Fate of *Fusarium* Toxins during the Malting Process

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ABSTRACT

Little is known about the fate of *Fusarium* mycotoxins during the barley malting process. To determine the fungal DNA and mycotoxin concentrations during malting, we used barley grain harvested from field plots that we had inoculated with *Fusarium* species that produce type A or type B trichothecenes or enniatins. Using a recently developed multi-mycotoxin liquid chromatography-tandem mass (LC-MS/MS) stable isotope dilution method, we identified *Fusarium* species-specific behaviors of mycotoxins in grain and malt extracts and compared toxin concentrations to amounts of fungal DNA in the same samples. In particular, the type B trichothecenes and *Fusarium culmorum* DNA contents dramatically were increased up to 5400% after kilning. By contrast, the concentrations of type A trichothecenes and *Fusarium sporotrichioides* DNA decreased during the malting process. These data suggest that specific *Fusarium* species that contaminate the raw grain material might have different impacts on malt quality.

KEYWORDS: *Fusarium* mycotoxins; stable isotope dilution assay; LC-MS/MS; qPCR; barley; malting process

INTRODUCTION

Small grain cereal crops such as wheat and barley are often infected by *Fusarium* spp. and are thus contaminated with various *Fusarium* mycotoxins. In barley, Fusarium head blight (FHB) disease can reduce both crop yield and grain quality.[1] Barley is primarily used as animal fodder and for malt production, which is used in the food industry, for example for baking and beer brewing. Regarding malt production, maltsters and brewers require high-quality raw barley material to ensure the impeccable quality of their final products. In addition to technological problems during brewing, fungal infection of the barley grain can cause food safety and human health risks. Accordingly, the European Union legislation has set maximum limits for the concentrations of zearalenone at 100 µg/kg and type B trichothecene deoxynivalenol at 1250 µg/kg in unprocessed cereals.[2] Several mycotoxins have been identified in beer such as aflatoxins, fumonisins, ochratoxin A, enniatins, zearalenone, type A trichothecene HT-2-toxin, deoxynivalenol, 3- and 15-acetyldeoxynivalenol, and modified mycotoxins such as deoxynivalenol-3-glucoside (Figure 1).[3–5] Deoxynivalenol and its derivatives, which have high levels of thermal stability and water solubility, are the most abundant mycotoxins in beer, with reported levels of deoxynivalenol and deoxynivalenol-3-glucoside as high as 36 and 51 µg/L, respectively [3,5]. Because plants use phase II metabolism as both a defense and detoxification mechanism, deoxynivalenol is exposed to glycosylation and can be metabolized to deoxynivalenol-3-glucoside, which is referred to as a modified mycotoxin.[6] In addition, deoxynivalenol-3-glucosidecan be hydrolyzed to deoxynivalenol in human and animal gastrointestinal tracts.[7] This process might

increase the total concentration of deoxynivalenol in beer and the overall exposure of consumers and livestock to deoxynivalenol.

Previous studies have investigated the fate of type B trichothecenes from natural *Fusarium*-infected samples during brewing.[5,8] Additionally, some information concerning the concentrations of *Fusarium* toxins during specific steps of the malting process is available.[3,9–11] In the present study, we used grain harvested from inoculated barley field plots from 2014. In contrast to previous investigations, this material should allow robust monitoring of the fates of fungal toxins throughout the malting process as the levels of fungal contaminants should be relatively high in the raw grain material. To accurately quantitate the fates of *Fusarium* toxins (deoxynivalenol, deoxynivalenol-3-glucoside, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, HT-2-toxin, T-2-toxin, enniatin B, B1, A1, A, and beauvericin) during the malting process, we used a recently developed multi-mycotoxin stable isotope dilution LC-MS/MS method intended for the identification of these toxins in cereals.[12] Additionally, quantitative polymerase chain reaction (qPCR) was used for fungal DNA analysis, which was not applied in earlier reports on the mycotoxins' fate during malting and enabled us here to evaluate fungal growth.. This laboratory-scale study was performed using grain from two different barley varieties ('Grace' and 'Scarlett') that had been inoculated with three different *Fusarium* species (*F. culmorum*, *F. sporotrichioides*, and *F. avenaceum*), respectively, and as well as two control batches. The aim of this study was to simultaneously monitor the behaviors of *Fusarium* DNA and 11 *Fusarium* mycotoxins from barley grain to malt at all key steps of the malting process.

MATERIALS AND METHODS

Chemicals and reagents.

Acetonitrile, methanol, and water (analytical grade) were purchased from VWR (Ismaning, Germany). Formic acid (>95%) was purchased from Sigma-Aldrich (Steinheim, Germany). Unlabeled reference compounds (deoxynivalenol-3-glucoside, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, HT-2-toxin, and T-2-toxin) and some labeled standards ($[^{13}C]_{15}$-deoxynivalenol and $[^{13}C]_{22}$-HT-2-toxin) were purchased from Coring System Diagnostix (Gernsheim, Germany). Enniatin B was obtained from Bioaustralis (New South Wales, Australia); enniatins B1, A1, and A were obtained from Enzo Life Sciences (Lörrach, Germany). Labeled internal standards ($[^{13}C]_2$-3-acetyldeoxynivalenol, $[^{13}C]_4$-T-2-toxin, $[^{15}N]_3$-enniatin B, $[^{15}N]_3$-enniatin B1, $[^{15}N]_3$-enniatin A1, $[^{15}N]_3$-enniatin A, and $[^{15}N]_3$-beauvericin) were synthesized in our laboratory as reported previously.[13–15]

Raw materials.

Malting experiments were conducted using grain of the spring barley cultivars ‘Scarlett’ (Sc; Saatzucht Josef Breun GmbH & Co. KG, Herzogenaurach, Germany), and ‘Grace’ (Gr; Ackermann Saatzucht GmbH & Co. KG, Irlbach, Germany). Barley was cultivated in 2014 under field conditions in Freising, Germany (450 m a.s.l., on average 7.7 °C, on average 814 mm, silt loam soil). A fungicide cover spray was applied during stem elongation (growth stage (GS) 32) to provide basic plant protection and foliar disease control. Fertilization was used according to regional standards. For each variety, the experimental setup, which included four treatments

with four repetitions, was organized in a completely randomized design. Field plots (11.25 m^2) were inoculated with *Fusarium* species-specific conidia suspensions twice within a 7-day range during the anthesis period (GS65-69). Individual inoculum contained a mixture of spores derived from different single-spore isolates of *F. culmorum* (Fc, Fc002, Fc06, Fc03), *F. avenaceum* (Fa, Fa002, Fa01), and *F. sporotrichioides* (Fs, Fs001, Fs002, Fs03). The inocula densities for each mixture depended on the spore production potential of each isolate and thus ranged from $6.4x10^6$ conidia/m^2 to $9.3x10^6$ conidia/m^2. A corresponding number of control plots remained non-inoculated. Mature plants (GS 92-95) were harvested using a single plot combine. Grain samples (around 7 kg each) were stored in jute bags at room temperature until further processing.

Malting process.

The malting process was performed according to the standard collection method described in the Mitteleuropäische Analysenkommission (MEBAK) procedure.[16] A three-day steeping process was used. On the first day, barley (around 1200 g each) was steeped for 5 h following a 19-h aeration period. On the second day, wet steeping took 4 h, with a subsequent 20-h aeration period. On the third day, the steeping degree of 44.5% was adjusted by spraying if needed. Subsequently, germination proceeded for three days in climate-controlled chambers (14.5 ± 0.5 °C and 95–98% relative humidity). Green malt was turned twice daily. The germinated barley grain were then withered at 50 °C for 16 h, followed by kilning at 60 °C for 1 h, at 70 °C for 1 h, and finally at 80 °C for 5 h. Rootlets were removed at the end of kilning.

Samples.

Samples were collected at each key step during the malting process. In detail, the barley grain, grain after steeping, green malt, malt after withering, malt with rootlets, malt after germ separation, and rootlets (around 50 g each) were sampled (Figure. 2). The total weight and sampled weight were registered for every sample. The grain after steeping, green malt, and malt after withering were freeze-dried for 24 h, and their water contents were calculated. The reported final mycotoxin concentrations indicated the sample wet weights.

Sample preparation.

Grain after steeping, green malt, and malt after withering were freeze-dried before extraction. Barley grain, malt with rootlets, malt after germ separation, and rootlets were used directly.

Sample preparation was performed according to a recent publication.[12] In brief, 1 g of finely ground sample and 10 mL acetonitrile/water (84:16, v/v) were combined and shaken in a laboratory shaker at 225 rpm for 2 h. The resulting slurry was filtered, and 4 mL of the filtrate was spiked with internal standards (50 µL of $[^{13}C]_{15}$-deoxynivalenol (1 µg/mL), 100 µL of $[^{13}C]_{2}$-3-acetyldeoxynivalenol (1 µg/mL), 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 each of $[^{15}N]_{3}$-enniatin B, $[^{15}N]_{3}$-enniatin B1, $[^{15}N]_{3}$-enniatin A1, and $[^{15}N]_{3}$-enniatin A (0.1 µg/mL)). The filtrate volume was reduced when necessary, to fall within the linear range of the response curves and to minimize standard consumption, but was

restored to 4 mL with acetonitrile/water (84:16, v/v) before purification. The entire mixture was vortexed for 20 s and applied on a Bond Elut Mycotoxin cartridge (500 mg, 3 mL) (Agilent Technologies, Santa Clara, CA) the liquid was drawn through the cartridge by vacuum suction. The eluate was collected and evaporated until dryness. Each sample was reconstituted with 200 µL of acetonitrile/water (1/1, v/v) and membrane filtered (0.45 µm). The LC-MS/MS analysis was performed thereafter.

Analog samples (1 g) free of the monitored mycotoxins were used as blank matrices for matrix-matched calibration and were spiked with six different concentrations of deoxynivalenol-3-glucoside (20–500 µg/kg). After solvent evaporation, these samples were subjected to the above-described sample preparation method.

Mycotoxin analysis via LC-MS/MS.

HPLC and MS/MS parameters from a previously published multi-mycotoxin LC-MS/MS stable isotope dilution method were used to determine the fates of the monitored *Fusarium* toxins during malting in the present study.[12]

In brief, LC-MS/MS was performed on an LC-20A Prominence system (Shimadzu, Kyoto, Japan) using a Hydrosphere RP-C_{18} column (150 mm × 3.0 mm i.d., 3 µm) (YMC Europe GmbH, Dinslaken, Germany) with a C_{18}-guard column (4 mm x 2 mm i.d., Polar-RP) (Phenomenex, Aschaffenburg, Germany) maintained at 40 °C as a stationary phase. The binary gradient system comprised (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 initiated and held at 10% B for 2 min, raised linearly from 10-99% 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 initiated and held at 10% B for 2 min, raised linearly from 10-87% 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 an API 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems Inc., Foster City, CA). It was operated in the negative ESI mode for the analyte deoxynivalenol-3-glucoside and in the positive ESI mode for the analytes deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, HT-2-toxin, T-2-toxin, enniatin B, B1, A1, and A. 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.

Isolation of genomic DNA from barley grain, malt, and rootlets.

Genomic DNA was isolated according to the procedure published earlier, [17] based on an official method report.[18]

In brief, 2 g of finely ground grain, malt, and rootlets were mixed with 10 mL of CTAB (cetyltrimethylammonium bromide) extraction buffer (2% CTAB, 1.4 mol/L NaCl, 0.1 mol/L Tris base (pH 8), 20 mmol/L EDTA (pH 8), 1% polyvinylpolypyrolidone 40).

206 After incubation for 10 min at 65 °C, the mixture was centrifuged (10 min, $2.1x10^3$x g,
207 RT), and 1 mL of the supernatant was mixed with 1 mL of chloroform/isoamyl alcohol
208 (24:1, v/v). After centrifugation (10 min, $16.2x10^3$ x g, RT), 850 µL of the supernatant
209 was mixed with 8.5 µL of RNAse A solution (10 mg/mL) (Qiagen, Hilden, Germany)
210 and incubated for 30 min at 37 °C. CTAB solution (85 µL of 10% CTAB, 0.7 mol/L
211 NaCl) and an equal volume (850 µL) of chloroform/isoamyl alcohol (24:1, v/v) were
212 added for extraction. After centrifugation (15 min, $16.2x10^3$ x g, RT) 500 µL of the
213 upper aqueous phase were transferred to clean tubes and mixed with 1.5 mL of
214 precipitation buffer (1% CTAB, 0.7 mol/L Tris base (pH 8), 0.01 mol/L EDTA (pH 8)).
215 The samples were mixed and kept at RT for 15 min. DNA was collected by
216 centrifugation (15 min, $16.2x10^3$ x g, RT). The resulting pellets were washed twice
217 with EtOH (1 mL, 70%), vacuum dried, and resuspended in double-distilled water
218 (150 µL). The DNA quantity and quality were determined using a NanoDrop ND 1000
219 (Peqlab, Wilmington, DE), and DNA concentrations were adjusted to 20 ng/µL with
220 double-distilled water.
221
222 **Quantification of *Fusarium* DNA in barley grain, malt, and rootlets.**

223 Fungal DNA in barley, malt, and rootlets was quantified using qPCR methods
224 published previously.[17,19] In brief, DNA amplification was performed in a total volume
225 of 20 µL, containing 10 µL of 2× Maxima SYBR Green qPCR Mastermix (Fermentas,
226 St. Leon Rot, Germany), 300 nmol/L each of forward and reverse primer, 10 µg of
227 bovine serum albumin, and 100 ng of genomic DNA. qPCR was performed using a
228 MX3000P Cycler (Stratagene, Santa Clara, CA), with an initial step at 50 °C for 2
229 min, a step at 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. A

melting curve analysis was subsequently conducted at 50–95 °C. For normalization, a barley DNA assay (Hor1f/Hor2r) with primers specific for *F. culmorum* (FculC561fwd/FculC614rev), *F. sporotrichioides (*FspoA18 fwd/FspoA85rev), and *F. avenaceum (*Fave574fwd/Fave627rev) was introduced. Quantification of barley and *Fusarium* DNA was performed by external calibration. Dilution series (100, 10, 0.1, 0.01 ng DNA) were included in each approach. To equalize matrix effects, pure fungal DNA was diluted in *Fusarium* DNA free barley DNA (20 ng/µl). Separate dilution series for quantitation barley DNA were generated with *Fusarium* DNA free barley DNA. Results for *Fusarium* DNA were normalized according to barley DNA contents and are presented as *Fusarium* spp. DNA pg/ng barley DNA.

RESULTS AND DISCUSSION

Inoculation of barley and determination of *Fusarium* DNA concentrations.

Our study of the fates of *Fusarium* toxins during the malting process used defined inoculated material. Two barley varieties, ‘Grace’ (Gr) and ‘Scarlett’ (Sc), were artificially infected with three different *Fusarium* species, *F. culmorum* (Fc, producing type B trichothecenes), *F. sporotrichioides* (Fs, producing type A trichothecenes), and *F. avenaceum* (Fa, producing enniatins and beauvericin), while flowering in the field. In addition to these six inoculated batches (Gr-Fc, Sc-Fc, Gr-Fs, Sc-Fs, Gr-Fa, Sc-Fa), two control batches (Gr-Co, Sc-Co) were grown under the same field conditions. Fungal DNA from the appropriate *Fusarium* spp. was quantified via qPCR analysis of DNA extracts from barley grain, grain after steeping, green malt, malt after withering, malt with rootlets, malt after germ separation, and rootlets. This process was expected to influence the infection rate of barley raw material and allow an

assessment of fungal biomass development during malting by monitoring the depletion or accumulation of fungal DNA relative to plant DNA. The non-inoculated samples had low levels of *Fusarium* spp. DNA (maximum of 1.90 pg *F. culmorum* DNA/ng plant DNA, 1.20 pg *F. sporotrichioides* DNA/ng plant DNA, and 17.1 pg *F. avenaceum* DNA/ng plant DNA. These levels indicated a low natural rate of *Fusarium* infection in 2014. By contrast, the DNA levels were much higher in the inoculated materials than in the naturally infected barley. The *Fusarium* spp. DNA concentrations ranged between 116 and 787 pg *F. culmorum* DNA/ng plant DNA, 11.0 and 145 pg *F. sporotrichioides* DNA/ng plant DNA, and 14.6 and 169 pg *F. avenaceum* DNA/ng plant DNA (Table 1, Table 2, Table 3). These results prove that the inoculation was successful, resulting in the corresponding contamination of raw material with the respective toxins. Interestingly, after field inoculation, significant correlations between the fungal DNA and respective mycotoxin concentrations associated with all three *Fusarium* spp. and both barley varieties were observed throughout the malting process. Nielsen et al. [20] also observed a significant positive correlation between the total amounts of *F. graminearum* and *F. culmorum* and the concentrations of deoxynivalenol and zearalenone in barley grain for 60% and 40% of the variance, respectively. However, a trend toward a positive association between the fungal DNA and respective mycotoxin concentration strongly depends on the cultivar, as well as on regional and seasonal effects, and cannot always be observed.[17,20]

Sample preparation and LC-MS/MS analysis.

277 The recently published multi-mycotoxin stable isotope dilution LC-MS/MS method
278 was used to determine the fate of 11 *Fusarium* toxins (deoxynivalenol;
279 deoxynivalenol-3-glucoside; 3-acetyldeoxynivalenol; 15-acetyldeoxynivalenol; HT-2-
280 toxin; T-2-toxin; enniatin B, B1, A1, A; and beauvericin) during malting.[12] Samples
281 were taken at the following key steps of the malting process (Figure 2): barley grain,
282 grain after steeping, green malt, malt after withering, malt with rootlets, malt after
283 germ separation, and rootlets. The sample preparation had to be altered slightly
284 because of high levels of contamination in some samples (up to 79 mg/kg
285 deoxynivalenol). As mentioned above, the filtrate volumes of the samples had to be
286 reduced to fall within the linear ranges of the response curves.[12] A LC-MS/MS
287 chromatogram in positive ESI mode of spiked malt with internal standards is shown in
288 Figure 3.

289

290 **Fate of fungal DNA and *Fusarium* toxins during malting.**

291 For our malting trials, the above-described six inoculated and two control barley
292 batches were used as raw material. Although *F. avenaceum* is a well-known
293 producer of enniatin and beauvericin, [21] our *F. avenaceum* strain did not produce
294 detectable levels of beauvericin. Therefore, beauvericin was not analyzed further in
295 our study. *F. sporotrichioides* and *F. culmorum* are responsible for the production of
296 type A and type B trichothecenes, respectively.[21] We accordingly investigated the
297 respective *Fusarium* DNA and mycotoxins associated with the *Fusarium* ssp. used
298 for inoculation in the raw materials. In addition, all monitored *Fusarium* DNA and
299 mycotoxin species were analyzed in the control barley batches. In most cases,
300 enniatins and *F. avenaceum* DNA were observed in control batches grown under

301 natural conditions without inoculation. By contrast, low levels of *F. sporotrichioides*
302 and *F. culmorum* were observed in these control batches, and accordingly type B and
303 type A trichothecenes were either detectable at low levels or were present at levels
304 below the limits of quantitation or even limits of detection.

305 The concentrations of *F. culmorum* DNA and of deoxynivalenol, 3-
306 acetyldeoxynivalenol, and 15-acetyldeoxynivalenol in samples collected throughout
307 the malting process from inoculated and control barley batches are shown in Table 1.
308 Deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol behaved
309 similarly throughout the malting process. After steeping, the levels of the latter
310 mycotoxins declined to 15–49% of the initial levels, independent of the barley cultivar
311 and inoculation type. Schwarz et al. [11] and Lancova et al. [3] reported similar or even
312 greater reductions in deoxynivalenol and acetyldeoxynivalenol derivatives (up to
313 90%) after steeping. The *F. culmorum* DNA content increased from 124 to 304 pg/ng
314 plant DNA in cultivar 'Grace' and from 127 to 238 pg/ng plant DNA in cultivar
315 'Scarlett' during germination. Similarly, contents of the mycotoxins deoxynivalenol, 3-
316 acetyldeoxynivalenol, and 15-acetyldeoxynivalenol increased by 3–7-fold in both
317 cultivars. Studies applying scanning electron microscopy also provided evidence of
318 an increase in *Fusarium* fungi during germination. [11] After withering (50 °C for 16 h),
319 a further 2-fold increase in fungal DNA and slight increases in deoxynivalenol, 3-
320 acetyldeoxynivalenol, and 15-acetyldeoxynivalenol were observed from the green
321 malt to the withered malt stage. The final kilning (60 °C for 1 h, 70 °C for 1 h, and 80
322 °C for 5 h), however, had no major influence on the levels of *F. culmorum* DNA or the
323 monitored mycotoxins. This might be attributable to the high thermostability of
324 deoxynivalenol and its acetylated derivatives at temperatures up to 120 °C.[3,22] After

325 removing the highly contaminated rootlets, in Gr-Fc 170% of deoxynivalenol, 510% of
326 3-acetyldeoxynivalenol, and 470% of 15-acetyldeoxynivalenol and in Sc-Fc 190% of
327 deoxynivalenol, 730% of 3-acetyldeoxynivalneol, and 690% 15-acetyldeoxynivalenol
328 that were originally present in the inoculated raw grain, remained in barley malt.
329 Lancova et al. [3] obtained comparable results for deoxynivalenol (up to a 215%
330 increase) and acetyldeoxynivalenol derivatives (up to a 115% increase) using
331 artificially infected material. In barley, Vaclavikova et al. [9] observed a slight reduction
332 in deoxynivalenol to 90% of the original level after kilning. Similar to our control batch
333 (Gr-Co), in which 32% of deoxynivalenol remained, Schwarz et al. [11] reported 30–
334 100% of the initial deoxynivalenol levels in naturally contaminated barley after
335 malting.

336 The concentrations of *F. culmorum* DNA and deoxynivalenol-3-glucoside in samples
337 collected throughout the malting of inoculated and control barley are shown in Table
338 1. The initial concentration of deoxynivalenol-3-glucoside in raw grain was very low in
339 all investigated barley batches. Steeping had no major influence on this
340 concentration. However, in line with previous studies [5,9,23], the content of
341 deoxynivalenol-3-glucoside increased by 4100% in cultivar 'Grace' and by 5100% in
342 cultivar 'Scarlett' in the green malt stage after germination. Obviously, higher
343 deoxynivalenol-glycosyl-transferase enzymatic activity during germination induces
344 the glycosylation of deoxynivalenol.[3] The highest amount of deoxynivalenol-3-
345 glucoside were observed in Sc-Fc and in Gr-Fc after kilning, with 5400% and 4800%
346 increases over the initial level, respectively. These results demonstrated that
347 deoxynivalenol-glycosyl-transferase remained stable and retained its enzymatic
348 activity under exposure to high temperatures during withering and at least the initial

349 stage of kilning. Vaclavikova et al. [9] reported a 370% increase in the deoxynivalenol-
350 3-glucoside content in barley malt over the initial level. However, that study did not
351 provide further information about the fate of toxins during withering or kilning. In
352 accordance with a previous report, [3] the discarded rootlets were highly contaminated
353 with deoxynivalenol, acetyldeoxynivalenol derivatives, and deoxynivalenol-3-
354 glucoside.

355 The concentrations of *F. sporotrichioides* DNA and the type A trichothecenes HT-2-
356 toxin and T-2-toxin in inoculated and control barley samples collected throughout the
357 malting process are shown in Table 2. HT-2-toxin and T-2-toxin were washed out
358 during steeping to an extent that only 31% of HT-2-toxin remained irrespective of
359 cultivar, or 24% and 88% of the T-2-toxin levels remained in Gr-Fs and Sc-Fs,
360 respectively, when compared to the initial levels. In line with the moderate increases
361 observed in *F. sporotrichioides* DNA during germination and withering, the T-2-toxin
362 level increased to a maximum of 220%, and a slightly lower increase was observed
363 for HT-2-toxin. After kilning, the levels of HT-2-toxin and T-2-toxin decreased by 18%
364 and 50% (average of both cultivars), respectively, compared to those in the malt after
365 withering. A decrease in *F. sporotrichioides* DNA during kilning was also observed.
366 After separating the discarded rootlets, 13% of the initial overall HT-2-toxin level
367 (both cultivars), 35% of the initial T-2-toxin level in cultivar ‘Grace’, and 65% of the
368 initial T-2-toxin level in cultivar ‘Scarlett’ remained in the kilned malt. *F.*
369 *sporotrichioides* DNA, HT-2-toxin, and T-2-toxin might have been subjected to either
370 thermal or biological degradation during kilning. However, Lancova et al. [3] refuted the
371 suggestion that HT-2-toxin is thermally unstable. In their studies, these authors
372 observed a different HT-2-toxin pattern during the malting of artificially infected

barley. Specifically, the HT-2-toxin level increased up to 430% during kilning, and approximately 200% of the HT-2-toxin remained in the barley malt. Although our raw material stemmed from barley plots that were artificially infected in the field, microorganisms other than *Fusarium* spp*.* might have been present and could have caused biological degradation, as described above.

The concentrations of *F. avenaceum* DNA and enniatins in the inoculated and control barley samples collected throughout the malting process are shown in Table 3. All four monitored enniatins exhibited similar behaviors throughout this process. As described recently [12,15], ENNs exist in barley in the following decreasing order of concentration: enniatin B, B1, A1, and A. In accordance with Vaclavikova et al. [9], we observed a rapid decrease in enniatins during steeping, to levels below 30% (Gr-Fa, Gr-Co) or 60% (Sc-Fa, Sc-Co) of the initial levels. The results of a qPCR analysis of *F. avenaceum* DNA revealed an increase in fungal biomass from germination. Mycotoxin production increased along with the subsequent moderate growth in *F. avenaceum*, reaching maximum levels either after withering or after kilning. By contrast, Hu et al. [10] reported the degradation of all enniatins during kilning. However, the present study did not confirm that earlier finding regarding the thermal instability of enniatins, as the concentrations of enniatins did not decrease after kilning (60 °C for 1 h, 70 °C for 1 h, 80 °C for 5 h). In our study the level of enniatins in the final malt of Sc-Fa exceeded the initial levels by a factor of 1.4–2.2. In Gr-Fa and the control batches, approximately 30% of the initial enniatin levels were detected in the barley malt after germ separation. The latter results are comparable to those obtained by Vaclavikova et al. [9]. The separated rootlets were heavily loaded with enniatins, similar to findings previously published.[10]

This was the first study to investigate the fates of *Fusarium* species and corresponding *Fusarium* mycotoxins starting from grain from two field-inoculated barley cultivars throughout the malting process including analyses both of fungal DNA and mycotoxins. In general, barley grain germination during malting favors the growth of *Fusarium* species and hence allows mycotoxin production and glycosylation. The continued growth of all investigated *Fusarium* species and mycotoxins was observed during withering (50 °C for 16 h). In addition, the temperature conditions during kilning (60 °C for 1 h, 70 °C for 1 h, 80 °C for 5 h) did not interfere with partly observed further increases in the concentrations of *F. culmorum* and *F. avenaceum* DNA and their respective secondary metabolites. Interestingly, these conditions failed to inhibit the enzymatic activity of deoxynivalenol-glycosyl-transferase, resulting in an increased deoxynivalenol-3-glucoside content in kilned malt relative to that in green malt. In contrast to HT-2-toxin, T-2-toxin, and *F. sporotrichioides* DNA, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, deoxynivalenol-3-glucoside, and *F. culmorum* DNA, as well as enniatins and *F. avenaceum* DNA, were found to be highly thermostable during malting.

In our comparison of two investigated barley cultivars (‘Grace’ and ‘Scarlett’) with regard to *Fusarium* DNA and corresponding mycotoxin levels, we observed the same output load for ‘Grace’ and ‘Scarlett’ to *F. culmorum* and type B trichothecenes, whereas ‘Scarlett’ exhibited a lower abundance of *F. sporotrichioides* and *F. avenaceum*, as well as type A trichothecenes and enniatins. The limited number of *Fusarium* species and barley cultivars does not allow a general statement regarding resistance. Therefore, future studies should investigate different barley cultivars in

421 combination with a broader spectrum of important *Fusarium* species (for example *F.*
422 *graminearum, F. poae*, or *F. langsethiae*) to which the FHB complex in barley has
423 also been attributed.[20] The frequency of *Fusarium* infection and the subsequent
424 production of mycotoxins in barley grain and malt could depend on several natural
425 factors, as well as annual variations in weather conditions. Further studies of malt
426 quality improvement should address factors that can be directly managed, such as
427 fungicide treatment and nitrogen fertilization. Technological parameters during
428 malting might also play an important role in fungal growth and mycotoxin production.
429 In addition to temperature program modifications, different malting schemes that
430 incorporate intensive steeping and shorter germination periods are conceivable.
431 During industrial steeping, water-soluble mycotoxins might be more thoroughly
432 removed by steeping water as a result of thorough mixing in the steeping vessel in
433 the presence of compressed air, injected through aeration pipes. Likewise, a shorter
434 germination period might reduce the formation of fungal biomass and metabolites.
435 Throughout the malting process, our investigated control batches, which were
436 comparable to commercially available brewing barley, contained low levels of
437 *Fusarium* DNA, as well as mycotoxin levels below legislatively set limits.[2] Further
438 studies about the fates of *Fusarium* mycotoxins during mashing and brewing are
439 expected to clarify issues of consumer health risks and might help reducing the
440 mycotoxin transfer from malt to beer.

441

Supporting Information Available:

S-Table 1. Pearson Correlation Coefficients for Comparisons of the *Fusarium* ssp. DNA and Respective Mycotoxin Concentrations During Malting from Field Trials (2014)

S-Table 2. Concentrations of *Fusarium culmorum* DNA and Type B Trichothecenes in Control Barley Grain, Grain After Steeping, Green Malt, Malt After Withering, Malt With Rootlets, Malt After Germ Separation, and Rootlets

S-Table 3. Concentrations of *Fusarium sporotrichioides* DNA and type A Trichothecenes in Control Barley Grain, Grain After Steeping, Green Malt, Malt After Withering, Malt With Rootlets, Malt After Germ Separation, and Rootlets

S-Table 4. Concentrations of *Fusarium avenaceum* DNA and Enniatins in Control Barley Grain, Grain After Steeping, Green Malt, Malt After Withering, Malt With Rootlets, Malt After Germ Separation, and Rootlets

This material is available free of charge via the Internet at http://pubs.acs.org.

ABBREVIATIONS

ESI, electrospray ionization; EtOH, ethanol; Fa, *Fusarium avenaceum*; Fc, *Fusarium culmorum*; FHB, Fusarium head blight; Fs, *Fusarium sporotrichioides*; Gr, 'Grace'; MEBAK, Mitteleuropäische Brautechnische Analysenkommission e.V.; qPCR, quantitative polymerase chain reaction; Sc, 'Scarlett'

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544
545

546 **FIGURE CAPTIONS**

547 Figure 1. Structures of Type B, Type A Trichothecenes, and Enniatins

548 Figure 2. Key Steps in the Malting Process

549 Figure 3. LC-MS/MS Chromatograms of a Spiked Malt Sample in Positive ESI Mode

550 of A. Analytes and B. Internal Standards

551

552

553

Tables

Table 1. Concentrations of *Fusarium culmorum* DNA and Type-B Trichothecenes in Inoculated Barley Grain, Grain After Steeping, Green Malt, Malt After Withering, Malt With Rootlets, Malt After Germ Separation, and Rootlets

Sample ID	Fc DNA	DON			D3G			3-ADON			15-ADON		
	pg/ng plant DNA	Total			Total			Total			Total		
		µg/kg	mg	%	µg/kg	mg	%	µg/kg	mg	%	µg/kg	µg	%
Gr-Fc													
Barley grain	124	4100	5.4	100	580	0.8	100	240	0.3	100	97	130	100
Grain after steeping	116	780	1.6	30	1100	2.3	310	23	0.05	15	9.3	19	15
Green malt	304	7400	14	250	17000	31	4100	840	1.5	490	300	550	440
Malt after withering	463	13000	13	240	28000	28	3700	1900	1.9	600	660	660	520
Malt with rootlets	516	12000	12	220	37000	36	4800	1900	1.9	600	690	670	530
Malt after germ separation	493	10000	9.3	170	19000	17	2300	1800	1.6	510	670	600	470
Rootlets	715	79000	6.8	130	120000	10	1400	5600	0.5	160	2100	180	140
Sc-Fc													
Barley grain	127	4500	5.4	100	500	0.6	100	210	0.3	100	83	100	100
Grain after steeping	182	1400	2.7	49	1300	2.5	420	33	0.06	25	13	25	25
Green malt	238	8300	14	260	18000	30	5100	1100	1.8	710	390	660	670
Malt after withering	681	17000	16	300	33000	31	5200	2700	2.6	1000	980	910	910
Malt with rootlets	524	15000	13	240	36000	32	5400	2500	2.2	870	860	760	770
Malt after germ separation	752	13000	10	190	28000	22	3700	2400	1.9	730	870	690	690
Rootlets	787	76000	7.3	130	110000	10	1700	7200	0.7	270	2500	240	240

DON=deoxynivalenol, D3G=deoxynivalenol-3-glucoside, 3-ADON=3-acetyldeoxynivalenol, 15-ADON=15-acetyldeoxynivalenol

Table 2. Concentrations of *Fusarium sporotrichioides* DNA and Type-A Trichothecenes in Inoculated Barley Grain, Grain After Steeping, Green Malt, Malt After Withering, Malt With Rootlets, Malt After Germ Separation, and Rootlets

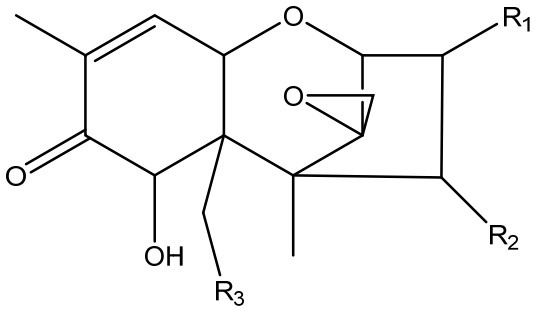
Sample ID	Fs DNA	HT-2-toxin			T-2-toxin		
	pg/ng plant DNA		Total			Total	
		µg/kg	µg	%	µg/kg	µg	%
Gr-Fs							
Barley grain	145	890	890	100	380	380	100
Grain after steeping	27.6	170	270	31	58	94	24
Green malt	39.9	240	350	39	300	430	110
Malt after withering	113	490	370	41	580	440	110
Malt with rootlets	54.3	420	320	36	260	190	50
Malt after germ separation	53.6	180	120	13	200	130	35
Rootlets	80.3	1900	140	16	790	58	15
Sc-Fs							
Barley grain	43.3	380	430	100	83	92	100
Grain after steeping	11.0	73	130	31	46	82	88
Green malt	13.1	70	120	27	50	83	90
Malt after withering	17.3	120	110	26	230	200	220
Malt with rootlets	15.0	99	86	20	130	110	120
Malt after germ separation	26.2	69	53	13	78	61	65
Rootlets	19.4	510	47	11	390	36	39

Table 3. Concentrations of *Fusarium avenaceum* DNA and Enniatins in Inoculated Barley Grain, Grain After Steeping, Green Malt, Malt After Withering, Malt With Rootlets, Malt After Germ Separation, and Rootlets

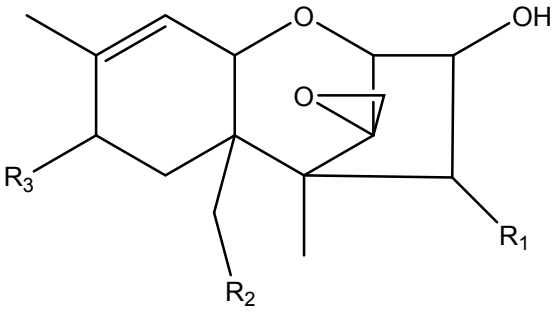
Sample ID	Fa DNA	enniatin B			enniatin B1			enniatin A1			enniatin A		
	pg/ng plant DNA	Total			Total			Total			Total		
		µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
Gr-Fa													
Barley grain	89.3	14000	14000	100	2300	2300	100	340	340	100	25	25	100
Grain after steeping	23.9	2100	3300	24	560	890	38	91	150	43	6.9	11	43
Green malt	70.4	2700	3800	27	730	1000	45	120	160	48	9.4	13	52
Malt after withering	88.4	5300	4000	28	1400	1000	45	220	160	49	16	12	47
Malt with rootlets	79.2	6100	4600	32	1300	960	41	200	150	45	16	12	46
Malt after germ separation	81.2	5100	3400	24	1200	820	35	160	100	31	12	8.1	32
Rootlets	169	14000	1100	7.9	3000	250	11	580	48	14	46	3.8	15
Sc-Fa													
Barley grain	15.0	1400	1800	100	390	500	100	54	69	100	4.4	5.7	100
Grain after steeping	14.6	500	1000	59	140	290	57	21	43	62	1.8[a]	3.7	65
Green malt	33.4	1000	2000	110	260	490	97	36	66	96	3.1	5.8	100
Malt after withering	59.3	2000	2200	120	470	490	98	72	76	110	6.7	7.1	120
Malt with rootlets	66.9	4200	4300	250	840	850	170	100	110	150	8.1	8.3	140
Malt after germ separation	37.3	4200	3900	220	890	820	160	100	97	140	8.4	7.8	140
Rootlets	81.4	3300	320	18	540	53	10	170	17	24	27	2.6	46

[a] below the limit of quantitation

Figure 1.



type B trichothecenes



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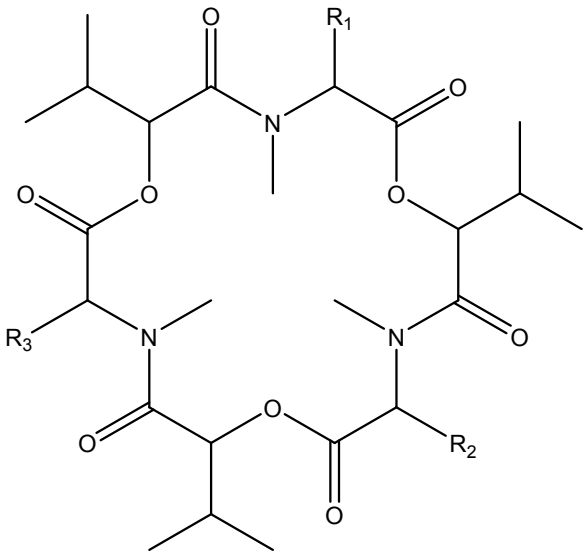


enniatins

	Analyte	R_1	R_2	R_3
type B trichothecenes	D3G	Glc	H	OH
	DON	OH	H	OH
	3-ADON	OAc	H	OH
	15-ADON	OH	H	OAc
type A trichothecenes	HT-2-toxin	OH	OAc	$OCOCH_2CH(CH_3)_2$
	T-2-toxin	OAc	OAc	$OCOCH_2CH(CH_3)_2$
enniatins	enniatin B	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$
	enniatin B1	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$
	enniatin A1	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$
	enniatin A	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$

D3G=deoxynivalenol-3-glucoside, DON=deoxynivalenol; 3-ADON=3-acetyldeoxynivalenol, 15-ADON=15-acetyldeoxynivalenol

Figure 2.

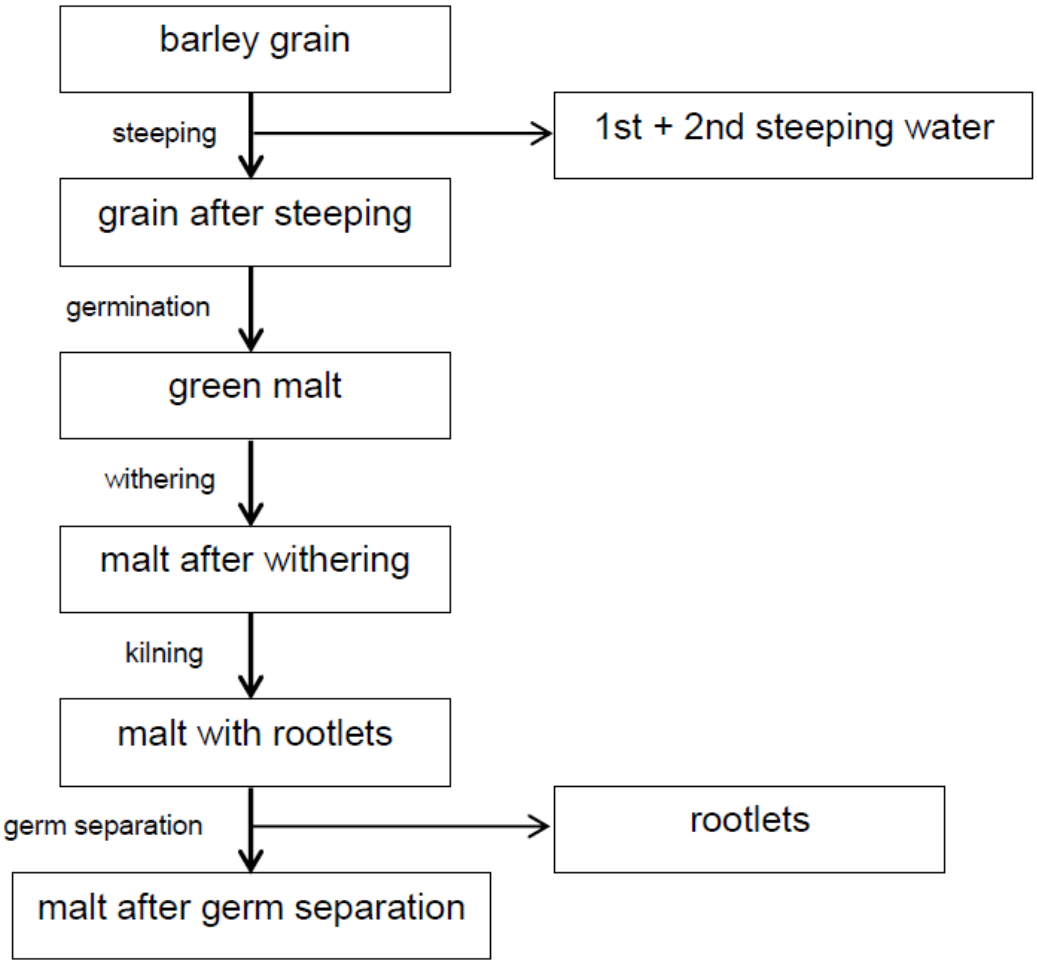
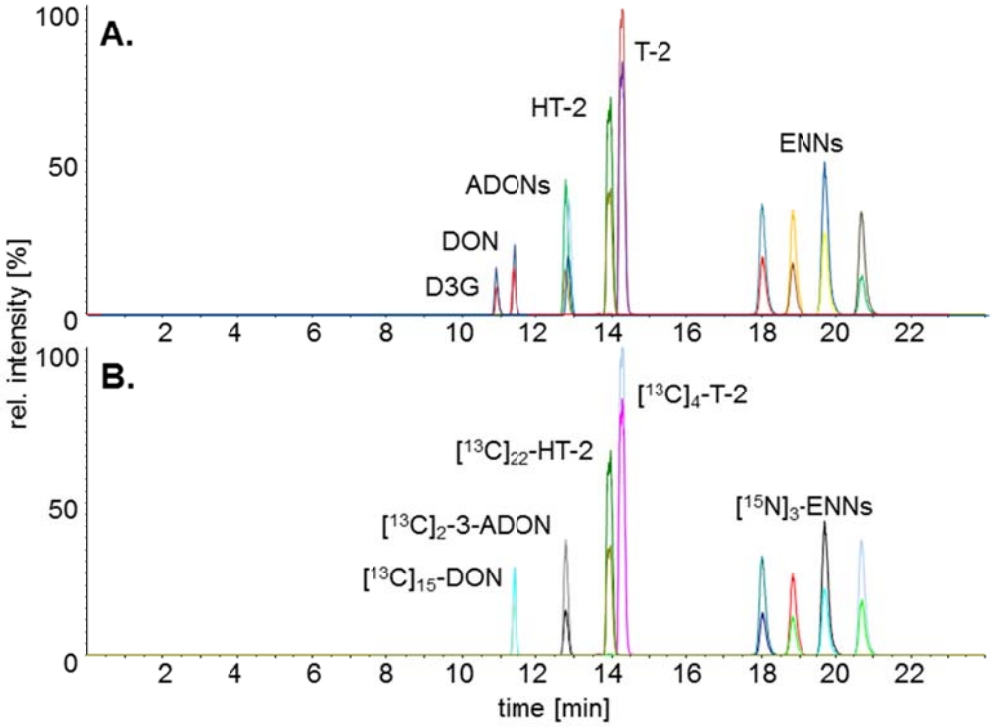

barley grain
steeping
1st + 2nd steeping water
grain after steeping
germination
green malt
withering
malt after withering
kilning
malt with rootlets
germ separation
rootlets
malt after germ separation

Figure 3.



D3G=deoxynivalenol-3-glucoside, DON=deoxynivalenol; ADONs=acetyldeoxynivalenol derivatives, HT-2=HT-2-toxin, T-2=T-2-toxin, ENNs=enniatins

For Table of Contents Only