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Abstract:	<p>This paper presents the application of liquid chromatography electrospray ionization ion trap - orbitrap mass spectrometry for the determination of 18 mycotoxins (aflatoxins, fumonisins, trichothecens, ochratoxin A, sterigmatocistine, beauvaricin, zearalenone and zearalenol) in beer. The extraction procedure was carried out by solid phase extraction (SPE): SPE columns were conditioned with acetonitrile/methanol and water. Then, beer was loaded and the SPE columns were washed with water. In these conditions, the recoveries were higher than 65 % and the relative standard deviation (RSD) were lower than 18 %. The lowest limits of quantification (LLOQ) ranged from 9 to 155 ng ml⁻¹. Matrix-matched calibration was performed for each beer, and reliable results were obtained from selected mycotoxins. The method was applied to the analysis of 25 commercial beers. Taking advantage of the hybrid capabilities, the presence of other mycotoxins were checked; enniatins (A, A1, B and B1) and fusaproliferin were studied in all the tested samples. The results confirmed the presence of zearalenone in one stout beer sample.</p>

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Application of hybrid linear ion trap-high resolution mass spectrometry to the analysis of mycotoxins in beer

J. Rubert^a, J. Mañes^a, K.J. James^b, C. Soler^{a*}

^aDepartment de Medicina Preventiva I Salut Pública. Facultat de Farmàcia. Universitat de València. Av. Vicent Andrés Estellés 46100 Burjassot (Spain);

^bPROTEOBIO. Mass spectrometry laboratory. Cork Institute of Technology. Rose Avenue, Cork (Ireland).

* Correspondence to: C. Soler, Department of Preventive Medicine and Public Health. Facultat de Farmàcia. Universitat de València. Av. Vicent Andrés Estellés 46100 Burjassot (Spain).
E-mail: carla.soler@uv.es

Abstract

This paper reports the application of liquid chromatography electrospray ionization ion trap - orbitrap mass spectrometry for the determination of 18 mycotoxins (aflatoxins, fumonisins, trichothecenes, ochratoxin A, sterigmatocystin, beauvaricin, zearalenone and zearalenol) in beer. The extraction procedure was carried out by solid phase extraction (SPE): SPE columns were conditioned with acetonitrile/methanol and water. Beer was loaded onto the column which was washed with water. In these conditions, the recoveries were more than 65 % and the relative standard deviation (RSD) were below 18 %. The lowest limits of quantification (LLOQ) ranged from 9 to 155 ng ml⁻¹. Matrix-matched calibration was performed for each beer, and reliable results were obtained from selected mycotoxins. The method was applied to the analysis of 25 commercial beers. Taking advantage of the hybrid capabilities, the presence of other mycotoxins were checked; enniatins (A, A₁, B and B₁) and fusaproliferin were studied in all the tested samples. The survey detected the presence of zearalenone in one stout beer sample.

Keywords: mycotoxins; Orbitrap; beer; high resolution mass; SPE

51 Introduction

52 During the 1970s, considerable efforts were directed toward perfecting high resolution
53 instruments. Fortunately, nowadays when liquid chromatography (LC) is combined with
54 a high resolution instrument, it can support a wide range of applications from routine
55 compound identification in complex mixtures (Thurman et al. 2006). One of these high
56 resolution instruments is LTQ Orbitrap MS; it combines a high resolution mass
57 spectrometer, as Orbitrap analyzer, with an external accumulation device such as a
58 linear ion trap, making possible multiple levels of fragmentation (MS^n) for the
59 elucidation of analyte structures. The use of the LTQ Orbitrap allows high-quality
60 accurate mass and acquisition of MS^n spectra. Fourier transformation of the acquired
61 transient allows wide mass range detection with high resolving power, mass accuracy,
62 and dynamic range. The LTQ Orbitrap MS has two main advantages: high sensitivity in
63 full scan and possibility of determination of accurate mass of product ions (Makarov et
64 al. 2010).

66 In the last years, the use of this instrument has been restricted to proteomic or metabolic
67 field (Bennett et al. 2011). In fact, there is scarce literature about its application in the
68 analysis of molecules with relative low molecular weight such as pesticides (Edison et
69 al. 2011), veterinary drugs and doping control (van der Heeft et al. 2009; Peters et al.
70 2010) or toxins. Hogenboom et al. (2009) applied Orbitrap technology to search for
71 target compounds and “unknown” compounds in different water samples. This study
72 demonstrated the presence of target compounds as pharmaceuticals, benzotriazoles and
73 illicit drugs, and they identified “unknown” compounds in groundwater sample and
74 landfill soil sample. Škrabáková et al. (2010) validated the use of high mass resolution
75 in full-scan mode for the determination of six azaspiracids in mussel tissue extracts.

In the field of mycotoxins analysis, Herebian et al. (2009) carried out a first approach for determining 31 mycotoxins in grain and they compared QqQ technology with Orbitrap instrument. They concluded that one of the major advantages of the high resolution full scan method is the possibility of screening unknown compounds. However, best sensitivity was obtained with triple quadrupole (QqQ).

Improving the results, Vaclavik et al. (2010) used Orbitrap technology to determine 11 mycotoxins in cereals and the same group extended the analysis to 32 mycotoxins in beer (Zachariasova et al. 2010). In the first study QuEChERS was used for extraction, while in the second study the extraction was carried up by classical liquid-liquid extraction.

Therefore, expanding upon this previous work, the objectives of the present study were; (i) to develop and to validate an analytical procedure based on the applicability of the LTQ Orbitrap for the simultaneous extraction of aflatoxins, fumonisins, trichothecenes, OTA, ZEN, ZON and BEA in beer. (ii) to apply the validated method for the routine analysis of 25 different Irish beer samples collected from Cork (Ireland). (iii) To apply the method to acquire data essential for the identification of other “non-target” mycotoxins that may be present in beer: enniatins (A, A₁, B and B₁) and fusaproliferin were checked.

Materials and methods

Reagents and chemicals

HPLC grade solvents, acetonitrile, methanol and water were supplied by ThermoFisher (Dublin, Ireland). Analytical grade reagent formic acid (purity > 98%) ammonium

formate was obtained from Panreac Quimica S.A.U. (Barcelona, Spain). All solvents were passed through a 0.45 μm cellulose filter purchased from Scharlau (Barcelona, Spain).

Stock solution

The certificate standards of aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), aflatoxin G₂ (AFG₂), Ochratoxin A (OTA), sterigmatocystin (STER), α -zearalenol (ZOL), zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 3-acetyldeoxynivalenol-3deuterated (3-ADON-d₃), diacetoxyscirpenol (DAS), neosolaniol (NEO), fumonisin B₁ (FB₁), fumonisin B₂ (FB₂) and beauvericin (BEA) were purchased from Sigma Aldrich (Madrid, Spain). T-2 and HT-2 toxin and aflatoxin M₁ (AFM₁) stock solutions (in acetonitrile) were obtained from Biopure referenzsubstanzen GmbH (Tulln, Austria). Fumonisin B₃ (FB₃) was supplied by the PROMEC unit (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, South Africa).

The individual stock solutions of aflatoxins (AFs) and OTA with concentration 500 $\mu\text{g/ml}$ were prepared in acetonitrile and STER, ZOL, ZEN, NIV, DON, 3-ADON, FB₁, FB₂, BEA were prepared on the same concentration in methanol. On the one hand, stock solutions FB₃, DAS, T-2 and HT-2 at concentration of 100 $\mu\text{g/ml}$ were prepared in acetonitrile. As internal standard (IS) compounds AFM₁ at 0.05 $\mu\text{g ml}^{-1}$ and 3-ADON-d₃, NEO at 0.150 $\mu\text{g ml}^{-1}$ were used; working solutions were prepared by dilution of individual stock solutions in methanol. All the standard solutions were kept in safety conditions and at -20°C.

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125 All other working standard solutions were prepared immediately before use by diluting
126 the stock solution with methanol:water (50:50) (V/V).

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128 Oasis HLB SPE (200 mg) cartridges were purchased from Waters (Milford, MA, USA).
129 An extraction manifold from Waters connected to a Büchi Vac V-500 (Flawil,
130 Switzerland) vacuum system was used for SPE experiments.

131
132 *Samples*
133 Beer samples were purchased from different stores and pubs of Cork (Ireland) and kept
134 at -20°C in a dark and dry place. The bottle samples were purchased in supermarkets
135 and conserved. On the other hand, pint (568 ml) samples were collected in sterile
136 beakers from each individual beer and stored until analysis. Samples with undetectable
137 levels of mycotoxins were used for spiking and recovery studies.

138
139 *Extraction Procedure*
140 The extraction procedure was developed according to Romero-Gonzalez et al. (2009).
141 Beer was degassed by sonication for 25 min. The Oasis HLB cartridges were
142 conditioned with 5 ml of acetonitrile/methanol (50:50 v/v) and 5 ml of water.
143 Consequently, 10 ml of beer was loaded onto C₁₈ cartridge. After that, SPE columns
144 were washed with 5 ml of water. Then the cartridges were dried for 30 min. In the last
145 step, the mycotoxins were eluted by adding 4 ml of acetonitrile/methanol (50:50 v/v).
146 Then, the extract was transferred into a 15 ml conical tube and evaporated to dryness at
147 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator
148 (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 ml

149 with methanol/water (50:50) (v/v), filtered through a 0.20 μm Millex-GN nylon filter
150 (Millipore, Carrigrohilly, Ireland) and collected into a vial.

151

152 *HPLC-Orbitrap XL*

153 Chromatographic separations were carried out using an Accela LC system (Thermo
154 Scientific, Hemel Hempstead, UK), equipped with a reversed-phase analytical column
155 (Gemini C₁₈, 150 mm, 2 mm i.d, 5 μm ; Phenomenex) maintained at 35°C. As mobile
156 phase, 5 mM ammonium formate and 0.1% formic acid in water (A) and 5 mM
157 ammonium formate in methanol (B) were used. The gradient was as follows: at the start
158 5 % solvent B was used: the percentage of solvent B was linearly increased up to 95 %
159 in 10 min and then the percentage of solvent B was linearly decreased to 80 % in 5 min.
160 The column was equilibrated to initial conditions for 5 min. The flow rate was 200 μL
161 min^{-1} and the injection volume was 10 μL .

162

163 The LC system was connected to a hybrid LIT–FT mass spectrometer (LTQ Orbitrap
164 XL; Thermo Scientific, Bremen, Germany), operating in positive ion mode. The linear
165 ion trap (LTQ) part of the hybrid MS system was equipped with heated electrospray
166 interface (H-ESI). Full-scan accurate mass spectra (mass range from 90 to 900 Da) were
167 obtained at high resolution (100,000 FWHM) and processed using Xcalibur v.2.0
168 software. The electrospray source conditions were: source voltage 4 kV, heated
169 capillary temperature 275 °C, capillary voltage 30 V, sheath gas and auxiliary gas 35 and
170 30 (arbitrary units), respectively.

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172 The mass spectrometer was operated in a data-dependent-acquisition (DDA) mode in
173 which both MS and MSⁿ spectra were acquired specifying parent masses of target

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3 174 compounds. In this mode, the acquisition software probed the MS spectra in real-time
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5 175 on a full scan allowed accurate mass. The accurate mass is capable to find true
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8 176 unknowns since the method does not require any pre-selection of masses. The
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10 177 instrument is initially set to operate in full-scan mode until a parent ion appears to preset
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12 178 the instrument, which switches into the product-ion mode (MSⁿ). The mass resolution
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15 179 was set at 100,000 FWHM for both screening and quantitative analysis. The products
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17 180 ions were generated in the LTQ trap at an optimized collision energy setting of
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20 181 mycotoxins. The scan type settings are presented in Table 1. No exclusion list was used.
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22 182 The total cycle time depends upon the resolution; at a resolution of 100,000 FWHM the
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24 183 total cycle time is about 1s. The results were used to create a (full-scan) accurate mass
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26 184 MS and an MSⁿ database to enable identification of compound in future screening.
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29 185 The Orbitrap instrument was calibrated using a solution containing caffeine, MRFA,
30
31 186 and Ultramark 1621, according to the manufacturer's instructions. Operation of the
32
33 187 entire LC/MS instrumentation was controlled using Xcalibur software (Thermo
34
35 188 Scientific).
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41 190 *Data processing*
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43 191 LTQ Orbitrap MS for target compound analysis is related to its high mass resolution
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45 192 potential, corresponding to a mass assignment accurate to four decimal places. The
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47 193 ability to determine the m/z of an ion to within a maximum deviation of 5 ppm allows
48
49 194 the determination of a unique elemental composition based on the mass defect of the
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51 195 constituent atoms (van der Heeft et al. 2009). The ability to closely match the
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53 196 measured/theoretical mass with the observed mass greatly increases the reliability of
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55 197 identification (table 1).
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The total list of accurate masses detected in a sample was corrected manually for masses also found in matrix. Based on the accurate mass, the elemental composition of the peaks of interest was calculated using the elemental composition tool within the Xcalibur software.

In this study, the calculated elemental compositions with a maximum deviation of 5 ppm from the measured exact mass were considered. To ensure correct mass calibration, the exact mass of the MS internal standard compounds were checked regularly. To obtain an elemental composition, all compounds were studied by chemical structure. So, theoretical mass was calculated by using ChemDraw 11.0 (CambridgeSoft) and www.chemspider.com.

Accurate mass screening for non target mycotoxins

Full-scan accurate mass measurements were compared with theoretical exact masses of known emergent mycotoxins, such as enniatins A, B, A₁, B₁ and fusaproliferin (Jestoi et al. 2004; Jestoi 2008; Jestoi et al 2009).

Method Validation

Confirmation of the identity was carried out by comparing the retention time and fragmentation pattern of the compound with that of a certified standard and quantification by accurate mass (European Community 2002). Linearity was assessed using standard solutions and matrix-matched calibrations by analyzing in triplicate six concentrations levels; matrix-matched calibration was prepared for each beer brand. To evaluate matrix effects, two strategies were studied: matrix-matched calibration and internal standard addition. The recovery experiments were carried out by spiking the

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sample in five replicates at two concentration levels: LLOQ level and 100 times LLOQ level (Table 2). In the same way, precision of the method was determined in fortified beer at LLOQ level and 100 times LLOQ level and calculated as RSD of measurements in quintuplicate, in the same day and five non-consecutive days.

Other analytical parameters, such as limits of detection (LODs) and lowest limits of quantification (LLOQs), were calculated by Xcalibur 2.0 software; both parameters were determined by decreasing concentration levels in fortified beer. When the signal-to-noise ratios (S/N) of 3 or above were considered acceptable for LOD. LLOQ was defined as the lowest concentration that could be quantified with coefficient of variation (%CV) and the absolute value of mean relative error (%MRE) [(spiked concentration–nominal concentration/nominal concentration) ×100] of less than or equal to 20%. (table 2).

RESULTS AND DISCUSSION

LC-LTQ Orbitrap optimization.

All ion source tune parameters were optimized manually by infusing mycotoxin standards and monitoring the [M+ NH₄]⁺ ion for trichothecenes type A and BEA, also [M+ H]⁺ ion for the other mycotoxins (table 1). This optimization was carried out by adjusting the parameters until no precursor ion fragmentation was observed. In this study mass resolution was set at 100 000 FWHM and the mean values of the mass measurements were all lower than 3 ppm of their theoretical *m/z* values, as it is shown in table 1.

Table 1 presents also the data-depending acquisition settings. MSⁿ measurements were performed to obtain information of main fragment ions generated in the linear ion trap (nominal mass product ions) within the same analysis. In addition, the accurate masses of these product ions were linked with precursor compound masses.

Confirmation of the identity was done by comparing the retention time, accurate mass and MSⁿ fragmentation pattern of the compound to that of a reference standard.

SPE Optimization.

The extraction was carried out according to Romero-Gonzalez et al. (2009). However, in this study the range of mycotoxin analyzed was extended and, in order to improve the recovery values, some parameters were studied. With this aim, blank beer was fortified at 2 times LLOQ level being this level the reference concentration level.

The organic extraction solvent was the parameter optimized due to the wide-range of mycotoxins analyzed with different polarities. Then, the use of 4 mL of methanol and 4 ml of acetonitrile were compared. Methanol provided good recoveries for fumonisins (FB₁, FB₂, and FB₃) and trichothecenes type B (DON, NIV, 3-ADON); however, the recoveries obtained for trichothecenes type A and AFs were lower than 50 %. On the other hand, acetonitrile reached acceptable recovery values for AFs and trichothecenes Type A (>65 %). For this reason, it was decided to use a solvent mixture elution of acetonitrile/methanol. The proportion acetonitrile/methanol (40:60 v/v) proposed by Romero et al. (2009) allowed acceptable recoveries between 63 % for STER to 95 % for OTA; however, in this study the mixture acetonitrile/methanol (50:50 v/v) improved absolute recoveries for trichothecenes Type A (108 % DON) , AFs (>66 % AFB₂) and BEA (>66 %), as shown table 2.

273 *Validation of the method*

274 To evaluate the ability of the developed method for quantitative analysis of the selected
275 mycotoxins in beers, the analytical performance of the proposed method was studied
276 and validated in terms of linearity, limits of detection and quantification, precision and
277 reproducibility of the technique. Validation experiments were performed with four types
278 of beer brands available in Ireland: (i) stout beer, (ii) red ale beer, (iii) ale beer and (iv)
279 pale lager beer. The analytical performance of the methodology for beer samples is
280 summarized in table 2.

281
282 Limits of detection (LODs) for selected mycotoxins ranged from 3 ng ml⁻¹ (AFB₁ and
283 AFB₂ in ale and pale lager beers) to 50 ng ml⁻¹ (NIV in stout beers). Lowest limits of
284 quantification (LLOQs) ranged from 9 ng ml⁻¹ (AFB₂ in ale and pale lager beers) to 155
285 ng ml⁻¹ (NIV in stout beers). Although these limits did not differ significantly between
286 the four matrices, in the case of stout and red ale beer seemed to be slightly higher. It
287 could be assumed that this phenomenon is caused by the greater roasted of the malt or
288 barley and the appearance of different new compounds that could interfere with the
289 selected mycotoxins, increasing these limits.

290
291 Electrospray ionization is usually susceptible to matrix-related signal suppression,
292 which is believed to result from the competition between the analyte ions and matrix
293 components. Therefore the presence of co-extracted matrix components may affect
294 analyte quantification. For this purpose, five calibration sets (standard in pure solvent,
295 and matrix-matched standards of stout, red ale, ale and pale lager beer) at six
296 concentration levels between LLOQ and 100 times LLOQ were prepared. As seen in
297 table 2, matrix effects were calculated in percentages as the ratio of matrix-matched

calibration slope and solvent calibration slope (Zachariasova et al. 2010). A value of 100% indicates that there is no absolute matrix effect. There exists signal enhancement if the value is >100% and signal suppression if the value is <100%. The matrix effects did not differ significantly between the matrices selected. Severe suppression occurred for aflatoxins, STER, trichothecenes type A (T-2 and HT-2) and trichothecenes type B (3-ADON).

The best way to compensate the matrix effect is the use of isotopic internal standards. Unfortunately, the labeled analogues or internal standard (IS) of these mycotoxins were inaccessible to us owing to their cost (Romero- González et al. 2009). Thus, other approaches were evaluated.

The first option was the addition of established internal standards used in mycotoxin field as AFM₁, NEO and 3-ADON-d₃. AFM₁ and NEO are analogue molecules of AFs and trichothecenes type A, respectively, since they are metabolites of these mycotoxins. STER and AFs were calibrated with AFM₁, since STER is supposed to be a precursor of the AFs and AFM₁ is a metabolite of AFB₁. 3-ADON-d₃ is the deuterated analogue of 3-ADON. With this calibration approach no significant improvement was achieved, probably due to the fact that the optimal SPE-HPLC-LTQ-ORBITRAP conditions for internal standards were different from those of the mycotoxins.

A second calibration alternative was tested according to Council decision 2002/657/EC (European Community 2002) and document SANCO/10684 (2009): external matrix-matched calibration. The use of matrix-matched standards compensated the suppression signal effects achieving an accurate quantification as reported the literature (Bacaloni et

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3 323 al. 2005; Romero et al. 2009; Zachariasova et al. 2010). Matrix-matched calibration
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5 324 curves were prepared in the four extract beers, showing good linearity between LLOQ
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8 325 and 100 times LLOQ concentration levels (linear range), with a correlation coefficient $\geq$
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10 326 0.9861.
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15 328 The inter-day precision and intra-day repeatability of the method were also evaluated on
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17 329 extracted beer samples at two different concentration levels (LLOQ and 100 times
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19 330 LLOQ). The RSD values for intraday analyses were in the range 5-15 % and the RSD
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21 331 for inter-day values were between 6 to 18 %, showing good reproducibility of the
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23 332 technique. The recovery ranges in low and high spiked levels were 66-108 % for the
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25 333 intraday test and 65-103 % for the inter-day test.
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31 335 In the light of these results, the developed method gave acceptable recoveries and these
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33 336 results were in good agreement with the performance criteria of Commission Regulation
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35 337 (EC) N° 401 of 23 February 2006 (European community 2006) laying down the
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37 338 methods of sampling and analysis for the official control of the levels of mycotoxins in
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39 339 foodstuffs.
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45 341 *Analysis of commercialized Irish beers.*
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48 342 In this study, the analyzed samples were divided into four main categories: stout, red
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50 343 ale, ale and pale lager. Twenty-five samples were analysed: 6 stouts, 3 red ale, 6 ale and
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52 344 10 pale lager beers. The first step was to identify target compounds (selected
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54 345 mycotoxins) in Irish beers. Red ale, ale and pale lager beers were analysed by LTQ
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56 346 Orbitrap XL; in these alcoholic beverages selected mycotoxins were not present.
57
58 347 However, ZEN was identified and quantified (37.8 ng ml⁻¹) in one stout beer. Figure 1
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depicts a full scan of stout beer sample where ZEN was identified by retention time (RT) (Figure 1A). After that, extracted ion chromatogram was checked for selected compound and ZEN was observed at appropriate RT of 12.19 (Figure 1(b)). This compound was unambiguously identified by accurate mass and pathway study. The measured m/z was 319.1547 and the calculated error (ppm) was 0.31 (Figure 1C). In order to improve the identification of the mycotoxin, ion trap spectrum was acquired, and characteristic pathway for ZEN was obtained. This mycotoxin (ZEN) lost one molecule of water in the first fragmentation of ion trap (MS^2); this fragment has been also reported by Frenich et al. (2009).

To our knowledge, this is the first time that ZEN is identified unambiguously by accurate mass and fragmentation study in beer. Previous works that screened ZEN in beer (Maragou et al. 2008; Romero et al. 2009) did not identify this mycotoxin in this food commodity.

The second step was to screen "non-target" mycotoxins in Irish beer samples: enniatins A, B, A₁, B₁ and fusaproliferin. The structures of these emergent mycotoxins were studied (www.chemspider.com) and the exact mass was calculated (ChemDraw 11.0). Several studies about emergent mycotoxins have reported two possibilities for molecular ion formation; $[M+H]^+$ and $[M+NH_4]^+$ (Uhlir et al. 2004; Jestoi et al. 2009). For this reason, exact mass for these ions were calculated and scanned on each sample by Xcalibur 2.0 software. In this study these emergent mycotoxins were not identified.

Conclusion

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372 The SPE-LC-LTQ-Orbitrap-MS method reported in this paper was able to determine 18
373 mycotoxins in beer providing high sensitivity, high recoveries and achieving low LODs
374 and LLOQs. Moreover, this technology allowed a screening of 6 emergent mycotoxins,
375 non-target compounds which could be checked.

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377 The developed method was applied for the analysis of selected mycotoxin in 25
378 different Irish beer samples. The results confirmed the presence of ZEN in one sample
379 of stout beer.

380

381 Even though it is true that the found concentration level was low, it is to be taken into
382 account that the risk of the exposition depends of the quantity of beer consumed for a
383 prolonged period of time; this makes necessary a mycotoxin control in beer production
384 chain.

385

386 To our knowledge, this work reports for the first time the application of LTQ-Orbitrap
387 technology to routine monitoring mycotoxins in commercial beers, demonstrating its
388 potential by accurate mass and pathway study.

389

390 **Acknowledgements**

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393 grant.

394

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FIGURE CAPTION:

Figure 1: Chromatogram of positive stout beer sample: A) Full scan MS 100-900 m/z.
B) extracted ions m/z 319,15. C) accurate mass ZEN and D) confirmation by ion trap
(MS2).

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Table 1. Ion formation, elemental formula, theoretical and measured m/z and error mass (ppm) for selected mycotoxins. Data-depending-acquisition parameters for ion trap acquisition.

Compound	Full Scan FTMS					Data-depending-acquisition (DDA)		
	Ion formation	Elemental formula	Theoretical m/z	Measured m/z	Error (ppm)	Retention time	Parent mass	CE
NIV	[M+H] ⁺	C ₁₅ H ₂₁ O ₇	313.1287	313.1289	0.63	4.99	313.13	18
DON	[M+H] ⁺	C ₁₅ H ₂₁ O ₆	297.1338	297.1337	-0.33	6.35	297.13	20
3-ADON	[M+H] ⁺	C ₁₇ H ₂₃ O ₇	339.1445	339.1444	-0.29	8.50	339.14	18
DAS	[M+NH ₄] ⁺	C ₁₉ H ₃₀ O ₇ N	384.2023	384.2021	-0.52	8.48	383.20	19
HT-2	[M+NH ₄] ⁺	C ₂₂ H ₃₆ O ₈ N	442.2441	442.2445	0.90	10.81	442.24	18
T-2	[M+NH ₄] ⁺	C ₂₄ H ₃₈ O ₉ N	484.2547	484.2545	-0.55	11.34	484.25	18
FB ₁	[M+H] ⁺	C ₃₄ H ₆₀ O ₁₅ N	722.3963	722.3962	-0.13	10.45	722.39	54
FB ₂	[M+H] ⁺	C ₃₄ H ₆₀ O ₁₄ N	706.4014	706.4017	0.42	11.62	706.40	25
FB ₃	[M+H] ⁺	C ₃₄ H ₆₀ O ₁₄ N	706.4014	706.4016	0.28	11.06	706.40	25
ZEN	[M+H] ⁺	C ₁₈ H ₂₃ O ₅	319.1546	319.1547	0.31	12.19	319.15	20
ZOL	[M+H] ⁺	C ₁₈ H ₂₅ O ₅	321.1702	321.1705	0.93	11.95	321.17	20
AFB ₁	[M+H] ⁺	C ₁₇ H ₁₃ O ₆	313.0711	313.0712	0.31	9.78	313.07	35
AFB ₂	[M+H] ⁺	C ₁₇ H ₁₅ O ₆	315.0869	315.0868	-0.31	9.63	315.08	35
AFG ₁	[M+H] ⁺	C ₁₇ H ₁₃ O ₇	329.0661	329.0662	0.30	9.30	329.06	29
AFG ₂	[M+H] ⁺	C ₁₇ H ₁₅ O ₇	331.0818	331.0816	-0.60	9.10	331.08	32
STER	[M+H] ⁺	C ₁₈ H ₁₃ O ₆	325.0712	325.0711	-0.30	12.43	325.07	35
OTA	[M+H] ⁺	C ₂₀ H ₁₉ O ₆ NCl	404.0901	404.0903	0.43	12.23	404.09	17
BEA	[M+NH ₄] ⁺	C ₄₅ H ₆₁ O ₉ N ₄	801.4439	801.4437	-0.24	14.01	801.44	50

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Table 2. Matrix effect, lowest limits of quantification (LLOQs), recovery values (RSD %) given in brackets calculated at two concentration levels (ng ml⁻¹), mean level for all four matrices is presented. Results show values for stout/red ale/ale/pale lager beers.

Compound	Matrix effect (%)	LOD (ng ml ⁻¹)	LLOQ (ng ml ⁻¹)	Intr-day precision		Inter-day precision	
				Low level (LLOQ)	High level (100xLLOQ)	Low level (LLOQ)	High level (100xLLOQ)
NIV	90/98/94/98	50/45/44/40	155/135/131/120	77/80/84/91 (10)	78/82/84/89 (10)	73/81/80/90 (10)	70/81/84/89 (11)
DON	83/105/85/94	20/18/16/16	60/54/48/48	83/84/87/108 (11)	80/81/86/103 (9)	78/83/81/97 (11)	81/81/83/100 (9)
3-ADON	43/55/48/59	30/27/25/25	90/80/75/75	83/84/87/108 (11)	82/86/85/102 (8)	80/84/85/101 (12)	82/85/85/102 (9)
DAS	107/101/102/102	13/13/12/10	40/40/35/30	93/96/95/103 (9)	95/96/98/101 (5)	92/95/95/102 (10)	98/98/98/103 (9)
HT-2	59/63/53/65	7/9/7/7	20/27/20/20	71/75/77/79 (9)	73/78/79/79 (6)	72/78/79/81 (10)	76/80/81/85 (11)
T-2	63/69/63/68	5/5/4/4	15/15/12/12	68/76/74/92 (10)	71/79/78/99 (8)	68/69/71/83 (10)	68/72/72/89 (11)
FB ₁	115/111/99/112	35/32/30/32	105/95/90/95	80/85/87/92 (11)	77/79/80/89 (9)	81/81/82/89 (12)	81/80/80/89 (10)
FB ₂	100/125/111/109	32/32/30/30	95/95/90/90	85/89/87/88 (10)	80/82/83/85(8)	86/88/87/88 (15)	80/81/80/86(14)
FB ₃	116/112/119/123	32/32/32/32	95/95/95/95	82/85/79/91 (12)	78/80/75/81 (11)	83/83/81/88 (15)	77/79/76/78 (11)
ZEN	87/79/82/83	12/10/12/10	36/30/36/30	75/77/85/88 (10)	72/74/85/83 (8)	77/77/81/85 (11)	71/70/81/82 (7)
ZOL	90/88/89/93	10/10/8/8	30/30/24/24	75/81/85/91 (8)	72/78/78/89 (5)	73/83/82/90 (9)	73/75/76/88 (5)
AFB ₁	56/60/59/61	5/4/3/3	15/12/9/9	77/79/73/78 (5)	79/79/78/81 (5)	78/79/76/80 (7)	77/79/77/78 (9)
AFB ₂	55/56/65/64	4/3/3/3	12/10/9/9	66/76/71/81 (8)	72/79/75/83 (6)	66/71/72/82 (10)	71/78/72/80 (9)
AFG ₁	51/57/67/65	5/4/4/4	15/12/12/12	70/73/71/79 (7)	76/75/77/79 (7)	70/70/70/79 (7)	72/71/72/81 (8)
AFG ₂	56/56/69/61	4/5/4/4	12/15/12/12	68/69/72/77 (9)	70/76/76/79 (6)	68/68/70/75 (9)	71/75/74/78 (9)
STER	66/67/69/71	7/6/6/5	21/18/18/15	69/79/72/81 (6)	72/80/76/84 (7)	65/73/76/80 (6)	71/77/75/87 (9)
OTA	77/71/75/79	5/4/4/4	15/12/12/12	70/77/75/94 (10)	78/82/85/97 (9)	71/72/78/89 (12)	79/80/81/90 (8)
BEA	55/66/59/64	4/5/5/4	12/15/15/12	69/75/91/99 (15)	71/73/88/93 (13)	66/77/88/93 (18)	70/73/90/95 (16)

^a Number of replicates:5

^b Different days: 5

Figure 1. Chromatogram of positive stout beer sample: A) Full scan MS 100-900 m/z. B) extracted ions m/z 319.15. C) accurate mass ZEN and D) confirmation by ion trap (MS²).

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