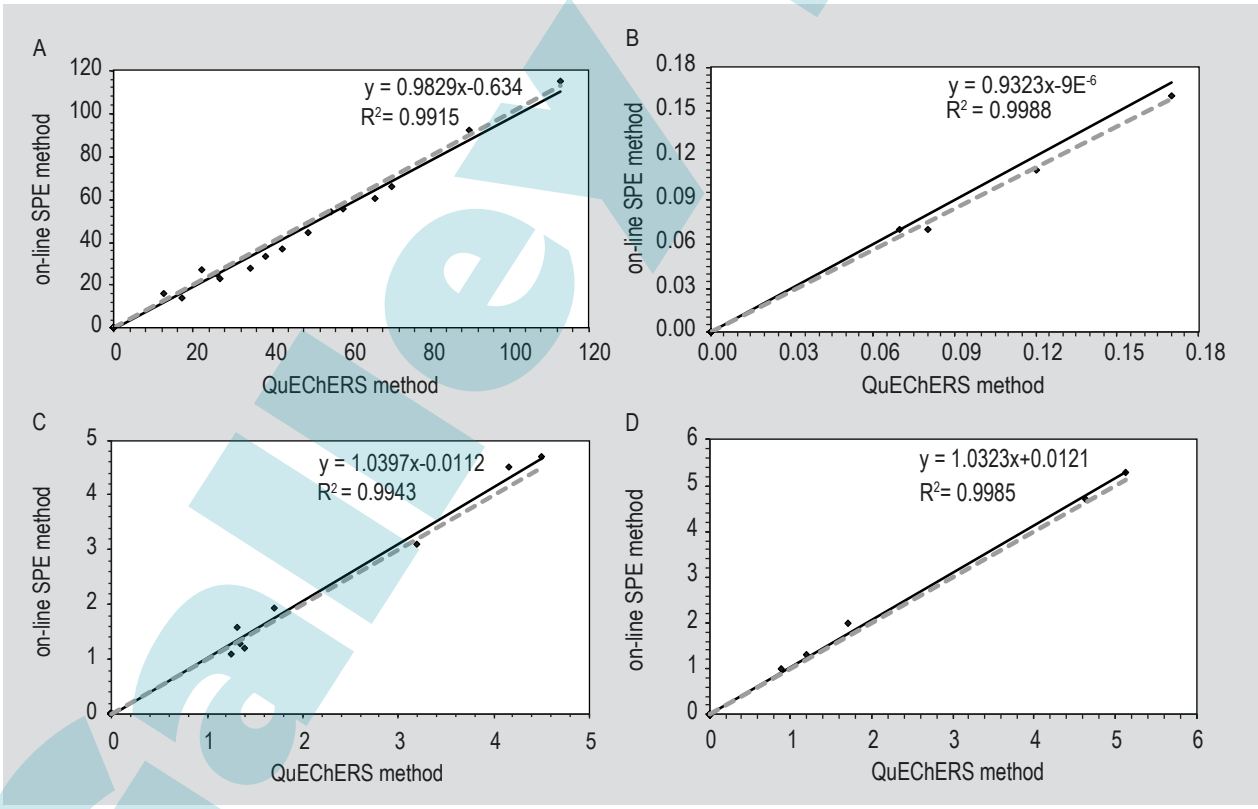

Figure 2. The functional relationships between the results obtained by our online solid-phase extraction (SPE) method and a modified QuEChERS methodology for the positive mycotoxin determinations in beer: (A) deoxynivalenol – 15 points; (B) aflatoxin B_1 – 4 points; (C) HT-2 toxin – 8 points; (D) T-2 toxin – 5 points).

µg/l. As the determined concentrations of OTA were very low, the obtained results confirmed the previously published assumption of negligible concern for consumers (Al-Taher *et al.*, 2013; Lhotská *et al.*, 2016; Skarkova *et al.*, 2013; Zhao *et al.*, 2017), while the observed concentrations still provide useful information in terms of long-term monitoring of food safety issues and health hazards.

In order to assess the efficiency of the elaborated online SPE method, the analytical results were compared to those obtained using a validated, well-established, and reliable modified QuEChERS methodology (Bogdanova *et al.*, 2018; Rodríguez-Carrasco *et al.*, 2015; Tamura *et al.*, 2011; Vaclavikova *et al.*, 2013). The functional relationship between these two methods for DON, AFB_1, T-2, and HT-2 is shown in Figure 2, whereas enniatins, BEA, ZEA, STE, and OTA were excluded from the comparison because of the totally different *m*-LODs and/or insufficient number of positive detections. For example, the *m*-LOQ for OTA in a previously published study was 0.46 µg/l (the established beer density was 1.032 g/cm^3) (Bogdanova *et al.,* 2018), whereas in the present study it was 0.05 µg/l. At the same time, the *m*-LOQ for ZEA was previously established at the level of 0.31 µg/l (Bogdanova *et al.,* 2018), whereas in the present study it was only 0.95 µg/l. Two regression curves were plotted: the bisecting line and the functional relationship between the methods calculated by linear regression. It was concluded that the curve for all selected mycotoxins practically merged with the bisecting line and the observed bias could be acknowledged to be in the range of method uncertainty, affirming the equivalency of the developed method in terms of providing reliable results. This clearly indicates that no significant variances occurred in HPLC-HRMS analysis, and affirms that the extraction steps, separation conditions, and detection of the mycotoxins in our developed online SPE-HPLC-TOF-MS method have been properly established and provide reliable results for rapid mycotoxin determination in beer samples.

4. Conclusions

The co-occurrence of different toxic compounds in one matrix implies a potential risk for additive, synergistic or antagonistic toxic effects. Considering the risks to human health, the determination of mycotoxins in food even at low levels is of special relevance to food safety. As to our knowledge, this is the first study on the application of fully automated online SPE-HPLC-TOF-MS method for the determination of mycotoxins in beers, which provides nearly equal or even superior detection limits and recoveries compared to those obtained from the more commonly used HPLC-MS/MS methods based on QuEChERS or offline SPE sample preparation. The developed method was successfully validated to meet the performance criteria set in EU guidelines and applied to the analysis of commercially available Latvian beers. A good agreement between the results obtained from the elaborated method and a well-established modified QuEChERS method emphasised the great potential of online SPE-HPLC-TOF-MS techniques in the determination of mycotoxins and provided a respectable and 'greener' alternative to the currently employed procedures for the analysis of these contaminants.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/WMJ2017.2298.

Figure S1. Separation of selected mycotoxins using different analytical columns: (A) Kinetex C18 analytical column; (B) Luna Omega Polar C18 analytical column.

Table S1. The concentrations of detected mycotoxins in analysed beer samples obtained by the online SPE-HPLC-TOF-MS method.

Conflict of interest

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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