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7 Fate of *Fusarium* Toxins during Brewing

8

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Feldfunktion geändert

24

25

26 **ABSTRACT**

27 Some information are available about the fate of *Fusarium* toxins during the brewing
28 process, but only little is known about the single processing steps in detail. In our
29 study we produced beer from two different barley cultivars inoculated with three
30 different *Fusarium* species, namely *Fusarium culmorum*, *Fusarium sporotrichioides*,
31 and *Fusarium avenaceum*, producing a wide range of mycotoxins such as type B
32 trichothecenes, type A trichothecenes, and enniatins. By the use of multi-mycotoxin
33 LC-MS/MS stable isotope dilution methods we were able to follow the fate of
34 *Fusarium* toxins during the entire brewing process. In particular, the type B
35 trichothecenes deoxynivalenol, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol
36 showed a similar behavior. Between 35 to 52% of those toxins remained in the beer
37 after filtration. The contents of the potentially hazardous deoxynivalenol-3-glucoside
38 and the type A trichothecenes increased during mashing, but a rapid decrease of
39 deoxynivalenol-3-glucoside content was found during the following steps of lautering
40 and wort boiling. Concentration of enniatins greatly decreased with the discarding of
41 spent grains or finally with the hot break. The results of our study show the retention
42 of diverse *Fusarium* toxins during the brewing process and allow for assessing the
43 food safety of beer regarding the monitored *Fusarium* mycotoxins.

44

45 **KEYWORDS:** *Fusarium* mycotoxins; stable isotope dilution assay; LC-MS/MS;
46 barley; brewing process

47

48

49 **INTRODUCTION**

50 With an average cultivation area of 50 million hectare and a crop size of 140 million
51 tons worldwide in 2015, barley can be rated along with wheat, rice and corn to the
52 most important cereals in the world.[1] Besides an increased production of cereals and
53 barley, the worldwide beer output increased in the last ten years from 1.55 billion to
54 1.96 billion hectoliters in 2014.[2] Therefore, beer can be considered an important
55 basic food. The quincentennial of the "German Beer Purity Law" and an average per
56 capita beer consumption in Austria, Germany and the Czech Republic over 100 liters
57 per person in 2014 [3] additionally underlines the need to ensure purity and quality of
58 beer. Beer is produced from natural cereal products and cereal crops are susceptible
59 to fungal infection. Fusarium head blight is a devastating fungal disease of small
60 grain cereals and in particular associated with *F. graminearum*, *F. culmorum,* and
61 *F. avenaceum.*[4] The infection of barley with *Fusarium* species leads to losses in yield
62 and grade and hence can result in declining end-use quality. Besides technological
63 problems during malting and brewing, a heavy fungal infestation of brewing barley is
64 mostly accompanied by a distinct mycotoxin contamination.[5,6] Food and feed heavily
65 loaded with *Fusarium* mycotoxins present a serious health risk for humans and
66 animals and compromise food safety. Therefore, impeccable barley and barley malt
67 quality is crucial for the production of high-quality beer. The maximum limits for
68 unprocessed cereals, being equivalent to brewing malt, are legislatively set at 1250
69 µg/kg for deoxynivalenol and at 100 µg/kg for zearalenone.[7] Maximum levels for type
70 A trichothecenes are recommended but have not yet been established.

71 The most abundant *Fusarium* toxins already observed in commercial beers are
72 zearalenone, the type B trichothecenes nivalenol, deoxynivalenol-3-glucoside,

73 deoxynivalenol, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol (Figure 1).[8–10]
74 The so-called modified mycotoxin deoxynivalenol-3-glucoside [11] that is mostly formed
75 during germination due to high enzymatic activity of
76 deoxynivalenolglycosyltransferase is an emerging issue concerning food safety and
77 risk assessments. Deoxynivalenol-3-glucoside can be cleaved to deoxynivalenol in
78 human and animal gastrointestinal tracts and thus can increase the initial amount of
79 deoxynivalenol.[12,13]

80 Earlier studies focused on fermentation and provide little information about the
81 influences of other key steps of the brewing process, especially mashing or lautering,
82 on the fate of *Fusarium* toxins. We applied the newly developed multi-mycotoxin
83 stable isotope dilution LC-MS/MS methods for cereals and beers [14,10] to monitor the
84 fate of *Fusarium* toxins (Figure 1) (deoxynivalenol; deoxynivalenol-3-glucoside; 3-
85 acetyldeoxynivalenol; 15-acetyldeoxynivalenol; HT-2-toxin; T-2-toxin; enniatin B, B1,
86 A1, A; and beauvericin) throughout mashing and brewing. Our study was based on
87 defined *Fusarium* species-specific contaminated basic material. Plants of two
88 different barley varieties (‘Grace’ and ‘Scarlett’) were artificially infected in field trials
89 with spores of three different *Fusarium* species (*F. culmorum*, *F. sporotrichioides* and
90 *F. avenaceum*, respectively). Resulting raw grain was malted as previously
91 reported.[15] Subsequently, these barley malts were brewed on a pilot scale. Samples
92 were taken at every key step of the brewing process. This study aimed at
93 simultaneously monitoring the fate of ten *Fusarium* mycotoxins throughout the
94 brewing process from barley malt grist to beer.

95

96 **MATERIALS AND METHODS**

97 **Chemicals and reagents.**

98 Acetonitrile, methanol, and water (analytical grade) were purchased from VWR
99 (Ismaning, Germany). Formic acid (>95%) was bought from Sigma-Aldrich
100 (Steinheim, Germany). The unlabeled reference compounds (deoxynivalenol-3-
101 glucoside, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, HT-2-
102 toxin, T-2-toxin and some labeled standards ($[^{13}C]_{15}$-deoxynivalenol, $[^{13}C]_{22}$-HT-2-
103 toxin) were bought from Coring System Diagnostix (Gernsheim, Germany). Enniatin
104 B was obtained from Bioaustralis (New South Wales, Australia) and the other
105 enniatins B1, A1 and A from Enzo Life Sciences (Lörrach, Germany). The labeled
106 internal standards ($[^{13}C]_{2}$-3-acetyldeoxynivalenol, $[^{13}C]_{4}$-T-2-toxin, $[^{15}N]_{3}$-enniatin B,
107 $[^{15}N]_{3}$-enniatin B1, $[^{15}N]_{3}$-enniatin A1, and $[^{15}N]_{3}$-enniatin A) were synthesized in our
108 laboratory as reported previously.[16–18]

109

110 **Raw material.**

111 Brewing experiments were carried out based on the same material as published
112 recently.[15] In brief, field experiments were conducted cultivating the spring barley
113 varieties 'Grace' (Gr) and 'Scarlett' (Sc) in 2014. At the time period of anthesis, field
114 plots (11.25 m^2) were artificially infected with *Fusarium* species-specific conidial
115 suspensions. Individual inocula were mixtures of spores from different isolates of *F.*
116 *culmorum* (Fc, Fc002, Fc06, Fc03), *F. avenaceum* (Fa, Fa002, Fa01) and *F.*
117 *sporotrichioides* (Fs, Fs001, Fs002, Fs03). Spore densities were dependent on
118 individual spore production potential of strains and, therefore, different for each

119 mixture (min.: 6.4×10^6 conidia/m^2, max.: 9.3×10^6 conidia/m^2). A respective number of
120 plots remained non-inoculated and served as control. Plant protection treatments and
121 fertilization were carried out according to regional standard rates. After harvesting
122 matured plants by using a single plot combine, obtained raw grain material was
123 stored in jute bags at room temperature in a dark and dry place. According to the
124 standard MEBAK [19] procedure and as already published [15] the malting process was
125 performed: steeping and germination time 6 days, germination temperature 15 °C,
126 95% relative humidity, steeping degree 45%, withering of germinated barley grains at
127 50 °C for 16 h, then kilning at 60 °C for 1 h, at 70 °C for 1 h, and finally at 80 °C for 5
128 h, followed by removing the rootlets at the end of kilning).

129

130 **Brewing process.**

131 Brewing was performed in a 10 L micro scale brewhouse at the Chair of Brewing and
132 Beverage Technology (TUM). Before mashing in, the malt was ground in a two-roll-
133 mill. The adjusted distance between crushing rollers was 1.8 mm. Grists (~1.5 kg
134 each) were added to 6 L of distilled water (62 °C). First rest was held on 62 °C with
135 continuous stirring. Afterwards mash was heated up to 72 °C (1 °C/min) and rest took
136 another 30 min while stirring. When the second rest was finished, mash was heated
137 up to 76 °C and the temperature was held for 5 min at 76 °C. Afterwards mash was
138 transferred in a preheated (76 °C) lauter tun, in which solid-liquid separation was
139 done. First wort was lautered by following two additions of 1.5 L of pre-heated
140 distilled water (76 °C) for recausticizing spent grains. After lautering, wort was
141 transferred into the wort kettle and boiled for 1 h. Hops were added (calc. as 20 IBU
142 in finished beer) when boiling started and wort boiling took another for 60 min until

143 the original extract of 11.5% w/w was reached. Hot trub was removed in a whirlpool
144 followed by wort cooling to 8 °C and pitching in a Cornelius container. The utilized
145 yeast was a Fermentis Saflager 34/70. Main fermentation was carried out at a
146 temperature of 12 °C till a gravity of 3.5% w/w was reached. Afterwards green beer
147 was hosed (transferred in a new Cornelius container) and maturation followed for 4
148 days. Beer (around 8 L each) was cold stored for 3 weeks (1 °C) and finally filtered
149 with depth filter sheets (Seitz K 200).

150

151 **Separate mashing and lautering process.**

152 The laboratory scale mashing and lautering process was performed with three
153 different malts of the variety 'Grace': The source material inoculated with *F.*
154 *culmorum* that has already been used in the brewing process was used directly and
155 further designated as "infected malt". Subsamples of this material were additionally
156 autoclaved (136 °C, 80 min, 3 bar) to inactivate barley and fungal enzymes and later
157 named "autoclaved malt". A so-called "mixed material" was prepared from 3 g
158 autoclaved grist and 7 g non-inoculated control grist to exclude fungal contamination
159 and to retain barley enzyme activities.

160 Ten g of each grist was mashed in with 40 mL distilled water (62 °C) and the same
161 mashing process and temperature program as described above was performed. For
162 solid-liquid separation mash was transferred in a funnel with glass wool. Lautering
163 was carried out by two additions of 10 mL pre-heated distilled water (76 °C) for
164 recausticizing spent grains.

165

166 **Samples.**

167 Samples were taken at each key step during the brewing process. In detail, grist,
168 mash, sweet wort, spent grains, original wort, hot break, green beer and finally
169 filtered beer were sampled (Figure 2). Grist, mash, sweet wort, and spent grains were
170 sampled during the laboratory scale mashing and lautering process. The total weight
171 and the sampled weight were recorded for every sample. Mash, spent grains, and hot
172 break were freeze-dried for 72 h before mycotoxin analysis and their water content
173 was calculated. All results and concentrations of mycotoxins refer to the wet weight of
174 the samples.

175

176 **Sample preparation.**

177 The sample preparations were performed as published recently.[14,10] In brief, between
178 10 mg and 1 g of finely ground homogenous solid samples (grist, freeze dried mash,
179 spent grains, and hot break) had 10 mL acetonitrile/water (84:16, v/v) added. The
180 samples were shaken in a laboratory shaker (225 rpm, 2 h). The resulting slurry was
181 filtered and 4 mL of the filtrate was spiked with the internal standards (50 µL of
182 $[^{13}C]_{15}$-deoxynivalenol (1 µg/mL), 100 µL of $[^{13}C]_{2}$-3-acetyldeoxynivalenol (1 µg/mL),
183 30 µL of $[^{13}C]_{22}$-HT-2-toxin (1 µg/mL), 30 µL of $[^{13}C]_{4}$-T-2-toxin (1 µg/mL) and 80 µL
184 each of $[^{15}N]_{3}$-enniatin B, $[^{15}N]_{3}$-enniatin B1, $[^{15}N]_{3}$-enniatin A1, and $[^{15}N]_{3}$-enniatin A
185 (0.1 µg/mL). The filtrate volume was reduced when necessary to fall into the linear
186 range of the response curves and to minimize standard consumption, but it was
187 restocked to 4 mL with acetonitrile/water (84:16, v/v) before purification.

188 To 5 mL of degassed liquid samples (sweet wort, original wort, green beer, and
189 filtrated beer), 10 mL acetonitrile and the internal standards were added (50 µL of
190 $[^{13}C]_{15}$-deoxynivalenol (1 µg/mL), 150 µL of $[^{13}C]_2$-3-acetyldeoxynivalenol (1 µg/mL),
191 60 µL of $[^{13}C]_{22}$-HT-2-toxin (1 µg/mL), 40 µL of $[^{13}C]_4$-T-2-toxin (1 µg/mL) and 70 µL
192 of $[^{15}N]_3$-enniatin B, $[^{15}N]_3$-enniatin B1, $[^{15}N]_3$-enniatin A1, and $[^{15}N]_3$-enniatin A,
193 respectively (0.1 µg/mL). Deoxynivalenol-3-glucoside was quantitated using $[^{13}C]_{15}$-
194 deoxynivalenol in liquid samples. The mixture was vortexed (20 sec) and centrifuged
195 (4000 rpm, 5 min, RT). The residue was extracted twice with 3 mL acetonitrile/water
196 (70:30, v/v), respectively, and the combined supernatants were evaporated to
197 dryness. Before purification the residue was dissolved in 4 mL acetonitrile/water
198 (84:16, v/v).

199 The solid phase extraction was performed equally for solid and liquid samples. The
200 samples were completely applied on a Bond Elut Mycotoxin cartridge, 500 mg, 3 mL,
201 (Agilent Technologies, Santa Clara, CA) and the liquids were passed through the
202 cartridges by vacuum suction. The eluates were collected and evaporated until
203 dryness. The samples were reconstituted with 200 µL acetonitrile/water (1:1, v/v) and
204 membrane filtered (0.45 µm). Afterwards, LC-MS/MS analysis was performed.

205 Analog solid samples free of the monitored mycotoxins (1 g) were chosen as blank
206 matrix for the matrix-matched calibration. The solid blank samples were spiked with
207 six different amounts of deoxynivalenol-3-glucoside (20–500 µg/kg). After
208 evaporation of the solvent the same sample preparation and purification for solids
209 was performed as described above.

210

211 **Mycotoxin analysis via LC-MS/MS.**

212 To follow the fate of the monitored *Fusarium* toxins during mashing and brewing
213 HPLC and MS/MS parameters of the previously published multi-mycotoxin LC-
214 MS/MS stable isotope dilution methods were used.[14,10]

215 In brief, LC-MS/MS was carried out on a Shimadzu LC-20A Prominence system
216 (Shimadzu, Kyoto, Japan) with a 150 x 3.0 mm ID, S-3 µm Hydrosphere RP-C_{18}
217 column (YMC Europe GmbH, Dinslaken, Germany) and a C_{18}-guard column
218 (Phenomenex, Aschaffenburg, Germany) as stationary phase that was kept at 40 °C.
219 The binary gradient system consisted of (A) 0.1% formic acid and (B) methanol with
220 0.1% formic acid at a flow rate of 0.2 mL/min. The gradient for the negative ESI mode
221 was started and held at 10% B for 2 min, raised linearly from 10-99% B during the
222 next 6 min, and then maintained at 99% B for 7.5 min. Next, the mobile phase
223 returned to 10% B within 2 min and the system was equilibrated for 9.5 min before
224 the next run. The gradient for the positive ESI mode was started and held at 10% B
225 for 2 min, raised linearly from 10-87% B during the next 6 min, held at 87% for 7 min,
226 raised to 100% B during the next 5 min, and then maintained at 100% B for 3.5 min.
227 Next, the mobile phase returned to 10% B within 2 min and the system was
228 equilibrated for 9.5 min before the next run. The injection volume was 10 µL.

229 The LC was interfaced with an API 4000 Qtrap hybrid triple quadrupole/linear ion trap
230 mass spectrometer (Applied Biosystems Inc., Foster City, CA). It operated in the
231 negative ESI mode for the analyte deoxynivalenol-3-glucoside in solid samples and
232 in the positive ESI mode for the analytes deoxynivalenol-3-glucoside in liquid
233 samples as well as deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol,
234 HT-2-toxin, T-2-toxin, enniatin B, B1, A1, and A independent of the sample

235 properties. The ion source parameters for the negative mode were set as follows:
236 curtain gas 20 psi, CAD gas pressure medium, ion spray voltage −4500 eV, spray
237 gas 50 psi, dry gas 65 psi, and temperature 525 °C. The ion source parameters for
238 the positive mode were set as follows: curtain gas 20 psi, CAD gas pressure high, ion
239 spray voltage 4500 eV, spray gas 80 psi, dry gas 75 psi, and temperature 450 °C.

240

241

242 **RESULTS AND DISCUSSION**

243 **Sample preparation and LC-MS/MS analysis.**

244 The recently published multi-mycotoxin stable isotope dilution LC-MS/MS methods
245 for cereals and beers [14,10] were applied to follow the fate of ten *Fusarium* toxins
246 throughout the brewing process. In detail, deoxynivalenol, deoxynivalenol-3-
247 glucoside, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, HT-2-toxin, T-2-toxin,
248 enniatin B, enniatin B1, enniatin A1, and enniatin A were analyzed in the samples at
249 the following key steps of the brewing process (Figure 2): grist, mash, sweet wort,
250 spent grains, original wort, hot break, green beer, and filtered beer. In the separate
251 laboratory scale mashing and lautering process deoxynivalenol, deoxynivalenol-3-
252 glucoside, and the acetylated deoxynivalenol derivatives were determined in grist,
253 mash, spent grains, and sweet wort of autoclaved, mixed, and inoculated malt grist,
254 respectively. The contents of 15-acetyldeoxynivalenol in sweet wort, original wort,
255 green beer, and beer were corrected for 72% recovery, according to the validation
256 results recently published.[10]

257

258 **Fate of *Fusarium* toxins during brewing.**

259 Our brewing trials were based on the above described six inoculated and two control
260 barley malts. In previous studies [15] these raw materials were malted according to the
261 standard MEBAK procedure.[19] Plants for the control barley samples were grown
262 under natural field conditions without inoculation. The mycotoxin levels of these
263 control samples collected throughout brewing were mostly below the limit of detection
264 or quantitation (data not shown). Hops and yeast added during brewing were also

265 analyzed and classified as free from the monitored mycotoxins. Hence, in the present
266 samples, all relevant toxin contents derived from inoculation with individual *Fusarium*
267 species.

268 The concentrations of deoxynivalenol, deoxynivalenol-3-glucoside, 3-
269 acetyldeoxynivalenol, and 15-acetyldeoxynivalenol in the samples collected
270 throughout the brewing process from inoculated barley batches are shown in Table 1.
271 The behavior of deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol
272 was similar in both cultivars throughout the brewing process. After mashing, the
273 residual amounts of the latter mycotoxins ranged between 76% (Gr-Fc) and 120%
274 (Sc-Fc) of the initial levels. The lautering step reduced the contents of
275 deoxynivalenol, deoxynivalenol-3-glucoside, 3-acetyldeoxynivalenol, and 15-
276 acetyldeoxynivalenol by a maximum of 13% (Sc-Fc) through the separation of the
277 spent grains. A similar amount of 14% deoxynivalenol in spent grains was observed
278 by Inoue et al..[20] In sweet wort around 84% deoxynivalenol, 94% 3-
279 acetyldeoxynivalenol, and 65% 15-acetyldeoxynivalenol of the initial level (average of
280 both cultivars) was observed. Lancova et al. [21] obtained similar ranges (70-90%) for
281 deoxynivalenol and acetyldeoxynivalenol derivatives in sweet wort made from
282 naturally contaminated and artificially infected raw material. After wort boiling, on
283 average 0.6% of each type B trichothecene was removed with the hot break. In line
284 with previous reports [20–22], we observed that fermentation, maturing, and filtration did
285 not change significantly the concentrations of deoxynivalenol, 3-
286 acetyldeoxynivalenol, and 15-acetyldeoxynivalenol in original wort, as well as in
287 green beer, and beer. In contrast to this, Nathanail et al. [23] reported a decrease of
288 maximum 15% for deoxynivalenol during fermentation. The absolute contents of

289 deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol were lower in
290 beer than in green beer as a result of losing beer during the filtration step. On
291 average for both cultivars, 46% deoxynivalenol, 49% 3-acetyldeoxynivalenol, and
292 35% 15-acetyldeoxynivalenol of the initial levels remained in filtered beer. Our results
293 on the contents of the latter toxins in beer are lower by a factor of 2 compared to
294 findings previously published by Lancova et al. [21] and Schwarz et al..[24] By contrast,
295 Kostelanska et al. [22] observed an increase of deoxynivalenol of 195-365% in final
296 beer. These authors assumed this to result from a release of bound mycotoxins due
297 to physiochemical and enzymatic processes.

298 Similar to the already described type B trichothecenes, the modified mycotoxin
299 deoxynivalenol-3-glucoside increased to maximum 120% of the initial level after
300 mashing, independent of the cultivar. But in contrast to deoxynivalenol, 3-
301 acetyldeoxynivalenol, and 15-acetyldeoxynivalenol, the content of deoxynivalenol-3-
302 glucoside rapidly decreased during lautering beyond expectation: Sweet wort had
303 only 26% deoxynivalenol-3-glucoside in cultivar 'Grace' and 23% deoxynivalenol-3-
304 glucoside in cultivar 'Scarlett' of the respective initial amounts. The spent grains were
305 only contaminated with maximum 3.5% deoxynivalenol-3-glucoside (Sc-Fc). To our
306 knowledge, this change of deoxynivalenol-3-glucoside during lautering has not been
307 reported before. Lancova et al. [21] and Kostelanska et al. [22] investigated
308 deoxynivalenol-3-glucoside from malt grist to final beer in several intermediate steps,
309 but not in mash. In their studies, an increase of deoxynivalenol-3-glucoside from grist
310 to sweet wort between 350-1400% was observed and again referred to a release of
311 bound toxins during mashing due to physiochemical and enzymatic processes.
312 However, no direct relation between deoxynivalenol-3-glucoside in mash and sweet

313 wort could be made. In our study hardly any fluctuations in the concentrations of
314 deoxynivalenol-3-glucoside in sweet wort, original wort, green beer, and beer could
315 be measured in both cultivars. About 1.3% deoxynivalenol-3-glucoside were removed
316 with the hot break from the remaining process (average of both cultivars). At the end
317 18% deoxynivalenol-3-glucoside (Gr-Fc) or 13% deoxynivalenol-3-glucoside (Sc-Fc)
318 were left in filtered beer. Hence, lautering emerged as the most relevant step during
319 brewing for removal of deoxynivalenol-3-glucoside. To verify and understand the
320 modification or degradation of deoxynivalenol-3-glucoside during lautering we
321 performed a laboratory scale mashing and lautering process. An enzyme based
322 modification of deoxynivalenol-3-glucoside could be assumed because optimal
323 temperatures for malt enzymes occurred during mashing. To investigate this
324 assumption in more detail, in the separate laboratory scale mashing and lautering
325 trial autoclaved malt was further used to exclude any enzyme activities of fungi and
326 barley. Mixed malt (a mixture of autoclaved malt and control malt) was used to
327 exclude fungal enzyme activity and retain barley enzyme activity. As repetition, the
328 same malt inoculated with *F. culmorum* as already used in the brewing process was
329 investigated to confirm our previous results.

330 We measured fungal and plant DNA of the infected, mixed and autoclaved malt via
331 quantitative polymerase chain reaction. As published recently,[15] in contrast to the
332 control malt the infected malt was heavily loaded with *Fusarium* spp. DNA. Neither
333 plant nor fungal DNA was detectable in the autoclaved malt (data not shown).
334 Therefore, we concluded that along with the DNA the malt and fungal enzymes were
335 destroyed during autoclaving.

The fate of deoxynivalenol, deoxynivalenol-3-glucoside, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol during mashing and lautering of autoclaved, mixed, and inoculated malt is shown in Figure 3. The behavior of deoxynivalenol, deoxynivalenol-3-glucoside, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol in inoculated malt of the laboratory scale mashing and lautering process was quite similar to the results of the brewing process. Consequently, the rapid decrease of deoxynivalenol-3-glucoside as well as the steady behavior of the other type B trichothecenes during lautering could be confirmed. However, during lautering of mixed malt the same phenomenon of deoxynivalenol-3-glucoside appeared: We observed deoxynivalenol-3-glucoside at 28% of the initial level in sweet wort, whereas only 2% was removed with the spent grains and the level of deoxynivalenol remained stable. In contrast to the latter results, deoxynivalenol-3-glucoside showed a different behavior during lautering of the autoclaved malt: of the initial levels, 43% in sweet wort and 35% in spent grains could be found. The sum of both percentages (78% deoxynivalenol-3-glucoside) could approximately reach the original content of deoxynivalenol-3-glucoside in mash and revealed a significantly higher level (at a two-sided p level < 0.01) than in inoculated or mixed malt (performing a statistical significance t-test with $n = 2$). The content of deoxynivalenol in the autoclaved malt trial again remained constant. 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol could not be detected in autoclaved and mixed malt. Thus, during mashing and lautering a modification or degradation of deoxynivalenol-3-glucoside to deoxynivalenol and acetylated deoxynivalenol derivatives or vice versa appeared rather improbable regarding our results. Hence, the decrease of deoxynivalenol-3-glucoside during lautering could be traced to enzyme activity in barley. A modification of deoxynivalenol-3-glucoside to deoxynivalenol-oligo/poly-glucosides [25], deoxynivalenol glutathione adducts [26] or

361 other reported biologically modified deoxynivalenol products, e.g. deoxynivalenol
362 cysteine, cysteine-glycine, malonylglucoside and hexoside derivatives [27], might be
363 possible.

364

365 The concentrations of HT-2-toxin and T-2-toxin in the samples collected throughout
366 the brewing process from Fs-inoculated barley batches are shown in Table 2. During
367 mashing we observed an increase of HT-2-toxin and T-2-toxin to a maximum of
368 200% and 330% of the initial levels, respectively. This increase can either be
369 attributed to a further production of toxins due to fungal activity or to a release of
370 bound mycotoxins due to physical or enzymatic processes. The latter hypothesis was
371 also suggested by Kostelanska et al. [28]. Independent of the cultivar, 150% HT-2-toxin
372 of the initial level was found in sweet wort. The analyzed spent grains were
373 moderately contaminated with 24% HT-2-toxin in cultivar 'Grace' and 14% HT-2-toxin
374 in cultivar 'Scarlett'. In accordance with Lancova et al., [21] the spent grains were
375 heavier loaded with T-2-toxin: 75% T-2-toxin in cultivar 'Grace' and 47% T-2-toxin in
376 cultivar 'Scarlett'. The mass difference of T-2-toxin between mash and sweet wort
377 could almost completely be attributed to spent grains. The absolute amount of 410 µg
378 (280% of the respective initial level) T-2-toxin (Gr-Fs) and 140 µg (230%) T-2-toxin
379 (Sc-Fs) remained in sweet wort. Maximum 2.5% HT-2-toxin (Sc-Fa) and 3.7% T-2-
380 toxin (Sc-Fa) were separated from wort with the hot break. In accordance with
381 Lancova et al. [21] and Inoue et al.,[20] the respective concentrations of HT-2-toxin and
382 T-2-toxin in original wort hardly differed or did not differ at all from those in green beer
383 or filtered beer. Nathanail et al. [23] provide contrasting results. They reported a
384 decrease of HT-2-toxin and T-2-toxin up to 34% during fermentation. In our study, the

385 level of type A trichothecenes in beer could be reduced neither by fermentation nor
386 by maturing, storage or filtration. Finally, 95% HT-2-toxin and 135% T-2-toxin of the
387 initial levels were retrieved in filtered beer (average of both cultivars).

388

389 The concentrations of enniatin B, B1, A1, and A in the samples collected throughout
390 the brewing process from inoculated barley batches are shown in Table 3. During
391 brewing all four monitored enniatins showed a similar behavior irrespective of barley
392 cultivar. As indicated previously, [15] enniatins were again present in following
393 descending order of concentration: enniatin B, B1, A1, and A. After mashing, we
394 could observe just a slight change of enniatins between 80% and 130% of the initial
395 levels in malt grist (Gr-Fa, Sc-Fa). The subsequent lautering could be deemed as the
396 most important step during brewing to separate enniatins from the remaining
397 process. The analyzed spent grains were highly contaminated with enniatins and
398 between 60% enniatin B (Sc-Fa) and 130% enniatin A (Gr-Fa) of the initial levels
399 were found in the spent grains. Vaclavikova et al. [29] and Hu et al. [30] reported similar
400 results of enniatins in spent grains. Accordingly, maximum 4.7% enniatin B (Gr-Fa)
401 was found in sweet wort, whereas enniatin A could no longer be detected in sweet
402 wort (Gr-Fa, Sc-Fa). The levels of enniatin B1 and A1 in sweet wort were in between
403 with 1.7% (Gr-Fa) to 2.8% (Sc-Fa). As expected and already published by Hu et al.
404 [30], the contents of enniatins resolved in sweet wort were analogous to their polarity in
405 the following decreasing order: enniatin B, B1, A1, and A. The remaining amounts of
406 enniatins were mostly removed with the hot break, in which between 0.3% enniatin A
407 (Gr-Fa, Sc-Fa) and 2.8% enniatin B (Gr-Fa) of the initial enniatin levels were found.
408 The concentrations of enniatin A in original wort, green beer, and beer (Gr-Fa, Sc-Fa)

409 were below the limit of detection. In Sc-Fa, enniatin B1 could not be evidenced in
410 green beer and beer and enniatin A1 was even in original wort no longer detectable.
411 The amount of enniatins was hardly influenced by fermentation, storage, and
412 filtration. A maximum of 0.5% enniatin of the initial level (Gr-Fa) remained in finished
413 beer.

414

415 With this study we could follow the fates of ten *Fusarium* mycotoxins from malt grist
416 to filtered beer during the barley brewing process based on material deriving from two
417 field-inoculated and subsequently malted barley cultivars. In general, all investigated
418 *Fusarium* toxins were not affected significantly by wort boiling, fermentation,
419 maturing, storage or filtration. In our study, mashing and lautering proved to be the
420 most important and interesting steps influencing mycotoxin levels during brewing.
421 Except for deoxynivalenol-3-glucoside, the other type B trichothecenes did not reveal
422 major changes during brewing and between 35% and 52% of the initial levels
423 remained in finished beer. Interestingly, deoxynivalenol-3-glucoside disappeared by
424 more than 90% during lautering performed with water pre-heated to 76 °C. These
425 results demonstrate that the (temperature) conditions during lautering maintain a
426 residual enzyme activity in barley suggesting further enzymatic modification of
427 deoxynivalenol-3-glucoside tocompounds for which reference substances are not yet
428 available. . Due to the inconsistent behavior of deoxynivalenol-3-glucoside during the
429 brewing process a potential future maximum limit for this compound will not be
430 accessible from a calculation based on the level of 18% for normal lager as it is
431 commonly applied for deriving the maximum permitted level for deoxynivalenol from
432 malt.

433 In this study we investigated the fate of *Fusarium* toxins during brewing of two
434 different barley cultivars 'Grace' and 'Scarlett'. 'Scarlett' showed a lower abundance
435 of type A trichothecenes and enniatins in malt and final beer up to 60% and 35% of
436 the respective amounts in 'Grace'. In contrast to this, the output load for 'Scarlett' with
437 type B trichothecenes was up to 50% higher than for 'Grace', but we could observe a
438 similar contamination level in final beer regardless of the cultivar. The retention or
439 accumulation of the respective *Fusarium* toxins during brewing was independent of
440 the two barley varieties and their output load. Therefore, the two brewing trials based
441 on the cultivars 'Grace' and 'Scarlett' could be taken as repetitions to confirm the
442 respective behavior of the toxins. A statement concerning resistances of barley
443 cultivars in respect of *Fusarium* toxins cannot be drawn from our results.
444 Trichothecene content in our inoculated samples by far exceeded (up to a factor of
445 ~200) the levels reported for most naturally infected spring barley samples.[5,6,15]

446 The spent grains removed from the brewing process were highly contaminated with
447 type A trichothecenes and especially with enniatins. For enniatins, concentrations
448 reported here can be similarly found in natural infected barley.[31,30]

449 Hence, spent grains or draff often used as animal feed might present a serious health
450 risk to livestock. In "*Fusarium*-years" an analysis of spent grains regarding *Fusarium*
451 toxins is recommended to prevent feeding of highly mycotoxin-loaded fodder. As
452 enniatins are completely removed with the spent grains or latest with the hot break, in
453 finished beer the type A and type B trichothecenes can be considered as more
454 relevant with respect to food safety. However, the beers brewed from our control
455 barley malts that are comparable to commercial brewing malt contained neither type
456 A nor type B trichothecenes in 2015. Additionally considering our recent study of

457 *Fusarium* toxins in beers[10], it appears that even regular consumption of beer only
458 moderately exposes consumers to *Fusarium* toxins. Although the contamination level
459 in beer can be classified as low in the year 2015, it is subject to significant annual
460 variations and hence has to be minimized in any case.

461

462

463 **ABBREVIATIONS**

464 Fa, *Fusarium avenaceum*; Fc, *Fusarium culmorum*; Fs, *Fusarium sporotrichioides*;
465 Gr, Grace; Sc, Scarlett

466

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474

475

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577

578 **FIGURE CAPTIONS**

579 Figure 1. Structure of Type B **(1-4)**, Type A Trichothecenes **(5, 6)**, and Enniatins **(7-**
580 **10)**

581 Figure 2. Key Steps of Brewing Process

582 Figure 3. Fate of Deoxynivalenol **(1)**, Deoxynivalenol-3-Glucoside **(2)**, 3-
583 Acetyldeoxynivalenol **(3)**, and 15-Acetyldeoxynivalenol **(4)** during Mashing and
584 Lautering of (A) Autoclaved Malt, (B) Mixed Malt (a mixture of autoclaved and non-
585 inoculated control malt), and (C) Infected Malt (derived from field-inoculated and
586 subsequently malted barley)

587

588

Tables

Table 1. Contents of Type B Trichothecenes in Grist, Mash, Sweet Wort, Spent Grains, Original Wort, Hot Break, Green Beer, and Beer Prepared from Two Barley Cultivars 'Grace' (Gr-Fc) and 'Scarlett' (Sc-Fc) Inoculated with *F. culmorum*

Sample ID	DON (1)			D3G (2)			3-AcDON (3)			15-AcDON (4)		
		Total			Total			Total			Total	
	µg/kg	mg	%	µg/kg	mg	%	µg/kg	mg	%	µg/kg	µg	%
Gr-Fc												
Grist	10000	9.3	100	19000	17	100	1800	1.6	100	670	600	100
Mash	1700	7.3	78	4900	21	120	380	1.6	100	110	450	76
Sweet wort	1500	7.8	84	830	4.3	26	290	1.5	93	68[b]	360	60
Spent grains	240	0.2	2.5	510	0.5	2.9	63	0.06	3.8	55	54	9.1
Original wort	1400	7.2	77	880	4.4	26	250	1.3	79	65[b]	320	54
Hot break	420	0.05	0.6	1900	0.2	1.4	68	0.01	0.5	21	4.6	0.5
Green beer	1500	7.1	76	1100	4.9	29	260	1.2	76	67[b]	310	52
Beer	1400	4.6	50	980	3.3	18	260	0.8	52	64[b]	210	35
Sc-Fc												
Grist	13000	10	100	28000	22	100	2400	1.9	100	870	690	100
Mash	2200	8.9	85	6500	26	120	530	2.1	110	200	800	120
Sweet wort	1900	8.6	83	1100	5.0	23	390	1.8	94	110[b]	490	71
Spent grains	300	0.3	2.8	800	0.8	3.5	98	0.1	5	93	89	13
Original wort	1800	8.5	81	990	4.6	21	360	1.7	90	94[b]	440	64
Hot break	1000	0.1	0.7	3500	0.3	1.2	180	0.01	0.7	53	3.9	0.6
Green beer	1900	8.3	80	1100	4.8	22	340	1.5	80	100[b]	440	64
Beer	1500	4.3	42	980	2.9	13	300	0.9	46	82[b]	240	35

[a] below limit of quantitation; [b] recovery-corrected (72%)

Table 2. Contents of Type A Trichothecenes in Grist, Mash, Sweet Wort, Spent Grains, Original Wort, Hot Break, Green Beer, and Beer Prepared from Two Barley Cultivars 'Grace' (Gr-Fc) and 'Scarlett' (Sc-Fc) Inoculated with *F. sporotrichioides*

Sample ID	HT-2-toxin (5)			T-2-toxin (6)		
		Total			Total	
	µg/kg	µg	%	µg/kg	µg	%
Gr-Fs						
Grist	180	120	100	200	130	100
Mash	72	270	200	130	490	330
Sweet wort	45	200	150	90	410	280
Spent grains	36	32	24	120	110	75
Original wort	38	170	120	72	320	210
Hot break	38	2.2	1.6	50	2.9	2.0
Green beer	42	170	130	79	320	220
Beer	41	120	92	71	210	140
Sc-Fs						
Grist	69	53	100	78	61	100
Mash	26	100	190	41	160	260
Sweet wort	18	82	150	30	140	230
Spent grains	7.9	7.7	14	29	28	47
Original wort	15	67	130	25	110	180
Hot break	22	1.3	2.5	37	2.3	3.7
Green beer	16	64	120	26	107	180
Beer	17	52	98	25	77	130

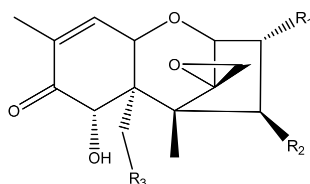
Table 3. Contents of Enniatins in Grist, Mash, Sweet Wort, Spent Grains, Original Wort, Hot Break, Green Beer, and Beer Prepared from Two Barley Cultivars 'Grace' (Gr-Fc) and 'Scarlett' (Sc-Fc) Inoculated with *F. avenaceum*

Sample ID	enniatin B (7)			enniatin B1 (8)			enniatin A1 (9)			enniatin A (10)		
		Total			Total			Total			Total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
Gr-Fa												
Grist	6100	4100	100	1200	820	100	160	100	100	12	8	100
Mash	1200	3900	97	310	1000	120	42	140	130	3.1	10	130
Sweet wort	38	160	4.7	4.7	20	2.4	0.4	1.8	1.7	-		
Spent grains	3800	3100	92	1100	940	110	150	120	120	12	10	130
Original wort	9.0	35	1.0	3.4	13	1.6	0.5	1.8	1.8	-		
Hot break	2600	94	2.8	210	7.7	0.9	15	0.6	0.5	0.7	0.03	0.3
Green beer	3.6	13	0.4	2.4	8.8	1.1	0.3	1.0	0.9	-		
Beer	2.6	6.9	0.2	1.4	3.7	0.5	0.2	0.5	0.5	-		
Sc-Fa												
Grist	4200	3900	100	890	820	100	100	97	100	8.4	7.8	100
Mash	720	3300	86	190	860	110	22	103	110	1.4	6.2	80
Sweet wort	19	100	2.7	3.2	17	2.1	0.5[a]	2.7	2.8	-		
Spent grains	2000	2300	60	560	640	78	65	75	78	5.3	6.1	78
Original wort	4.5	24	0.6	0.2	1.3	0.2	-			-		
Hot break	1600	70	1.8	150	6.4	0.8	11	0.5	0.5	0.6	0.03	0.3
Green beer	1.0	4.9	0.1	-			-			-		
Beer	0.9	2.6	0.07	-			-			-		

[a] below limit of quantitation; - not detectable

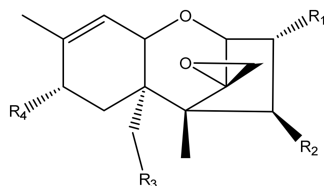
Figure 1.

Type B Trichothecenes



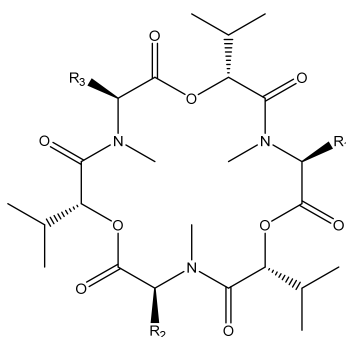
Deoxynivalenol, DON **(1)**	$R_1 = R_3 = OH, R_2 = H,$
Deoxynivalenol-3-glucoside, D3G **(2)**	$R_1 = Glc, R_2 = H, R_3 = OH$
3-Acetyldeoxynivalenol, 3-AcDON **(3)**	$R_1 = OAc, R_2 = H, R_3 = OH$
15-Acetyldeoxynivalenol, 15-AcDON **(4)**	$R_1 = R_3 = OAc, R_2 = H$

Type A Trichothecenes



HT-2-toxin **(5)**	$R_1 = R_2 = OH, R_3 = OAc, R_4 = OCOCH_2CH(CH_3)_2$
T-2-toxin **(6)**	$R_1 = OH, R_2 = R_3 = OAc, R_4 = OCOCH_2CH(CH_3)_2$

Enniatins



Enniatin B **(7)**	$R_1 = R_2 = R_3 = CH(CH_3)_2$
Enniatin B1 **(8)**	$R_1 = R_2 = CH(CH_3)_2, R_3 = CH(CH_3)CH_2CH_3$
Enniatin A1 **(9)**	$R_1 = CH(CH_3)_2, R_2 = R_3 = CH(CH_3)CH_2CH_3$
Enniatin A **(10)**	$R_1 = R_2 = R_3 = CH(CH_3)CH_2CH_3$

Figure 2.

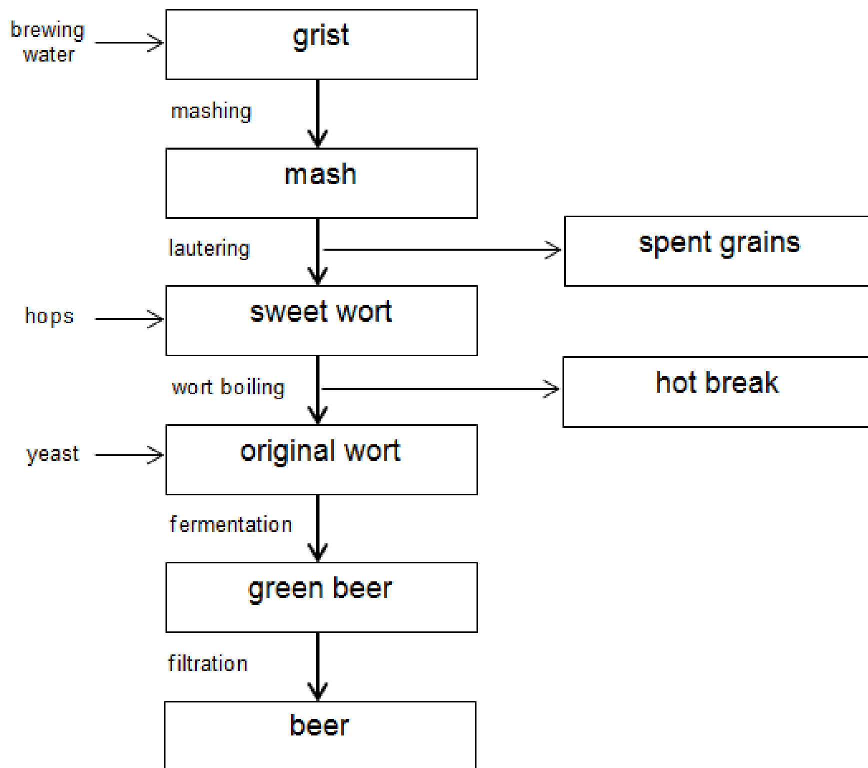


Figure 3.

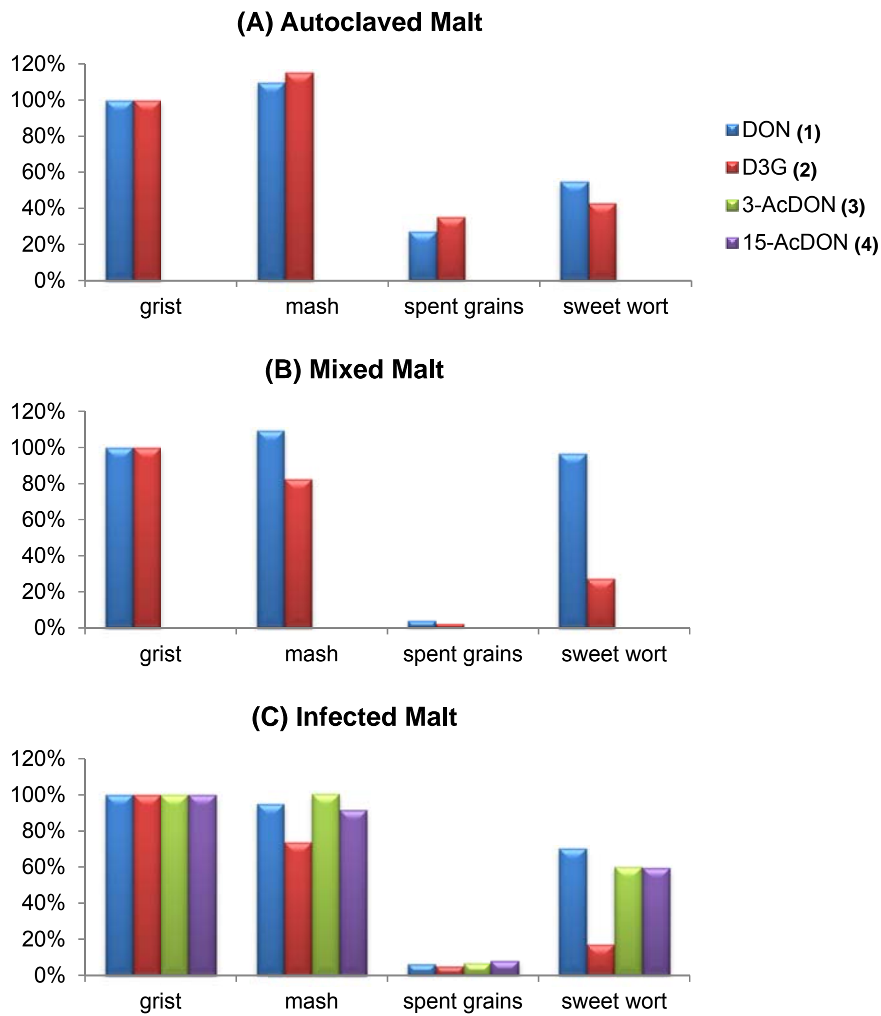


Table of Contents Graphic