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Multi-mycotoxin Stable Isotope Dilution LC-MS/MS Method for *Fusarium* Toxins in Beer

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1. Introduction

Due to a worldwide beer production of 1.96 billion hectoliter in 2014 (Barth-Haas Group, 2014/2015), beer can be considered a staple food. With the quincentennial of the German “Beer Purity Law” beer quality is more topical than ever and fungal infestation is a serious threat of quality.

The infection of small grain crops with *Fusarium,* known as *Fusarium* head blight, causes enormous losses in yield, grade and end-use quality worldwide (Parry et al., 1995). Moreover, *Fusarium* species produce a wide range of mycotoxins, e.g trichothecenes, enniatins (ENNs), zearalenone (ZEA), and thus can induce a severe contamination of cereals (Desjardins, 2006). Heavily loaded food and feedstuff can impair human and animal health (Desjardins, 2006). Fungal infestations of brewing barley and in particular metabolites produced by fungi can have a negative influence on solving, malting and brewing properties as well (Schapira et al., 1989; Oliveira et al., 2012; Sarlin et al., 2005).

To protect the health of consumers the regulatory limit in the European Union was set to 1250 µg/kg for deoxynivalenol (DON) in unprocessed cereals, which is applicable to malting barley (European Union, 2006). Along with DON also ENNs, beauvericin (BEA), ZEA, type A trichothecenes, other type B trichothecenes and modified mycotoxins (Rychlik et al., 2014) play an important role in barley and derived products. Due to their water solubility and thermal stability some *Fusarium* toxins, especially DON and the masked mycotoxin DON-3-glucoside (D3G), were found in beer samples analyzed in previous studies (Kostelanska et al., 2009; Lancova et al., 2008; Vaclavikova et al., 2013; Scott, 1996; Schwarz & Howard, 1995; Varga et al., 2013; Malachova et al., 2012).

In cereal products a sufficient removal of disturbing matrix components and the use of internal or isotope labeled standards (Klötzel et al., 2005) were highly recommended to minimize matrix interferences such as ion suppression or enhancement. As published in recent studies purification of beer samples was commonly based on precipitation of polar matrix components after addition of acetonitrile (Kostelanska et al., 2009; Lancova et al., 2008; Zachariasova et al., 2010; Vaclavikova et al., 2013; Malachova et al., 2012).

The aim of this study was to develop a generally applicable solid phase extraction for trichothecenes including modified mycotoxins, enniatins, beauvericin and zearalenone to analyze mycotoxins in beer samples. The resulting multi-mycotoxin stable isotope dilution LC-MS/MS method could be applied to beers from different continents to provide a current survey of *Fusarium* toxins in beer worldwide.

2. Materials and methods

2.1 Chemicals and reagents

Acetonitrile, methanol and water (analytical grade) were purchased from VWR (Ismaning, Germany). Formic acid (>95%) was bought from Sigma-Aldrich (Steinheim, Germany). The unlabeled reference compounds D3G, DON, 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), HT2-toxin (HT2), T2-toxin (T2), ZEA and some labeled standards ($[^{13}C]_{15}$-DON, $[^{13}C]_{22}$-HT2) were bought from Coring System Diagnostix (Gernsheim, Germany), BEA was obtained from AnaSpec (San Jose, USA), ENN B from Bioaustralis (New South Wales, Australia) and the other ENNS B1, A1 and A from Enzo Life Sciences (Lörrach, Germany). The labeled internal standards ($[^{13}C]_2$-3-ADON, $[^{13}C]_4$-T2-toxin, $[^{15}N]_3$-ENN B, $[^{15}N]_3$-ENN B1, $[^{15}N]_3$-ENN A1, $[^{15}N]_3$-ENN A and $[^{15}N]_3$-BEA) were synthesized in our laboratory as reported previously (Asam & Rychlik, 2006, 2007; Hu & Rychlik, 2012).

2.2 Preparation of standard solutions

Stock solutions of labeled and unlabeled toxins were prepared in concentrations of 10–100 µg/mL in acetonitrile (trichothecenes, zearalenone) or methanol (enniatins) and further diluted to a final concentration of 1 µg/mL and 0.1 µg/mL. All solutions were stored at 4 °C in the dark.

2.3 LC-MS/MS

LC-MS/MS was performed on a Shimadzu LC-20A Prominence system (Shimadzu, Kyoto, Japan) using a Hydrosphere RP-C_{18} column (150 x 3.0 mm^2, S-3 µm, 12 nm, YMC Europe GmbH, Dinslaken, Germany) with a C_{18}-guard column (Phenomenex, Aschaffenburg, Germany) as stationary phase that was kept at 40°C. The binary gradient system consisted of (A) 0.1% formic acid and (B) methanol with 0.1% formic acid at a flow rate of 0.2 mL/min. The gradient for the negative ESI mode was started and held at 10% B for 2 min, raised linearly from 10% B to 99% B during the next 6 min, and then maintained at 99% B for 7.5 min. Next, the mobile phase returned to 10% B within 2 min and the system was equilibrated for 9.5 min before the next run. The gradient for the positive ESI mode was started and held at 10% B for 2 min, raised linearly from 10% B to 87% B during the next 6 min, held at 87% for 7 min, raised to 100% B during the next 5 min, and then maintained at 100% B for 3.5 min. Next, the mobile phase returned to 10% B within 2 min and the system was equilibrated for 9.5 min before the next run. The injection volume was 10 µL.

The LC was interfaced with a hybrid triple quadrupole/linear ion trap mass spectrometer (API 4000 QTrap; Applied Biosystems Inc., Foster City, CA, USA). It operated in the negative ESI mode for the analyte ZEA and in the positive ESI mode for the analytes D3G, DON, 3-ADON, 15-ADON, FUSX, HT2, T2, ENN B, B1, A1, A, and BEA. The ion source parameters for the negative mode were set as follows: curtain gas 20 psi, CAD gas pressure medium, ion spray voltage −4500 eV, spray gas 50 psi, dry gas 65 psi, and temperature 525°C. The ion source parameters for the positive mode were set as follows: curtain gas 20 psi, CAD gas pressure high, ion spray voltage 4500 eV, spray gas 80 psi, dry gas 75 psi, and temperature 450°C. MS parameters were optimized by direct infusion of each standard solution (50 ng/mL to

1 µg/mL) into the source. The mass spectrometer was operated in the scheduled multiple reaction monitoring (MRM) mode for MS/MS measurements at the conditions detailed in **Table 1**. A waste valve diverted the column effluent to the mass spectrometer from 7 to 16 min in the negative mode and from 7 to 24 min in the positive mode. The remainder of the run was diverted to the waste. Data acquisition was performed with Analyst 1.6.2 software (Applied Biosystems Inc., Foster City, CA, USA).

2.4 Calibration and quantitation

For the response curves constant amounts of internal standard (S) were mixed with varying amounts of analyte (A) in molar ratios between 0.02 and 50 (1:50, 1:20, 1:10, 1:5, 1:2, 1:1; 2:1, 5:1, 10:1, 20:1, 50:1). Except for D3G, 15-ADON, and ZEA for all other analytes the [^{13}C]- or [^{15}N]-labeled isotopologues were used. D3G and 15-ADON were quantified by [^{13}C]$_{15}$-DON and [^{13}C]$_2$-3-ADON as internal standards, respectively. After LC-MS/MS measurement the response curves were calculated from molar ratios [n(S)/n(A)] versus peak area ratios [A(S)/A(A)]. Response functions were obtained using linear regression.

Six matrix calibration points were prepared for the matrix calibration curve of ZEA as detailed below. The matrix calibration curves were calculated from concentrations of the analyte [c(A)] versus peak area [A(A)] after LC-MS/MS measurement.

The contents of the analytes in beer samples were either calculated by using the respective response function (D3G, DON, 3-ADON, 15-ADON, HT2, T2, ENN B, B1, A1, A, BEA) or for ZEA by using the matrix calibration function.

2.5 Sample preparation

To 5 mL degassed beer, 10 mL acetonitrile and the internal standards were added (50 µL of $[^{13}C]_{15}$-DON (1 µg/mL), 150 µL of $[^{13}C]_{2}$-3-ADON (1 µg/mL), 60 µL of $[^{13}C]_{22}$-HT2 (1 µg/mL), 40 µL of $[^{13}C]_{4}$-T2 (1 µg/mL) and 70 µL of $[^{15}N]_{3}$-ENN B, $[^{15}N]_{3}$-ENN B1, $[^{15}N]_{3}$-ENN A1, $[^{15}N]_{3}$-ENN A, and $[^{15}N]_{3}$-BEA, respectively (0.1 µg/mL)). The mixture was vortexed for 20 sec and centrifuged at 4000 rpm for 5 min. The residue was extracted two more times with 3 mL acetonitrile/water (70/30, v/v), respectively. The combined supernatants were evaporated to dryness. The residue was solved in 4 mL acetonitrile/water (84/16, v/v), vortexed for 20 sec and completely applied on a Bond Elut Mycotoxin cartridge (500 mg, 3 mL, Agilent Technologies, Santa Clara, CA, USA). The liquid was passed through the cartridge by vacuum suction, the eluate was collected and evaporated to dryness. After reconstitution with 200 µL acetonitrile/water (1/1, v/v), the sample was membrane filtered (0.45 µm). The LC-MS/MS analysis was performed as described below.

For the matrix-matched calibration beer free of zearalenone (5 mL) chosen as blank matrix was spiked with 6 different amounts of analyte (4-150 g/kg ZEA). Then the same preparation as for samples as described above was performed.

2.6 Method validation

The following described validation of our new multi-mycotoxin stable isotope dilution LC-MS/MS method for *Fusarium* toxins was performed similar to the validation

155 procedure Habler & Rychlik (2016), Liu & Rychlik (2013), and Hu & Rychlik (2012)
156 published.

157 **2.6.1 Limits of Detection (LODs) and Quantitation (LOQs)**

158 Beer free of the monitored mycotoxins was chosen as blank matrix and used to
159 determine LODs and LOQs as suggested by Vogelgesang and Hädrich
160 (Vogelgesang & Hädrich, 1998). Therefore, the blank matrix was spiked with the
161 unlabeled analytes at four different amounts (10, 40, 70, 100 µg/L for D3G; 5, 20, 35,
162 50 µg/L for DON; 3, 10, 20, 30 µg/L for 3-ADON; 4, 15, 30, 40 µg/L for 15-ADON; 7,
163 30, 50, 70 µg/L for HT2; 3, 10, 20, 30 µg/L for T2; 0.1, 0.2, 0.5, 1 µg/L for ENN B),
164 each in triplicate. The LOD and LOQ were determined by signal to noise ratio for ZEA
165 quantified by matrix-matched calibration.

166

167 **2.6.2 Precision**

168 Naturally contaminated beer brewed from inoculated malting barley at the Chair of
169 Brewing and Beverage Technology, TU München, with contents of D3G at 202 µg/L,
170 DON at 245 µg/L, 3-ADON at 32 µg/L, 15-ADON at 10 µg/L, HT2 at 21 µg/L, T2 at 22
171 µg/L, 0.2 µg/L ENN B was spiked with ZEA (10 µg/L) and used for intra-day (n=3)
172 and inter-day (during n=3 weeks) precision measurements.

173

174 **2.6.3 Recovery of SIDA and recovery of matrix-matched calibration**

175 Blank beer was spiked in triplicate with different amounts of analytes (40, 70, 100
176 µg/L for D3G; 20, 35, 50 µg/L for DON; 10, 20, 30 µg/L for 3-ADON; 15, 30, 40 µg/L

for 15-ADON; 30, 50, 70 μg/L for HT2; 10, 20, 30 μg/L for T2; 0.5, 1, 4 μg/L for ENN B, and 4, 10, 20 μg/L for ZEA) and analyzed as described previously. Recoveries were calculated as the ratio of detected and spiked contents.

3. Results and discussion

3.1 Method development

3.1.1 LC-MS/MS

To ensure optimal MS conditions for all monitored analytes, two chromatographic runs in the positive and negative ESI modes were performed for each sample (Habler & Rychlik, 2016). ZEA was analyzed in the negative ESI mode and the abundant $[M-H]^-$ deprotonated ion was used as precursor ion. Either the protonated molecules of type B trichothecenes, ENNs and BEA or sodium adducts of HT2 and T2 were used as precursor ions in the positive ESI mode. The labeled standards had fragmentation patterns similar to the respective unlabeled compounds. More detailed explanations of MS/MS fragmentation of the monitored analytes and their respective unlabeled compounds have been described previously (Asam & Rychlik, 2006, 2007; Hu & Rychlik, 2012).

Using a Hydrosphere RP-C_{18} column (YMC Europe GmbH, Dinslaken, Germany) we were able to assure baseline separation for all analytes including D3G and DON except for BEA coeluting with ENN B1 and 3-ADON coeluting with 15-ADON.

Due to in source fragmentation and loss of glucose from D3G in the positive ESI mode D3G showed the same fragmentation pattern than DON (**Figure 1**). A chromatographic separation was necessary to allow individual quantification of D3G and DON. Considering the different fragmentation patterns of ENN B1 and BEA a coelution was not critical. The use of a Synergi Polar RP column would have avoided a coelution of ADONs but would have not been able to separate D3G and DON (Asam & Rychlik, 2007). The position isomers 3-ADON and 15-ADON with their

206 common precursor $[M+H]^+$ ion (*m/z 339.1*) are differentiated by the partially different
207 product ions and especially by their intensities (**Figure 2**). The three most intensive
208 fragments of 3-ADON are *m/z* 213.1, *m/z* 231.1, and *m/z* 137.1 and those of 15-
209 ADON are *m/z* 261.1, *m/z* 137.1, and *m/z* 231.1. The respective product ion was
210 chosen as quantifiers that were not produced by the other isomer (*m/z* 213.1 for 3-
211 ADON and *m/z* 261.1 for 15-ADON). Both ADONs revealed the same qualifiers in
212 different intensities (*m/z* 231.1 and *m/z* 137.1). Quantification of 3-ADON and 15-
213 ADON was possible without baseline separation as Berger et al. (1999) have already
214 described as well as our validation data showed.

215

216 **3.1.2 Sample purification**

217 Beer is a complex ethanolic solution of carbohydrates, proteins and phenols. For LC-
218 MS, the latter compounds are likely to shorten the lifetime of the HPLC column and to
219 impair the performance of an ESI-MS source when a simple dilution of the beer is
220 applied. A purification of beer samples using solid phase extraction (SPE) was
221 developed to maximize sensitivity and to reduce the consumption of labeled internal
222 standards.

223 Either for liquid samples a dilute-and-shoot method was often reported (Al-Taher et
224 al., 2013; Hu et al., 2014; Malachova et al., 2012). However, a precipitation of polar
225 matrix components after addition of acetonitrile as also reported (Kostelanska et al.,
226 2009; Lancova et al., 2008; Zachariasova et al., 2010; Vaclavikova et al., 2013) may
227 also precipitate polar mycotoxins and thus impair recovery. In our study, non-polar
228 mycotoxins like zearalenone or enniatins are hardly affected by beer matrix. But

matrix effects independent of the beer type especially resulted in ion suppression of polar analytes like D3G and DON. Therefore, we developed the first precipitation method with sequentially recovering of the target toxins followed by an unprecedented stable isotope dilution multi-mycotoxin method for trichothecenes combined with zearalenone, enniatins, beauvericin and the modified mycotoxin D3G that was based on a non-retentive solid phase extraction as purification of beer samples. In contrast to other provided and tested cartridges mostly designed for trichothecene analysis the Bond Elut Mycotoxin cartridge (Agilent Technologies, Santa Clara, CA, USA) contained no activated carbon. To achieve good recoveries and to prevent adsorption of the non-polar toxins (ZEA, ENNs, BEA) the already described Bond Elut Mycotoxin cartridge (Agilent Technologies, Santa Clara, CA, USA) had to be mandatorily used, as published recently (Habler & Rychlik, 2016).

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3.1.3 Calibration and quantitation

Response functions were obtained using linear regression. The response factors (calculated as follows: A(S)/A(A) x n(A)/n(S)) were between 0.75 (3-ADON) and 1.30 (ENN B1, A1, A). Due to the 3 to 4 times lower sensitivity of D3G compared to $[^{13}C]_{15}$-DON in the positive ESI mode the response factor of D3G was 3.0.

The coefficients of determination ranged between 1 and 0.9982. According to the Mandel test the calibration curves show linearity for D3G within the molar ratios 0.05-3, DON, 15-ADON, 3-ADON and HT2 within 0.1-10, T2, ENN B1, A1 and A within 0.05–20, ENN B within 0.05-10, and BEA within 0.05-5. A wide range of linearity for the matrix-matched calibration was given for ZEA between 4-150 µg/kg.

252

253 **3.2 Method validation**

254 Due to similar precisions, recoveries, LODs and LOQs of enniatins and beauvericin
255 (Habler & Rychlik, 2016; Hu & Rychlik, 2012) and low contaminations of non-polar
256 ENNs in beer to be expected (Hu et al., 2014; Vaclavikova et al., 2013) the method
257 validation was just performed for trichothecenes, ZEA and ENN B and validation data
258 of the latter were adopted for the other ENNs and BEA, if necessary.

259

260 **3.2.1 LODs and LOQs**

261 For the SIDAs the LODs and LOQs were calculated as suggested by Vogelgesang
262 and Hädrich (Vogelgesang & Hädrich, 1998). For the matrix-matched calibration the
263 LOD and LOQ were calculated from signal (S) to noise (N) ratio (LOD=3xS/N,
264 LOQ=10xS/N). Beer free of the monitored mycotoxins was used as blank matrix. The
265 LODs range between 0.05 and 6.9 µg/L and the LOQs range between 0.15 and 20
266 µg/L (**Table 2**). The LOD and LOQ of ENN B of the presented method showed 15
267 times more sensitivity to those reported by Hu & Rychlik (2012) or Vaclavikova et al.
268 (2013), which obviously is due to missing enrichment steps in the latter methods. The
269 sensitivities for type A and type B trichothecenes as well as for ZEA are comparable
270 with recently reported methods and thus should be sufficiently low (Al-Taher et al.,
271 2013; Harcz et al., 2007; Kostelanska et al., 2009).

272

273 **3.2.2 Precision**

The intra-day (n=3) and inter-day (n=3) coefficients of variation are shown in **Table 2**. The intra-day precision varied between 1 and 5% and the inter-day precision between 2 and 8%.

The precisions the stable isotope dilution assays and the matrix-matched calibration presented here were similar or even better to those previously reported (Hu et al., 2014; Al-Taher et al., 2013; Kostelanska et al., 2009; Zachariasova et al., 2010).

3.2.3 Recovery of SIDA and recovery of matrix-matched calibration

The recoveries of the SIDAs and the matrix-matched calibration were determined at 3 different spiking levels for each mycotoxin. The recoveries range between 72 and 117% with relative standard deviations (RSD) below 7% (**Table 2**). Labeled $[^{13}C]_2$-3-ADON was used as internal standard to quantify 15-ADON. This might be the reason of low recoveries between 72 and 77% for 15-ADON. Comparable recoveries around 78% for ADONs were reported by Kostelanska et al. (2009). The LC-MS/MS chromatogram of a spiked beer sample in the positive ESI mode is shown in **Figure 3**.

3.3 Analysis of (modified) mycotoxins in beer

In total, 30 different German organic and conventional beer samples (**Table 3**) as well as 31 beers from 12 different countries, including the USA, Canada, Mexico, Belgium, Iceland, Ireland, the Czech Republic, Israel, Japan, Thailand, Taiwan and China, were collected in 2015 (**Table 3**). Apart from 3 non-alcoholic beers, the alcohol content of the other investigated beers varied between 4.5 vol% and 10.5

297 vol%. The analyzed beer samples were partly contaminated with DON, D3G, 3-
298 ADON or ENN B. The other monitored mycotoxins like 15-ADON, HT2 and T2 as well
299 as ZEA, BEA and the other enniatins B1, A1 and A could not be detected in any of
300 the samples. Seven beer samples from Germany and eight beer samples from
301 Japan, Israel, Thailand, Iceland, the Czech Republic and Mexico were free of the
302 monitored mycotoxins.

303 In 1977 Lovelace et al. (1977) published an average concentration of 940 µg/L ZEA
304 in 15 “corn beers” from Zambia. The highest level of ZEA in these beers reached
305 4600 µg/L. In our investigated “corn beer” from 2015 ZEA could not be detected
306 above the LOD and the concentration of DON was very low with 3.40 µg/L.

307 26% of the analyzed beer samples were contaminated on average with 11.6 µg/L
308 DON, 13% with 19.6 µg/L D3G, 1.6% with 1.6 µg/L 3-ADON and 25% with 0.17 µg/L
309 ENN B. In these samples often DON and D3G occurred together, but with a varying
310 ratio of DON/D3G between 0.5 and 3.

311 In German beers the highest contamination could be found in one organic wheat
312 beer with a content of 28.8 µg/L DON, 10.6 µg/L D3G, and 1.6 µg/L 3-ADON.
313 However, due to the low sample number of analyzed organic beers, this result do not
314 allow the conclusion that organic beers posed a higher risk for consumers than
315 conventional beers. Harcz et al. (2007) investigated between 2003 and 2005
316 ochratoxin A and DON in conventional and organic beers. In average a higher
317 contamination of the organic beers with DON could not be found. In contrast to this
318 ochratoxin A could be analyzed less frequently but with higher concentrations in
319 organic beers. One pale ale from the USA had an even higher level of 34.5 µg/L
320 DON and 67.3 µg/L D3G. Other studies from Kostelanska et al. (2009), Zachariasova

et al. (2008) or Varga et al. (2013) showed greater incidences but similar contents of DON and D3G between 1.0 and 81.3 µg/L and 1.2 and 89.3 µg/L, respectively, in beers from European and North American markets. In contrast to Kostelanska et al. (2009) there was no trend discernible of higher DON concentrations with increasing alcohol content.

Due to their low polarity enniatins were not expected in beer (Hu et al., 2014; Vaclavikova et al., 2013). Our study showed negligibly small ENN B contents below 0.9 µg/L and no other enniatins or beauvericin were detectable.

The Tolerable Daily Intake for DON was set at 1 µg/kg body weight (Scientific Committee on Food 2005). Calculated with the DON level of 11.6 µg/L averaged found, a normal person weighing 70 kg could consume 6 liters of beer to reach the tolerable daily intake of 70 µg. This high consumption is abnormal and almost impossible. At least for the analyzed beer samples, it seems as even for heavy drinkers the exposure to these mycotoxins can be classified as low, but has to be minimized in any case.

4. Conclusions

This newly developed multi-mycotoxin method for *Fusarium* toxins in beer samples provides an approach involving precipitation of matrix compounds without decreasing recovery, which has the potential to increase specificity and sensitivity ~~applicable to~~ for all sorts of LC-MS/MS equipment.

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In the EU the maximum regulatorily permitted content of DON is 1250 µg/kg in unprocessed cereals, which usually is also applicable to brewing malt (European

Union, 2006). A charge around 15% can be assumed for normal lager, which means numerically that the maximum permitted content of DON would be 188 µg/L (1250 µg/kg x 15%) in beer ready to drink. All investigated beers were at least 5 times below that limit.

Likewise, a toxicological assessment reveals a negligible risk for consumers of beers imposed by the mycotoxins analyzed. Assuming a worst-case scenario with the highest contamination of 34.5 µg/L DON and the TDI set to 1 µg/kg body weight by the Scientific Committee on Food (2005), a person with a standard body weight of 70 kg would have to drink 2 L of beer to reach the TDI. However, the contribution of D3G to DON toxicity is still open and may require also maximum limits for this modified toxin.

In this regard, it is intended to follow the fate of mycotoxins during the entire brewing process and along with the multi-method for cereals (Habler & Rychlik, 2016), now the respective tool for liquid samples and beer is available and will be applied to studies on the carry-over of mycotoxins and options for reducing contamination.

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Conflict of interest

The authors declare that they have no competing interests.

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