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Multi-mycotoxin Screening and Quantitation Using UHPLC, High Resolution and Accurate Mass

Milena Zachariášová, Jana Hajšlová, Institute of Chemical Technology, Prague, Czech Republic
Michal Godula, Thermo Fisher Scientific, Prague, Czech Republic

Key Words

- Exactive LC-MS
- Beer
- Cereals
- Mycotoxins

Introduction

Mycotoxins are the toxic secondary metabolites produced by many species of microscopic filamentary 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.[1] In addition to the relatively common micro mycetes mentioned above, *Claviceps purpurea* which causes ergot disease, belongs to numerous barley pathogens.

Although the carry-over of aflatoxins, ochratoxin A, zearalenone, fumonisins, and ergot alkaloids from malted grains into beer was documented, the main research in this area focused on deoxynivalenol, the most frequent Fusarium mycotoxin.[2,3] In recent years, the presence of deoxynivalenol's main metabolite, deoxynivalenol-3-glucoside, has been reported at relatively high levels in malt and beer. This fact was further confirmed in the follow-up study, in which both deoxynivalenol and its glucoside were identified as the main contaminants of beers retailed on the European market.[4] As beer is a significant dietary constituent to a large portion of the population, control of mycotoxins in this commodity is very important. For this purpose, reliable analytical methods for fast and effective monitoring of mycotoxins during the beer production chain are needed.

There is a trend toward the simplification of sample preparation procedures as much as possible. Full spectral data acquisition techniques are also preferred because of their ease of usage, along with the possibility of retrospective archived data mining. Until now, the most common full spectral mass-spectrometric approach has been the time-of-flight technology (TOF-MS), with typical resolving power of approx. 12,500 FWHM (full width 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.[5,6] Mass spectrometry systems based on the Thermo Scientific Orbitrap technology routinely achieve mass resolving power of up to 100,000 FWHM and maintain excellent mass accuracy up to <5 ppm without the use of internal mass correction.[7]

The aim of this study was to introduce a multi-mycotoxin method for analysis of 32 mycotoxins in beer based on very simple sample preparation and ultra high performance liquid chromatography coupled with full spectral Orbitrap™ MS detection.

Mycotoxin standards of (i) Fusarium toxins, major conjugate and other products of transformation (nivalenol, deoxynivalenol, deoxynivalenol-3-glucoside, deepoxydeoxy - nivalenol, 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, ergocornine, ergocryptine, ergocristine) were provided by The Czech Agricultural and Food Inspection Authority. 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-methylether were dissolved in acetonitrile. Liquid standards of deepoxyde-oxynivalenol, diacetoxyscirpenol, HT-2 toxin, alfa-zear-alenole, beta-zearalenole, ochratoxin α, and ergot alkaloids were supplied in acetonitrile, and deoxynivalenol-3-glucoside was delivered in acetonitrile:water (1:1, v/v) solution. All of the standards were stored at -20 °C. For spiking experiments and calibration purposes, a composite working standard solution in acetonitrile (1000 µg L^{-1}) was prepared. All of the standards were brought to room temperature before use. The organic solvents acetonitrile and methanol (HPLC grade) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Ultra-pure water was produced by Milli-Q system (Millipore Corporation, Bedford, MA, USA).

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Sample Preparation

The aliquot of 4 mL of beer sample in PTFE cuvette was degassed in the ultrasonic bath, and after addition of 16 mL acetonitrile, the content was vigorously shaken for approximately 1 min. The dark colored matrix precipitated under these conditions and was then separated by centrifugation (10 min, 11,000 rpm). In the next step, the 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 UHPLC system, microfiltration was performed prior to injection (centrifugation through the 0.2 µm microfilter, (PVDF Zentrifugenfilter, Alltech, USA)).

To control potential losses due to partition between precipitate and aqueous phase, aliquots of ^{13C}-labelled deoxynivalenol and ^{13C}-labelled zearalenone standard solution were added as the surrogates prior to processing (^{13C}-deoxynivalenol and ^{13C}-zearalenone for correction of more and less polar analytes, respectively).

Instrument Setup and Conditions

The Thermo Scientific Accela UHPLC system was used for the separation of target analytes. Detection was carried out using a Thermo Scientific Exactive benchtop single stage mass spectrometer, powered by Orbitrap technology and operated in full scan mode at different resolution settings. The use of internal mass axis calibration (lock mass) was not necessary. Conditions used are summarized in Table 1. The capillary and tube lens were set for ±45 and ±115 V respectively.

For the mass accuracy estimation, the mass at the apex of the chromatographic peak, obtained as the extracted ion chromatogram, was used. The calculated (exact) masses of quantification ions are summarized in Table 2.

Results and Discussion

Considering the current trend of analyzing for multiple food contaminants while maintaining high throughput and simplified sample preparation, direct analysis of a liquid sample may seem like the preferred option. However, in this case, direct injection of the matrix directly on the chromatographic column was not feasible because of its very high complexity. Direct injection also provided poor detectability of target analytes due to high matrix interference. In addition to this limitation, direct injection also lowered the analytical column lifetime and rapidly contaminated the ion source. Because of the complex properties of the 32 mycotoxins and their metabolites, neither adsorption nor immunoaffinity chromatography represented a feasible sample preparative strategy. The only simple approach to eliminating at least part of the matrix components, while keeping target analytes in solution, was by reducing the polarity of beer sample by addition of water-miscible solvent – acetonitrile.

It should be noted, that until now, most published studies concerned with determination of multiple mycotoxins in a single analysis used electrospray source ionization (ESI). However, the detection limits obtained by ESI were still rather poor for several Fusarium toxins, particularly for DON and its conjugate. Due to the importance of reliable analysis of these very common natural beer contaminants, the capability of atmospheric pressure chemical ionization (APCI) was evaluated. The optimal flow rate of mobile phase was determined to be 5 mL min^{-1} and the vaporizer temperature was set to 250 °C. Under APCI conditions, the enhancement in detectability of Fusarium toxins was as high as 1200% of the value achievable by ESI.

UHPLC Conditions		**MS Conditions (APCI)**	
Column	Hypersil GOLD aQ, 100 mm × 2.1 mm i.d., 1.9 µm	Sheath Gas	35 units
Mobile phase A	5 mM NH_4COOH in water	Auxiliary Gas	10 units
Mobile phase B	Methanol	Capillary Temperature	250 °C
Flow Rate	500 µL/min	Vaporizer Temperature	250 °C
Column Temperature	40 °C	Capillary Voltage	+60/-50 V
Injection Volume	5 µL	Discharge Current	5 µA
Gradient Elution Program		Scan Range	100-1000 *m/z*
0.0 min	5% B	Resolution Settings	10,000
6.0 min	50% B	(FWHM)	25,000
10.0 min	95% B		50,000
15.0 min	95% B		100,000
15.1 min	5% B		
18.0 min	5% B		

Table 1: Accela™ UHPLC/Exactive MS settings

Recommended Thermo Fisher Scientific Supplies

- Hypersil GOLD aQ, *p/n 25302-102130*, Thermo Scientific
- Methanol Optima LC/MS Grade, *p/n A456-212*, Fisher Scientific
- Acetonitrile Optima LC/MS Grade, *p/n A955-212*, Fisher Scientific
- Water, *p/n W6-212*, Fisher Scientific
- Ammonium Formate, *p/n A666-500*, Fisher Scientific
- Fisherbrand™ Higher-Speed Easy Reader Plastic Centrifuge Tubes, *p/n 06-443-19*, Fisher Scientific

Analyte	Retention Time (min)	Elemental Formula	Molecular Weight Da	Exact Mass $[M+H]^+$ *m/z*	Exact Mass $[M+NH_4]^+$ *m/z*	Exact Mass $[M-H]^-$ *m/z*	Exact Mass $[M+HCOO]^-$ *m/z*
Nivalenol	2.4	$C_{15}H_{20}O_7$	312.1209				357.1191
Deoxynivalenol	3.3	$C15H_{20}O_6$	296.1260				341.1242
Deoxynivalenol-3-glucoside	3.4	$C_{21}H_{30}O_{11}$	458.1788				503.1770
Deepoxydeoxynivalenol	4.5	$C_{15}H_{20}O_5$	280.1311				325.1293
Fusarenon-X	4.5	$C_{17}H_{22}O_8$	354.1315				399.1297
Neosolaniol	4.9	$C_{19}H_{26}O_8$	382.1628		400.1966		
Verrucarol	5.2	$C_{15}H_{22}O_4$	266.1518		284.1856		
3-acetyldeoxynivalenol	5.7	$C_{17}H_{22}O_7$	338.1366				383.1348
Ochratoxin α	5.7	$C_{11}H_9ClO_5$	256.0139			255.0061	
Aflatoxin G2	6.5	$C_{17}H_{14}O_7$	330.0740	331.0812			
Aflatoxin G1	6.8	$C_{17}H_{12}O_7$	328.0583	329.0656			
Altenuene	7.1	$C_{15}H_{16}O_6$	292.0947				337.0924
Aflatoxin B2	7.2	$C_{17}H_{14}O_6$	314.0790	315.0863			
Aflatoxin B1	7.5	$C_{17}H_{12}O_6$	312.0634	313.0707			
Diacetoxyscirpenol	7.6	$C_{19}H_{26}O_7$	366.1779		384.2017		
Ochratoxin A	8.5	$C_{20}H_{18}ClNO_6$	403.0823	404.0901			
Alternariol	8.7	$C_{14}H_{10}O_5$	258.0528			257.045	
HT-2 Toxin	8.7	$C_{22}H_{32}O_8$	424.2097		442.2435		
β-zearalenol	9.2	$C_{18}H_{24}O_5$	320.1624			319.1546	
T-2 Toxin	9.6	$C_{24}H_{34}O_9$	466.2203		484.2541		
α-zearalenol	9.9	$C_{18}H_{24}O_5$	320.1624			319.1546	
Ergosin	10.2	$C_{30}H_{37}N_5O_5$	547.2795	548.2867			
Zearalenone	10.2	$C_{18}H_{22}O_5$	318.1467			317.1394	
Sterigmatocystin	10.6	$C_{18}H_{12}O_6$	324.0634	325.0712			
Alternariol-methylether	10.7	$C_{15}H_{12}O_5$	272.0685			271.0607	
Ergocornine	10.7	$C_{31}H_{39}N_5O_5$	561.2951	562.3024			
Ergosinine	11.8	$C_{30}H_{37}N_5O_5$	547.2795	548.2867			
Ergocryptine	11.1	$C_{32}H_{41}N_5O_5$	575.3108	576.3180			
Ergocristine	11.2	$C_{35}H_{39}N_5O_5$	609.2951	610.3024			
Ergocorninine	11.8	$C_{31}H_{39}N_5O_5$	561.2951	562.3024			
Ergocryptinine	12.1	$C_{32}H_{41}N_5O_5$	575.3108	576.3180			
Ergocristinine	12.3	$C_{35}H_{39}N_5O_5$	609.2951	610.3024			

Table 2: Overview of the most intensive ions used for quantification by the Exactive

The lone exception was ochratoxin A, which showed better ionization efficiency under the electrospray conditions, APCI was chosen for use because it provided significant improvement of detection limits for most of the tested analytes. The extracted ion chromatograms of individual mycotoxins shown in Figure 1 document very good and fast separation achieved on the Accela™ UHPLC system.

In a routine trace analysis, both high mass resolving power and high mass accuracy play an important role in the unbiased identification and reliable quantification of target analytes.[5] Figure 2 illustrates the benefits of high resolving power setting on the discrimination of isobaric interferences. The importance of optimal choice of extraction window width is demonstrated here mainly for the use of lower mass resolution. While the use of a wide mass window typically results in worsened selectivity, using a narrow mass window presents a risk of removing some analytes from the chromatogram.

As demonstrated in Figure 3, the risk of false negative results occurs, especially for low intensity ions. While 50 µg L^{-1} of deoxynivalenol-3-glucoside was still detectable at the mass resolving power setting of 10,000 FWHM, almost no signal was detected by the same mass resolution at level 5 µg L^{-1}. At resolving power of 25,000 FWHM, the peak shape was improved. When the resolving power of 50,000 and/or 100,000 FWHM was enabled, optimal peak shape of deoxynivalenol-3-glucoside at 5 µg L^{-1} was obtained. As demonstrated, the higher resolving power, the better mass accuracy of deoxynivalenol-3-glucoside is obtained.

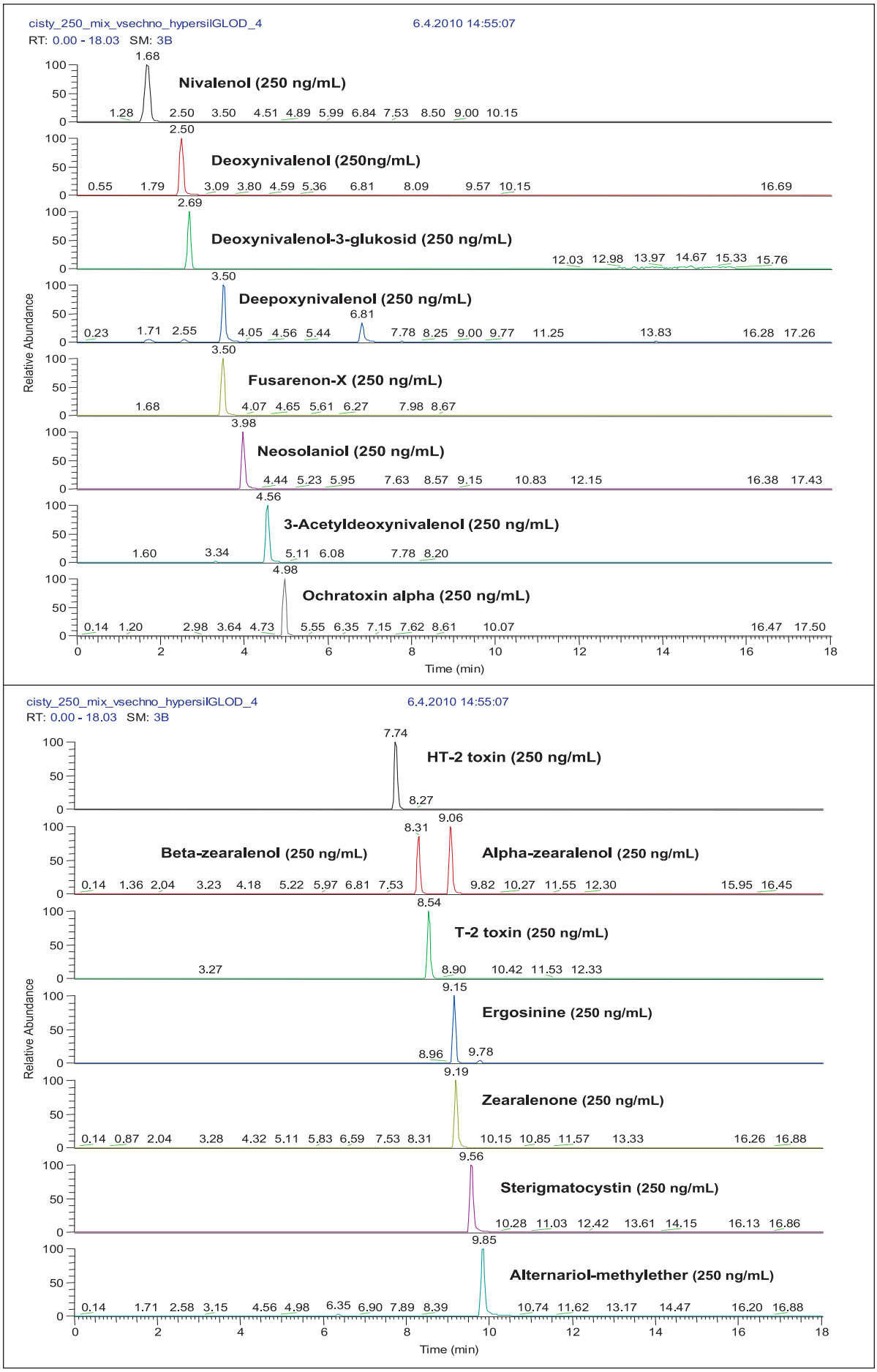

Figure 1: Extracted ion chromatograms of analyzed mycotoxins

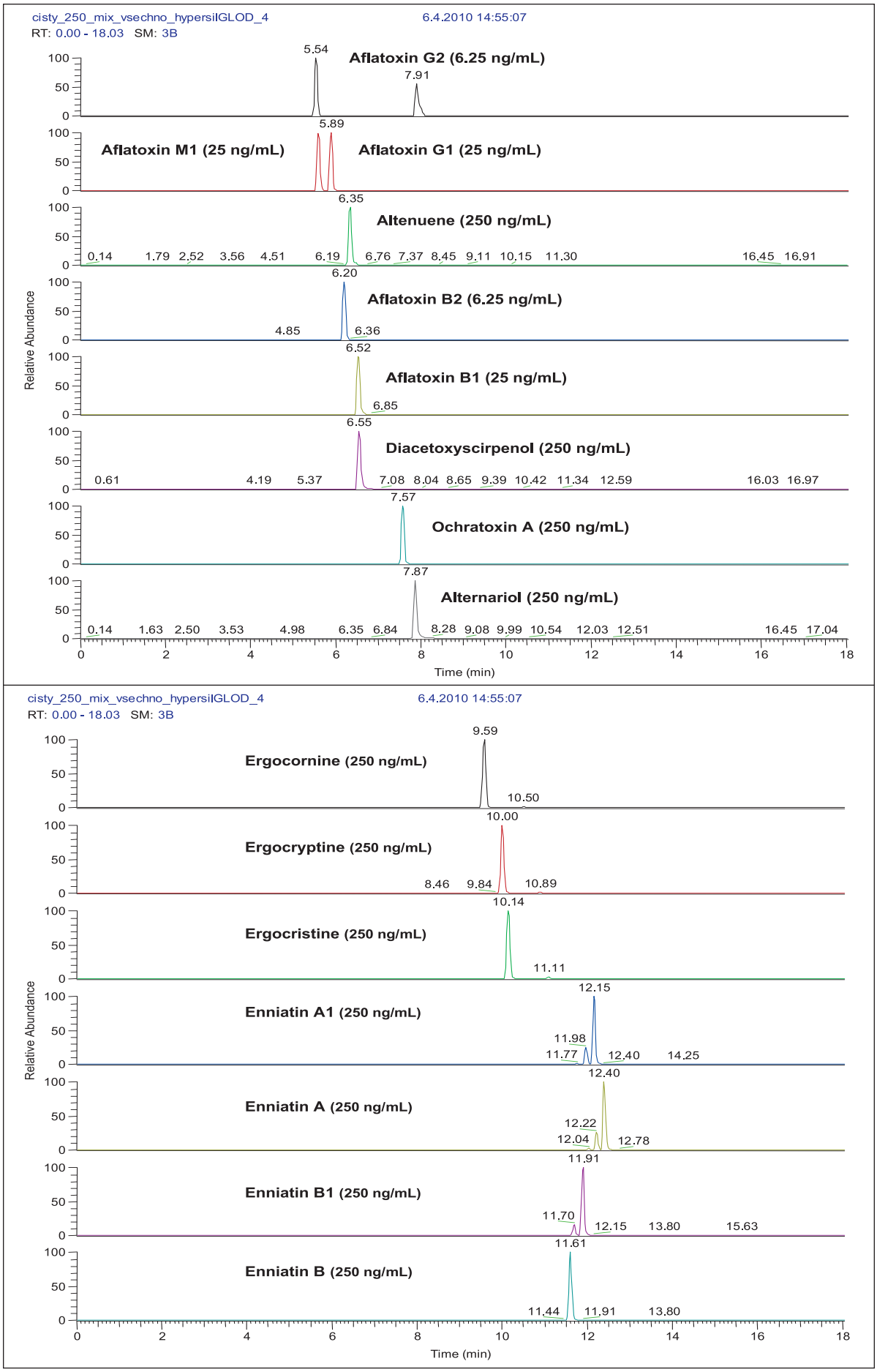

Figure 1 Continued: Extracted ion chromatograms of analyzed mycotoxins

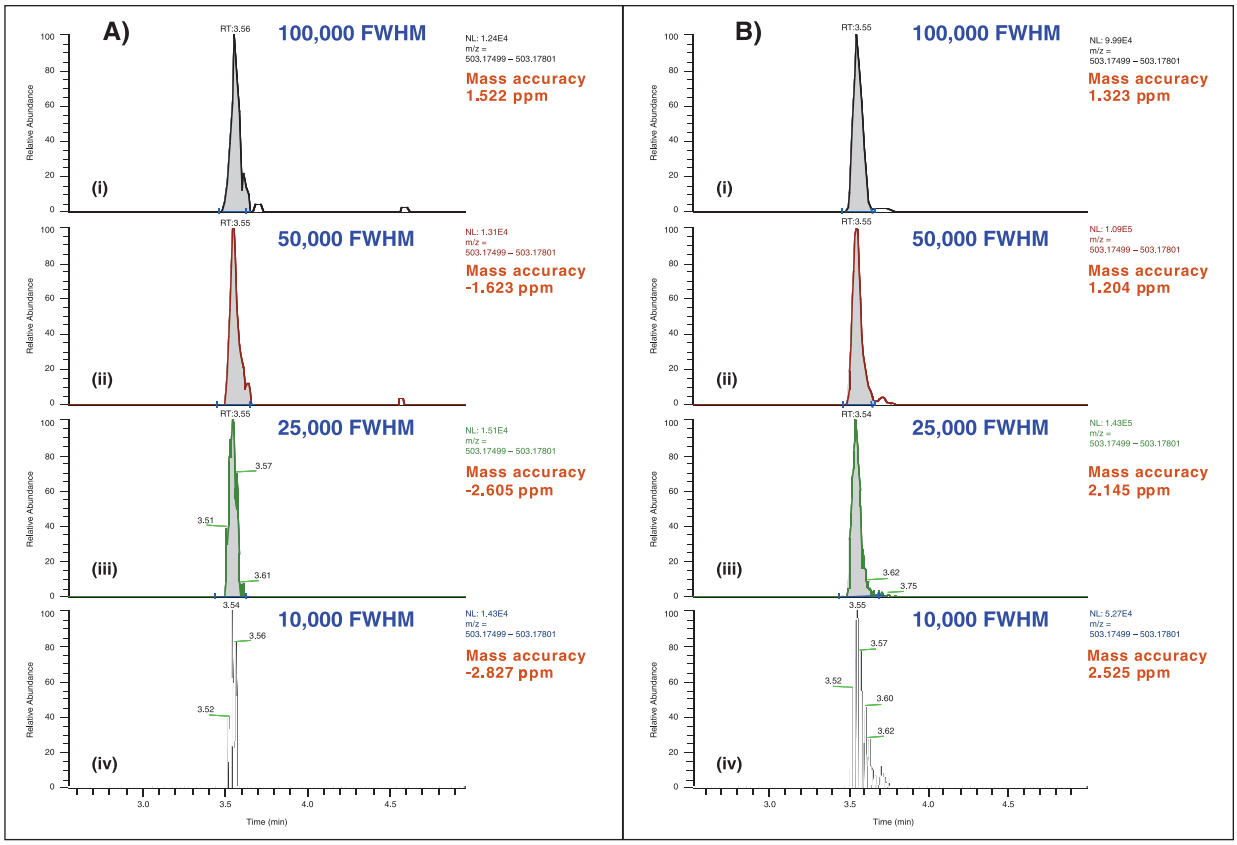

Figure 2: Extracted ion chromatograms of deoxynivalenol-3-glucoside in beer when performing four different resolving power settings (10,000; 25,000; 50,000; and 100,000 FWHM), mass extraction window ±3 ppm. The spiking levels were 5 µg L^{-1} (A) and 50 µg L^{-1} (B).

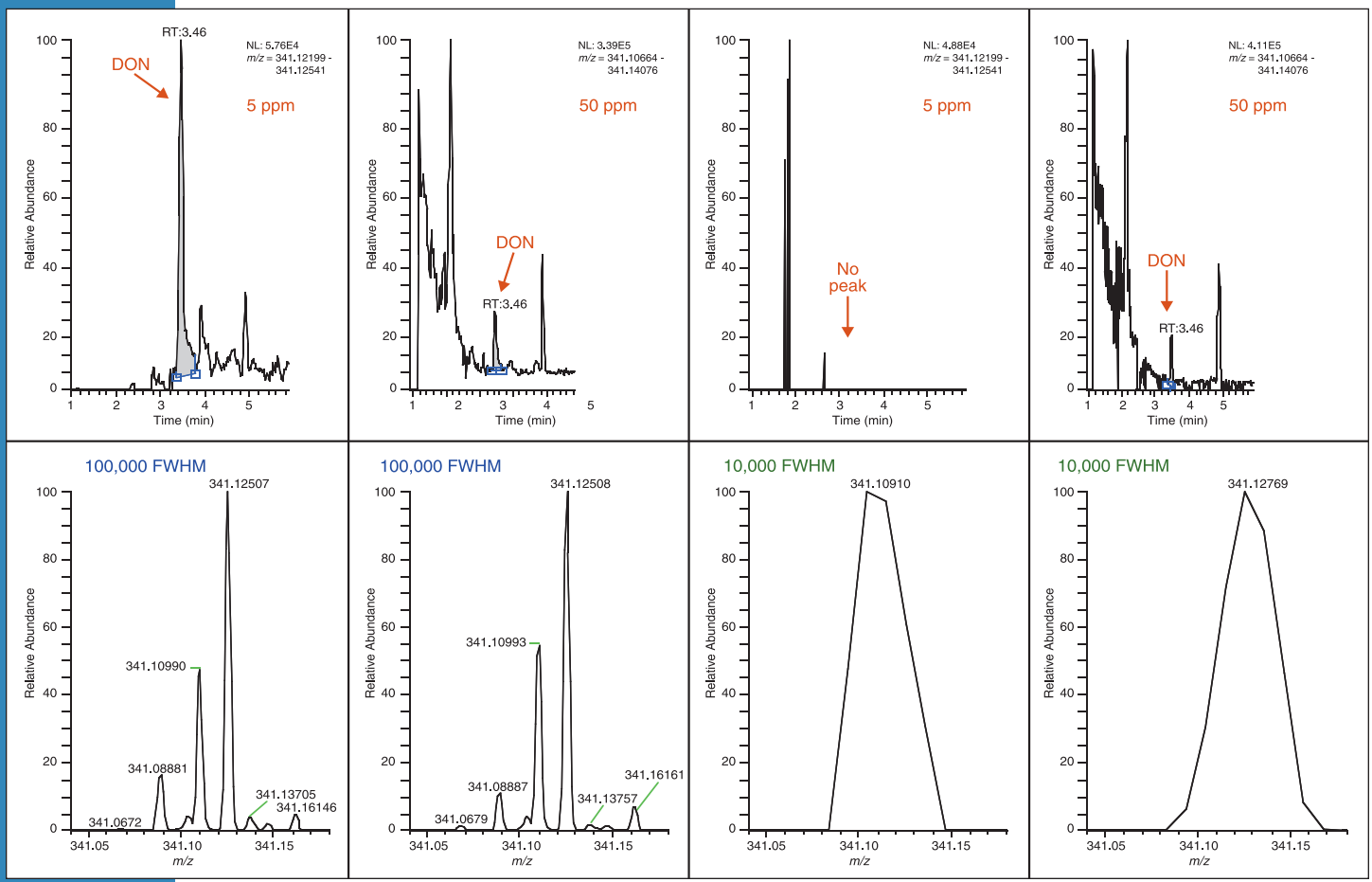

Figure 3: Extracted ion chromatograms and the mass spectra of deoxynivalenol in beer (10 µg L^{-1}) when performing two different resolving power settings (10,000 and 100,000 FWHM) and two different mass extraction windows (±5 and ±50 ppm).

Method Validation

The optimized multi-mycotoxin UHPLC-MS method was thoroughly validated. Prior to analysis of spiked samples, the extent of matrix effects was investigated in order to determine the quantification strategy. For this purpose, two calibration sets were prepared: (i) standards net solvent; (ii) matrix-matched standards. In both cases, the concentration of target mycotoxins was in the range 0.5–250 µg L^{-1}. Although the signal suppression/enhancement (SSE) range was not too broad (63–112%) matrix-matched calibration standards were used.

An important issue to address is calculating an equivalent to limit of quantification (LOQ). Tandem mass spectrometry's classical definition of LOQs based on signal to noise ratio (typically S/N > 6) is not always applicable in high resolution MS because a chemical noise is, in fact, absent in the chromatogram. Due to that fact, lowest calibration levels (LCL) were determined to be the most suitable option. The LCLs of analytes in our study were experimentally established as the lowest concentrations of matrix-matched standards repeatedly identified over time. The relative standard deviations of measurement calculated from nine repeated injections ranged between 11–28% (see Table 3). While these lowest calibration levels for 91% of analytes were at 1-10 µg L^{-1} level, a relatively high LCL level was found for ochratoxin A, which showed much better ionization under electrospray conditions (less than 5 µg L^{-1}).

Mycotoxin	LCL Pure Standard (µg L^{-1})	LCL Matrix-matched Standard (µg L^{-1})	Recovery %			RSD (%) at the Spiking Level 10 µg L^{-1} [1]	RSD (%) at the LCL Level[2]	SSE (%)[3]
			Spike 10 µg L^{-1}	Spike 30 µg L^{-1}	Spike 60 µg L^{-1}			
Nivalenol	2	6	107	97	103	8.9	19	92
Deoxynivalenol	2	3	104	112	99	4.9	24	112
Deoxynivalenol-3-glucoside	2	2	96	103	100	4.3	23	92
Deepoxydeoxynivalenol	4	15	102	116	104	7.2	19	94
Fusarenon-X	2	4	105	113	119	10.3	16	75
Neosolaniol	2	2	99	111	112	10.5	14	93
Verrucarol	3	4	98	99	101	8.4	18	84
3-acetyldeoxynivalenol	4	8	103	96	102	13.7	24	86
Ochratoxin α	4	31	102	98	108	9.8	21	67
Aflatoxin G2	1	2	103	106	99	10.9	25	65
Aflatoxin G1	1	4	117	94	107	8.9	19	63
Altenuene	0.5	1	119	120	113	8.4	22	93
Aflatoxin B2	0.5	1	111	106	104	5.5	12	91
Aflatoxin B1	0.5	2	107	90	92	5.2	13	105
Diacetoxyscirpenol	0.5	1	116	113	124	7.4	17	94
Ochratoxin A[4]	60	60	105	96	97	9.15[5]	26	84
Alternariol	0.5	2	101	107	98	8.5	16	76
HT-2 Toxin	2	4	117	116	104	6.9	19	87
β-zearalenol	1	2	111	92	98	9.1	11	85
T-2 Toxin	1	2	99	119	105	7.9	17	88
α-zearalenol	1	1	114	107	97	8.9	16	84
Ergosin	1	3	111	109	106	12.9	26	78
Zearalenone	1	1	106	117	105	9.4	19	91
Sterigmatocystin	0.5	0.5	118	98	110	11.6	16	107
Alternariol-methylether	1	1	114	109	113	9.1	14	88
Ergocornine	1	2	115	121	102	9.6	20	81
Ergosinine	1	2	98	114	102	8.4	12	91
Ergocryptine	1	2	103	111	101	14.9	23	101
Ergocristine	2	8	95	112	94	6.1	24	81
Ergocorninine	1	2	114	124	104	11.7	15	95
Ergocryptinine	1	2	88	113	101	11.4	26	97
Ergocristinine	2	8	104	119	99	9.1	28	103

Table 3: Validation data for the developed UHPLC-Orbitrap-MS method

1. RSD at the spiking level 10 µg L^{-1} was calculated from 6 spikes
2. RSD at the LCL level was calculated from 11 repeated injections of the particular matrix-matched standard
3. SSE (%) = matrix-matched calibration slope/solvent calibration slope * 100; SSE value of 100% means no effect of matrix on the ion signal
4. The spiking levels of ochratoxin A were 80, 100, and 120 µg L^{-1}
5. The RSD of ochratoxin A was determined at the spiking level of 100 µg L^{-1}

The linearity of the new method was tested for solvent as well as matrix-matched calibration curve constructed in the ranges LCL to 250 µg L^{-1}. The majority of analytes showed linearity in the range 0.9960–0.9999 (R^2). The recoveries of analytes tested at levels 10, 30, and 60 µg L^{-1} ranged from 92–124%, with no losses of analytes during the sample preparation occurred (Table 3).

Conclusion

The UHPLC-MS technology represents the most interesting alternative equivalent to tandem mass spectrometry with the possibility of retrospective data mining. Our UHPLC-MS operated in APCI mode enables rapid determination of trace levels of multiple mycotoxins occurring in complex beer samples. At the highest resolving power setting, 100,000 FWHM, the mass error up to 5 ppm (without the use of internal mass correction) enables the use of a very narrow mass extracting window, ±5 ppm, for the routine work, which significantly improves the selectivity of detection.

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