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Fate of Enniatins and Beauvericin During the Malting and Brewing Process Determined by Stable Isotope Dilution Assays

Ling Hu [a], Martina Gastl [b], Andrea Linkmeyer [c], Michael Hess [c], and Michael Rychlik [a,d *]

[a] Chair of Analytical Food Chemistry, Technical University of Munich, Alte Akademie 10, D-85354 Freising, Germany

[b] Chair of Brewery and Beverage Technology, Technische Universität München, D-85354 Freising, Germany

[c] Chair of Phytopathology, Technische Universität München, D-85354 Freising, Germany

[d] BIOANALYTIK Weihenstephan, , Technische Universität München, Alte Akademie 10, D-85354 Freising, Germany

* Corresponding Author.

Phone: + 49 8161 71 3153. Fax: + 49 8161 71 4216. E-Mail: michael.rychlik@tum.de.

ABSTRACT The fate of enniatins A, A1, B, B1 and beauvericin during the malting and brewing process was investigated. Three batches of barley grains were used as starting material, one was naturally contaminated, two were artificially inoculated with *Fusarium* fungi. Samples were taken from each key step of the malting and brewing procedure, the levels of the toxins were determined with stable isotope dilution assays using liquid chromatography-tandem mass spectrometry detection. Significant increases of the toxins were found during germination of two batches of barley grains, resulting in green malts contamination up to a factor of 3.5 compared to grains before germination. Quantitative PCR analyses of fungal DNA revealed in all batches growth of *F. avenaceum* during germination. After kilning, only 41-72% of the total amounts of the toxins in green malts remained in kilned malts. In subsequent mashing stage, the toxins in kilned malts predominantly were removed with spent grains. In the final beer, only one batch still contained 74 and 14 µg/kg of enniatin B and B1, respectively. Therefore, the carryover of these enniatins from the initial barley to final beer was less than 0.2% with the main amounts remaining in the spent grains and the malt rootlets.

KEYWORDS Enniatins; beauvericin; *Fusarium*; stable isotope dilution assay; LC-MS/MS; beer; brewing; barley; malt; qPCR

1. Introduction

Beer is a popular and widely consumed drink in the world. In some countries such as the Czech Republic, Germany and Austria, the annual per capita consumption exceeds 100 L (the Brewers of Europe, 2010). Unfortunately, the major raw material for beer production, barley, is frequently infected with mycotoxin-producing fungi either in the field or during storage (Medina et al., 2006). Some residues of the mycotoxins accumulated in barley grains may survive the beer production chain and contaminate the final product.

A number of mycotoxins have been analyzed in beer, including aflatoxins, fumonisins, T-2 and HT-2 toxins, ocharatoxin A, zearalenone, zearalenol, deoxynivalenol, 3- and 15-acetyldeoxynivalenol, and deoxynivalenol-3-glucoside (Romero-González, Vidal, Aguilera-Luiz, & Frenich, 2009;; Zöllner, Berner, Jodlbauer, & Lindner, 2000; Kostelanska et al., 2009). Among them, many were not detectable or existed only in traces, with the exception of deoxynivalenol and its derivatives, the levels of which were as high as 37 μg/L.

Some earlier studies have focused on the fate of aflatoxin, ochratoxin A, citrinin, zearalenone, fumonisins, as well as deoxynivalenol and 15-acetyldeoxynivalenol during the beer making process (Chu, Chang, Ashoor, & Prentice, 1975; Krogh, Hald, Gjertsen, & Myken, 1974; Scott, 1996; Schwarz, Casper, & Beattie, 1995), which mainly involves malting and brewing. In recent years, the predominance of deoxynivalenol and its derivatives in beer has drawn more attention of researchers to follow their fate during beer making in detail. (Lancova et al., 2008; Kostelanska et al., 2011). The latter authors studied the influence of the key steps such as steeping, germination, kilning, mashing, and fermentation on the behavior of mycotoxins belonging to the deoxynivalenol group. They found DON-3-glucoside to be the most

prevalent compound being transferred into beer with concentrations amounting to approx. 40 µg/L.

On the other hand, the existence of the emerging *Fusarium* mycotoxins enniatins and beauvericin in beer has rarely been reported except one very recently published study on changes of enniatins during beer making by Vaclavikova et al. (2013) Enniatins and beauvericin are cyclic hexadepsipeptides consisting of three D-2-hydroxycarboxylic acid and *N*-methylamino acid moieties, they are often found in cereals such as wheat, barley, maize, and oats, as well as cereal-based products, and contamination levels of several hundred mg/kg have been reported (Uhlig, Torp, & Heier, 2006; Mahnine et al., 2011; Ritieni et al., 1997).

Enniatins and beauvericin possess a wide range of biological activities. They are toxic to brine shrimp, with enniatin B showing an acute (6 h) LC_{50} of 21 µg/mL (Hamill, Higgens, Boaz, & Gorman, 1969; Tan, et al., 2011). Their insecticidal activity towards adults of the blowfly *Calliphora erythrocephala* and larvae of the mosquito *Aedes aegypti* have also been reported (Grove & Pople, 1980). Besides, they are known to have inhibitory effects on acyl-CoA: cholesterol acyltransferase (ACAT), which is involved in cholesterol storage, with beauvericin exhibiting an IC_{50} of 3.0 µM (Tomoda et al., 1992). Furthermore, they are reported to be toxic to cell lines of human origin such as hepatocellular carcinoma-line Hep G2 and fibroblast-like foetal lung cell line MRC-5, IC_{50} values for enniatins A, A1, B1, and beauvericin were all in the lower micromolar-range (Ivanova, Skjerve, Eriksen, & Uhlig, 2006).

Recently, we biosynthesized the $^{15}N_3$-labeled enniatins and beauvericin and developed stable isotope dilution assays for their determination in cereals and relating food samples (Hu & Rychlik, 2012). In the current study, we applied the stable isotope dilution assays of enniatins and beauvericin to monitor their fate during the whole beer production process on a laboratory scale. The aims of this study were to elucidate the

behavior and transfer of enniatins and beauvericin from barley grains through malts to beer by using acurrate and precise stable isotope dilution assays, and to assess the risk of enniatins and beauvericin contamination in beer.

2. Materials and methods

2.1 Chemicals and reagents

Acetonitrile (MeCN), chloroform, isoamylalcohol, ethanol and sodium chloride were purchased from Merck (Darmstadt, Germany), MeCN was of analytical-reagent grade. CTAB, Tris base, polyvinylpolypyrolidone-40, EDTA, malt extract, peptone were obtained from Sigma (Steinheim, Germany). Water for HPLC was purified by a Milli-Q-system (Millipore GmbH, Schwalbach, Germany). BEA was obtained from AnaSpec (San Jose, USA), ENN B was obtained from Bioaustralis (New South Wales, Australia), and ENNs A, A1, B1 were purchased from Enzo Life Sciences (Lörrach, Germany). The internal standards $[^{15}N]_3$-enniatin A, $[^{15}N]_3$-enniatin A1, $[^{15}N]_3$-enniatin B, $[^{15}N]_3$-enniatin B1, and $[^{15}N]_3$-beauvericin were synthesized as reported recently (Hu & Rychlik, 2012).

2.2 Raw materials

For malting and brewing experiments, grain of the spring barley variety Quench (Syngenta Seeds, Bad Salzufflen, Germany) was used. Barley was grown under field conditions in Weihenstephan, Freising (Germany). A basic fungicide treatment was applied at the end of stem elongation to control foliar leaf diseases. In a completely randomized experimental design including three variants with four replicates, 12 square meter plots were artificially inoculated at flowering with macroconidia suspensions of highly aggressive single spore isolates of *Fusarium avenaceum* (Fa002) and *F. culmorum* (Fc002) in a density of $75x10^6$ conidia qm^{-1}. Control plots

remained un-inoculated and were exposed to natural infestation. At dead ripening, grains of the core of each plot were harvested individually to avoid cross-contamination. Four repeated plots were mixed to the batches QC (control), QFc (inoculated with *Fusarium culmorum)* and QFa (inoculated with *Fusarium avenaceum)*. As for malting and brewing more than 1 kg of barley was required, only one malting and brewing trial was possible for each batch. However, as the results of the different batches (contents of fungal DNA, decrease of mycotoxins during malting and brewing) are sound, the trials were considered to be representative. Bottom fermenting yeast *Saccharomyces cerevisiae* W 34/70 was supplied by the brewery Hofbräuhaus Freising. Hallertau Hallertauer Select hop (5.1% alpha acids) was purchased from Simon H. Steiner GmbH (Mainburg, Germany).

2.3 Malting process

Malting was performed according to the standard MEBAK procedure: steeping and germination time: 6 days, germination temperature: 14.5 °C, steeping degree: 45 % (Anger, 2006). The germinated barley grains, i.e. the green malts, were then kilned at 50°C for 16 h, followed by kilning at 60°C for 1 h, at 70 °C for 1h, and finally at 80 °C for 5 h. At the end of kilning, the brittle rootlets were removed from the kilned malts.

2.4 Brewing process

For each batch of malt, the wort production was carried out in a 10 L (scale: 10 L cast-out wort) scale pilot brewing plant. Kilned malt (1.2 kg) was milled with a two-roll mill using a 0.8 mm gap. The temperature profile of the infusion mashing was 62 °C for 30 min, then 72°C for another 30 min, and finally 76°C for 5 min. The malt/liquor ratio was 1.2 kg:5 L. No adjuncts were used according to the German “Reinheitsgebot”. The wort was boiled for 90 minutes at atmospheric pressure. Hop

addition was done at the beginning of wort boiling with Hallertau Hallertauer Select hop (5.1 % alpha acids) in order to reach 20 bitter units (BU) in beer. The sweet wort was boiled until the wort reached 11.5 °P (degree Plato, specific gravity of the extract, equivalent to grams of sucrose in 100 g solution at 20 °C). After the whirlpool rest of 20 min, the trub (i.e. the precipitate) was separated from the hopped wort and the wort was cooled. To the latter, 70 g of yeast (equivalent to 15 x 10^6 yeast cells/mL) was added, and the subsequent 6 d fermentation took place at 12°C. At the end of fermentation, the brewing tanks containing the green beer were kept at 16 °C for 3 d, followed by 10 d at 0°C for maturation.

Fermentation and storage time is displayed in **Fig**. **1**. After maturation, the beer was filtered through a filter sheet SEITZ-KS 80 (Pall Filtersystems GmbH, Bad Kreuznach, Germany). Thereafter, bottling was done with a single-organ long-tube filler with CO_2-flushing and pre-evacuation.

2.5 Sampling

Samples were taken during each key step of the malting and brewing processes (**Fig. 1**), including barley grains, first and second steeping water, green malt, kilned malt, rootlets, sweet wort, spent grains, cool wort, and trub. During the fermentation period, samples were taken every day. In addition, samples were taken after the three-day maturation at 16°C, as well as after the ten-day maturation at 0°C of the green beer. Filtered beer, yeast sediment, and hop were also analyzed.

2.6 Extraction for mycotoxin analysis

The green malt samples were sterilized with 70% ethanol and then dried at room temperature for 2 d before being ground and extracted. The rest of solid samples were ground and homogenized before extraction. The liquid samples were used

directly. The three trub samples, which were separated from the boiled wort by precipitation, were dried at 80 °C in an oven for 12 h before extraction, as they contained variable contents of liquid. 1 g of each sample was spiked with 10 ng (100 µL x 100 ng/mL solution in MeCN) of each of the labeled standards, the sample was suspended in 10 mL of MeCN-H_2O (84:16, v/v), vortexed (Ika Vortex Genius 3, Staufen, Germany) for 1 min and extracted by shaking for 4 h, after which the sample was centrifuged at 4000 rpm for 10 min, and 1 mL of the supernatant was filtered through a membrane filter (SPARTAN 13/0.45 RC, Whatman, Dassel, Germany) prior to HPLC. For the samples which fell out of the linear range (0.1-10) of the calibration curves, a second analysis was carried out, the extraction procedure was repeated, but the labeled standards were not added in the beginning. Instead, depending on the contamination levels, 0.1 mL of the extract was blended with 10 or 100 ng of each standard after extraction. Completeness of extraction and equilibration with the internal standards was verified by a comparison experiment using a barley sample (QFc). 20 ng of labeled enniatin A1 and 200 ng of labeled enniatin B1 were added to one gram of the barley sample before extraction. In comparison, another 1 g of the same sample was extracted without addition of labeled standards. After the extraction, 0.1 mL of the latter extract was blended with 10 ng of labeled enniatin A1 and 10 ng of labeled enniatin B1.

2.7 Mycotoxin analysis

Liquid chromatography was carried out on a Shimadzu LC-20A Prominence system (Shimadzu, Kyoto, Japan) using a YMC-Pack ProC18 column (150 × 3.0 mm i.d., 3 µm particle size, YMC Europe GmbH) coupled to a C-18 guard column (4.0 × 2.0 mm i.d., Phenomenex) The starting mobile phase MeCN-H_2O (80:20, v/v) was kept

constant for 5 min, then linearly raised to 100% MeCN in 10 min, and held for 3 min before returning to the starting conditions. The injection volume was 10 µL, flow rate was 0.2 mL/min, and equilibration time between two runs was 5 min. Data acquisition and processing were carried out using Analyst 1.5 software (Applied Biosystems Inc., Foster City, CA, USA).

For routine measurement, the LC was interfaced to a hybrid triple-quadrupole/linear ion trap mass spectrometer (API 4000 QTrap; Applied Biosystems Inc., Foster City, CA, USA) operated in the positive ESI and MRM (multiple reaction monitoring) mode. MS parameters were identical with those of our previous study (Hu & Rychlik, 2012). The effluent from the column was directed to the mass spectrometer from 11 to 21 min and to the waste for the rest of the run using a switching valve.

To check whether the sample extract would pose a potential hazard to the mass spectrometer, a kilned malt sample was measured on the LC-MS/MS system combined with a Shimadzu PDA detector. A Shimadzu companion software was used in addition to Analyst 1.5 software for data acquisition.

Method validation was performed analogously to that reported recently (Hu & Rychlik, 2012). For the determination of limits of detection (LODs), limits of quantitation (LOQs), as well as for recoveries, a blank potato starch was spiked with enniatins and beauvericin at four different levels (2, 5, 15, and 20 µg/kg), each in triplicate. Intraday (n = 5) and interday precision (n = 3) were determined using the barley batch QC.

2.8 Isolation of genomic DNA from fungi

Isolates of *F. avenaceum* (TMW 4.1863) and *F. tricinctum* (TMW 4.0479) used for standard curves and positive controls were provided by Prof. Dr. Ludwig Niessen (Chair of Technical Microbiology, Technische Universität München). Fungal mycelia, grown for seven days in 100 mL liquid malt broth (3 % malt extract, 0.3 % peptone) at

ambient temperature on a rotary shaker (70 rpm), was filtered through folded filters (Schleicher & Schuell), washed twice with 50 mL sterile tap water and ground intensely using mortar and pistil and adding some sterile sea sand. Isolation of genomic DNA from the ground mycelium was carried out according to Niessen and Vogel (1997). Quantity and quality of DNA were measured by use of a microvolume spectrophotometer NanoDrop ND-1000 (Thermo Fisher Scientific).

2.9 Isolation of genomic DNA from grains and green malts

Genomic DNA of grains and green malts was extracted according to the DNA extraction method recommended by the European Community Reference Laboratories for the isolation of maize DNA (Joint Research Centre, 2007) with some modifications. Two g powdered grain or 200 mg milled and homogenized green malt were mixed vigorously with 10 mL or 1.2 mL CTAB extraction buffer (2 % CTAB, 1.4 mol/L NaCl, 0.1 mol/L Tris base (pH 8), 20 m mol/L EDTA (pH 8), 1 % polyvinylpolypyrolidone-40), respectively. The mixture was incubated for 10 min at 65°C. After centrifugation for 10 min (2.1 x 10^3 x g, RT), 1 mL of the supernatant was transferred to a new reaction tube. The solution was then mixed with chloroform:isoamylalcohol (CIA, 24:1, 1 volume) and centrifuged (10 min, 16.2 x 10^3 x g, RT). Subsequently, a volume of 850 µL of the supernatant was mixed with a RNAse A solution (8.5 µL, 10 mg/mL Qiagen, Hilden, Germany) and incubated for 30 min at 37 °C. Thereafter, a CTAB solution (85 µL, 10 % CTAB, 0.7 mol/L NaCl) was added followed by extraction with the equal volume of CIA (24:1). After centrifugation (15 min, 16.2 x 10^3 x g, RT), 700 µL of the supernatant was mixed with 1/10 volume of a 10 % CTAB solution (10 % CTAB, 0.7 mol/L NaCl), followed by extraction with equal volume of CIA (24:1). After centrifugation (15 min, 16.2 x 10^3 x g, RT), 500 µL of the upper aqueous phase was transferred to a new reaction tube and precipitation buffer

(1.5 mL, 1 % CTAB, 0.05 mol/L Tris-base (pH 8), 0.01 mol/L EDTA (pH 8)) was added. The samples were mixed gently and kept at RT for 15 min. The DNA was collected by centrifugation for 15 min (16.2 x 10^3 x g, RT). The supernatant was discarded and the pellet was washed twice with EtOH (1 mL, 70 %). The pellet was vacuum-dried and resuspended in double distilled water (120 µL). DNA quantity and quality was determined by using the NanoDrop ND-1000 (Peqlab, Wilmington, USA) and the DNA concentration was adjusted to 20 ng/µL in double distilled water. Genomic DNA was extracted once of each grain sample (four replicates of each variant) and three times of the green malt samples.

2.10 Quantification of *Fusarium* DNA in barley grains and green malts

Quantification of fungal DNA in barley grains and green malts by quantitative polymerase chain reaction (qPCR) was carried out according to Nicolaisen et al. (2009). DNA amplification was performed in a total volume of 20 µL containing 10 µL 2x Maxima® SYBR Green qPCR Master mix (Fermentas, St. Leon Rot, Germany), 300 nmol/L forward and reverse primer each, 10 µg bovine serum albumin and 100 ng genomic DNA. PCR reaction was carried out in duplicate for each sample. The qPCR was performed in a MX3000P Cycler (Stratagene, Santa Clara, USA) and consisted of an initial step at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles with 95°C for 15 s and 60 °C for 1 min. The subsequent melting curve analysis was performed at 55 – 95 °C. In addition to primers specific for *F. avenaceum* and *F. tricinctum*, a barley DNA assay was introduced for normalization (Nicolaisen et al., 2009). Absolut quantification of barley and *Fusarium* DNA was carried out by external standard calibration. Therefore, dilution series (100, 10, 1, 0.1, 0.01 ng DNA) of pure fungal DNA, diluted in 20 ng/µL *Fusarium* DNA-free barley DNA, or *Fusarium* DNA-free barley DNA were generated and included in the qPCR analysis. Barley DNA

quantities served for normalization of fungal DNA contents, which were calculated as pg fungal DNA per ng plant DNA. Concentrations of *F. avenaceum* and *F. tricinctum* DNA in barley grains and green malt presented in **Table 1** were analyzed by one-way analysis of variance (ANOVA) and Post-Hoc test Tukey-B using PASW Statistics 18.0 (SPSS Inc., Chicago, USA) at $p < 0.05$.

2.11 Data analysis

All determinations were made in triplicate. Concentrations of enniatins and beauvericin presented in **Tables 3-5** were means of the three determinations. The concentrations were given on an “as is” basis, i.e. without correction for moisture contents. The total amount of green malt of each batch was corrected based on moisture content of respective batch of barley grains. Total amount of enniatins and beauvericin in barley grains, green malt, and kilned malt, respectively, was analyzed by one-way analysis of variance (ANOVA) using PASW Statistics 18.0 (SPSS Inc., Chicago, USA) at $p < 0.05$.

3. Results and discussion

3.1 Inoculation of barley with *Fusarium* species and quantitative PCR of fungal DNA

To follow the path of enniatins and beauvericin from barley to beer, our study started with defined inoculations of the cereal to obtain a targeted contamination with the mycotoxins. Apart from a control batch of un-inoculated and naturally contaminated barley (QC), two further barley batches were produced after inoculation with the species *Fusariom culmorum* (QFc) and *Fusarium avenaceum* (QFa), respectively. In order to verify the infestation of the barley under study, a specific quantification of fungal DNA of the enniatin and beauvericin producing species *F. avenaceum* and *F.*

299 *tricinctum* in grains and green malts was done by qPCR analysis (**Table 1**) and
300 allowed the correlation with mycotoxin levels in grain and its accumulation during
301 germination. Grains of the un-inoculated control plots (QC) revealed DNA contents of
302 0.85 pg/ng plant DNA of *F. avenaceum* and 0.39 pg/ng plant DNA *F. tricinctum*. These
303 results indicate a latent natural infection of grains with these species. In response to
304 artificial inoculation with the deoxynivalenol (DON) producing species *F. culmorum*
305 (QFc), DNA content of *F. avenaceum* was approximately half of the amount when
306 compared to the un-inoculated control. Obviously, inoculation with the highly
307 aggressive *F. culmorum* reduced infestation levels with *F. avenaceum* and *F.*
308 *tricinctum* which is likely to be due to competition among the different species.
309 Artificial inoculation with *F. avenaceum* resulted in high infection rates (34.2 pg/ng
310 plant DNA) with this species in harvested grains (QFa) when compared to grains of
311 the un-inoculated plots (QC) or plots inoculated with *F. culmorum* (QFc).
312
313 **3.2 Sample preparation and analysis**
314 The stable isotope dilution assays for enniatins and beauvericin previously developed
315 (Hu & Rychlik, 2012) were adopted in this study and the method validation was
316 updated. As shown in **Table 2**, the method reveals good recoveries (90-110%),
317 precisions (CV = 0.9-5.5%), and sensitivities, with LODs and LOQs in range between
318 0.4-1.2 µg/kg and 1.2-3.5 µg/kg, respectively. Thus, the sensitivity should be
319 sufficiently low according to previous reports on contamination of cereals with these
320 toxins at contents exceeding 10 µg/kg (Mahnine et al., 2011; Sørensen et al, 2008). In
321 contrast to our method, the recently reported study on enniatins (Vaclavikova et al.,
322 2012) was based on a less thoroughly validated method. Due to the lack of suitable
323 internal standards, the latter revealed lower recoveries of the toxins in barley, malt,

wort and beer. Moreover, a 10-fold higher spiking level than in the present study was used and does not represent the occurring contents in the samples.

In the study presented here, accuracy is achieved by the addition of isotope-labeled internal standards that compensate for losses and the matrix effects during ESI-MS/MS measurement. Nevertheless, due to the lack of extensive cleanup, it was still suspected that the sample extract might contaminate the mass spectrometer. To settle this question, an undiluted kilned malt (QFc) extract regarded as one of those loaded with most matrix interferences, was measured on the LC-MS/MS system combined with a Shimadzu PDA detector covering the wavelengths from 190 to 360 nm. As shown in **Fig. 2 (A)**, the major peaks, i.e. the unwanted contaminants, were eluted between 3 and 6 min, during which time period the effluent from the column was not directed to the mass spectrometer but to the waste. Therefore, these contaminants would cause no harm to the mass spectrometer. Moreover, extracts of samples such as steeping water, wort and beer, which contained less matrix load, would pose an even smaller risk to the mass spectrometer.

Some samples contained more than 1 mg/kg enniatins, which would require an addition of more than 1 µg of the labeled standards to fall into the linear range of calibration. As these additions would consume too much of our stock of standards, an alternative approach had to be pursued. As we did not want to reduce the sample weight due to reasons of homogeneity, we tested the addition of labeled standards after sample extraction to an aliquot of the extract. In a comparison experiment, equivalence was demonstrated to the addition at the beginning of the extraction, as the coefficients of variance between the two methods were below 1.3 % for the enniatins under study.

As shown in **Fig. 2 (B** & **C)**, the YMC-Pack ProC18 column used here rendered narrow peaks and separated the enniatins well and, although the peak of beauvericin

overlapped with that of enniatin B1, they can be distinguished using their specific mass transitions.

3.3 Behavior of fungal species, enniatins and beauvericin during malting

The three batches of barley grains QC, QFc and QFa, mentioned above were used as the starting materials for malting. The natural infection of QC with *F. avenaceum* detected by qPCR was confirmed by the mycotoxin analyses, as its contamination level of enniatins and beauvericin was higher than that of QFc, which was artificially inoculated with *Fusarium culmorum* and the latter of which is known to be no enniatin producer (Desjardins, 2006). As expected, QFa presented the highest contamination level of the mycotoxins and confirmed to be a very potent producer of enniatins. However, the strain used in our study did not produce beauvericin in a similar dimension as the enniatins.

The concentrations of enniatins and beauvricin in the barley grains, green malt, as well as first and second steeping water are given in **Table 3**, in addition, the total contents of each mycotoxin in green malt and steeping water were compared to those in the barley grains. The two steeping steps removed 23-38% of enniatin B from the barley grains, while the reduction of enniatins A1 and B1 was less (2.5-22.5%). The enniatin A and beauvericin in the steeping water were below the limits of detection. Unlike deoxynivalenol and 15- and 3-acetyl-deoxynivalenol, which were largely reduced by steeping to below quantitation limits (Schwarz, Casper, & Beattie, 1995; Lancova et al., 2008), the major part of enniatins and beauverin remained in the barley grains, obviously due to their low water solubility. Our results are partly contrary to those of Vaclavikova et al (2013), who observed that the levels of enniatins A and A1 decreased to 10-20% of their initial levels in the barley used as raw material. According to the data delivered by the latter authors, steeping must have reduced

substantially the toxin load. Unfortunately, no analyses of the steeping water were presented to support this conclusion, but our analyses of the steeping water and the material in the different malting stages contravene this hypothesis.

Similar to the reports of Schwarz et al. (1995) and Lancova et al. (2008), production of mycotoxins occurred in our study during germination. For QC, the amount of enniatins and beauvericin in green malts increased by about 50%. The increases of enniatins were much higher for QFc, which were between 103 to 244%, while beauvericin did not change significantly ($p < 0.05$). On the contrary, for QFa, there was a slight decrease of enniatin A1, while the rest four mycotoxins did not change significantly ($p < 0.05$). In contrast to prior reports on the fate of *Fusarium* toxins during malting (Schwarz et al.,1995; Lancova et al., 2008; Vaclavikova et al., 2013), we analysed the growth of fungi along with their mycotoxin production. The results of qPCR of fungal DNA are shown in **Table 1**. For batch QC showing an increase of mycotoxins of about 50%, the qPCR revealed likewise an increase of *F. avenaceum* DNA of about 50%. Analoguously, the highest relative mycotoxin increase of over 100% in QFc was paralleled by an increase of *F. avenaceum* DNA of over 300% during germination. It appears that *F. avenaceum* was still able to grow and to produce mycotoxins although *F. culmorum* was highly abundant. In contrast to this, *F. tricinctum* appeared to be suppressed by *F.culmorum* as can be seen from its low DNA levels in QFc before and after germination (**Table 1**). In contrast to the latter two batches of barley, QFa showed the highest *F. avenaceum* DNA and mycotoxin content before malting. However, although its DNA still increased about 4.5 fold during germination, the mycotoxins showed no further increase. Obviously, *F. avenaceum* was not able to produce higher mycotoxin levels or stopped production when the amounts reached these high levels. These results allow the conclusion that germination favors *Fusarium* growth and further production of enniatins and the responsible species

appears to be *F. avenaceum.* In contrast to this, beauvericin was also produced, but at decisively lower levels.

The concentrations of enniatins and beauvericin in green malt, kilned malt, and rootlets were listed in **Table 4**, and the total amounts of each mycotoxin in the latter two fractions were compared to those in green malt.After kilning, only 41-72% of the enniatins and beauvericin originally present in green malts remained in kilned malts. 2.5-13.5% of enniatins and a higher percentage (14-28%) of beauvericin were removed from the kilned malts along with the discarded rootlets. Therefore, 21-54% of enniatins and 9-40% of beauvericin were eliminated during the kilning stage, possibly by thermal or biological degradation. Meca et al. (2012) reported that beauvericin was degraded by 20-90% after being heated at 160, 180, and 200 °C for 20 min, respectively. Loss of the phenylalanine and hydroxyvaleric acid units was proposed by the authors according to fragments observed in full scan LC-MS. However, the kilning of green malts was carried out at lower temperatures (between 50 and 80 °C), albeit for a longer time (in total, 23 h). Thus, the thermal degradation of beauvericin cannot be substantiated by the findings of the latter authors, but, nonetheless, it is a likely pathway. With regard to the thermal degradation of enniatins, no detailed information was reported as far as we know. To shed light on this phenomenon, a simulation experiment was carried out. 100 ng of enniatin B and beauverin each were added to 1 g of a barley grain sample originally containing none of the mycotoxins above their LOQ. Then, the sample was heated in an oven with the same heating times and temperatures used for kilning. The losses of enniatin B and beauvericin after the treatment were 29% and 16%, respectively, which fell within the range of the losses found during kilning and confirmed thermal degradation to be the main cause for the decrease of the toxins. These results are contradictory to those recently reported by Vaclavikova et al. (2013), who partly observed an increase of some

enniatins during kilning. Unfortunately, no explanations for this unexpected finding were presented by the authors. Regarding the effect of discarding the toxins with the rootlets, the data of the latter authors cannot be evaluated as, in contrast to our data, only the concentrations and no absolute amounts or balances were given.

Biological degradation of enniatins and beauvericin could be another possible explanation. As demonstrated by Abrunhosa et al. (2002), a number of *Aspergillus* fungi were able to degrade more than 80% of ochratoxin A in culture medium, among which were some producers of ochratoxin A. The same group later isolated the enzyme responsible for the degradation process (Abrunhosa & Venâncio, 2007). Therefore, the *Fusarium* fungi that were active during the kilning stage might have played a role in the degradation of enniatins and beauvericin. To fully understand the mechanisms for the degradation of enniatins and beauvericin during kilning, further researches would be necessary. In comparison, Lancova et al. (2008) reported that kilning did not change the levels of the trichothecenes deoxynivalenol and acetylated deoxynivalenols, neither did thermodegradation as they are stable up to 120 °C. In contrast to this, a study on the fate of five triazole fungicides during beer making by Navarro et al. (2011) revealed that kilning lowered their contents by 2.5-9.5%.

3.4 Disposition of enniatins and beauvericin during brewing

The kilned malt was regarded as the starting point of the brewing process. In the beginning of brewing, it was ground into fine grits and extracted by water in the mashing procedure, after which the sweet wort was separated from the spent grains. As shown in **Table 5**, 64-98% of enniatins and 53-85% of beauvericin originally present in kilned malts were retained in spent grains, the sweet wort contained no more than 6% of enniatins and no detectable beauvericin. The percentages of enniatins retained in the spent grains were in accordance with those of Vaclavikova et

al. (2012), who reported 64-91% of the enniatins to remain in spent grains. On the contrary, according to previous researches, the highly water-soluble deoxynivalenol was either not detected or detected only in traces in spent grains (Kostelanska et al., 2011; Lancova et al., 2008; Schwarz et al., 1995) and, most of this toxin was transferred into sweet wort. In a study of the fate of 312 pesticides during beer brewing, Inoue et al. (2011) observed that the more hydrophobic compounds were adsorbed more rapidly onto spent grains. Therefore, given their low water solubility, the high contamination levels of enniatins and beauvericin in spent grains were not unexpected. These spent grains could pose possible risks to animals, as they are used as a buffer, forage or concentrate replacer in feed for ruminant animals (Navarro, Pérez, Vela, Mena, & Navarro, 2005).

In the following step, the sweet wort was boiled with hops, then the trub was precipitated and the wort was cooled. Although no enniatin A was detectable in sweet wort, it was found in the trub, ranging from 1-8% of that in the kilned malts. Probably the enniatin A in sweet wort was too diluted to be detected. The same was evident for beauvericin, as it was detected in the trub of QC and QFa in spite of the fact that no beauvericin was detectable in the respective sweet worts. No target mycotoxins were quantifiable in the cool wort of batch QC and no more than 1.6% of them were found in QFc and QFa.

Those residues of enniatins and beauvericin in trub exceeding their LOQs accounted for 0.9 to 8.1% of those in kilned malts. Losses may have happened during sample preparation of the trub as it was heated at 80 °C for 12 h in an oven. Therefore, the true amounts of these mycotoxins in trub could be higher than detected.

As the next step in brewing, yeast was added into the cool wort to start the 6-day fermentation. At the end of fermentation, no enniatins A, A1, and B1 and beauvericin were detected in the green beer of QC, with enniatin B being below LOQ. For QFc, no

enniatin A and beauvericin were found, enniatin A1 being below its LOQ and enniatins B and B1 were only 9 and 4 µg/kg, respectively. For QFa, no enniatin A and beauvericin were detected, the concentration of enniatin A1 decreased from 9 µg/kg in the cool wort to 6 µg/kg in the green beer, enniatin B declined from 297 µg/kg to 219 µg/kg, and enniatin B1 dropped from 121 µg/kg to 61 µg/kg. Similarly, decreases of ochratoxin A and fumonisins were observed by Scott et al. (1995) when added to wort and fermented for 8 days by *Saccharomyces cerevisiae*, losses were between 2 and 28%. Strains of *Saccharomyces cerevisiae* were also reported to degrade patulin during the alcoholic fermentation of apple juice (Moss & Long, 2002).

Small fluctuations of the concentrations of enniatins B and B1 were found during the 6 days of fermentation, possibly due to adsorption of the mycotoxins by yeast or due to inhomogenous sampling.

Finally, the maturation and filtration of green beer saw further decreases of enniatins A1, B and B1 in QFa, which had been treated with *Fusarium avenaceum* and was the only batch still containing enniatins above limits of quantitation. In the final beer, the concentration of enniatin A1 in QFa declined to not detectable, enniatin B and B1 declined to 74 (i.e. by 66% after maturation and filtration) and 14 µg/kg (i.e. by 77% after maturation and filtration), respectively. The carryover of enniatins B and B1 from the initial barley grains to final beer was 0.2% and 0.1%, respectively. In the report by Navarro et al. (2005), the fungicides myclobutanil and propiconazole were lessened by 50% and 25%, respectively, after maturation and filtration, and the authors suggested surface adsorption as a probable cause. Scott et al. (1995) also reported up to 21% of ochratoxin A taken up by yeast during fermentation of wort. On the basis of this notion, the yeast sediment after filtration of beer was analyzed and up to 1045 µg/kg (by dry weight) of enniatins were detected. However, as yeast was partially lost during filtration, its total amount was unknown. Therefore, the finding can only confirm

that some of the enniatins were adsorbed by yeast, but the exact percentage cannot be calculated. As yeast residue is used as raw material for feeds and foods, further attention has to be drawn to occurrence of enniatins in respective samples. Moreover, unfiltered beer could contain detectable amounts of these toxins.

In hop, the enniatin B level was 7 µg/kg, the other enniatins and beauvericin were either not detected or below limits of quantitation. In any case, their origin from hop could be neglected as only 8 g were used for each batch.

4. Conclusion

The fate of enniatins and beauvericin during beer making was studied in detail by taking three heavily contaminated batches of barley grains as starting material. The previously developed stable isotope dilution assays were applied for the sample measurement.

In general, the disposition of the toxins appeared to be governed by their low water solubility and their lability at elevated temperatures. Therefore, steeping was not effective in removing enniatins and beauvericin. Fungal growth and mycotoxins production occurred during the subsequent germination stage, resulting in more heavily contaminated green malts for two batches. The present study is the first to prove the growth of *Fusarium avenaceum* during germination by qPCR. Fungal growth was paralleled by mycotoxin production until a maximum content, which was not exceeded even at higher fungal infestation. Kilning along with the removal of rootlets (reduction range 28 – 59 %) contributed significantly to reduce the amount of enniatins and beauvericin. During the brewing process, a decisively great part of 53 – 98 % of these mycotoxins was retained on spent grains. The few of the toxins left in the sweet wort was mostly removed with trub afterwards, the following fermentation and maturation stages had them further degraded. By the end of the whole beer

making process, no more than 0.2% of the enniatins and beauvericin originally present in barley grains was detected in QFa batch of beer (74 µg/L and 14 µg/L of enniatin B and enniatin B1, respectively); in the other two batches of beer, none of them was detected above the limits of quantitation. Regarding a risk assessment of enniatins and beauvericin in beer, there is a lack of valid data on toxicity in mammals (Tan et al., 2011). Nevertheless, due to their low contents, we conclude that enniatins and beauvericin contamination on barley grains should pose little if any risk to beer drinkers. However, the spent grains along with the yeast sediment could be risky if fed to animals.

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656

Tables

Table 1. DNA contents of the enniatin and beauvericin producing species *F. avenaceum* and *F. tricinctum* in grain and green malt.

Sample ID[a]	*F. avenaceum* DNA [pg/ng plant DNA]				*F. tricinctum* DNA [pg/ng plant DNA]			
	grain		green malt		grain		green malt	
QC	0.85^{A}	(100%)	1.33^{A}	(155%)	0.39^{A}	(100%)	1.72^{B}	(445%)
QFc	0.38^{A}	(100%)	1.72^{A}	(448%)	0.25^{A}	(100%)	0.29^{A}	(118%)
QFa	34.20^{B}	(100%)	152.19^{C}	(445%)	0.25^{A}	(100%)	3.29^{C}	(1318%)

[a]Samples derived from field plots and were exposed to natural infestation (QC), artificial inoculation with *F. culmorum* (QFc) and *F. avenaceum* (QFa). Contents with different superscripts are significantly different (Tukey-B $p < 0.05$).

Table 2. Limits of detection (LODs), limits of quantitation (LOQs), coefficients of variation, and recoveries of enniatins and beauvericin

	LOD	LOQ	Precision (coefficients of variation)		Recovery (3 spiking levels)		
	(µg/kg)	(µg/kg)	Inter-day (n = 3)	Intra-day (n = 5)	5 µg/kg	15 µg/kg	20 µg/kg
Enniatin A	1.2	3.5	4.12%	3.47%	101 ± 4.0%	100 ± 4.7%	100 ± 5.4%
Enniatin A1	0.4	1.2	1.36%	2.53%	99 ± 6.6%	103 ± 1.1%	104 ± 0.4%
Enniatin B	0.8	2.2	1.23%	4.28%	102 ± 5.3%	104 ± 4.6%	105 ± 2.1%
Enniatin B1	1.2	3.5	0.93%	3.68%	94± 1.5%	99 ± 4.3%	102 ± 5.0%
Beauvericin	0.8	2.4	5.52%	4.92%	99 ± 3.9%	97 ± 2.6%	94 ± 2.6%

Table 3. Concentrations (µg/kg) of enniatins and beauvericin in the barley grains, green malt and first and second steeping water, and their contents compared to those in the barley grains (in total and in percentages)

QC	ENN A			ENN A1			ENN B			ENN B1			BEA		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
barley	13.8	23.9	100.0%	883.3	1528	100.0%	5222	9034	100.0%	3515	6081	100.0%	9.9	17.1	100.0%
1st steeping	-	-	-	9.0	53.9	3.5%	367.6	2206	24.4%	105.3	632.0	10.4%	-	-	-
2nd steeping	-	-	-	nq	nq	nq	67.5	540.1	6.0%	23.9	191.0	3.1%	-	-	-
green malt	20.4	34.8*	145.8%	1474	2516*	164.6%	7973	13610*	150.6%	5967	10180*	167.5%	16.0	27.3*	159.5%
QFc	**ENN A**			**ENN A1**			**ENN B**			**ENN B1**			**BEA**		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
barley	4.5	7.7	100.0%	252.9	431.7	100.0%	2949	5033	100.0%	1576	2690	100.0%	8.0	13.7	100.0%
1st steeping	-	-	-	nq	nq	nq	102.7	821.7	16.3%	22.2	177.4	6.6%	-	-	-
2nd steeping	-	-	-	4.0	31.6	7.3%	136.3	1090	21.7%	53.6	428.7	15.9%	-	-	-
green malt	12.2	20.6*	268.4%	877.8	1484*	343.7%	6052	10230*	203.2%	3540	5984*	222.4%	8.5	14.4	105.2%
QFa	**ENN A**			**ENN A1**			**ENN B**			**ENN B1**			**BEA**		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
barley	38.8	67.7	100.0%	4046	7064	100.0%	119400	208400	100.0%	40690	71050	100.0%	14.8	25.8	100.0%
1st steeping	-	-	-	-	-	-	2863	22900	11.0%	105.5	844.3	1.2%	-	-	-
2nd steeping	-	-	-	40.5	323.7	4.6%	3038	24300	11.7%	113.2	906.0	1.3%	-	-	-
green malt	36.1	64.2	94.7%	3449	6131*	86.8%	117800	209400	100.5%	37480	66640	93.8%	14.5	25.8	99.8%

-, not detectable; nq, not quantifiable

QC, control batch of barley; QFc, batch of barley inoculated with *Fusarium culmorum*; QFa, batch of barley inoculated with *F. avenaceum*

*total content in green malt which was significantly different ($p < 0.05$) from that in barley

Table 4. Concentrations (µg/kg) of enniatins and beauvericin, and their contents in kilned malt and rootles compared to those in green malt (in total and in percentages)

QC	ENN A			ENN A1			ENN B			ENN B1			BEA		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
green malt	20.4	34.8	100.0%	1474	2516	100.0%	7973	13600	100.0%	5967	10180	100.0%	16.0	27.3	100.0%
kilned malt	10.5	14.7*	42.3%	734.3	1030*	40.9%	5226	7327*	53.8%	3633*	5093*	50.0%	8.3	11.7*	43.2%
rootlets	37.1	2.3	6.7%	2150	135.5	5.4%	10300	648.9	4.8%	7756	488.6	4.8%	72.6	4.6	16.7%
QFc	**ENN A**			**ENN A1**			**ENN B**			**ENN B1**			**BEA**		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
green malt	12.2	20.6	100.0%	877.8	1484	100.0%	6052	10230	100.0%	3540	5983	100.0%	8.5	14.3	100.0%
kilned malt	11.1	14.7*	71.4%	670.3	888.8*	59.9%	4690	6219*	60.8%	3211	4258*	71.2%	6.8	9.0*	62.8%
rootlets	17.1	1.5	7.3%	994.0	87.0	5.9%	2946	257.8	2.5%	2909	254.5	4.3%	46.3	4.1	28.2%
QFa	**ENN A**			**ENN A1**			**ENN B**			**ENN B1**			**BEA**		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
green malt	36.1	64.2	100.0%	3449	6132	100.0%	117800	209400	100.0%	37480	66640	100.0%	14.5	25.8	100.0%
kilned malt	25.7	34.7*	54.1%	2915	3939*	64.2%	92690	125200*	59.8%	35460	47910*	71.9%	12.7	17.2*	66.6%
rootlets	95.5	8.7	13.5%	5635	512.8	8.4%	107300	9765	4.7%	44820	4079	6.1%	40.9	3.7	14.4%

QC, control batch of barley; QFc, batch of barley inoculated with *Fusarium culmorum*; QFa, batch of barley inoculated with *F. avenaceum*

*total content in kilned malt which was significantly different ($p < 0.05$) from that in green malt

Table 5. Concentrations (µg/kg) of enniatins and beauvericin in different stages of brewing, and their contents remaining to kilned malt (in total and in percentages)

QC	ENN A			ENN A1			ENN B			ENN B1			BEA		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
kilned malt	10.5	14.7	100.0%	734.3	1030	100.0%	5226	7327	100.0%	3633	5093	100.0%	8.3	11.6	100.0%
spent grains	33.3	13.9	94.8%	1905	796.1	77.3%	15530	6493	88.6%	10030	4194	82.3%	21.4	8.9	76.9%
sweet wort	-	-	-	nq	nq	nq	36.9	325.5	4.4%	12.6	111.1	2.2%	-	-	-
trub	31.6	1.2	8.1%	250.6	9.5	0.9%	7944	301.9	4.1%	3117	118.4	2.3%	7.8	0.3	2.5%
cool wort	-	-	-	-	-	-	nq	-	-	-	-	-	-	-	-
QFc	**ENN A**			**ENN A1**			**ENN B**			**ENN B1**			**BEA**		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
kilned malt	11.1	14.7	100.0%	670.3	888.8	100.0%	4690	6219	100.0%	3211	4258	100.0%	6.8	9.0	100.0%
spent grains	24.8	11.9	80.7%	1420	681.5	76.7%	12510	6003	96.5%	7046	3382	79.4%	16.0	7.7	85.2%
sweet wort	-			3.7	31.9	3.6%	44.0	379.1	6.1%	28.7	247.3	5.8%	-	-	-
trub	3.8	0.2	1.4%	304.1	16.1	1.8%	5782	306.5	4.9%	2743	145.4	3.4%	-	-	-
cool wort	-			-	-	-	13.9	90.0	1.4%	8.7	56.4	1.3%	-	-	-
QFa	**ENN A**			**ENN A1**			**ENN B**			**ENN B1**			**BEA**		
		total			total			total			total			total	
	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%	µg/kg	µg	%
kilned malt	25.7	34.7	100.0%	2915	3939	100.0%	92690	125200	100.0%	35460	47910	100.0%	12.7	17.2	100.0%
spent grains	83.0	34.0	98.0%	6140	2518	63.9%	251500	103100	82.3%	104200	42700	89.1%	22.3	9.1	53.3%
sweet wort	-	-	-	27.7	241	6.1%	713.8	6220	5.0%	331.8	2891	6.0%	-	-	-
trub	11.2	0.6	1.8%	1626	91.1	2.3%	43270	2423	1.9%	21400	1199	2.5%	21.5	1.2	7.0%
cool wort	-	-	-	10.5	64.8	1.6%	297.4	1836	1.5%	121.3	748.7	1.6%	-	-	-

QC, control batch of barley; QFc, batch of barley inoculated with *Fusarium culmorum*; QFa, batch of barley inoculated with *F. avenaceum*

-, not detectable; nq, not quantifiable

Legends to the figures

Figure 1 Scheme of key steps of malting and brewing processes.

Figure 2 The combined HPLC-DAD (A) and LC-MS/MS (B & C) chromatograms of a kilned malts (QFc) sample.

Fig. 1.

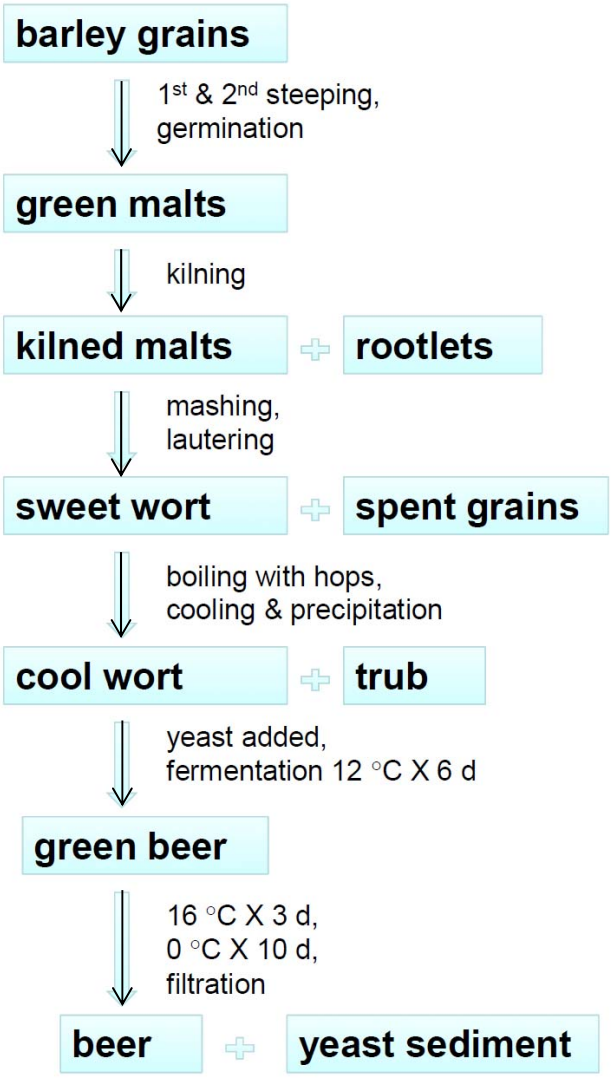
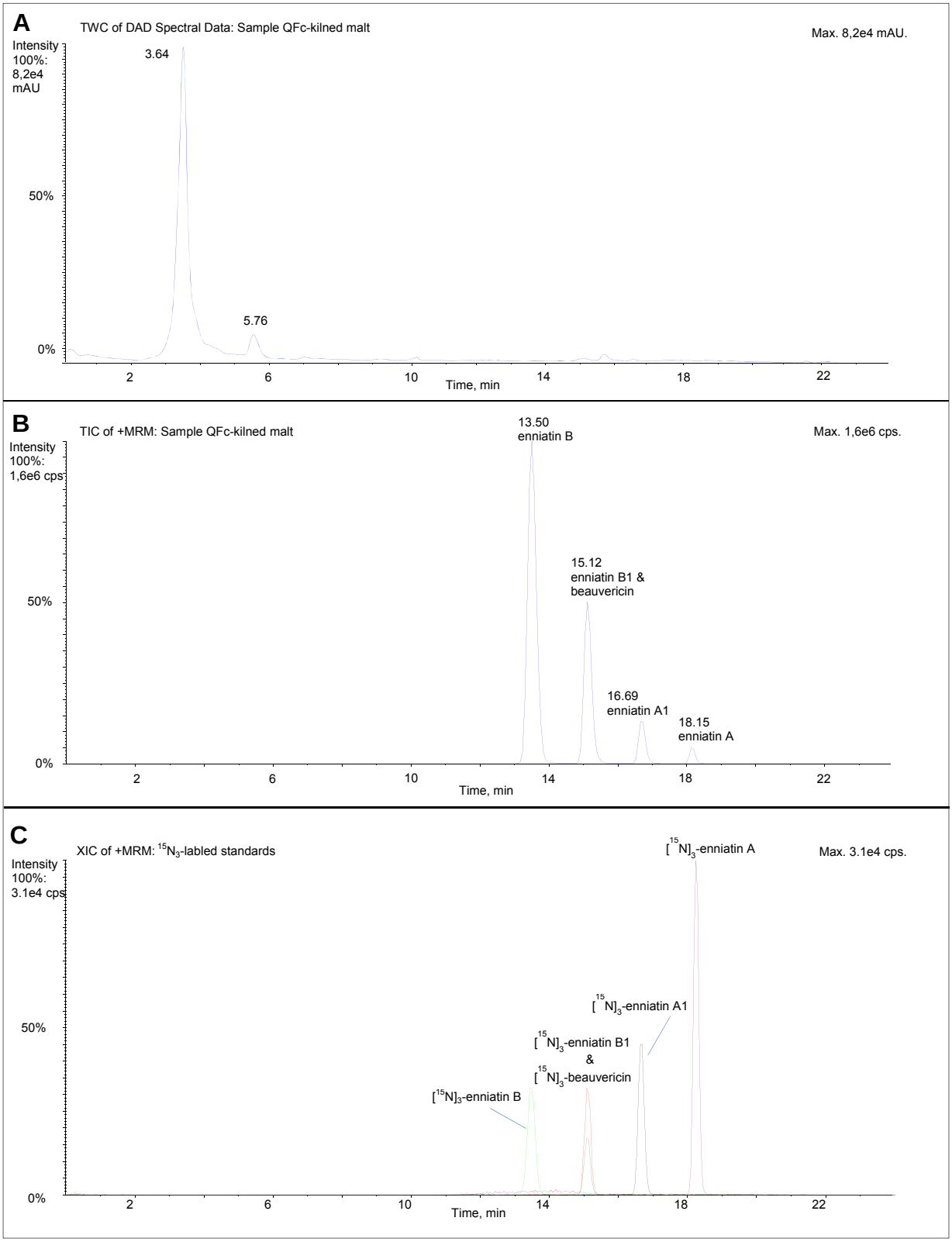

barley grains
1st & 2nd steeping, germination
green malts
kilning
kilned malts
rootlets
mashing, lautering
sweet wort
spent grains
boiling with hops, cooling & precipitation
cool wort
trub
yeast added, fermentation 12 °C X 6 d
green beer
16 °C X 3 d, 0 °C X 10 d, filtration
beer
yeast sediment

Fig. 2.


A
TWC of DAD Spectral Data: Sample QFc-kilned malt
Max. 8,2e4 mAU.
Intensity 100%: 8,2e4 mAU
3.64
5.76
50%
0%
2
6
10
14
18
22
Time, min
B
TIC of +MRM: Sample QFc-kilned malt
Max. 1,6e6 cps.
Intensity 100%: 1,6e6 cps
13.50 enniatin B
15.12 enniatin B1 & beauvericin
16.69 enniatin A1
18.15 enniatin A
50%
0%
2
6
10
14
18
22
Time, min
C
XIC of +MRM: $^{15}N_3$-labled standards
Max. 3.1e4 cps.
Intensity 100%: 3.1e4 cps
[^{15}N]$_3$-enniatin A
[^{15}N]$_3$-enniatin A1
[^{15}N]$_3$-enniatin B1 & [^{15}N]$_3$-beauvericin
[^{15}N]$_3$-enniatin B
50%
0%
2
6
10
14
18
22
Time, min