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Analysis of multiple mycotoxins in beer employing (ultra)-high-resolution mass spectrometry

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The objective of the presented study was to develop and optimize a simple, high-throughput method for the control of 32 mycotoxins (*Fusarium* and *Alternaria* toxins, aflatoxins, ergot alkaloids, ochratoxins, and sterigmatocystin) in beer. Due to the broad range of their physicochemical properties, the sample preparation step was simplified as much as possible to avoid analyte losses. The addition of acetonitrile to beer samples enabled precipitation of abundant matrix components. The clean-up efficiency was controlled by ambient mass spectrometry employing a direct analysis in real time (DART) ion source. For determination of analytes, ultra-high-performance liquid chromatography hyphenated with high-resolution mass spectrometry utilizing an orbitrap (U-HPLC–orbitrapMS) or time-of-flight (TOFMS) technology was used. Because of significantly better detection capabilities of the orbitrap technology, the U-HPLC–orbitrapMS method was chosen as a determinative step and fully validated. To compensate matrix effects, matrix-matched calibration was employed. The lowest calibration levels for most of the target mycotoxins ranged from 1 to 8 $\mu g\,L^{-1}$ beer and the recoveries of analytes were in range from 86 to 124%. Copyright © 2010 John Wiley & Sons, Ltd.

Mycotoxins are toxic secondary metabolites produced by many species of microscopic filamentous fungi occurring on field cereals, including barley. The most abundant fungal genera affecting the malting barley are *Alternaria*, *Aspergillus*, *Penicillium*, and *Fusarium*, which simultaneously showed relatively high producing potential for a wide range of mycotoxins (almost 30% of *Alternaria*, 20% of *Aspergillus*, and 88% of *Fusarium* fungi isolated from barley grains was able to produce alternaria toxins, aflatoxins, ochratoxin A, deoxynivalenol and zearalenone).[1] Additionally to the relatively common micromycetes mentioned above, also the *Claviceps purpurea* causing the ergot disease belongs to frequent barley pathogens.[2]

Although the carry-over of aflatoxins, ochratoxin A, zearalenone, fumonisins, and ergot alkaloids from malted grains into beer has been documented,[2,3] the main research in this area has been focused on deoxynivalenol, the most frequent *Fusarium* mycotoxin.[4–8] In recent years, the presence of its main metabolite, deoxynivalenol-3-glucoside, in malt and beer has been reported at relatively high levels (the deoxynivalenol-3-glucoside/deoxynivalenol molar ratio was mostly even $\geq$1).[9] This was further confirmed in our follow-up study, in which both deoxynivalenol and its glucoside were determined as the main contaminants of beers retailed on the European market.[10] Since beer significantly contributes to the diet of a population, control of the presence of mycotoxins in this commodity is very important. For this purpose, reliable analytical methods for fast and effective monitoring of mycotoxins in the beer production chain are needed.

Over the years, several multidetection methods for the determination of mycotoxins in beer have been developed (an overview is summarised in Table 1). The first of them employed the gas chromatographic (GC) approach with derivatisation of analytes,[11] which is nowadays practically out of the use. All of the later published methods have already been based on liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS);[12–15] however, employing a more-or-less specific clean-up step limits the number of analytes it is possible to determine within the analysis. Nowadays, there is a trend towards simplifying the sample preparation procedure as much as possible, and, simultaneously, the full spectral data acquisition techniques are preferred because of their ease of use, and the possibility of retrospective mining of archived data. Until recently, the most common full spectral MS approach has been time-of-flight (TOF)MS, with a typical mass resolving power of benchtop instruments of 10 000–12 000 FWHM (full width at half maximum). However, in complex food matrices such as beer, this rather limited mass resolving power leads to the risk of inaccurate mass measurements caused by unresolved background matrix interferences.[16,17] Contrary to that, recently introduced benchtop systems based on the orbitrap technology, a special type of ion trap with a central spindle-shaped electrode around which the trapped ions oscillate,

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Table 1. Overview of published methods for the determination of multiple mycotoxins in beer[11–15]

Scott *et al.*[11]			Ventura *et al.*[12]			Rudrabhatla *et al.*[13]			Belajova *et al.*[14]			Romero-Gonzalez *et al.*[15]		
Analyte[a]	LOD[b] (μg L^{-1} of beer)	LOD[b] (μg L^{-1} of injected sample)	Analyte[a]	LOD[b] (μg L^{-1} of beer)	LOD[b] (μg L^{-1} of injected sample)	Analyte[a]	LOQ (μg L^{-1} of beer)	LOQ (μg L^{-1} of injected sample)	Analyte[a]	LOQ (μg L^{-1} of beer)	LOQ (μg L^{-1} of injected sample)	Analyte[a]	LOQ (μg L^{-1} of beer)	LOQ (μg L^{-1} of injected sample)
DON	0.1–1.5	0.4–6	**AFB1**	0.02	0.1	**DON**	i.n.p.[c]	i.n.p.[c]	**DON**	7	140	**DON**	0.45	2.25
NIV	0.01–0.3	0.04–1.2	**AFB2**			**HT-2**	i.n.p.[c]	i.n.p.[c]	**ZEA**	7.4	29.6	**HT-2**	0.2	1
α-ZOL	2.5–3	10–12	**AFG1**			**T-2**	i.n.p.[c]	i.n.p.[c]				**T-2**	0.23	1.15
β-ZOL	2.5–3	10–12	**AFG2**			**ZEA**	i.n.p.[c]	i.n.p.[c]				**ZEA**	0.3	1.5
ZEA	1.5–2	6–8	**OTA**			**FB1**	i.n.p.[c]	i.n.p.[c]				**FB1**	0.23	1.15
						FB2	i.n.p.[c]	i.n.p.[c]				**FB2**	0.3	1.5
						FB3	i.n.p.[c]	i.n.p.[c]				**AFB1**	0.13	0.65
						AFB1	i.n.p.[c]	i.n.p.[c]				**AFB2**	0.17	0.85
						AFB2	i.n.p.[c]	i.n.p.[c]				**AFG1**	0.1	0.5
						AFG1	i.n.p.[c]	i.n.p.[c]				**AFG2**	0.27	1.35
						AFG2	i.n.p.[c]	i.n.p.[c]				**AFM1**	0.07	0.35
						OTA	i.n.p.[c]	i.n.p.[c]				**OTA**	0.07	0.35
Sample preparation														
SPE, derivatisation			SPE clean-up (Oasis HLB)			Immunoaffinity clean-up			Immunoaffinity clean-up			SPE clean-up (C-18)		
Beer equivalent per 1 mL of injected solvent (injected volume)														
4 mL (1 μL)			5 mL (10 μL)			i.n.p.[c] (i.n.p.[c])			20 mL (50 μL) – DON 4 mL (20 μL) – ZEA			5 mL (5 μL)		
Instrumentation														
GC–MS Vista 6000 (Varian)/VG 7070 EQ (VG Analytical)			UPLC–MS/MS Acquity UPLC (Waters)/Quattro Premier triple quadrupole (Waters)			LC–MS/MS Pro Star 210 (Varian)/320-MS (Varian)			LC–DAD (DON), LC–FLD (ZEA) 1100 Series (Agilent Technologies)			UPLC–MS/MS Acquity UPLC (Waters)/Acquity TQD tandem quadrupole (Waters)		

[a] Abbreviations: DON, deoxynivalenol; NIV, nivalenol; HT-2, HT-2 toxin; T-2, T-2 toxin; α-ZOL, α-zearalenol; β-ZOL, β-zearalenol; ZEA, zearalenone; FB1, fumonisin B1; FB2, fumonisin B2; FB3, fumonisin B3; AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; AFG2, aflatoxin G2; AFM1, aflatoxin M1; OTA, ochratoxin A.
[b] The LOQ value was not available.
[c] i.n.p. – information was not provided

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allow to achieve routinely a mass resolving power of up to 100 000 FWHM and maintain excellent mass accuracy even <5 ppm without the use of continuous internal mass correction.[18–20]

The aim of this study was to introduce a fast multiple mycotoxin method for the analysis of 32 mycotoxins in beer based on a very simple sample preparation and ultra-high-performance liquid chromatography (U-HPLC) coupled with full spectral orbital trapping MS detection, and to explore the potential of such a system for routine work.

EXPERIMENTAL

Reagents and chemicals

Mycotoxin standards of (i) *Fusarium* toxins, major conjugates and other products of transformation (nivalenol, deoxynivalenol, deoxynivalenol-3-glucoside, deepoxydeoxynivalenol, fusarenon-X, neosolaniol, 3-acetyldeoxynivalenol, diacetoxyscirpenol, HT-2 toxin, T-2 toxin, verrucarol, zearalenone, α-zearalenole, β-zearalenole); (ii) aflatoxins (aflatoxin G1, aflatoxin G2, aflatoxin B1, aflatoxin B2); (iii) sterigmatocystin; and (iv) ochratoxins (ochratoxin A, and ochratoxin α) were purchased from Biopure (Tulln, Austria); standards of (v) *Alternaria* toxins (altenuene, alternariol, and alternariol-methylether) were obtained from Sigma–Aldrich (Taufkirchen, Germany), and standards of (vi) ergot alkaloids (ergosine, ergosinine, ergocornine, ergocorninine, ergocryptine, ergocryptinine, ergocristine, ergocristinine) were donated by The Czech Agricultural and Food Inspection Authority (Czech Republic). Isotopically labelled $^{13}C_{15}$-deoxynivalenol and $^{13}C_{18}$-zearalenone were purchased from Biopure (Tulln, Austria). The purity of standards was declared in the range 96–98.9%.

Solid standards of nivalenol, deoxynivalenol, fusarenon-X, neosolaniol, 3-acetyldeoxynivalenol, T-2 toxin, verrucarol, zearalenone, α-zearalenole, β-zearalenole, sterigmatocystin, ochratoxin A, altenuene, alternariol and alternariol methyl ether were dissolved in acetonitrile. Liquid standards of deepoxydeoxynivalenol, diacetoxyscirpenol, HT-2 toxin, ochratoxin α, and $^{13}C_{15}$-deoxynivalenol and $^{13}C_{18}$-zearalenone were supplied in acetonitrile, and deoxynivalenol-3-glucoside was delivered in a mixture of acetonitrile/water (1:1, v/v). The ergot alkaloid standards were dissolved in a mixture of acetonitrile/water/acetic acid (79:20:1, v/v/v). For the spiking experiments and calibration purposes, composite working standard solutions of (i) all native mycotoxins in acetonitrile (1000 μg L^{-1}), and (ii) isotopically labelled deoxynivalenol and zearalenone in acetonitrile (1000 μg L^{-1}) were prepared. All of the standards were stored at −20°C and were brought to room temperature before use.

The organic solvents acetonitrile, methanol, and ethanol (HPLC grade) were obtained from Sigma–Aldrich (Taufkirchen, Germany). Ultra-pure water was produced by Milli-Q system (Millipore Corporation, Bedford, MA, USA).

Sample preparation for U-HPLC–orbitrapMS

An aliquot of 4 mL of beer sample in a PTFE cuvette was degassed in an ultrasonic bath, and, after addition of 16 mL acetonitrile, the contents were vigorously shaken for 1 min. The dark coloured matrix precipitated under these conditions was then separated by centrifugation (10 min, 11 000 rpm). In the next step, a 5-mL aliquot of the supernatant was evaporated to dryness and reconstituted in 1 mL of methanol/water (50:50, v/v). To avoid obstruction of the U-HPLC system, microfiltration was performed prior to injection using centrifugation through a 0.2-μm microfilter PVDF Zentrifugenfilter (Alltech, Deerfield, IL, USA).

To control potential losses, e.g. due to the partition between a precipitate and an aqueous phase, an aliquot of $^{13}C_{15}$-labelled deoxynivalenol and $^{13}C_{18}$-labelled zearalenone standard solution corresponding to a contamination level of 20 μg L^{-1} was added as the surrogates to beer prior to processing ($^{13}C_{15}$-deoxynivalenol and $^{13}C_{18}$-zearalenone for correction of more and less polar analytes, respectively).

U-HPLC separation

An Accela U-HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) was used for the separation of target analytes. It was equipped with an Acquity UPLC HSS T3 analytical column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, Milford, MA, USA) held at 40°C for the separation of sample components. As the mobile phase, 5 mM ammonium formate in water (A) and methanol (B) was used. The gradient was as follows: start with 5% B, linear increase to 50% B in 6 min, for next 4 min another linear increase to 95% B, keep up to 15 min, switching to 5% B in 15.1 min, and column equilibration for 3 min before the next injection start. The flow rate was dependent on the employed ionisation technique, i.e. 300 or 500 μL min^{-1} for electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), respectively. The injection volume was 5 μL and the partial loop was used as an injection technique.

OrbitrapMS detection and the evaluation set-up

The operation parameters of the single-stage orbitrap mass spectrometer (Exactive; Thermo Fisher Scientific, Bremen, Germany) were optimised for two types of ion sources. Optimal conditions for the *heated electrospray interface* (HESI-II; Thermo Fisher Scientific, Bremen, Germany) were as follows: sheath gas/aux gas: 35/10 arbitrary units, capillary temperature: 250°C, heater temperature: 250°C, capillary voltage: +60/–50 V, and spray voltage +4/−3.1 kV. Regarding the *atmospheric pressure chemical ionisation interface* (APCI; Thermo Fisher Scientific, Bremen, Germany), the following optimal parameters settings were used: sheath gas/aux gas: 55/10 arbitrary units, capillary temperature: 250°C, vaporiser temperature: 320°C, capillary voltage: +60/−50 V, discharge current: 5 μA.

The system was operated in the full spectral acquisition mode in the mass range of *m/z* 100–1000 at different resolving power settings of 10 000, 25 000, 50 000, and 100 000 FWHM at fixed acquisition rates of 10, 4, 2, and 1 spectrum s^{-1}, respectively, in both positive and negative ionisation mode. The external mass axis calibration without the use of the specific lock mass was employed. For the mass accuracy estimation, mass at the apex of the chromatographic peak obtained as the extracted ion chromatogram was used. The calculated (exact) masses of analytes ions are summarised in Table 2.

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Table 2. Overview of the quantification (in bold) and confirmation ions together with their relative intensity (in parentheses) obtained for particular mycotoxins by the orbitrapMS instrument

Analyte	Retention time (min)	Elemental formula	Exact mass $[M+H]^+$ *m/z*	Exact mass $[M+NH_4]^+$ *m/z*	Exact mass $[M+Na]^+$ *m/z*	Exact mass $[M-H]^-$ *m/z*	Exact mass $[M+HCOO]^-$ *m/z*	Exact mass $[M+CH_3COO]^-$ *m/z*
Nivalenol	2.4	$C_{15}H_{20}O_7$				311.1136 (0.02)	**357.1191 (1)**	371.1348 (0.05)
Deoxynivalenol	3.3	$C_{15}H_{20}O_6$				295.1187 (0.11)	**341.1242 (1)**	355.1398 (0.09)
Deoxynivalenol-3-glucoside	3.4	$C_{21}H_{30}O_{11}$				457.1715 (0.02)	**503.1770 (1)**	517.1927 (0.11)
Deepoxydeoxynivalenol	4.5	$C_{15}H_{20}O_5$	281.1348 (0.98)	298.1649 (0.6)			**325.1293 (1)**	
Fusarenon-X	4.5	$C_{17}H_{22}O_8$	**355.1387 (1)**				399.1297 (0.75)	413.1453 (0.05)
Neosolaniol	4.9	$C_{19}H_{26}O_8$		**400.1966 (1)**	405.1520 (0.02)			
Verrucarol	5.2	$C_{15}H_{22}O_4$	**267.1591 (1)**	284.1856 (0.32)			311.1500 (0.16)	
3-Acetyldeoxynivalenol	5.7	$C_{17}H_{22}O_7$	**339.1438 (1)**				383.1348 (0.41)	
Ochratoxin α	5.7	$C_{11}H_9ClO_5$	257.0211 (0.1)			**255.0061 (1)**		
Aflatoxin G2	6.5	$C_{17}H_{14}O_7$	**331.0812 (1)**			329.0667 (0.13)		
Aflatoxin G1	6.8	$C_{17}H_{12}O_7$	**329.0656 (1)**			327.0510 (0.14)		
Altenuene	7.1	$C_{15}H_{16}O_6$	**293.1020 (1)**			291.0874 (0.07)	337.0924 (0.51)	351.1085 (0.07)
Aflatoxin B2	7.2	$C_{17}H_{14}O_6$	**315.0863 (1)**			313.0718 (0.02)		
Aflatoxin B1	7.5	$C_{17}H_{12}O_6$	**313.0707 (1)**			311.0561 (0.05)		
Diacetoxyscirpenol	7.6	$C_{19}H_{26}O_7$	367.1751 (0.06)	**384.2017 (1)**				
Ochratoxin A	8.5	$C_{20}H_{18}ClNO_6$	404.0901 (0.42)			**402.0749 (1)**		
Alternariol	8.7	$C_{14}H_{10}O_5$	259.0601 (0.06)			**257.045 (1)**		
HT-2 toxin	8.7	$C_{22}H_{32}O_8$	425.2170 (0.25)	**442.2435 (1)**				
β-Zearalenol	9.2	$C_{18}H_{24}O_5$	321.1697 (0.21)			**319.1546 (1)**		
T-2 toxin	9.6	$C_{24}H_{34}O_9$		**484.2541 (1)**	489.2095 (0.003)			
α-Zearalenol	9.9	$C_{18}H_{24}O_5$	321.1697 (0.19)			**319.1546**		
Ergosine	10.2	$C_{30}H_{37}N_5O_5$	**548.2867 (1)**			546.2722 (0.75)		
Zearalenone	10.2	$C_{18}H_{22}O_5$	319.1540 (0.25)			**317.1394**		
Sterigmatocystin	10.6	$C_{18}H_{12}O_6$	**325.0712 (1)**			323.0561 (0.03)		
Alternariol-methylether	10.7	$C_{15}H_{12}O_5$	273.0758 (0.71)			**271.0607 (1)**		
Ergocornine	10.7	$C_{31}H_{39}N_5O_5$	**562.3024 (1)**			560.2878 (0.96)		
Ergosinine	11.8	$C_{30}H_{37}N_5O_5$	**548.2867 (1)**			546.2722 (0.72)		
Ergocryptine	11.1	$C_{32}H_{41}N_5O_5$	**576.3180 (1)**			574.3035 (0.82)		
Ergocristine	11.2	$C_{35}H_{39}N_5O_5$	610.3024 (0.93)			**608.2878 (1)**		
Ergocorninine	11.8	$C_{31}H_{39}N_5O_5$	**562.3024 (1)**			560.2878 (0.89)		
Ergocryptinine	12.1	$C_{32}H_{41}N_5O_5$	**576.3180 (1)**			574.3035 (0.91)		
Ergocristinine	12.3	$C_{35}H_{39}N_5O_5$	610.3024 (0.86)			**608.2878 (1)**		

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Sample preparation for DART–orbitrapMS profiling

The use of acetonitrile for the purification was demonstrated for a pale lager beer brand. The mass spectra of (i) raw beer together with (ii) the purified supernatant obtained after acetonitrile addition and centrifugation, as well as (iii) the dark coloured matrix precipitate were acquired employing ambient MS using a direct analysis in real time (DART)[21] ion source. To reliably compare the mass spectra of these three fractions, they were introduced into the ion source in the same solvent (water containing 5% of ethanol) to prevent signal suppression/enhancement due to presence of acetonitrile. Samples for the DART–orbitrapMS profiling were processed as follows:

(i) Beer: raw degassed beer without any adjustment;
(ii) Upper fraction: 5 mL of supernatant was evaporated and redissolved in 1 mL of 5% ethanol in water (the matrix equivalent was 1 mL of beer per 1 mL of solution);
(iii) Bottom fraction: The supernatant was poured off and the sediment was redissolved in 4 mL of 5% ethanol in water (the matrix equivalent was 1 mL of beer per 1 mL of solution).

DART–orbitrapMS conditions

The DART–MS system consisted of a DART ion source (DART-SVP) with a 12 Dip-It tip scanner autosampler (IonSense, Saugus, MA, USA) coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). A Vapur interface (IonSense, Saugus, MA, USA) was employed to hyphenate the ion source and the mass spectrometer. Low vacuum in the interface chamber was maintained by a membrane pump (Vacuubrand, Wertheim, Germany).

The DART–MS instrument was operated in both positive and negative ionisation modes. The settings of the system parameters were as follows: (i) DART ionisation: helium flow: 2.5 L/min; gas temperature: 250°C; discharge needle voltage: −5000 V; grid electrode: ±350 V; (ii) mass spectrometric detection: capillary voltage: −50 V (negative mode); +30 V (positive mode); tube lens voltage: −110 V (negative mode); +110 V (positive mode); capillary temperature: 250°C. The acquisition rate was set to 2 spectra/s corresponding to a mass resolving power of 50 000 FWHM (*m/z* 200). A constant speed of 0.5 mm/s was used for the Dip-It tip scanner autosampler.

UPLC–TOFMS method

For UPLC an Acquity system (Waters, Milford, MA, USA) was used. The flow rate of mobile phase was 300 μL min^{-1}, other chromatographic conditions were the same as described in the section ''U-HPLC separation''.

For the TOFMS detection, an LCT Premier XE instrument (Waters Milford, MA, USA) with a dual ESI source was used. Parameters of the ion source were as follows: capillary voltage 3.5 kV, cone voltage 40 V, desolvation temperature 350°C, source temperature 120°C, cone gas flow 10 L h^{-1}, and desolvation gas flow 750 L h^{-1}. The leucine-enkephalin lock mass calibrant (flow 10 μL min^{-1}) was measured every 40 scans. The mass spectrometer was used in W– and W+ mode with a mass resolving power of 12 500 FWHM. Acquisition of the mass range *m/z* 100–1000 was done with the applied dynamic range enhancement (DRE) function and an acquisition time of 0.4 s. The detector was operated in negative ionisation mode.

Validation of the method

Validation experiments were performed with three types of beer brands available at the Czech market, i.e. (i) pale lager beer (alcohol content: 4.9% volume, grading: 11.9% mass), (ii) non-alcoholic beer (alcohol content: 0.5% volume), and (iii) dark lager beer (alcohol content: 4.7% volume, grading: 11.9% mass). For calibration purposes, beer matrix-matched calibration standards at concentrations of 0.5, 1, 3, 5, 10, 15, 20, 40, 60, 100, 200, and 250 μg L^{-1} were prepared for each beer brand (aliquots of multi-mycotoxin composite working standard solution were mixed with corresponding blank beer samples, i.e. the sample with undetectable levels of mycotoxins, processed according to the section ''Sample preparation for U-HPLC–orbitrapMS''), each vial contained the $^{13}C_{15}$-labelled deoxynivalenol and $^{13}C_{18}$-labelled zearalenone as the internal standards at a level of 20 μg L^{-1}. For assessment of matrix effects, solvent standards dissolved in a methanol/water mixture (50:50, v/v) at concentration levels of 0.5, 1, 3, 5, 10, 15, 20, 40, 60, 100, 200, and 250 μg L^{-1} were prepared. To determine method trueness, analyses of spiked beer samples (spiking levels 15, 30 and 60 μg L^{-1}, each in six repetitions) were carried out (the concentration of added isotopically labelled deoxynivalenol and zearalenone was, similarly, as above, 20 μg L^{-1}).

All of the validation measurements were realised under the APCI conditions with a mass resolving power of 100 000 FWHM in two chromatographic runs, one in positive and the second in the negative ionisation mode. For evaluation of quantitative performance, a mass extraction window of ±8 ppm was used.

RESULTS AND DISCUSSION

Sample preparation

Considering the current trend in the analysis of multiple food contaminants emphasising high-throughput approaches, maximal simplification of the sample preparation step, or, preferably, direct analysis of liquid sample such as beer, might seem a challenging option. However, in this particular case, injection of such a matrix directly onto the chromatographic column was not feasible because of its very high complexity (beer contains a wide range of low molecular compounds representing various structure classes with different polarity). A poor detectability of target analytes due to a relatively high chemical noise and matrix suppression of their signal was encountered. In addition to this limitation, the lowering of analytical column lifetime together with rapid contamination of ion source might be experienced as a consequence of omitting the sample clean-up. With regard to the wide range of physicochemical properties of the 32 mycotoxins and their metabolites involved in our study, neither adsorption nor immunoaffinity chromatography represented a conceivable strategy. The only simple approach to eliminate at least part of the matrix components, while keeping target analytes in solution, was to reduce the polarity

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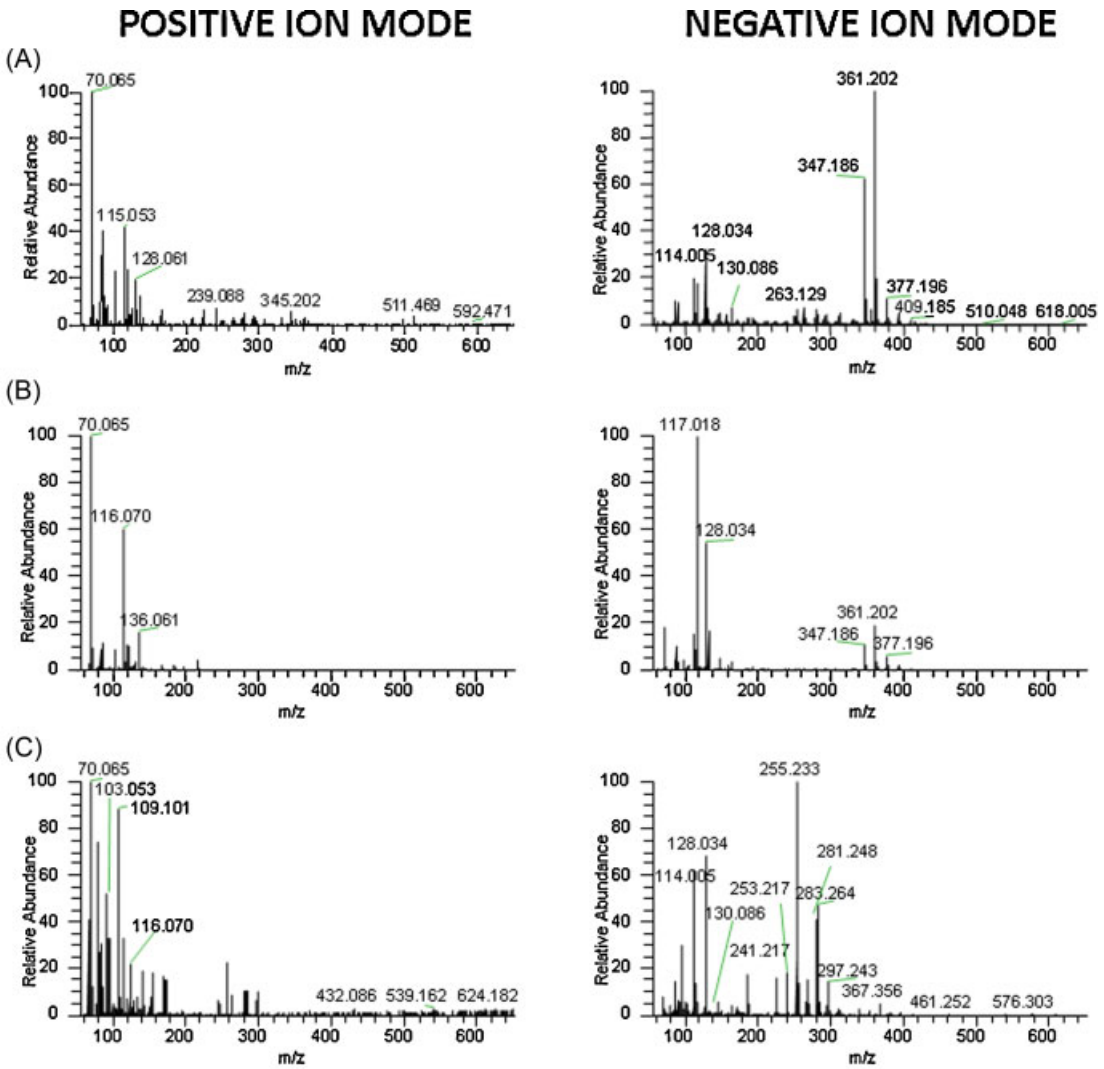
of beer samples by addition of the water-miscible solvent, acetonitrile. To document rapidly the purification effect achieved by precipitation of insoluble bulky matrix components, ambient MS employing a DART ion source coupled with orbitrapMS was employed. As shown in Fig. 1, most of the low molecular weight compounds yielding the ions in the range *m/z* 50–300 were removed from the liquid phase and transferred into the precipitate (among them, mainly fatty acids such as palmitic, palmitoleic, oleic, and stearic were identified), whereas the hop acids, e.g. humulone, cohumulone, and humulinone, remained in the liquid phase. In addition to brown pigments (melanoidins), it may be assumed that also hydrocoloids such as proteins and dextrins, not amenable to DART-MS, were removed by precipitation. As discussed below, no reduction in recoveries due to sorption of analytes on matrix precipitate occurred.

MS detection

In our previous experiments concerned with occurrence of *Fusarium* mycotoxins in beer, tandem mass spectrometry (MS/MS) was employed for detection/quantification of target analytes.[9,10,22] Unfortunately, retrospective data mining was impossible by this approach. To explore the potential of high-resolution full spectral mass analysers enabling comprehensive profiling of respective sample, and, if needed, also realisation of the non-target search, TOFMS and orbitrapMS technologies were employed and critically assessed. Figure 2 demonstrates the quality of data acquired by these two MS



Positive ion mode

$[C_7H_{12}O–H_2O+H]^+$ (*m/z* 95.085), $[C_7H_{14}O–H_2O+H]^+$ (*m/z* 97.101), $[C_8H_8O–H_2O+H]^+$ (*m/z* 103.053), $[C_8H_{14}O–H_2O+H]^+$ (*m/z* 109.101), proline (*m/z* 116.070)

Negative ion mode

Proline (*m/z* 114.005), succinic acid (*m/z* 117.018), pyroglutamic acid (*m/z* 128.034), leucine/isoleucine (*m/z* 130.086), palmitoleic acid (*m/z* 253.217), palmitic acid (*m/z* 255.233), oleic acid (*m/z* 281.248), stearic acid (*m/z* 283.264), cohumulone (*m/z* 347.186), n-/ad-humulone (*m/z* 361.202), humulinone (*m/z* 377.196)

Figure 1. Positive and negative DART–orbitrapMS profiles of (A) original beer, (B) purified beer, and (C) brown-coloured precipitate (for details concerning the sample preparation, see Experimental section).

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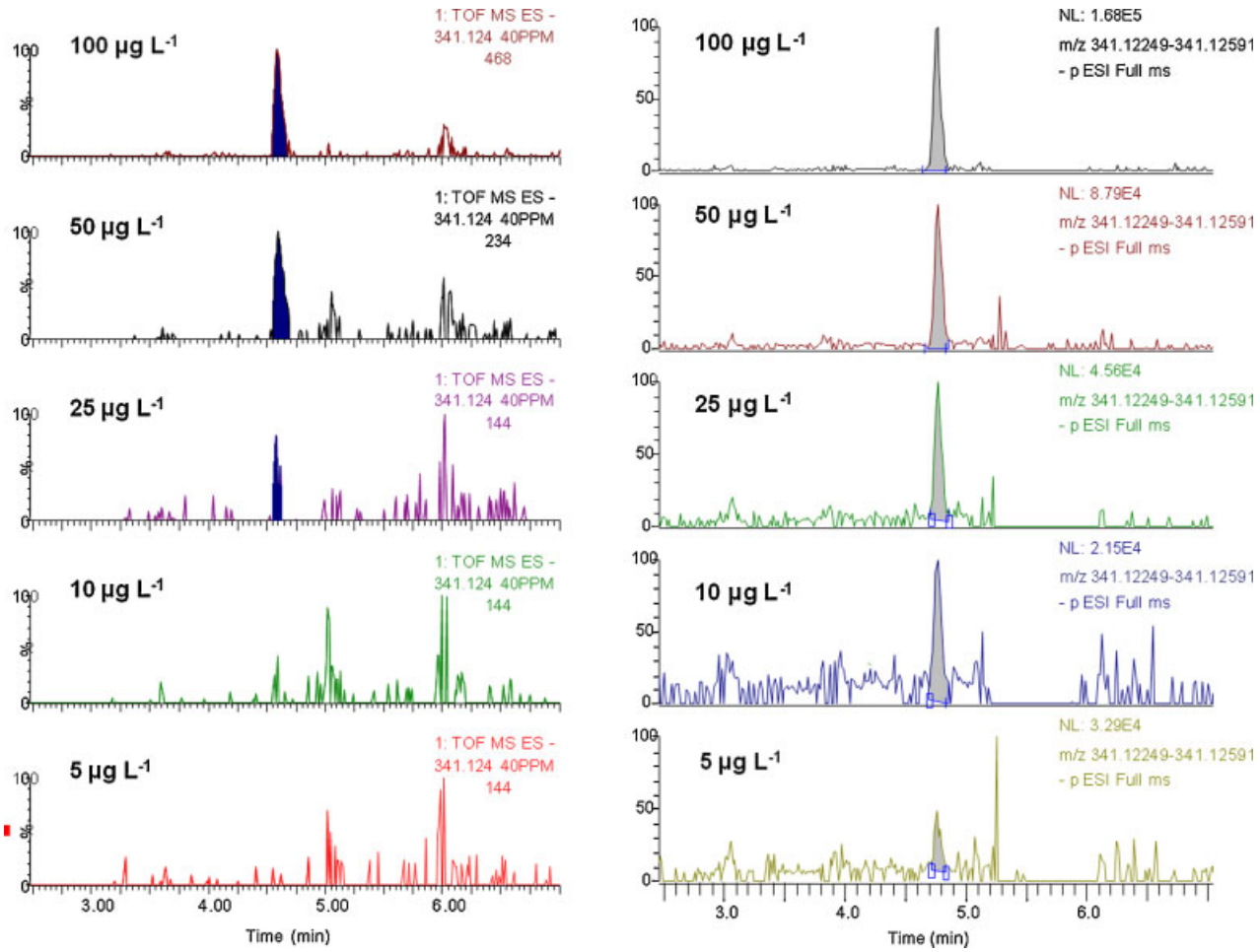

Figure 2. Comparison of detection capabilities of two mass spectrometric systems: (A) UPLC–ESI-TOFMS (12 500 FWHM, mass extraction window 40 ppm) and (B) U-HPLC–ESI-orbitrapMS (100 000 FWHM, mass extraction window ±5 ppm).

systems under their best achievable mass spectrometer settings (LC conditions, as well as the sample preparation, were identical). The TOFMS instrument operated at its maximum attainable mass resolving power (approximately 12 500 FWHM), and orbitrapMS at the 'ultra-high' mass resolving power of 100 000 FWHM (maximum setting). As can be seen in Fig. 2, employing the TOFMS system it was not possible to detect DON at concentration levels below 25 µg L^{-1}, while orbitrapMS (at its highest resolving power) enabled detection of even 5 µg L^{-1}. Based on these facts, the follow-up work was focused on implementation of the orbitrapMS system as the detection tool.

Optimisation of orbitrapMS detection

The set of experiments with different settings of the Exactive, the orbitrapMS instrument, was realised with the aim of assessing its capability for multiple mycotoxins analysis. The impact of examined parameters is discussed in the following paragraphs.

ESI vs. APCI

Most of the published studies concerned with determination of multiple mycotoxins used an electrospray ionisation (ESI) source for their ionisation; therefore, in the first phase of the experiments, we also focused on this ionisation principle. However, as we discovered, the quantification limits were rather poor for several *Fusarium* toxins, especially for deoxynivalenol and its glucoside. With regard to the importance of detecting these most common natural beer contaminants at very low levels, the capability of atmospheric pressure chemical ionisation (APCI) was tested. The increase in the signals under the APCI conditions for almost all of analytes (with the exception of ochratoxin A, which showed better ionisation efficiency under the ESI conditions) is illustrated in Fig. 3. As shown in this figure, up to a 12-fold enhancement in detectability of *Fusarium* toxins was achieved.

Mass resolving power and extraction window width setting

In the routine trace analysis, both high mass resolving power and high mass accuracy play important roles in the unbiased identification and reliable quantification of target analytes (this topic is discussed in detail by Kellmann *et al.*[16]).

When measuring the validation samples under the maximum mass resolving power setting of 100 000 FWHM (i.e. 'ultra-high resolution' mode with an acquisition rate of 1 spectrum s^{-1}), 86% of analytes showed a mass error less than 3 ppm, and the worst value did not exceed 5 ppm. However, no significant worsening of mass accuracy was observed under the conditions of 'high resolution' mode 50 000

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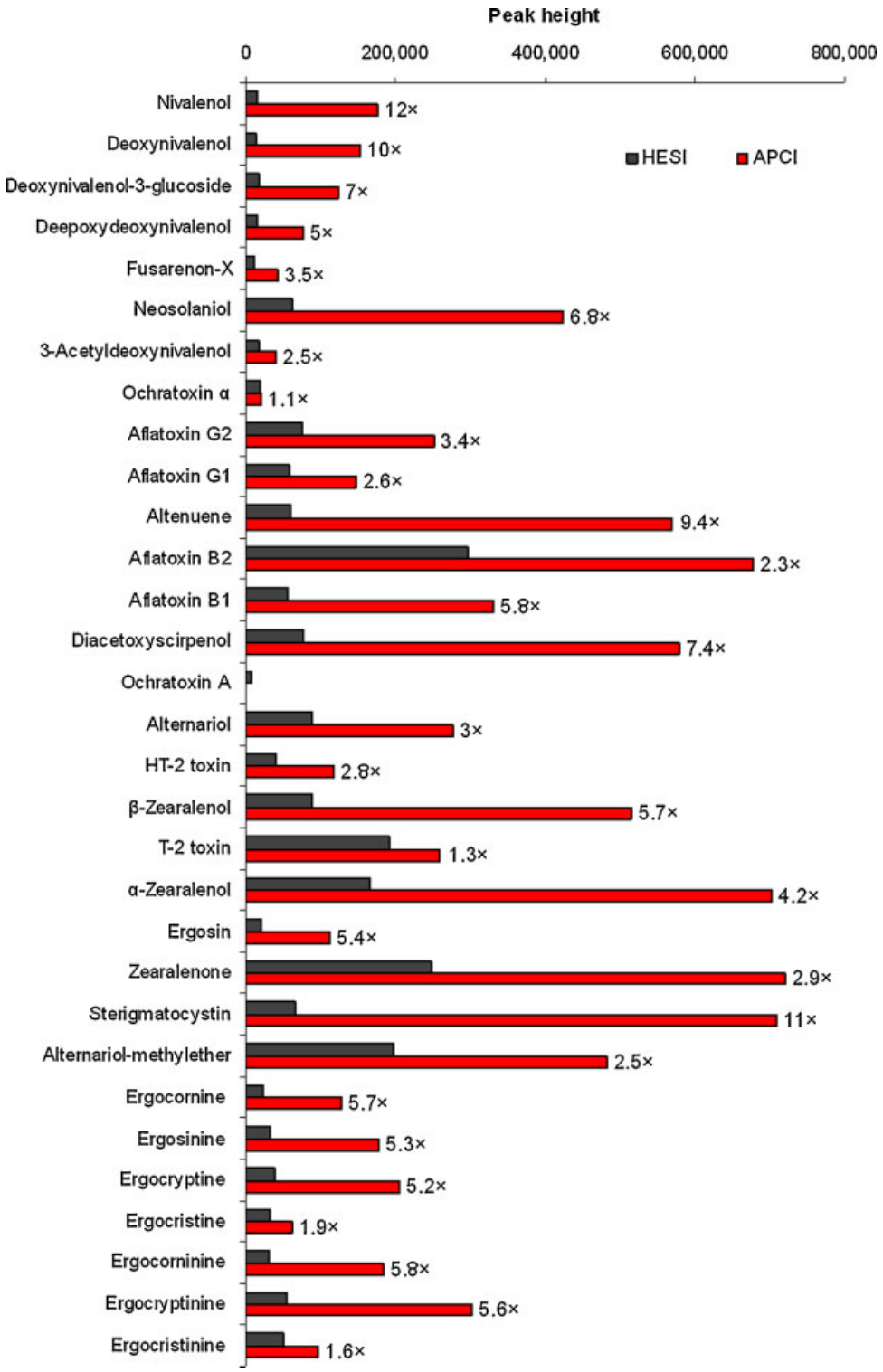

Figure 3. Efficiency of ESI and APCI demonstrated at the spiked beer using 100 000 FWHM mass resolving power setting (the increase of signal in APCI compared to ESI is indicated). Spiking level was 20 $\mu g\,L^{-1}$ for each mycotoxin with the exception of deepoxynivalenol, 3-acetyldeoxynivalenol, ochratoxin alpha, and ochratoxin A, where it was 80 $\mu g\,L^{-1}$.

FWHM. Thanks to the higher acquisition rate facilitated by this detection mode (2 spectra s^{-1}), acquiring both positive and negative ions within a single run was possible, thus allowing a higher sample throughput. Under these conditions, approximately eight points per each U-HPLC peak (with widths around 8 s) were obtained, which enabled optimal shape without the need for significant peak smoothing.

For the purpose of evaluation of data, an optimal width of mass extraction window common to all of the analyses was sought to discriminate isobaric matrix interferences, and, at the same time, avoid false negatives when data processing. Employing too high a mass accuracy tolerance resulted in a worsened detection selectivity. On the other hand, using too narrow mass extraction window led either to distortion of the

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peak shape (caused by leaving out of some data point due to an exceeded mass tolerance of particular one) or, in some cases to false negatives. Based on our data, the window width of ±8 ppm was chosen as a compromise for all of the analytes.

Validation of the U-HPLC–orbitrapMS method

In the final phase, the optimised multiple mycotoxin U-HPLC–orbitrapMS method was thoroughly validated. Prior to analyses of spiked samples, the extent of matrix effects was investigated in order to decide on the quantification strategy. For this purpose, four calibration sets (standards in pure solvent, and matrix-matched standards of pale lager, non-alcoholic, and dark lager beer) in the concentration range 0.5–250 $\mu g\,L^{-1}$ were prepared. As seen in Table 3, the extent of matrix effects calculated in percentages as the ratio of the solvent calibration slope minus matrix calibration slope and solvent calibration slope was not too high, in maximum 37%, which documents good effectiveness of the matrix precipitation procedure involved in the sample preparation step. The matrix effects did not differ significantly between pale lager and non-alcoholic beer; in the case of dark beer, they seemed to be slightly higher. However, to compensate the analytical bias to the maximal extent, the use of corresponding matrix-matched standards was preferred in subsequent validation experiments. Besides the effort of avoiding the underestimation of results caused by the ion suppression of co-eluting matrix, use of standards in pure solvent was impossible also because of a risk of an opposite problem, the 'matrix-induced signal enhancement'. Immediately after the routine cleaning of the ion optic, particularly the ion transfer capillary, some analytes injected in pure solvent were partially lost (see Fig. 4, where calibration curves of deoxynivalenol-3-glucoside in solvent and matrix-matched standards are compared both under the standard conditions, and immediately after the cleaning). It can be assumed that the above phenomenon is caused by similar processes as those occurring in a hot GC splitless injector where adsorption and/or thermal degradation of analytes takes place on active sites of the system unless a (protective) matrix is present.[23]

Another issue we attempted to address was defining an equivalent to the limit of quantification (LOQ), performance characteristics commonly employed in MS/MS. Contrary to this detection approach, the 'classic' definition based on signal-to-noise (S/N) ratio (typically S/N ratio >10) is not always applicable in high-resolution MS detection mode because chemical noise is in fact absent in the chromatogram, especially when a narrow extraction window is employed (under such conditions, unrealistic, very high values of S/N ratio might be calculated by the software). Due to that fact, we decided to use a lowest calibration levels (LCLs) approach as the most suitable option.[24,25]

The LCLs of analytes in our study were experimentally established based on the (most intensive) quantification ion (see Table 2) as the lowest concentrations of matrix-matched standards which it was possible to repeatedly determine during a longer time period. In fact, the single-stage MS lacks more than one intensive (identification/quantification) ion with sufficiently high intensity (as shown in Table 2, relative intensities of appropriate confirmatory ions are mostly at least one order of magnitude lower), and thereby confirmation at such low levels is not possible. Gaining of additional confirmation ions by means of non-selective fragmentation in the ion source or in the collision cell in the parallel run also did not fix this problem, since the fragment ions did not reach even one-tenth of the intensity of the precursor ion (data not shown). However, in spite of those facts, reliability of measurement based on the quantification ions was thoroughly verified; the relative standard deviations (RSDs) calculated from nine repeated injections at the LCLs were for particular analytes as low as 11–28%, see Table 3 (retention time of target analytes did not fluctuate more than ±2%). While these LCLs for 91% of analytes were at the $10^0\ \mu g\ L^{-1}$ level, a relatively high LCL was found for ochratoxin A (approx. 60 $\mu g\,L^{-1}$), which showed much better ionisation under ESI conditions (LCL less than 5 $\mu g\,L^{-1}$).

Through the validation experiments, good trueness of the generated data was documented for all three beer matrices tested: the recoveries of analytes tested at levels of 15, 30, and 60 $\mu g\,L^{-1}$ ranged from 86 to 124%; no losses of analytes during the sample preparation occurred, RSDs did not exceed 11% (Table 3). Recoveries as well as RSD values obtained during validation are in compliance with Commission Regulation EC No. 401/2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. The linear range of the presented method was tested for solvent as well as for matrix-matched calibration curves constructed in the ranges from LCL to 250 $\mu g\,L^{-1}$. The majority of analytes showed linearity in the range 0.9960–0.9999 (R^2), and any decline in R^2 value caused by the presence of matrix was noticed.

As already mentioned in the section ''ESI vs. APCI'', APCI was chosen for all realised validation experiments owing to enhanced detection sensitivity, especially for deoxynivalenol and its glucosylated form. However, we should also note some limitations of this approach. Fumonisins, relatively high molecular weight compounds with a carboxylic function group, also belonging to the *Fusarium* mycotoxins, do not show ionisation efficiency under APCI conditions at all. Moreover, these relatively problematic compounds require acidic chromatographic conditions (0.1% formic acid in water) to prevent their dissociation during separation, which is, however, limiting for other *Fusarium* toxins, mainly for type B trichothecenes.[17] From this reason, fumonisins have to be determined separately anyway. Afterwards, if a separate analytical run for fumonisins analysis is performed, an isochronal analysis of ochratoxin A is typically conducted to achieve better ionisation efficiency under ESI.

Special attention has to be paid to quantification of ergot alkaloids: the '-ine' forms of the ergopeptides (biologically active *R*-forms) are transformed into the '-inine' epimers (biologically inactive *S*-forms) when they are solvated. The rate of the equilibration process depends on the type of solvent and on the temperature,[26] and it is also influenced by the pH value.[27,28] According to our findings, the equilibrium ratio of respective epimers (-inine to -ine form) is approximately 1.5 to 1 in a methanol/water (50:50, v/v) mixture as well as in the presence of beer matrix. As far as a single solid epimer is available as the calibrant, then some 'aging' of its standard solution should be allowed before its use to avoid incorrectness in quantification.

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Table 3. Performance characteristics of developed U-HPLC–orbitrapMS method

Mycotoxin	LCL of pure standard; μg L^{-1}	LCL of matrix-matched standard (pale lager/ non-alcoholic/ black lager); μg L^{-1}	RSD (%) at the LCL for pale lager/ non-alcoholic/ black lager[a]	Recoveries for pale lager/non-alcoholic/ black lager; (mean RSD)[b] (%)			ME (%)[c] for pale lager/ non-alcoholic/ black lager
				spike 15 μg L^{-1}	spike 30 μg L^{-1}	spike 60 μg L^{-1}	
Nivalenol	2	6/4/6	19/21/18	107/96/103 (8.9)	97/92/106 (6.2)	103/91/89 (6.1)	8.1/13/-10
Deoxynivalenol	2	3/3/3	24/17/21	104/93/95 (4.9)	108/103/97 (8.4)	99/94/101 (7.6)	-11/-12.2/-27
Deoxynivalenol-3-glucoside	2	2/3/3	23/25/19	96/111/107 (6.3)	103/105/88 (4.6)	100/92/97 (5.4)	-8.1/-14/-19
Deepoxydeoxynivalenol	4	15/15/20	19/21/26	102/107/91 (7.2)[d]	116/87/104 (9.6)[d]	104/101/99 (7.2)[d]	6.1/8/14
Fusarenon-X	2	4/3/9	16/19/18	105/115/96 (9.3)	113/102/96 (9.8)	119/109/92 (9.0)	25/11/-19
Neosolaniol	2	2/1.5/3	14/21/16	99/94/108 (7.6)	111/92/101 (6.7)	112/88/98 (7.7)	9.1/13/32
Verrucarol	3	4/4/6	18/19/20	98/91/89 (8.4)	99/94/99 (8.5)	101/96/97 (6.8)	16/18/27.4
3-Acetyldeoxynivalenol	4	8/8/18	24/26/28	103/99/112 (14)[d]	96/103/113 (7.7)[d]	102/89/101 (6.1)[d]	14/16/-2
Ochratoxin α	4	30/30/38	21/26/19	102/94/97 (9.8)[d]	98/97/102 (9.9)[d]	108/104/92 (6.6)[d]	37/21/28
Aflatoxin G2	1	2/0.5/3	25/19/24	103/101/105 (11)	106/94/98 (7.3)	99/94/96 (6.1)	15/19/20
Aflatoxin G1	1	3/1/3	19/16/27	117/102/99 (8.9)	94/94/99 (8.3)	107/101 /104 (9.5)	9/13/16
Altenuene	0.5	1/1.5/6	22/24/18	119/95/92 (8.4)	120/117/101(4.2)	113 /86/97 (8.7)	7.1/11/30
Aflatoxin B2	0.5	0.5/0.5/0.5	12/19/14	111/107/93 (6.5)	106/88/89 (3.9)	104/96/99 (4.8)	9.1/7/18
Aflatoxin B1	0.5	2/1.5/3	13/18/17	107/92/98 (5.2)	90/97/101 (6.8)	92/99/96 (6.4)	-5.0/15/17
Diacetoxyscirpenol	0.5	1/1.5/1.5	17/21/24	116/98/117 (9.4)	113/107/96 (7.2)	124/114/95 (8.1)	6.1/5/26
Ochratoxin A	60	60/45/65	26/32/27	105/107/87 (9.1)[d]	96/101/99 (8.8)[d]	97/92/91 (6.2)[d]	16/19/22
Alternariol	0.5	2/1.5/2	16/24/29	101/89/97 (8.5)	107/96/99 (9.3)	98/102/104 (8.9)	24/27/28
HT-2 toxin	2	4/4/6	19/29/26	117/95/90 (6.9)	116/101/92 (4.3)	104/89/96 (6.4)	13/14/-18
β-Zearalenol	1	2/1.5/6	11/26/18	111/107/92 (9.1)	92/89/99 (7.9)	98/101/97 (9.3)	15/11/-6.2
T-2 toxin	1	2/1.5/3	17/16/21	99/91/104 (8.5)	119/112/88 (7.8)	105/99/106 (10)	12/23/20
α-Zearalenol	1	1/1.5/5	16/18/23	114/112/97 (8.9)	107/107/96 (9.2)	97/88/89 (7.2)	16/6/28
Ergosine	1	3/1.5/3	26/24/17	111/114/94 (12.9)	109/99/92 (8.8)	106/101/111 (6.6)	22/18/-8
Zearalenone	1	1/1/3	19/11/15	106 104/89 (9.4)	117/92/97 (8.2)	105/94/93 (6.9)	9.1/14/16
Sterigmatocystin	0.5	0.5/0.5/0.5	16/19/20	118/91/95 (10.1)	98/96/102 (8.7)	110/107/95 (6.8)	-7.0/-11/19
Alternariol-methylether	1	1/1/3	14/18/23	114/91/93 (9.1)	109/106/94 (9.5)	113/102/92 (8.9)	12/9.2/17
Ergocornine	1	2/1.5/3	20/27/24	115/104/97 (9.6)	121/97/87 (7.6)	102/102/96 (7.2)	19/14/24
Ergosinine	1	2/2/4	12/18/20	98/107/111 (8.4)	114/102/89 (6.9)	102/99/91 (6.8)	9/11/-6
Ergocryptine	1	2/0.5/2	23/15/19	103/95/91 (12.2)	111/92/94 (6.2)	101/94/95 (7.9)	-1.0/5.6/11
Ergocristine	2	5/4/5	24/26/17	95/90/112 (6.1)	112/114/95 (8.4)	94/89/87 (5.6)	19/18/22
Ergocorninine	1	2/1.5/3	15/19/24	114/105/97 (11.7)	124/97/101 (11)	104/105/93 (7.3)	5.1/4.8/6
Ergocryptinine	1	2/1.5/3	26/19/21	88/89/101 (8.6)	113/103/99 (8.2)	101/99/107 (6.6)	3.1/4.5/9.2
Ergocristinine	2	5/2/5	28/24/29	104/102/95 (7.1)	119/102/97 (7.6)	99/92/86 (8.3)	-3.0/-6/8

[a] RSD at the LCL was calculated from 11 repeated injections of the particular matrix-matched standard.
[b] RSD at each spiking level was calculated from 6 spikes; mean level for all three matrices is presented.
[c] ME(%) = (solvent calibration slope – matrix matched calibration slope)/solvent calibration slope * 100%.
[d] The spiking levels of ochratoxin A and ochratoxin alpha were 80, 100, 120 μg L^{-1}; for deepoxydeoxynivalenol and 3-acetyldeoxynivalenol 25, 50, 100 μg L^{-1}.

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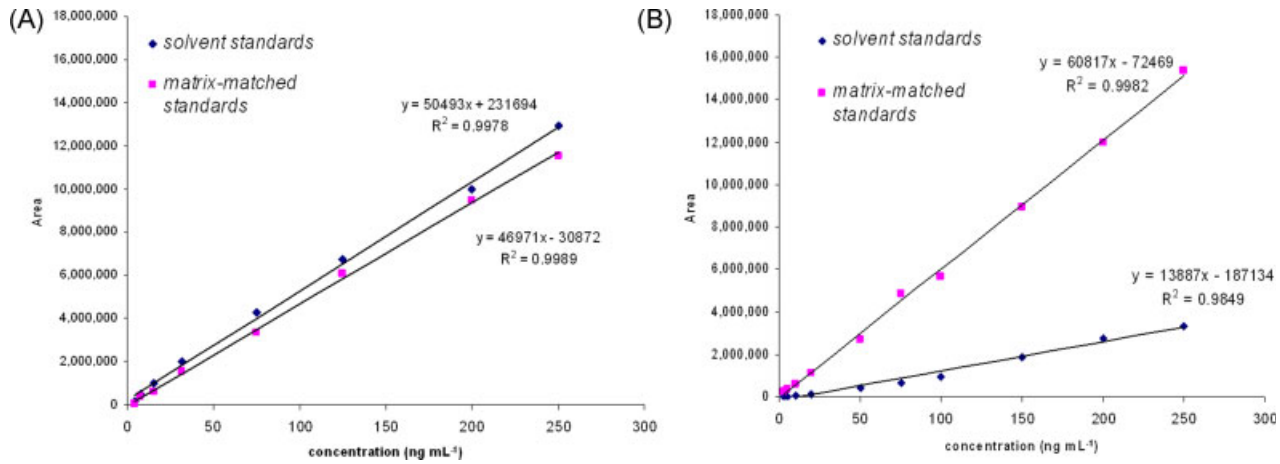

Figure 4. (A) The matrix-induced signal suppression of deoxynivalenol-3-glucoside under the standard conditions. (B) The matrix-induced signal enhancement of deoxynivalenol-3-glucoside when employing the ion transfer capillary immediately after its cleaning.

CONCLUSIONS

The U-HPLC–orbitrapMS technology represents a progressive alternative equivalent to MS/MS. Although the file sizes of this full spectral detection tool are approximately 500 times higher as compared with MS/MS, it provides the very profitable possibility of retrospective data mining. The U-HPLC–orbitrapMS system used within this study operating in APCI mode enabled rapid determination of trace levels of multiple mycotoxins potentially occurring in beer samples. Achievement of good trueness of obtained data for analytes occurring at low $\mu g\ L^{-1}$ levels was only possible under the following conditions:

(i) To reduce the ion suppression phenomenon, and the isobaric interferences originating from abundant co-eluting matrix components, they had to be removed by precipitation achieved by decreased of sample polarity.
(ii) At the (ultra)high mass resolving power settings of 100 000 or 50 000 FWHM, the mass error not exceeding 5 ppm (without the use of internal mass correction) enabled the use of a very low mass extraction window ±8 ppm for the routine work, which significantly improved the selectivity of detection.
(iii) For sensitive detection of ochratoxin A, ESI has to be employed.
(iv) For reliable quantification of analytes, the matrix-matched calibration should be preferred. To control potential losses due to partitioning between precipitate and aqueous phase during the sample preparation, the ^{13}C-labelled internal standard has to be employed as a surrogate.

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