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**TRANSFER OF FUSARIUM MYCOTOXINS AND
“MASKED” DEOXYNIVALENOL
(DEOXYNIVALENOL-3-GLUCOSIDE) FROM FIELD
BARLEY THROUGH MALT TO BEER**

Katerina Lancova, Jana Hajslova, Jan Poustka, Alexandra Krplova, Milena Zachariasova, Pavel Dostálek, Lenka Sachambula

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TRANSFER OF FUSARIUM MYCOTOXINS AND MASKED DEOXYNIVALENOL (DEOXYNIVALENOL-3-GLUCOSIDE) FROM FIELD BARLEY THROUGH MALT TO BEER

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For Peer Review Only

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3 1 **TRANSFER OF *FUSARIUM* MYCOTOXINS AND “MASKED” DEOXYNIVALENOL**
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5 2 **(DEOXYNIVALENOL-3-GLUCOSIDE) FROM FIELD BARLEY THROUGH MALT**
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7 3 **TO BEER**
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12 5 K. Lancova¹, J. Hajslova^{1*}, J. Poustka¹, A. Krplova¹, M. Zachariasova¹, P. Dostalek², L.
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37
38 16 **ABSTRACT**
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41 17 The fate of five *Fusarium* toxins- deoxynivalenol (DON), sum of 15- and 3-acetyl-
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43 18 deoxynivalenol (ADONs), HT-2 toxin (HT-2) representing the main trichothecenes and
44
45 19 zearalenone (ZON) during the malting and brewing processes was investigated. In addition to
46
47 20 these “free” mycotoxins, the occurrence of deoxynivalenol-3-glucoside (DON-3-Glc) was for
48
49 21 the first time, monitored in beer production chain (currently, only DON and ZON are
50
51 22 regulated). Two batches of barley, naturally infected and artificially inoculated with *Fusarium*
52
53 23 *Spp.* during the time of flowering, were used as a raw material for processing experiments. A
54
55 24 highly sensitive procedure employing high performance liquid chromatography coupled with
56
57 25 tandem mass spectrometry (LC-MS/MS) was validated for analysis of “free” *Fusarium*
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3 1 mycotoxins and DON-conjugate in all types of matrices. The method was able to detect also
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5 2 nivalenol (NIV), fusarenon-X (FUS-X) and T-2 toxin (T-2), nevertheless, none of these toxins
6
7
8 3 were found in any of samples. While steeping of barley grains (the first step in malting
9
10 4 process) apparently reduced *Fusarium* mycotoxin levels to below their quantification limits (5
11
12 5 - 10 µg/kg), their successive accumulation occurred during germination. In malt, the content
13
14
15 6 of monitored mycotoxins was higher compared to the original barley, the most significant
16
17 7 increase was found for DON-3-Glc. During the brewing process, significant further increases
18
19 8 in levels occurred. Concentrations of this “masked” DON in final beers exceeded “free”
20
21 9 DON, while in malt grists, this trichothecene was the most abundant, with the DON/DON-3-
22
23 10 Glc ratio being approx. 5:1 in both sample series. When calculating mass balance, no
24
25 11 significant changes were observed during brewing for ADONs, the content of DON and ZON
26
27 12 slightly decreased, by a maximum of 30%. Only traces of HT-2 were detected in some
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29 13 processing intermediates (wort after trub removal and green beer).
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36 **KEYWORDS**

37
38 16 Deoxynivalenol, deoxynivalenol-3-glucoside, conjugated mycotoxins, *Fusarium* mycotoxins,
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40 17 barley, malt, beer, processing.
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45 **INTRODUCTION**

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48 20 Beer contributes significantly to the diet of the population world-wide. The British
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50 21 Beer and Pub Association has reported recently (2006) that the annual per-capita consumption
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52 22 of this fermented drink in 2004 exceeded 100 litres in many countries, with a maximum of
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54 23 157 litres in the Czech Republic. Unfortunately, barley, the major raw material used in beer
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56 24 production, like other cereals, can often be infected under field conditions by toxinogenic
57
58 25 *Fusarium* species, causing the disease called *Fusarium* head blight and, consequently,
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1 contaminated by mycotoxins. It should be noted, that hops, an ingredient supplying bittering
2 and aroma components to beer, can be also contaminated with *Fusarium* Spp. (Solarska
3 2007), the disease is called in this case *Fusarium* cone tip blight (Bienapfl et al. 2001).
4 However, until now hops have seldom been investigated as a possible source of *Fusarium*
5 mycotoxins (Hazel and Patel 2004).

6 In 2003, a report on task for Scientific Cooperation (SCOOP) concerned with
7 occurrence of *Fusarium* toxins in a wide range of food commodities, the data needed for
8 assessment of dietary intake by the population of EU Member States become available
9 (http://ec.europa.eu/food/fs/scoop/index_en.html). The selected information on the occurrence
10 of these mycotoxins in barley and malt reported by participating countries (Finland, France,
11 Italy, The Netherlands, Norway, UK) is summarised in Table I. As documented here, DON,
12 the representative of trichothecenes *B* group, is the most abundant *Fusarium* toxin in these
13 key raw materials used for beer production. The extent of its transfer into beer analysed in
14 various countries worldwide is shown in Table II (Omurtag et al. 2007, Papadopoulou-
15 Bouraoui et al. 2004, Odhav and Naicker 2002, Molto et al. 2000, Shim et al. 1997, Ruprich
16 and Ostry 1995, Weddeling et al. 1994, Niessen and Donhauser 1993, Scott et al. 1993,
17 Trucksess et al. 1986, Payen et al. 1983). In the two most extensive available studies focused
18 on DON behaviour during malting and/or brewing processes (Schwarz et al. 1995, Niessen et
19 al. 1993), fairly contradictory results, both DON increase and its decrease as compared to
20 barley used for processing, were found, see Table III. Besides DON, the presence of ZON and
21 15-ADON was found in malts employed for brewing experiments, however, in the later
22 stages, their occurrence above the method LOD, was documented only in spent grains, not in
23 beers (Schwarz et al. 1995). It should be noted, that ZON is metabolised to α - and β -
24 zearalenol by brewing strains of *Saccharomyces cerevisiae*. However, the occurrence of ZON
25 and its metabolites in beer was reported only rarely, Zöllner et al. (2000) detected these

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3 1 mycotoxins in only one of 23 beer samples collected in different countries, produced from
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5 2 different grains and under different brewing conditions. Overall, no more information on the
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8 3 fate of *Fusarium* toxins, others than DON, 15-ADON and ZON, during the malting and
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10 4 brewing processes has been available until now. Also the occurrence of conjugated forms of
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12 5 mycotoxins in beer has previously not been investigated, although their presence in cereals is
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14 6 known. The first report on “masked” mycotoxins represented typically by toxin bound to a
15
16 7 more polar molecule like glucose, amino acids, or sulphate, appeared in the mid of the 80s
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18 8 when the severity of mycotoxicoses in animals was found to be greater than their determined
19
20 9 levels in feedstuffs. It was suggested, that hydrolysis of toxin conjugates occurs in the
21
22 10 digestive tracts releasing the levels of precursor toxins, thus increasing animals’ exposure
23
24
25 11 (Gareis 1994). Later on (Berthiller et al. 2005), deoxynivalenol-3-glucoside (DON-3-Glc) was
26
27 12 reported as the major form of “masked” DON constituting up to 12% of total content of this
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29 13 mycotoxin in examined cereals, wheat and maize.

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34 14 To avoid consumers’ health risk due to unacceptably high dietary intake of *Fusarium*
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36 15 toxins, the European Commission has established the maximum levels for DON (1250 µg/kg
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38 16 in cereals; 200 µg/kg in processed cereal-based foods and baby foods) and ZON (100 µg/kg in
39
40 17 cereals; 20 µg/kg in processed cereal-based foods and baby foods). The regulation for other
41
42 18 fusariotoxins, the sum of HT-2 and T-2 toxin, is currently under preparation and is expected
43
44 19 to come into the force in July 2008 (European Commission 2006). The temporary tolerable
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46 20 daily intakes (TDI) 1, 0.7, 0.2, and 0.06 µg/kg b.w. have been proposed for DON, NIV, ZON
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48 21 and the sum of T-2 and HT-2, respectively (European Commission 2006). However, it does
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50 22 not appear that the occurrence and significance of “masked” mycotoxins has been considered
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52 23 in the formulation of these limits.

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57 24 The study reported here has investigated the fate of *Fusarium* mycotoxins within all
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59 25 steps involved in the beer production chain. The recent introduction of a pure DON-3-Glc

1 standard on to the market allowed the LC-MS/MS method to be established for this analyte in
2 the matrices collected within the current malting and brewing experiments. In this way, it was
3 possible for the first time to obtain information on the possible transfer of “masked”
4 mycotoxins from barley infected by *Fusaria* into beer.

6 MATERIALS AND METHODS

7 *Chemicals*

8 Standards of analysed mycotoxins (deoxynivalenol, DON; deoxynivalenol-3-
9 glucoside, DON-3Glc; nivalenol, NIV; fusarenon-X, FUS-X; sum of 15- and 3-acetyl-
10 deoxynivalenol, ADONs; T-2 toxin, T-2; HT-2 toxin, HT-2 and zearalenone, ZON) were
11 purchased from Biopure (Austria). To ensure the accuracy of measurements certified
12 reference material, DON naturally contaminated wheat (R-Biopharm, UK), ZON naturally
13 contaminated milo (R-Biopharm, UK) and DON in wheat flour (BCR, Belgium), were used.
14 The BCR reference materials were produced and certified in accordance with internationally
15 accepted rules laid down in the various guides by BCR (Bureau Communautaire de
16 Référence), the ISO (International Organisation for Standardisation) and the WHO (World
17 Health Organisation).

18 *Samples*

19 The levels of *Fusarium* toxins in barley and malt grists that were used for processing
20 experiments are summarized in Table IV (analytical method used for analysis is described
21 below).

22 *Barley for malting*

23 For the malting experiments conducted within the first part of the presented study, two
24 batches of barley (each 4 kg; variety Kompakt) were supplied by the Agrotest Fyto Ltd.
25 (Kromeriz, Czech Rep.): (i) series NAT - sample containing “low levels” of mycotoxins –

1 naturally infected with *Fusarium* Spp., and (ii) series ART - sample with “appreciable levels
2 of mycotoxins” inoculated in the field by spraying with a spores suspension containing a
3 mixture of *F. culmorum* and *F. graminearum* (1:1; 10^6 conidia/ml suspension; 250-300 l/ha)
4 at the time of barley flowering.

5 *Malt grist for brewing*

6 Two batches of malt grists (each 6.5 kg) were prepared by the Research Institute of
7 Brewing and Malting (division Brno, Czech Rep.) from barleys (variety Kompakt)
8 representing again both series, NAT and ART. It should be noted, that because of concurrence
9 of processing experiments, mycotoxin levels in malt grists taken for brewing were not
10 identical with those determined in products obtained in malting.

11 *Processed products*

12 *Malted barley*

13 Micro-malting experiments that were carried out by the Malting Institute of the
14 Research Institute of Brewing and Malting (Brno, Czech Rep.) in line with traditional
15 processes. Barley grains (3.5 kg) were micromalted in a small-scale malting equipment of the
16 company KVM (CR). Malt was produced from barley within 3 key steps: i) steeping, ii)
17 germination and iii) kilning. To initiate germination, the barley must be adequately supplied
18 with both water and oxygen during a steeping stage and this was achieved in this study. The
19 production of enzymes is the main purpose of the germination step. During the kilning stage,
20 water is removed from the green malt and the malt then becomes stable and storable. Detailed
21 descriptions of the experimental malting conditions and a list of samples taken during this
22 process are summarised in Table V.

23 *Beer*

24 Beer was produced from malt grist according to a standard procedure in the brewery
25 pilot plant at the Department of Fermentation Chemistry and Bioengineering (Institute of

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3 1 Chemical Technology - ICT, Prague). In brief, the malt grist was mashed in a double mash
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5 2 process. After mashing, the liquid portion was separated from the spent grains by the classical
6
7 3 lautering process. Then the wort was boiled together with added hop. After hot trub
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9 4 separation, fermentation was carried out with bottom-fermenting yeast. Detailed experimental
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11 5 brewing conditions together with a description of the samples taken throughout the beer
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13 6 production is summarised in Table VI.
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20 8 *Mycotoxin analysis*

21 9 *Sampling*

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23 10 500 g of grains were taken from thoroughly mixed barley batches delivered to the
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25 11 laboratory. These representative samples were ground in the laboratory mill (LM 120; Perten
26
27 12 Instruments). For mycotoxin analysis, two sub-samples (each 12.5 g) were taken. Similar
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29 13 approach was employed for sampling of representative malt grists and other processing
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31 14 intermediates. Regarding liquid brewing intermediates and beers, 16 ml aliquots used for
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33 15 mycotoxin analysis were taken from 100 ml samples collected within process.
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40 17 *Extraction and clean-up procedure for solid samples*

41 18 Representative samples (12.5 g) were extracted with 50 ml acetonitrile:water mixture
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43 19 (84:16, v/v) for one hour using an automatic shaker (IKA Laborortechnik, Germany). The
44
45 20 crude extracts were then filtered (Filtrak No. 390, VEB Freiburger, Germany) and 8 ml
46
47 21 aliquots transferred into sample tubes to which 80 µl of acetic acid (99%, Sigma-Aldrich)
48
49 22 were added. Purification was achieved by solid phase extraction (SPE) employing
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51 23 MycoSepTM 226 cartridges (Romer, Austria). 4 ml of purified extract were evaporated to
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53 24 dryness and the solid residues transferred into 1 ml of a water: methanol mixture (50:50, v/v),
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55 25 and passed through a 0.2 µm microfilter (Alltech, USA) prior to further analysis.
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60 26 *Extraction and clean-up procedure for liquid samples*

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3 1 The aliquots (16 ml) of processing intermediates (sweet wort, warm wort, wort after
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5 2 trub removal, green beer) as well as the final product (beer), were shaken after the addition of
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7 3 84 ml acetonitrile and 3.2 g Celite for one hour using an automatic shaker. The extract
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9 4 obtained was then filtered and 5 ml aliquots were evaporated to dryness. The residues were
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11 5 transferred into 1 ml of a water: methanol mixture (50:50, v/v) prior to the analysis.
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15 6 *High performance liquid chromatography coupled with tandem mass spectrometry*
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17 7 *(LC-MS/MS)*
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19
20 8 High performance liquid chromatography (HP1100 Binary Series LC system, Agilent
21
22 9 Technologies, USA) coupled with mass spectrometer (Finnigan LCQ Deca, USA) were used
23
24 10 for the analysis. Chromatographic separation of sample components was carried out on a
25
26 11 reverse phase column with polar end-capping (Synergi Hydro RP, 150mm x 3mm x 4µm,
27
28 12 Phenomenex, USA) held at 40 °C and operated under gradient conditions. The mobile phase
29
30 13 was composed of 10 mM ammonium acetate in purified water (A) and methanol (B). The flow
31
32 14 rate of the mobile phase was set to 0.5 ml/min and the injection volume was 20 µl. Gradient
33
34 15 elution was performed starting from A:B (80:20, v/v) and reaching A:B (30:70, v/v) in 8 min.
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36 16 From 8 to 15 min the ratio A:B (30:70, v/v) was stable and then jumped to A:B (80:20, v/v).
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38 17 The time of post run lasted 7min.
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43 18 Identification and quantification of analytes was performed using a tandem in time mass
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45 19 spectrometry (MS/MS) realised by ion trap with the following parameters (ion source type –
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47 20 APCI operated both in negative and positive ion modes under following conditions: capillary
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49 21 temperature - 150 °C, vaporizer temperature - 450 °C, nitrogen sheath gas flow – 1.2 l/min,
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51 22 nitrogen auxiliary gas flow – 3 l/min, source voltage - 6 kV, collision gas - helium, scan type -
52
53 23 selected reaction monitoring). APCI positive ions in form $[M+NH_4]^+$ were used for type A
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55 24 trichothecenes. The ion transitions: parent > daughters (quantifier-Q, identifier- I) were as
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57 25 follows: HT-2: 442>273(Q),299(I) and T-2: 488>305(Q),245(I). For the type B
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1 trichothecenes, the fragmentation of $[M+CH_3COO]^-$ in case of NIV: 371>311(Q),281(I);
2 DON: 355>295(Q),265(I); DON-3-Glc: 517>457(Q),427(I); ADON: 397>337(Q),307(I);
3 FUS-X: 413>353(Q),263 (I) and $[M-H]^-$ in the case of ZON: 317>273(Q),299(I) were
4 performed.

5 *Performance characteristics of analytical method / Quality assurance*

6 Limits of detection (LODs) and quantification (LOQs), recoveries and repeatabilities
7 expressed as RSDs that were obtained within the validation process are reported in Table VII.
8 Calibration curves for all analytes were linear within the working range from 5 to 5000 $\mu\text{g}/\text{kg}$.
9 Squared correlation coefficients (R^2) were under 0.999 for 10 point calibration curves. The
10 analyses of all examined samples were performed in duplicate. The data shown in Tables VIII
11 and IX are mean values corrected for analytes recoveries.

12 The method used for samples analysis of was accredited (ISO 17025) for cereals and,
13 as a part of external quality control, the trueness of generated data was successfully
14 demonstrated through participation in proficiency testing Food Analysis Performance
15 Assessment Scheme (FAPAS) organized by Central Science Laboratory (CSL, York UK).
16 The z-scores for analyses of HT-2 and T-2 in oat (Proficiency Test (PT) 2234), ZON in baby
17 food (PT 2236) and maize (PT 2219), DON in wheat flour (PT 2229) and maize (PT 2224)
18 were in the range ± 2 .

19 *Moisture determination*

20 Moisture content was determined gravimetrically by drying samples in an oven for 2
21 hours at 131 ± 2 °C according to ISO Standard No. 712 “Moisture determination in cereals
22 and cereal products”.

23 **RESULTS AND DISCUSSION**

1 *Fusarium* mycotoxins are almost unavoidable natural contaminants of barley which is
2 the key raw material for beer production. To estimate consumers' exposure to *Fusarium*
3 toxins from this commodity and to support identification of measures aimed at its reduction
4 (as in the case of other contaminants), the knowledge on the fate of these hazardous fungal
5 secondary metabolites through the production of beer is essential. The currently available
6 information on the changes of DON and other *Fusarium* toxins during barley processing is
7 rather contradictory and of new interest is none of the available studies was concerned with
8 "masked" mycotoxins issue. The results obtained within this research on the behaviour of
9 *Fusarium* toxins both during malting and brewing addresses some of these issues and should
10 contribute to a better understanding of their fate during the beer making process.

12 ***Malting process***

13 It is well established that, barley, depending on a crop year and agricultural practices
14 employed, is often to some extent infected by *Fusarium* fungi, and consequently by their toxic
15 secondary metabolites. The dynamics of *Fusarium* toxins documented by the analysis of 10
16 samples collected during the malting of both naturally (NAT) and artificially (ART)
17 contaminated barleys shown in Table VIII. The relative transfer of DON, DON-3-Glc,
18 ADONs and HT-2 (when taking their content in processed barley grains equal to 100%) are
19 illustrated for sample series ART in Figure 1.

20 During the steeping stage, DON as well as ADONs levels were either eliminated
21 (series NAT) or largely reduced to below 10% (series ART) of their original amount in the
22 sample of barley. Similarly, a decline of DON in samples examined during this malting step,
23 was earlier observed by Schwarz et al.(1995). Interestingly, there was a slight increase of
24 DON-3-Glc in sample series ART occurred within steeping. While "free" mycotoxins were
25 obviously extracted by steep water, the removal of DON conjugate, probably due to its release

1 from polysaccharides, was not complete. Further work is required to examine the processes
2 involved with the DON conjugate at this and other processing stages.

3 During the germination stage, significant increases of mycotoxin concentrations in
4 processing intermediates occurred in both series of experiments (NAT and ART), see Table
5 VIII. Although DON-3-Glc, ADONs, HT-2 were below the detection limits in the NAT
6 barley sample, these mycotoxins were detected in this germination stage. The experimental
7 growth of *Fusarium* toxins shown in Figure 1 and Table VIII might be associated with a rapid
8 onset of enzymatic activities in the germination of barley kernels, alternatively, *de novo*
9 formation of fungal secondary metabolites is another conceivable cause. Worth to notice, that
10 not only increase, but also reduction of DON in malt as compared to barley used for its
11 production was reported in one of few older studies (Schwarz et al. 1995).

12 The final malting stage, kilning, did not change “free” trichothecenes levels
13 significantly, neither thermodegradation (*Fusarium* mycotoxins are stable up to 120°C), or
14 their additional formation as fungal growth was halted by the high temperature. The trend for
15 DON-3-Glc was slightly different in the samples series NAT compared with ART, see Table
16 VIII. While its levels dropped in the samples NAT, no distinct changes were observed in
17 series ART and information on DON-3-Glc stability at higher temperatures is not clear from
18 these data.

19 The final barley malt was obtained by removing the rootlets. It should be noted, that
20 this waste product contained the highest mycotoxin levels of all intermediates taken during
21 malting process (see Table VIII). Considering its use as a nutritive component of feeding
22 stuffs and/or “healthy” food supplements exposure resulting from rootlets by both humans
23 and farm animals should be taken into consideration.

24 Regarding the comparison of *Fusarium* toxins in the raw material (barley, series ART)
25 and the final product (malt), the levels of DON, DON-3-Glc, ADONs and HT-2 were 2.1, 8.6,

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3 1 1.1 and 2.1 times higher in malt than in barley, respectively, see Figure 1. In series NAT,
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5 2 DON was the only mycotoxin detected in barley, nevertheless, the malt prepared from this
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7 3 sample contained all the spectrum of mycotoxins (although at lower levels) as that found in
8
9 4 ART. DON increased 3.8 times. The release from conjugated forms and/or their de novo
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11 5 production by growing fungi is likely to be the cause of trichothecenes levels being elevated
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13 6 during the malting process.
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20 8 ***Brewing process***

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22 9 The ingredients needed for beer production are malt grist, hops, yeast and water.
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24 10 Whereas the last two items are hardly the source of mycotoxins, malt grist, as shown above,
25
26 11 may supply significant amounts of these toxins to beer. Theoretically, hops may also contain
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28 12 toxic secondary metabolites produced by fungi, but no information suggesting this commodity
29
30 13 as a source of contamination is available. In any case, hop extract is largely diluted to beer
31
32 14 (3.4 g of hops per 1 l of beer) so their likely contribution to the overall load of *Fusarium*
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34 15 toxins is likely to be low. Within this part of the study, two series of malt grists prepared from
35
36 16 NAT and ART together with respective final beers, were analysed for 7 trichothecenes and
37
38 17 ZON; and the levels of “masked” DON, DON-3-Glc, were also determined. The results
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40 18 obtained are summarised in Table IX, and illustrated in Figures 2 and 3, in which the transfer
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42 19 of DON, DON-3-Glc, ADONs and HT-2 was calculated as a percentage according to the
43
44 20 mass balance from the starting raw material (malt grist; 6 kg) into final beer (50 l). For better
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46 21 understanding of mass balance calculation based on data shown in Table VI, an example of
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48 22 DON-3-Glc transfer from malt grist to sweet wort follows: conjugate concentration in the
49
50 23 NAT malt grist was 349 µg/kg giving a total amount of 2,094 µg in the 6 kg needed for
51
52 24 preparation of 59 l sweet wort, in that DON-3-Glc level was 561 µg/kg giving a total amount
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1 of 29,441 μg . Employing these figures for “transfer” of DON-3-Glc from the raw material to
2 the first brewing intermediate was $29,441 / 2,094 = 14.05$.

3 As expected, most of DON was removed from malt grist to beer, this toxin was not
4 detected (LOQ = 5 $\mu\text{g}/\text{kg}$) in NAT spent grains (the waste from mashing step) and only 1% of
5 the total DON was found in more contaminated ART sample. DON transfers to sweet wort
6 were 70 and 67% in NAT and ART series, respectively. In hot wort and wort after trub
7 removal corresponding to the ART series, DON transfer exceeded even 100%. In green beer
8 as well as in the final beer, the total content of DON in both sample series, NAT and ART,
9 was lower as compared to its total content in the malt grist batch taken for brewing
10 experiments. These results are in a good agreement with an earlier study by Schwarz et al.
11 (1995), who reported similar DON transfer rates where the values were in the range of 80-
12 93% in four parallel brewing experiments resulting from highly contaminated malt grists (see
13 Table III in Introduction). A distinctly opposite trend, huge increases of DON in beers, as
14 high as 300 and 600%, were reported by Niessen and Donhauser (1993), who used two malt
15 grists with similar DON levels (see Table III) as those in the current study. It should be noted;
16 that a relatively accurate method (although not enabling low detection limits), gas
17 chromatography coupled with mass spectrometry (GC/MS), was used by Schwarz et al.
18 (1995) for target analytes quantification whereas a screening enzyme-linked
19 immunoabsorbent assay (ELISA) based on a polyclonal antibody was employed in the
20 Niessen’s and Donhauser’s study. Large discrepancies between the results of these studies can
21 be due to possible cross-reactions associated with the ELISA method. Fairly beyond
22 expectation, the content of this “masked” DON increased dramatically in sweet wort, hot wort
23 and wort after trub removal prepared from both sample series, NAT and ART, as compared to
24 its level in malt grist taken for processing. In the later case, DON transfer in these
25 intermediates exceeded even $10^3\%$. The dynamics of DON-3-Glc was rather different

1
2
3 1 between the two experimental series; nevertheless, in terms of transfer to the final product, it
4
5 2 was equal at 600% in both beers derived from NAT and ART malt grists. Interestingly, this
6
7 3 value closely corresponded to “DON increase” reported by Niessen and Donhauser (1993),
8
9 4 see Table III. Based on preliminary tests, for which several types of commercial ELISA kits
10
11 5 were employed, we showed the cross-reactivity towards DON-3-Glc to be comparable to that
12
13 6 of DON. With regard to this, until now not reported observation, it can be assumed that the
14
15 7 data reported in Niessen’s study were significantly overestimated due to the presence of DON
16
17 8 conjugate. Its hydrolysis from bounds to higher molecular constituent is probably supported
18
19 9 by high temperatures, 70 and 100°C, occurring in sweet wort and hot wort technological
20
21 10 stages. To get more knowledge on “masked” mycotoxin transformation, we have initiated
22
23 11 intensive research in this area.
24
25
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28

29 12 In effect, ADONs, represent, another form of masked DON. As shown in Figure 2 and
30
31 13 3, their levels in experimental samples were almost equal or slightly higher as compared to
32
33 14 DON. Transfer into final beers were slightly above 100%. Unfortunately, under the
34
35 15 chromatographic conditions employed in our method (several reversed phase columns were
36
37 16 tested), we were not able to separate 3- and 15-acetyldeoxynivalenol. It should be noted that
38
39 17 in most of commercial DON ELISA kits, high cross-reactivity (sometimes even higher than
40
41 18 100%) towards 3-acetyl DON is occur is declared by producers (no declaration for DON-3-
42
43 19 Glc). Theoretically, this trichothecene could also contribute to overestimated data shown in
44
45 20 the study by Niessen et al. (1993).
46
47
48
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50 21 As shown in Table VI, series ART, ZON was also transferred into beer and this
51
52 22 estrogenic mycotoxin can be largely metabolised by *Saccharomyces cerevisiae* to β -
53
54 23 zearalenol. However, this component was not monitored in our experiments. Also considering
55
56 24 the possible occurrence of zearalenon-4-glucoside (ZON-4-Glc) in cereals (Engelhardt *et al.*,
57
58 25 1999), it is difficult to assess the apparent ZON transfer at 80%, since both its transformation
59
60

1 and/or elimination may be due to it being bound and released from conjugates during brewing
2 process.

3 4 5 6 7 8 9 10 **CONCLUSIONS**

11
12 This study concerned with the fate of *Fusarium* toxins in the beer production chain
13 starting from barley has shown many interesting results of which some have never been
14 reported before.

15
16 Significant increases of *Fusarium* toxins (DON, ADONs and HT-2) occurred during
17 malting process, mainly in the germination step. In addition to these trichothecenes, the
18 presence and formation of high levels of DON-3-Glc was documented. Further significant
19 increases in levels of this DON conjugate occurred during the brewing process. In sweet wort
20 its relative content was ten times higher as compared to that in malt grist taken for the
21 processing experiment. Generally, two different phenomena should be considered when
22 explaining high levels of DON-3-Glc that can be found in beer:

23
24 (i) *De novo* growth of *Fusarium* micromycetes (either coming from spores or
25 hyphae invading the kernels, or due to cross-contamination during this process)
26 favoured under certain malting conditions (3 days, 14.5°C, moisture 45%) is
27 accompanied by production of additional mycotoxins and their transformation
28 products.

29
30 (ii) Enzymes produced during the mashing of malt grist degrade cell walls,
31 membrane bound proteins, and starch depots in kernels, thus releasing DON-3-Glc
32 from insoluble forms.

33
34 To get a deeper knowledge on the effect of processing on *Fusarium* toxins and their
35 “masked” forms, more research is needed. Besides beer making other processing practices,
36 namely those employing fermentation such as bread making, should also be investigated to

1
2
3 1 identify both additional risk sources as well as possibilities to reduce the total mycotoxin load
4
5 2 in foods. Although the bio-availability of mycotoxin conjugates has not been fully
6
7 3 documented yet, the possibility of approaching, or even exceeding TDI of 1 µg/kg body
8
9 4 weight of DON established by Scientific Committee on Food (SCF) (European Commission
10
11 5 2006), could occur. By supposing similar toxicological profile for DON and DON-3-Glc,
12
13 6 these conditions could occur for a 60 kg person when drinking approx. 2 glasses of
14
15 7 “experimental” beer prepared in our study from naturally infected barley in which the sum of
16
17 8 both “free” and conjugated DON was 77 µg/l.
18
19
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21

22 9 There are discrepancies between the data in the literature on DON content in the beer
23
24 10 production chain obtained by ELISA, and our results which were established by LC-MS/MS.
25
26 11 This and other preliminary work at our laboratory lead us to assume that in part may be due to
27
28 12 cross-reactivity of commercial test kits towards DON-3-Glc and other forms of DON. In any
29
30 13 case, challenges in mycotoxins analysis, including the possibility to employ immunoaffinity
31
32 14 columns for simultaneous pre-concentration of both “free” and “masked” mycotoxins, have
33
34 15 been identified.
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51
52 23 are incorporated in conclusions. Authors wish to express appreciation of his scientific input.
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Table I Survey of *Fusarium* toxins in barley and malt in some EU countries; P/T = number of positive samples/total number of examined samples; mean = mean concentration ($\mu\text{g}/\text{kg}$) calculated from positive samples; max = maximum concentration ($\mu\text{g}/\text{kg}$) (*Scientific Cooperation Report, 2003*)

| Country | Food Commodity | DON | | | NIV | | | 3-ADON | | | T-2 | | | HT-2 | | | ZON | | |
|----------------|----------------|---------|------|-----|--------|------|-----|--------|------|-----|-------|------|-----|-------|------|-----|------|------|-----|
| | | P/T | Mean | Max | P/T | Mean | Max | P/T | Mean | Max | P/T | Mean | Max | P/T | Mean | Max | P/T | Mean | Max |
| Finland | barley | 46/77 | 111 | 619 | 4/77 | * | 351 | 6/77 | * | 101 | 0/77 | * | * | 3/77 | 23 | 41 | 6/77 | 18 | 53 |
| | malt | 28/68 | 106 | 394 | 21/68 | 135 | 293 | 0/68 | * | * | 5/68 | 58 | 153 | 13/68 | 45 | 47 | 0/50 | * | * |
| France | barley | 9/9 | 15 | 35 | 9/9 | * | 40 | 0/9 | * | * | 0/9 | * | * | 0/9 | * | * | * | * | * |
| | malt | 185/350 | * | 550 | 0/194 | * | * | 0/194 | * | * | 0/194 | * | * | 0/194 | * | * | * | * | * |
| Italy | barley | * | * | * | * | * | * | * | * | * | 1/1 | 280 | 280 | * | * | * | 1/1 | 5 | 5 |
| Netherlands | barley | 5/40 | 299 | 510 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Norway | barley | 6/20 | 47 | 60 | 0/20 | * | * | 0/20 | * | * | * | * | * | * | * | * | 1/20 | 11 | 11 |
| United Kingdom | barley | 26/153 | 12 | 53 | 10/153 | 25 | 109 | 9/153 | 7 | 37 | 0/153 | * | * | 0/153 | * | * | * | * | * |

*this information was not available

Table II Survey of DON levels ($\mu\text{g/l}$) in beers as reported by countries worldwide; mean was calculated from positive samples

| <i>Country</i> | <i>Incidence</i> | <i>Mean</i> | <i>Range</i> | <i>Reference</i> |
|----------------|------------------|-------------|--------------|-----------------------------------|
| Afrika | 0/35 | * | * | Odhav and Naicker 2002 |
| Argentina | 22/50 | 14.3 | 4 - 221 | Molto et al. 2000 |
| Canada ** | 29/50 | 5.4 | 0.3-50 | Scott et al. 1993 |
| Czech Republic | 59/77 | 13 | 7-70 | Ruprich and Ostry 1995 |
| France | 1/49 | 20 | * | Payen et al. 1983 |
| Germany | 85/196 | 205 | up to 569 | Niessen and Donhauser 1993 |
| Germany | 18/18 | 5 | up to 9 | Weddeling et al. 1994 |
| Korea | 14/54 | * | 1-23 | Shim et al. 1997 |
| USA | 0/14 | * | * | Trucksess et al. 1986 |
| Turkey | 0/50 | * | * | Omurtag et al. 2007 |
| Austria | 25/33 | 10.2 | 4.5-29.5 | |
| Belgium | 47/47 | 18.1 | 4.1-56.7 | |
| Cyprus | 5/6 | 8 | 4-12.2 | |
| Czech Republic | 17/17 | 21.5 | 4.6-55.3 | |
| Denmark | 9/9 | 19.9 | 6-47.1 | |
| France | 24/27 | 11 | 4.1-30.2 | |
| Finland | 3/4 | 7.4 | 5.2-10.6 | |
| Germany | 45/46 | 4.7 | 4-40.5 | |
| Greece | 4/4 | 17 | 16.2-16.8 | |
| Hungary | 2/2 | 10.8 | 10.5-11.1 | Papadopoulou-Bouraoui et al. 2004 |
| Ireland | 2/2 | 8.7 | 7.7-9.6 | |
| Italy | 16/16 | 10.5 | 5-29.4 | |
| Netherlands | 3/4 | 8 | 5.9-9.7 | |
| Norway | 3/4 | 7.7 | 6-9.9 | |
| Poland | 10/10 | 17.2 | 5-32.9 | |
| Sweedden | 4/7 | 5.1 | 5.1-14.6 | |
| Slovakia | 9/12 | 13.5 | 5.5-36.9 | |
| Spain | 6/13 | 7.3 | 5.1-12.2 | |
| United Kingdom | 25/33 | 10.9 | 4.1-30.8 | |

* this information was not available

** including 17 imports

Table III Overview of literature data on changes of DON during barley processing, malting and brewing; in parenthesis DON (mg/kg) in raw material - barley)

| <i>Process</i> | <i>Sample</i> | <i>% Remaining DON in processing intermediates and products</i> | | | | | | |
|------------------|----------------------|---|------------|-----------|-----------|----------------------------|------------|-------------|
| Malting | Barley | 100 (10.6) | 100 (22.5) | 100 (8.5) | 100 (4.8) | 100 (<0.2) | * | * |
| | Steep-out | 10 | 11 | 0 | 0 | 0 | * | * |
| | Green Malt | 36 | 55 | 18 | 115 | 0 | * | * |
| | Kilned Malt | 33 | 55 | 16 | 100 | 0 | * | * |
| | Malt Rootlets | 48 | 225 | 46 | 300 | 0 | * | * |
| Brewing | Malt Grist | 100 (3.5) | 100 (17.3) | 100 (1.8) | 100 (6.2) | * | 100 (0.44) | 100 (0.034) |
| | Mash, 50°C | * | * | * | * | * | 266 | 500 |
| | Mash, 62°C | * | * | * | * | * | 320 | 529 |
| | Mash, 72°C | * | * | * | * | * | 405 | 765 |
| | First Wort | * | * | * | * | * | 234 | 588 |
| | Sparging | * | * | * | * | * | 157 | 441 |
| | Kettle full Wort | * | * | * | * | * | 384 | 735 |
| | Last Sparging | * | * | * | * | * | 23 | 0 |
| | Spent Grains | 3.4 | 1.8 | 0.0 | 0.0 | * | 0 | 0 |
| | Hot Wort | * | * | * | * | * | 364 | 794 |
| | Fermentation, day 2 | * | * | * | * | * | 355 | * |
| | Fermentation, day 6 | * | * | * | * | * | 2625 | * |
| | Fermentation, day 14 | * | * | * | * | * | 309 | 588 |
| | Beer | 93.4 | 85.0 | 90.0 | 80.0 | * | 323 | 588 |
| <i>Reference</i> | | Schwarz et al. 1995 | | | | Niessen and Donhauser 1993 | | |

*this information was not available

Table IV *Fusarium* toxins* (µg/kg dry weight) in barley grains and malt grist used for processing experiments

| <i>Commodity</i> | <i>Sample Code</i> | <i>Fusarium Infection</i> | <i>Sample Series Code</i> | <i>Mycotoxin</i> | | | | |
|------------------|--------------------|---------------------------|---------------------------|------------------|------------------|--------------|-------------|------------|
| | | | | <i>DON</i> | <i>DON-3-Glc</i> | <i>ADONs</i> | <i>HT-2</i> | <i>ZON</i> |
| Barley Grains | BG | natural | NAT | 12 | < 5 | < 10 | < 10 | < 5 |
| | | artificial | ART | 238 | 14 | 140 | 11 | < 5 |
| Malt Grist | MG | natural | ART | 316 | 67 | 133 | < 10 | < 5 |
| | | artificial | NAT | 1712 | 349 | 933 | < 10 | 77 |

* NIV, FUS-X and T-2 were not detected in any analysed samples

Table V Characterization of pilot malting experiments

| <i>Malting Sstage</i> | <i>Sample Name</i> | <i>Sample Code</i> | <i>Hours elapsed between start of processing stage and sampling</i> | <i>Experimental Conditions</i> |
|-----------------------|--------------------|--------------------|---|--|
| Raw material | Barley Grains | BG | - | - |
| 1. Steeping | - | SI | 24 | Steeping lasted 3 days to achieve a required degree of steeping 45.5%; temperature of the steeping water and air was 14.5°C; the samples "SI, SII, SIII" were taken on three following days. |
| | - | SII | 48 | |
| | - | SIII | 72 | |
| 2. Germination | - | GI | 24 | Germination stage lasted 3 days at the temperature 14.5°C; the samples "GI, GII" and the product of germination "Green Malt" were taken on three consecutive days of this stage. |
| | - | GII | 48 | |
| | Green malt | GM | 72 | |
| 3. Kilning | - | KI | 6 | Kilning lasted 22 hours at kilning temperature 80°C for the period of 4 hours; the samples "KI, KII" were taken after 6 and 12 hours, respectively. |
| | - | KII | 12 | |
| 4. Malt cleaning | Rootlets | R | - | The rootlet fraction attached to the kilned malt was removed. |
| | Malt | M | - | |

Table VI Characterization of pilot brewing experiments

| <i>Brewing Stage</i> | <i>SampleName</i> | <i>Sample Code</i> | <i>Experimental Conditions</i> |
|--------------------------------|--|--------------------|--|
| 1. Mashing | Malt Grist (sample before mashing) | MG | Malt Grist (6 kg) was mixed with water (38°C); the total volume was 45 l; double mash process (3 hours) was used; first infusion mash at 55°C, second infusion mash at 75°C. |
| 2. Lautering | Sweet Wort | SW | Sweet Wort (59 l) obtained by mixing of the first and the second wort (extract after sparging) was separated from Spent Grains (1.2 kg). |
| | Spent Grains | SG | |
| 3. Wort boiling | Hot Wort | HW | Hops (170 g) were added to the wort during boiling (90 min); the volume of obtained Hot Wort was 50 l. |
| 4. Wort cooling and clarifying | Wort after Trub Removal | WTR | Wort after Trub Removal was obtained after trub removal and cooling at 8°C for 1 day; the volume was approx. 50 l. |
| 5. Fermentation | Green Beer | GB | Green Beer obtained after 10 days of fermentation at 12°C for; the volume was approx. 50 l. |
| 6. Maturation | Beer | B | Beer after following 21 days of fermentation at 1°C ; the volume was approx. 50 l. |

Table VII LC-MS/MS method validation data (n=5; sample codes are shown in Tables V and VI)

| <i>Parameter</i> | <i>Matrix</i> | <i>Unit</i> | <i>Mycotoxin</i> |
|------------------|---------------|-------------|------------------|
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| | | | <i>DON</i> | <i>DON-3-Glc</i> | <i>NIV</i> | <i>ADONs</i> | <i>HT-2</i> | <i>T-2</i> | <i>ZON</i> | <i>FUS-X</i> |
|---------------------|----------------|-------|------------|------------------|------------|--------------|-------------|------------|------------|--------------|
| <u>LOD</u> | solid samples | µg/kg | 0.5 | 0.5 | 1 | 1 | 1 | 0.5 | 1 | 1 |
| | liquid samples | µg/l | 1 | 1 | 5 | 5 | 5 | 1 | 1 | 1 |
| <u>LOQ</u> | solid samples | µg/kg | 5 | 5 | 10 | 10 | 10 | 5 | 5 | 10 |
| | liquid samples | µg/l | 5 | 5 | 10 | 10 | 10 | 5 | 5 | 10 |
| | BG | % | 88 | 45 | 64 | 96 | 91 | 86 | 75 | 61 |
| | SI | % | 80 | 38 | 61 | 101 | 87 | 101 | 71 | 63 |
| | SII | % | 87 | 40 | 66 | 97 | 90 | 82 | 69 | 70 |
| | SII | % | 86 | 34 | 65 | 92 | 96 | 83 | 75 | 62 |
| | GI | % | 79 | 48 | 62 | 101 | 98 | 79 | 71 | 59 |
| | GII | % | 79 | 38 | 69 | 106 | 88 | 89 | 67 | 62 |
| | GM | % | 89 | 45 | 61 | 87 | 90 | 95 | 72 | 61 |
| | KI | % | 82 | 46 | 65 | 92 | 72 | 99 | 67 | 69 |
| <u>Recovery</u> *** | KII | % | 78 | 48 | 64 | 103 | 92 | 106 | 69 | 68 |
| | M | % | 79 | 50 | 62 | 94 | 93 | 89 | 72 | 73 |
| | R | % | 80 | 38 | 68 | 90 | 87 | 82 | 81 | 64 |
| | MG | % | 84 | 39 | 71 | 92 | 97 | 105 | 79 | 71 |
| | SG | % | 81 | 44 | 62 | 87 | 98 | 84 | 84 | 68 |
| | SW | % | 105 | 115 | 102 | 118 | 105 | 118 | 109 | 103 |
| | HW | % | 108 | 121 | 118 | 117 | 111 | 124 | 114 | 104 |
| | WTR | % | 112 | 120 | 103 | 108 | 103 | 111 | 108 | 110 |
| | GB | % | 109 | 109 | 103 | 101 | 117 | 108 | 115 | 103 |
| | B | % | 117 | 102 | 102 | 116 | 110 | 129 | 104 | 101 |

* solid and liquid samples were spiked with analytes standards on levels 100 µg/kg and 50 µg/l, respectively

** uncertainty of analysis of each matrix expressed as RSD was in all cases below 10%

Table VIII *Fusarium* toxins* (µg/kg dry weight) in samples collected during malting process (Sample codes are shown in Table V)

| Sample Code | Sample Series NAT | | | | Sample Series ART | | | |
|-------------|-------------------|------------------|--------------|-------------|-------------------|------------------|--------------|-------------|
| | <i>DON</i> | <i>DON-3-Glc</i> | <i>ADONs</i> | <i>HT-2</i> | <i>DON</i> | <i>DON-3-Glc</i> | <i>ADONs</i> | <i>HT-2</i> |
| BG | 12 | <5 | <10 | <10 | 238 | 14 | 140 | 11 |
| SI | <5 | <5 | <10 | <10 | 27 | <5 | 11 | <10 |
| SII | <5 | <5 | <10 | <10 | 21 | 8 | 12 | <10 |
| SIII | <5 | <5 | <10 | <10 | 24 | 13 | 18 | <10 |
| GI | 11 | 16 | <10 | <10 | 105 | 56 | 146 | 12 |
| GII | 21 | 52 | 19 | 16 | 204 | 106 | 148 | 24 |
| GM | 49 | 68 | 44 | 24 | 601 | 90 | 699 | 20 |
| KI | 46 | 17 | 22 | 37 | 575 | 82 | 314 | 47 |

| | | | | | | | | |
|-----|-----|-----|-----|------|------|------|-----|-----|
| KII | 44 | 10 | 20 | 32 | 540 | 109 | 228 | 33 |
| M | 45 | 10 | 17 | 17 | 510 | 125 | 160 | 22 |
| R | 553 | 460 | 571 | 1061 | 1067 | 1911 | 641 | 493 |

* NIV, FUS-X, T-2 and ZON were not detected in any analysed samples

Table IX *Fusarium* toxins* in samples collected during brewing process (Sample codes are shown in Table VI)

| Sample Code | Unit | Sample Series NAT | | | | | Sample Series ART | | | | |
|-------------|-------|-------------------|-----------|-------|------|-----|-------------------|-----------|-------|------|-----|
| | | DON | DON-3-Glc | ADONs | HT-2 | ZON | DON | DON-3-Glc | ADONs | HT-2 | ZON |
| MG | µg/kg | 316 | 67 | 133 | < 10 | < 5 | 1 712 | 349 | 933 | < 10 | 77 |
| SG | µg/kg | 6 | < 5 | < 10 | < 10 | < 5 | 103 | 838 | 50 | < 10 | 59 |
| SW | µg/l | 23 | 56 | 12 | < 10 | < 5 | 116 | 499 | 65 | < 10 | < 5 |
| HW | µg/l | 17 | 35 | < 10 | < 10 | < 5 | 219 | 561 | 131 | < 10 | < 5 |
| WTR | µg/l | 21 | 39 | < 10 | < 10 | < 5 | 237 | 401 | 104 | 11 | < 5 |
| GB | µg/l | 27 | 52 | 14 | 12 | < 5 | 180 | 267 | 115 | 10 | < 5 |
| B | µg/l | 26 | 51 | 18 | < 10 | < 5 | 178 | 265 | 117 | < 10 | 7 |

* NIV, FUS-X and T-2 were not detected in any analysed samples

Figures 2 and 3 Transfer of DON, DON-3-Glc and ADONs from malt grist to beer (total amount of mycotoxins in malt grist taken for respective beer batch production = 100%; contents of these mycotoxins in analysed samples are shown in Table IX; sample codes are specified in Table VI)

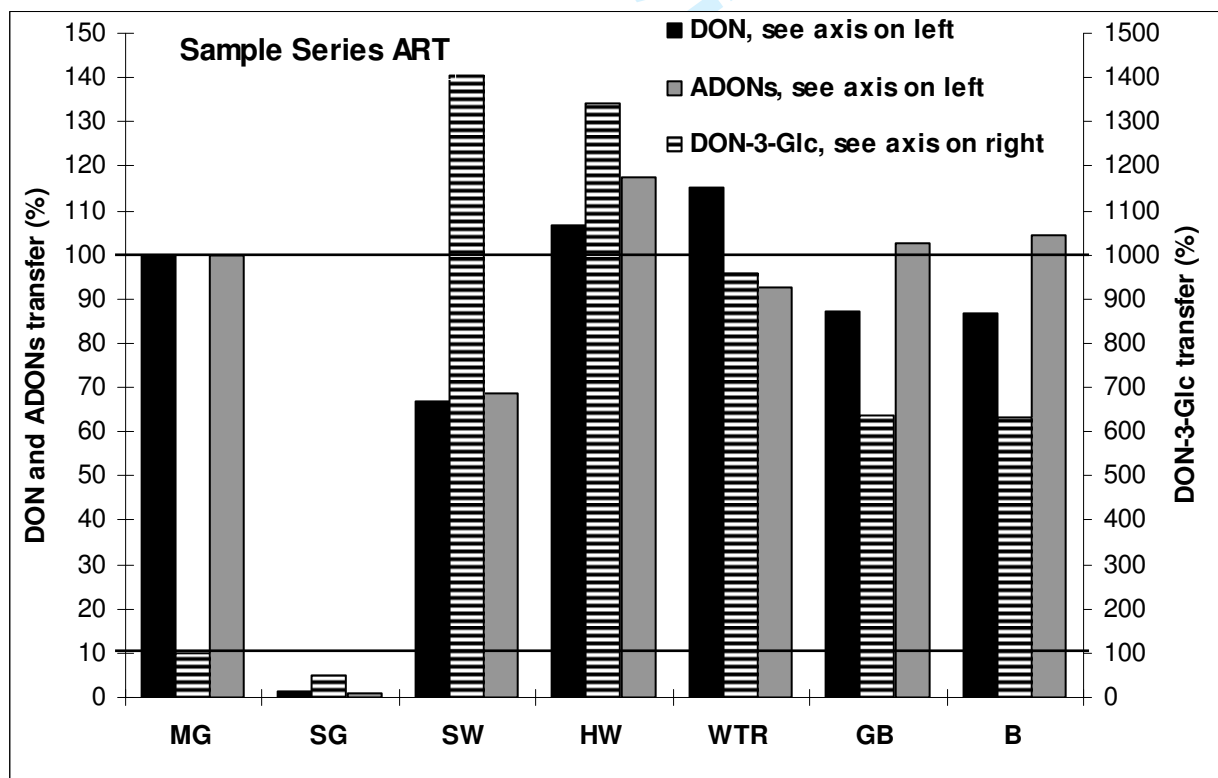
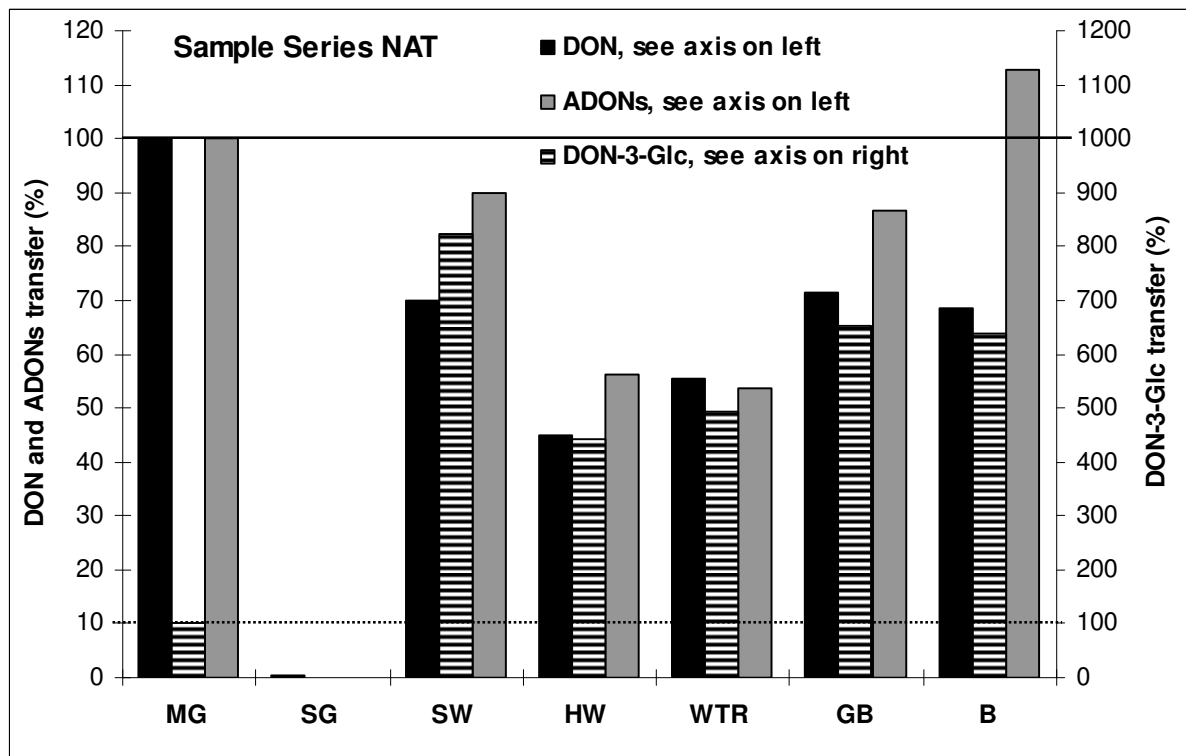


Figure 1 Transfer of DON, DON-3-Glc, ADONs and HT-2 from barley to malt, sample series ART (total amount of mycotoxins in process batch of barley grains = 100%; contents of these mycotoxins in analysed samples are shown in Table VIII; sample codes are specified in Table V)

