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Deoxynivalenol Oligoglycosides: New "Masked" *Fusarium* Toxins Occurring in Malt, Beer, and Breadstuff

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Supporting Information

ABSTRACT: The co-occurrence of deoxynivalenol-3-glucoside with its parent toxin, deoxynivalenol, has been recently documented in many cereal-based foods, especially in those produced by enzyme-catalyzed processes. The presence of this masked mycotoxin in the human diet has become an issue of health concern, mainly because of its assumed bioavailability. A selective immunoaffinity-based preconcentration strategy, followed by ultrahigh-performance liquid chromatography coupled with high-resolution orbitrap mass spectrometry, revealed that, in addition to the most common deoxynivalenol-3-glucoside, also oligoglycosylated deoxynivalenols with up to four bound hexose units were present in cereal-based products. The structure, origination, and fate of these deoxynivalenol conjugates during malt/beer production and bread baking have been thoroughly investigated. Special attention has been paid to the changes of deoxynivalenol conjugates enabled by industrial glycosidase-based enzymatic preparations. To the authors' best knowledge, this is the first study documenting the complexity of masked deoxynivalenol issue.

KEYWORDS: *deoxynivalenol, deoxynivalenol-3-glucoside, deoxynivalenol oligoglucosides, masked mycotoxins, malt, beer, breadstuffs*

INTRODUCTION

Deoxynivalenol (Figure 1, **1**), the major representative of *Fusarium* mycotoxins, is a common natural contaminant of small-grain cereals. The co-occurrence of conjugated "masked" deoxynivalenol forms has been documented in crops infected by toxinogenic fungi.[1−4]

Miller and Young,[5] who monitored deoxynivalenol levels in wheat artificially infected in the field with *Fusarium graminaerum*, were probably the first to speculate on deoxynivalenol "degradation in vivo by plant cells". They assumed that the rapid decline in deoxynivalenol concentration, observed after its peak in naturally infected winter wheat, was due to the "mycotoxin breakdown by plant enzymes". A similar suggestion was formulated by Scott et al.,[6] who observed a decline in the concentration of deoxynivalenol in *Fusarium*-infected wheat in the field and suggested that the toxin may somehow be metabolized. This assumption was confirmed in the follow-up studies employing wheat cell suspension cultures and ^{14}C-radiolabeled deoxynivalenol, in which the presence of polar deoxynivalenol glycosides was proven.[7] However, the most significant knowledge was provided by Sewald et al.,[8] whereby the transformation product, 3-β-D-glucopyranosyl-4-deoxynivalenol (deoxynivalenol-3-glucoside; Figure 1, **2**), was completely identified. Two-dimensional NMR spectroscopy and electrospray mass spectrometry were employed for this purpose.

Dall'Asta et al.[9] chemically synthesized various deoxynivalenol glucosides including deoxynivalenol-3-glucoside, the formation of which was later confirmed in wheat ears artificially treated with deoxynivalenol at anthesis. At the same time, the occurrence of this deoxynivalenol conjugate was documented by Berthiller et al.,[1,2] not only in deoxynivalenol-treated plants but also in naturally contaminated cereals. Its content found in naturally infected wheat and/or maize was up to 7.7 mol % of parent deoxynivalenol;[1] in the more extensive follow-up study, levels as high as 46 mol % of total deoxynivalenol were determined in naturally contaminated wheat and maize samples.[2]

Interestingly, a correlation between the field susceptibility to *Fusarium* head blight (FHB) disease and deoxynivalenol-3-glucoside/deoxynivalenol ratio in agricultural crops was found.[10] The nature of processes influencing the accumulation of deoxynivalenol and other *Fusarium* trichothecenes in grains has been comprehensively reviewed.[11]

Several other studies conducted after 2007, when a deoxynivalenol-3-glucoside analytical standard became commercially available, confirmed the ubiquitous occurrence of deoxynivalenol-3-glucoside in cereals.[2−4] Until now, great attention has been paid also to the fate of deoxynivalenol-3-glucoside during household/industrial cereal-processing practices. In this context, one of the most surprising observations was made by Lancova et al.[12] Those authors reported the dramatic increase of deoxynivalenol-3-glucoside during malting and brewing experiments, up to approximately 880 and 630% for malt and beer, respectively, as compared to the deoxynivalenol-3-glucoside content in input barley and malt. Within a discussion of possible reasons for this phenomenon, rapid onset of enzymatic activities within the germination of barley kernels, causing release of mycotoxins from their bonding with polysaccharides, and de novo formation of fungal secondary metabolites, were proposed as the most probable explanations.

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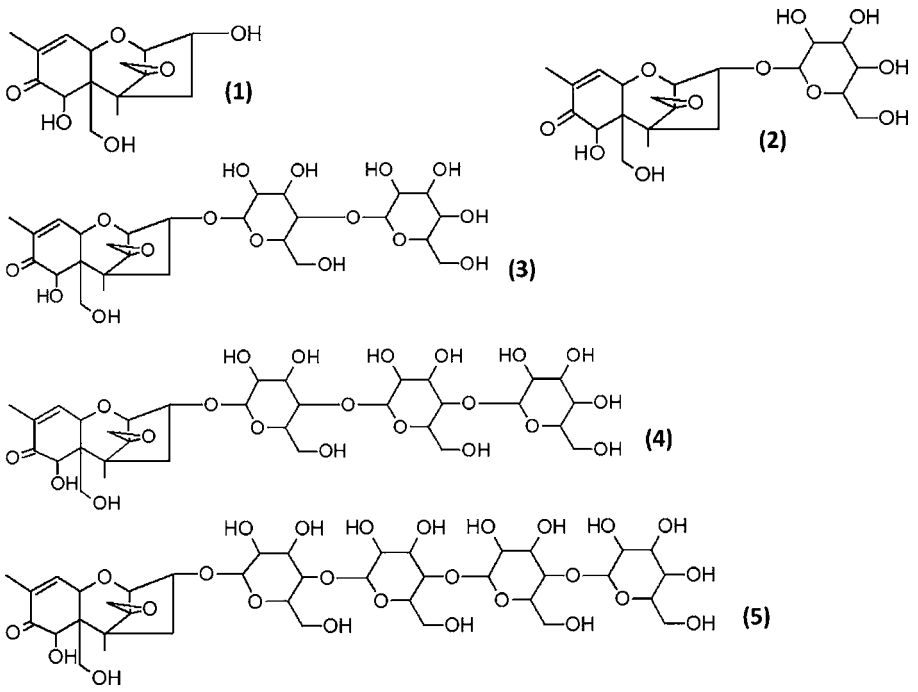

Figure 1. Structures of deoxynivalenol (**1**), deoxynivalenol-3-glucoside (**2**), deoxynivalenol-3-diglucoside (**3**), deoxynivalenol-3-triglucoside (**4**), and deoxynivalenol-3-tetraglucoside (**5**).

Very recently, Maul at al.[13] have carried out a study concerned with germination of deoxynivalenol-treated barley and documented an occurrence of an extensive "masking" of this mycotoxin resulting in the increase of deoxynivalenol-3-glucoside. They hypothesized that the formation of deoxynivalenol-3-glucoside is connected with an increased activity of glucose-related enzymes such as the glycosyltransferases, which occurs during the germination process of barley. The dynamics of deoxynivalenol and deoxynivalenol-3-glucoside during the brewing technology was further studied by Kostelanska et al.[14] An intensive increase of both compounds during the malt-mashing phase, a process whereby an intensive enzymatic hydrolysis occurs, was confirmed under real brewery house conditions. The ubiquitous occurrence of deoxynivalenol-3-glucoside, with concentrations in many cases exceeding those of free deoxynivalenol, was earlier clearly documented in various beers purchased at the world market.[15] Breadmaking represents another cereal technology that may, under certain conditions, lead to a relative increase of deoxynivalenol-3-glucoside levels in some processing intermediates. As demonstrated in a study by Kostelanska et al.,[16] this can be mainly due to the use of so-called "bakery improvers", mixtures of hydrolytic enzymes containing preparations commonly used in bakeries to improve dough rheology.

On the basis of the above-mentioned facts, it can be assumed that consumption of both beer and breadstuff may significantly contribute to the dietary intake of deoxynivalenol, which may approach or even exceed a tolerable daily intake (TDI) of 1 μg/kg.[17] The seriousness of the situation might be further escalated by the existence of deoxynivalenol conjugates with two and three glucose units, deoxynivalenol di- and triglucosides, which were for the first time identified in beer produced from artificially *Fusarium*-contaminated malt by means of time-of-flight mass spectrometry (TOF-MS) technology.[18] Because the glycosidic bond between deoxynivalenol and its glucoside in the deoxynivalenol-3-glucoside molecule can be cleaved by some bacterial strains present in the gastrointestinal tract,[19] thereby making deoxynivalenol glycosides bioavailable, an intensive effort has focused on getting as much information about these substances as possible. The biological availability of deoxynivalenol glycosides is indirectly supported also by Warth et al.,[20] who present the results of deoxynivalenol glucuronides, the main deoxynivalenol human metabolites, in the urine of volunteers. Unexpectedly high levels of these substances were determined, especially in people who consumed high amounts of beer in their diet.

The aim of this study was to provide a summary of until now unpublished masked deoxynivalenol research results concerning the structure elucidation of deoxynivalenol oligoglucosides found in various cereal-based foods, such as malt, beer, and breadstuffs. We also monitored their fate within the food-processing technologies, to explain their origination pathway during malt and beer production. Modern immunochemical-based analytical strategies were employed to purify and preconcentrate target deoxynivalenol oligoglucosides. Hydrophilic interaction liquid chromatography (HILIC) and reversed phase liquid chromatography in ultrahigh-performance mode hyphenated with an orbitrap mass spectrometer (orbitrapMS) were used for instrumental analysis of the target compounds. Additionally, characterization of the nature of glycosidic bonds in the above-mentioned deoxynivalenol conjugates was enabled by specific enzymatic hydrolytic reactions.

MATERIALS AND METHODS

Standards and Chemicals. Standards of deoxynivalenol and deoxynivalenol-3-glucoside were obtained from Romer Labs (Tulln, Austria). The DONtest HPLC immunoaffinity columns (IACs) were obtained from Vicam (Milford, MA, USA); cross-reactivities declared by the producer were as follows: 40−50% for 15-acetyldeoxynivalenol and 0% for 3-acetyldeoxynivalenol, nivalenol, T-2 toxin, and fusarenone-X.[21] The DONPREP IACs were purchased from R-Biopharm AG (Darmstadt, Germany); cross-reactivity of DONPREP IACs was declared only for 3-acetyldeoxynivalenol; nevertheless, no

specific value was provided. The immunoaffinity columns were handled according to the particular manufacturer's instructions.

The organic solvents, acetonitrile, methanol, and ethanol (all of HPLC grade), were obtained from Sigma-Aldrich (Taufkirchen, Germany). Ultrapure water was produced by a Milli-Q system (Millipore Corp., Bedford, MA, USA).

Brewing enzyme preparations (Termamyl BerwQ containing heat-stable α-amylase, AMG 300 L BrewQ containing amyloglucosidase, and NS 50010 containing β-glucosidase) were obtained from Novozymes A/S (Bagsvaerd, Denmark). The origin, specificity, temperature and pH optima, and activity of particular enzymes are as follows: (i) heat-stable α-amylase, isolated from *Bacillus licheniformis*, specific to α-1,4 glycosidic bounds, temperature optimum between 85 and 95 °C, pH optimum 5−8, activity = 240 KNU-T/g (1 KNU-T is the amount of α-amylase that under the standard conditions dextrinizes 5.26 g of starch per hour); (ii) amyloglucosidase isolated from *Aspergillus niger*, specific to α-1,4 and α-1,6 glycosidic bounds, temperature optimum between 70 and 75 °C, pH optimum 5−8; activity = 300 AGU/mL (1 AGU is the amount of enzyme that under the standard conditions hydrolyzes 1 μmol of maltose per minute); (iii) β-glucosidase isolated from *A. niger*, specific to β-1,4 glycosidic bounds, temperature optimum between 58 and 63 °C, pH optimum 5−8, activity = 225 CBU/g (1 CBU is the amount of enzyme that under the standard conditions hydrolyzes 1 μmol of cellobiose per minute). The products comply with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

Fungicide preparations Fandango (active ingredients prothioconazole and fluoxastrobin), Prosaro (active ingredients prothioconazole and tebuconazole), and Caramba (active ingredient metconazole) were obtained from Bayer CropScience (Monheim am Rhein, Germany).

Samples. *Experimental Barley, Malt, and Beer; Description of Malting and Brewing Technology.* Malting barley of Bojos variety was artificially infected by *Fusarium* ssp. solution (5×10^6 spores/mL) in the field when flowering. Approximately 32 kg of grains was harvested. Grains were split into three batches, and mycotoxins were analyzed to obtain mean levels of 2467 ± 305 μg/kg of deoxynivalenol and 939 ± 296 μg/kg of deoxynivalenol-3-glucoside. This barley was further used for malt production in an experimental small-scale malting house; approximately 10 kg of homogeneous barley grains was micromalted by the following technology in three repetitions: 1 day of steeping at 14 °C with one air break to gain a water content of 46.5%, 6 days of germination at 18 °C and kilning at 55 and 80 °C for 22 and 4 h, respectively. Mycotoxin levels in the final malt were 11638 ± 895 μg/kg for deoxynivalenol and 20912 ± 784 μg/kg for deoxynivalenol-3-glucoside. All three malt batches were further processed with a standard brewing double-mash technology to obtain three batches of final beer containing 2760 ± 95 μg/L of deoxynivalenol and 3883 ± 137 μg/L of deoxynivalenol-3-glucoside.

Experimental Wheat and Bread; Description of Baking Technology. For laboratory-scale baking experiments, white wheat flour derived from artificially *Fusarium*-infected winter wheat of Akteur cultivar was used. Inoculation was performed in the field when flowering with *Fusarium* ssp. concentrate (5×10^6 spores/mL). Approximately 7 kg of wheat kernels was harvested. Deoxynivalenol and deoxynivalenol-3-glucoside levels in this flour were 729 ± 119 and 160 ± 59 μg/kg, respectively. Wheat was homogenized and used for bread baking in three repetitions. The following technology was used: 300 g of wheat flour, 12 g of leavener, 3 g of fat, 5.1 g of salt, 4.5 g of saccharose, 160 mL of water, and 12 g of bakery improver were used. After kneading of all ingredients, dough was left to ferment for 45 min at 30 °C. Then the dough was kneaded again and finally proofed for 50 min at 30 °C. After the fermentation phase, the bread loaves were baked in a laboratory oven for 14 min at 240 °C. The mean deoxynivalenol and deoxynivalenol-3-glucoside levels analyzed in the final breads were 624 ± 96 and 81 ± 19 μg/kg, respectively.

Experimental Barley, Fungicide Treatment, and Green Malt Production. For the explanation of masked mycotoxin origin, germination of two barley batches was performed. The first batch was treated by *Fusarium* fungicide preparations from Bayer CropScience with active ingredients widely used to prevent *Fusarium* fungus growth, and the second set was a control variant. Barley grains were washed with the 1000 times diluted fungicide cocktail immediately before malting to destroy the surface fungal infection. Washing was done for approximately 10 min; a longer time period could lead to inhibition of the barley germinating activity. The effectiveness of the fungicide treatment was controlled microbiologically by cultivation of treated grains on the potato dextrose agar.

Germination of 500 g of barley grains was carried out for 6 days at 18 °C in three repetitions. Barley of Bojos and Sebastian varieties naturally, as well as artificially, infected in the field when flowering with 5×10^6 spores/mL was used for this purpose. Concentrations of mycotoxins were as follows: artificially infected Bojos, 2263 μg/kg deoxynivalenol and 731 μg/kg deoxynivalenol-3-glucoside; naturally infected Bojos, 56 μg/kg deoxynivalenol, and 8 μg/kg deoxynivalenol-3-glucoside; artificially infected Sebastian, 997 μg/kg deoxynivalenol and 239 μg/kg deoxynivalenol-3-glucoside; naturally infected Sebastian, 18 μg/kg deoxynivalenol and 12 μg/kg deoxynivalenol-3-glucoside.

Real-Life Samples. The presence of masked mycotoxins was also investigated in naturally contaminated malt and beer samples and various commercial bakery products. Malt samples (n = 6) were obtained from Czech beer producers; beer samples (n = 15) and various baguette samples (n = 15) were purchased from Czech retail markets.

Structure Elucidation of Deoxynivalenol Oligoglucosides. For elucidation of the deoxynivalenol oligoglucosides structure, that is, identification of the glycosidic bond position within the deoxynivalenol molecule and characterization of glycosidic bonds types, the experimental Bojos malt characterized above was employed.

Two complementary approaches were used for elucidation of the structure of masked deoxynivalenol conjugates. The first one was based on MS monitoring of specific fragment ions formed upon in-source fragmentation of respective pseudomolecular ions; the second one enabled specific enzymatic hydrolysis reactions. Both of these strategies share the need for sufficient deoxynivalenol glycoside preconcentration.

The optimal way to accomplish this task was the use of cross-reacting IACs. In this particular case, with regard to the strong antibody affinity of deoxynivalenol, it was desirable that the free deoxynivalenol was removed from the sample extract and the deoxynivalenol glycosides were selectively preconcentrated. For this purpose, the following procedure was performed: 1.8 mL of malt extract, obtained by shaking of 30 g of malt with 60 mL of distilled water, was applied on the "non-cross-reacting" deoxynivalenol-specific IAC (DONtest HPLC). Deoxynivalenol present in the sample extract was selectively trapped by the IAC antibodies, whereas deoxynivalenol glycosides together with matrix coextracts were washed by distilled water and caught in the glass cuvette placed under the DONtest column. Taking into account the total deoxynivalenol amount in the extract (10474 ng) and the DONtest column capacity of 3300 ng, after completion of the procedure, a residual deoxynivalenol amount of 7174 ng remained in the extract. For this reason, the whole procedure had to be repeated at least twice again. The passed-through fraction was uploaded on the second DONtest column to obtain an extract with 3874 ng of deoxynivalenol and further to the third DONtest IAC to obtain a final extract containing only 574 ng of residual deoxynivalenol. Then, for follow-up purification and simultaneous preconcentration of desired deoxynivalenol glycosides, the fraction passed through the DONtest cascade was applied on the "cross-reactive" immunoaffinity columns (DONPREP). Deoxynivalenol glycosides caught with the column antibodies were eluted by methanol. This was further evaporated, and deoxynivalenol glycosides were redissolved in the appropriate solvent, depending on the follow-up purpose described below.

UHPLC−OrbitrapMS Analysis; In-Source Fragmentation. Deoxynivalenol glycosides were dissolved in 0.8 mL of acetonitrile/water (90:10, v/v). Chromatographic separation of analytes was performed

Table 1. Recoveries of Deoxynivalenol and Deoxynivalenol-3-glucoside from the IACs Obtained by Model Aqueous Standard Mixtures (n = 5)

	DONPREP[a]		DONtest HPLC[b]	
amount of standards in the mixture	deoxynivalenol (%)	deoxynivalenol-3-glucoside (%)	deoxynivalenol (%)	deoxynivalenol-3-glucoside (%)
250 ng of deoxynivalenol + 250 ng of deoxynivalenol-3-glucoside	101 ± 6.1	99 ± 4.3	94 ± 5.8	0
500 ng of deoxynivalenol + 500 ng of deoxynivalenol-3-glucoside	100 ± 5.2	102 ± 5.1	98 ± 4.7	0
1000 ng of deoxynivalenol + 1000 ng of deoxynivalenol-3-glucoside	98 ± 4.3	65 ± 4.8	104 ± 6.8	0
1500 ng of deoxynivalenol + 1500 ng of deoxynivalenol-3-glucoside	92 ± 5.9	30 ± 9.6	96 ± 6.2	0
2000 ng of deoxynivalenol + 2000 ng of deoxynivalenol-3-glucoside	75 ± 5.1	9 ± 4.7	102 ± 4.8	0
2500 ng if deoxynivalenol + 2500 ng of deoxynivalenol-3-glucoside	58 ± 4.9	4 ± 1.9	99 ± 3.6	0

[a]Declared deoxynivalenol binding column capacity is 1800 ng. [b]Declared deoxynivalenol binding column capacity is 3300 ng.

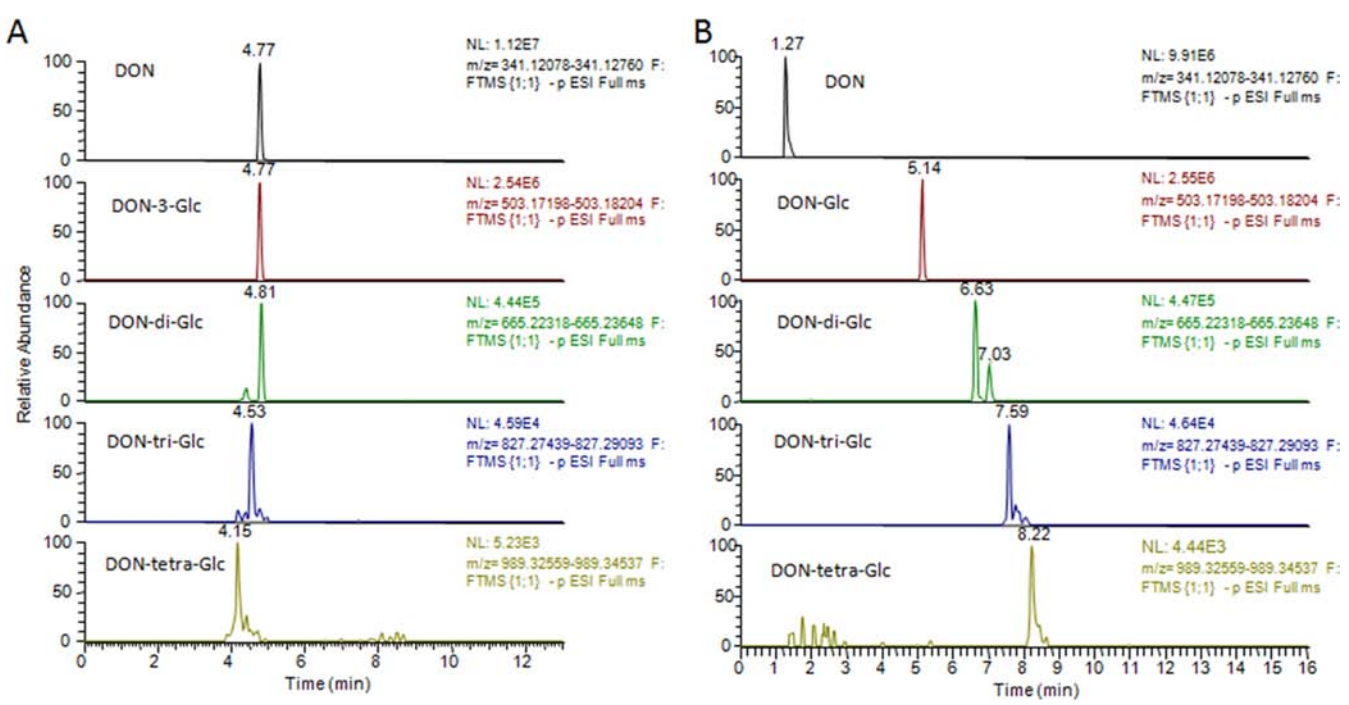

Figure 2. Separation of deoxynivalenol and its glycosides present in the beer sample (produced from Bojos barley, artificial *Fusarium* infection) under two different chromatographic conditions: (A) reverse-phase chromatography; (B) HILIC phase chromatography; ESI negative ionization.

by an Accela UHPLC liquid chromatograph (Thermo Fisher Scientific, San Jose, CA, USA). Separation of analytes was performed by a 100 mm × 2.1 mm i.d., 1.7 μm, Acquity UPLC BEH amide column (Waters, Milford, MA, USA). Column temperature was held at 40 °C. The mobile phases consisted of 2 mM ammonium formate in acetonitrile/water (99:1, v/v) (A) and deionized water (B). The gradient was as follows: start with 5% B, then linearly increase to 30% B in 6 min; for the next 4 min, perform another linear increase to 50% B, hold for 15 min; switch to 5% B at 15.1 min, and equilibrate the column for 3 min before the next injection. The flow rate was 300 μL/min, and an injection volume of 20 μL was enabled.

For mass spectrometric detection, ultrahigh-resolution orbitrap technology, Exactive MS system (Thermo Fisher Scientific, Bremen, Germany), was employed. The operation parameters of the orbitrap MS were optimized for heated electrospray interface in both positive and negative ionization modes. Sheath gas/auxiliary gas flow was 35/10 arbitrary units, capillary temperature was held at 250 °C, heater temperature was 250 °C, capillary voltage of +60/−50 V, and spray voltage of +4/−3.1 kV. The system was operated in the full spectrum acquisition in the mass range of m/z 120−2000 at a resolving power setting of 100000 fwhm (m/z 200) and fixed acquisition rate of 1 spectrum/s. Fragmentation experiments were performed in a separate analysis run with the use of 25 V in-source collision-induced dissociation (CID). For the mass accuracy estimation, the mass at the apex of the chromatographic peak obtained as the extracted ion chromatogram was used.

Enzymatic Treatment; Investigation of Glycosidic Bonds. Deoxynivalenol glycosides were dissolved in 0.8 mL of degassed mineral water. The pH of 6.5 was in line with optimal enzymatic reaction conditions, and the increased concentration of Ca^{2+} ions (70 ppm) was recommended for supporting the heat stability of α-amylase. To the vial with 0.8 mL of deoxynivalenol glycoside concentrate was added 8 μL of a particular enzyme preparation. The vials were placed into the laboratory ovens and held at their optimal temperature, that is, 85 °C for heat stable α-amylase, 69 °C for amyloglucosidase, and 65 °C for β-glucosidase. After 1, 6, and 22 h of the enzymatic treatment, 100 μL of the incubation was mixed with 900 μL of water, ultracentrifuged by using a 0.2 μm filter, and stored at −20 °C until UHPLC-orbitrapMS analysis. All of the experiments were carried out in triplicate.

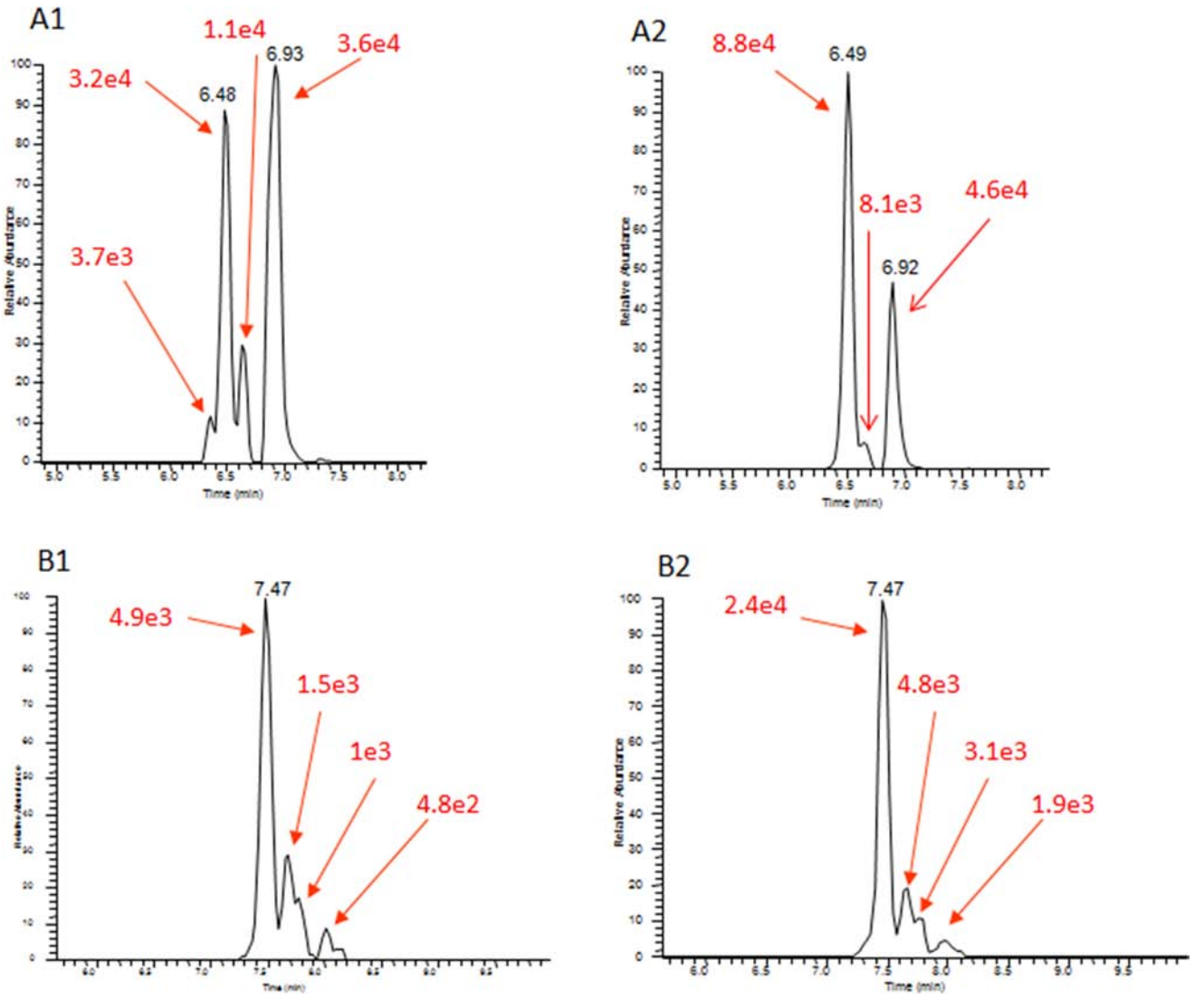

Figure 3. Isomeric pattern of deoxynivalenol diglucosides determined in malt (A1) and beer (A2) and deoxynivalenol triglucosides determined in malt (B1) and beer (B2) under the HILIC phase chromatography conditions (malt and beer produced from the same Bojos barley, artificial *Fusarium* infection). Isomers with their relative intensities are indicated by red arrows.

Determination of Deoxynivalenol and Its Glycosides in Samples. For quantitation of deoxynivalenol and deoxynivalenol-3-glucoside in samples, the sample preparation method published by Kostelanska et al.[14] employing the cross-reactive DONPREP IACs was used. If necessary, dilution of sample extracts was performed with respect not to exceed the DONPREP column capacity (1800 ng of deoxynivalenol). Due to the lack of analytical standards for respective deoxynivalenol oligoglucosides, their content is presented in terms of the deoxynivalenol conjugate to deoxynivalenol peak area ratio.

For the chromatographic separation of analytes, an Accela UHPLC liquid chromatograph (Thermo Fisher Scientific, San Jose, CA, USA) in reverse phase chromatography setting was used. A 100 mm × 2.1 mm i.d., 1.8 μm, Acquity UPLC HSS T3 (Waters) analytical column held at 40 °C was employed. The mobile phases consisted of 5 mM ammonium formate in water (A) and methanol (B). The gradient elution was performed as follows: start with 5% B, then linearly increase to 50% B in 6 min; perform another linear increase to 95% B in the next 4 min, hold for 15 min, switch to 5% B in 15.1 min, and equilibrate the column for 3 min before the next injection start. The flow rate was kept at 300 μL/min, and the injection volume of aqueous sample was 20 μL.

For the mass spectrometric detection, ultrahigh-resolution orbitrap technology, Exactive MS system (Thermo Fisher Scientific, Bremen, Germany), was employed. The operation parameters of the orbitrap mass spectrometer were optimized for heated electrospray interface in both positive and negative ionization modes. Sheath gas/auxiliary gas flow was 35/10 arbitrary units, capillary temperature was held at 250 °C, heater temperature was 250 °C, capillary voltage was +60/−50 V, and spray voltage was +4/−3.1 kV. The system was operated in full spectrum acquisition in the mass range of m/z 120−2000 at a resolving power setting of 100000 fwhm (m/z 200) and fixed acquisition rate of 1 spectrum/s.

RESULTS AND DISCUSSION

Structure Elucidation of Deoxynivalenol Glycosides. For the in-depth investigation of deoxynivalenol oligoglucoside structure, specific sample pretreatment including purification and preconcentration steps was necessary. The first approach we tested was application of adsorption chromatography; commercial mycotoxin-dedicated MycoSep cartridges were employed for this purpose. Our experiments showed that deoxynivalenol-3-glucoside was partly trapped together with other polar matrix impurities from the crude acetonitrile/water extract; its recovery in purified extract was only around 50% regardless of whether MycoSep 225 Trich, dedicated for

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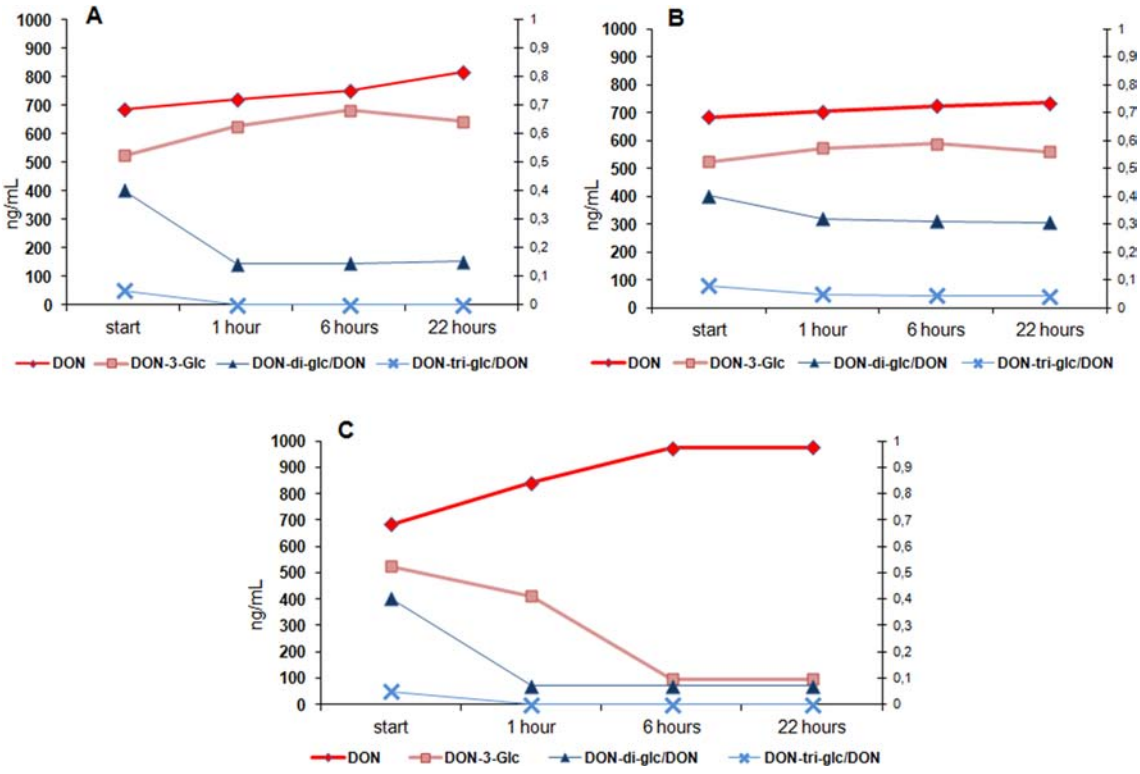

Figure 4. Changes of deoxynivalenol and deoxynivalenol-3-glucoside content (ng/mL) during enzymatic treatment of aqueous malt extract (left *y*-axis); relative changes in deoxynivalenol di- and triglucosides content expressed as deoxynivalenol diglucoside/deoxynivalenol and deoxynivalenol triglucoside/deoxynivalenol chromatographic peak areas ratios (right *y*-axis): (A) AMG 300 L BrewQ, amyloglucosidase; (B) Termamyl BerwQ, α-amylase; (C) NS 50010, β-glucosidase. Experiments were carried out in three repetitions; the RSDs of the obtained deoxynivalenol and deoxynivalenol-3-glucoside concentrations were up to 7.2 and 8.3%, respectively.

analysis of free trichothecene, or MycoSep 226 AflaZon, intended for aflatoxin and zearalenone analysis, was used. Considering the increased polarity of corresponding deoxynivalenol conjugates with the number of glucose units in their molecule, we assumed much higher affinity of these polar compounds to the cartridge sorbent. To confirm this hypothesis, a small-scale preliminary experiment with MycoSep 225 Trich in three repetitions was carried out. As we found, our expectation was only partly met because the binding of deoxynivalenol di-, -tri-, and tetraglucosides by the MycoSep cartridge was as low as 52 ± 5, 55 ± 5, and 57 ± 6%, respectively.

Another preconcentration/purification option was to employ immunoaffinity cleanup cartridges. During our previous research, we found that some of the commercial deoxynivalenol-dedicated immunoaffinity columns (DONPREP) were highly cross-reactive, not only to deoxynivalenol-3-glucoside but also to other deoxynivalenol oligoglucosides. On the other hand, obviously due to the use of more selective antibodies, the DONtest HPLC IACs did not show any cross-reactions to deoxynivalenol conjugates. Similar differences in cross-reactivity were reported also in our earlier study involving deoxynivalenol-dedicated enzyme-linked immunosorbent assays (ELISAs) obtained from various producers.[22] On the basis of this knowledge, we proposed an efficient strategy for selective isolation of masked deoxynivalenol glycosides from complex food extracts.

The first consideration was that deoxynivalenol conjugates in the experimental malt sample co-occur with high concentration of parent deoxynivalenol. Under such condition, it was obvious that the dominating free form would occupy most of the IAC binding sites and reduce target deoxynivalenol glycosides yield, whenever IAC binding capacity is exceeded, as illustrated in Table 1. To avoid the problem, we decided to first remove excessive deoxynivalenol by multistep passing of the malt extract through a cascade of non-cross-reactive DONtest HPLC cartridges and then preconcentrate the unbound target deoxynivalenol glycosides by employing the cross-reactive IACs represented by DONPREP.

UHPLC–OrbitrapMS Analysis; In-Source Fragmentation. Prior to the start of masked deoxynivalenol structure investigation, we focused on the optimization of their separation and detection conditions. High-performance liquid chromatography coupled with an ultrahigh-resolution orbitrapMS system was employed for this purpose. In the first phase, reversed phase chromatography, which is the most common separation approach in mycotoxin analysis, was tested. To our surprise, in addition to deoxynivalenol-3-glucoside and deoxynivalenol di- and triglucosides detected in our earlier pilot study,[18] also deoxynivalenol tetraglycoside was present in the examined beer sample (Figure 2A). Also, several other small signals, presumably various isobaric isomers, were detected around the major peaks at respective m/z values. However, the baseline separation of deoxynivalenol and its glycosides was very poor; the capacity factors (k') decreased only slightly with a number of sugar units in respective molecules. Moreover, some overlap of minor isomeric species occurred. Under such conditions, unambiguous identification of mass spectrometric fragments would not be possible. Therefore, as an alternative separation system, HILIC, suitable for the separation of polar

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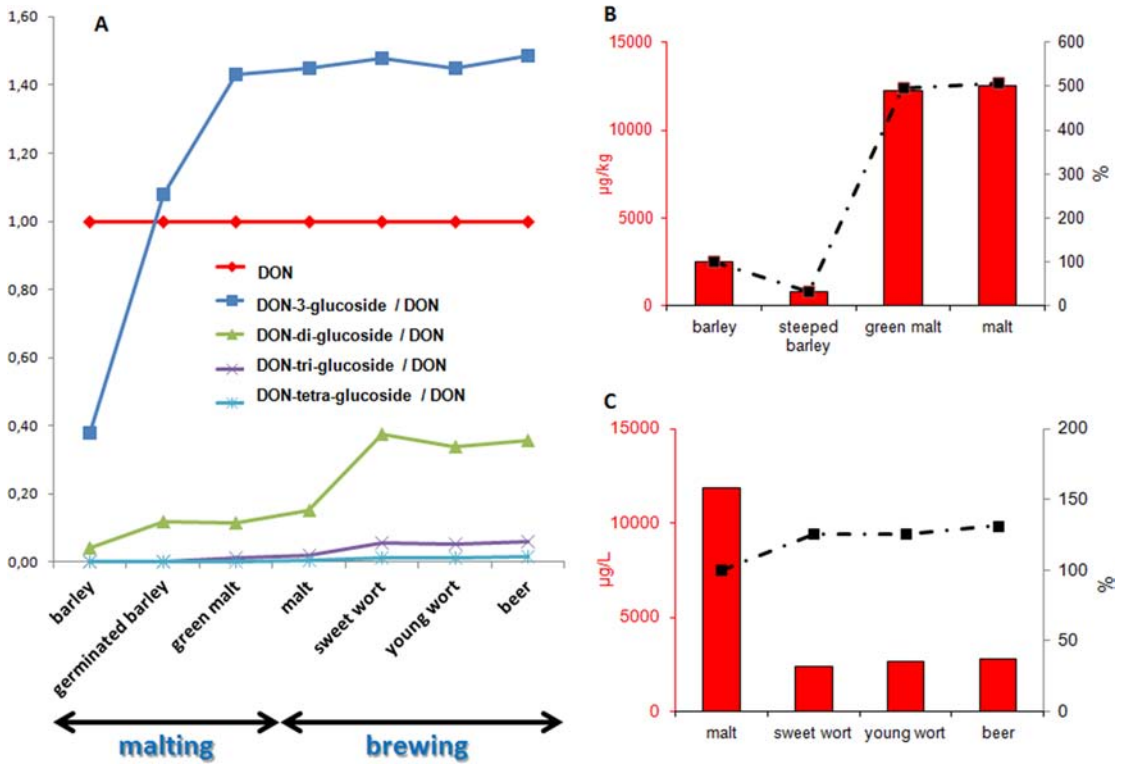

Figure 5. (A) Relative changes of deoxynivalenol glycosides content during malting and brewing; (B) deoxynivalenol concentration during malting technology (left *y*-axis) and deoxynivalenol percentage balance during malting technology (right *y*-axis); (C) deoxynivalenol concentration during brewing technology (left *y*-axis) and deoxynivalenol percentage balance during brewing technology (right *y*-axis). Experiments were done in three repetitions; the RSDs for deoxynivalenol and deoxynivalenol-3-glucoside concentrations in the processing intermediates were up to 17 and 19.2%, respectively.

analytes, was chosen for follow-up experiments. Figure 2B shows improved retention of deoxynivalenol glycosides, which increase with the size of the sugar moiety. The only limitation associated with the application of HILIC was the need to inject relatively polar analytes in an organic solvent, acetonitrile. To overcome the poor solubility problem, a small amount of water should be added to the acetonitrile, in our particular case, to a maximum of 10% (v/v); otherwise, worsened chromatography (tailing peak shapes) would occur.

Before starting the MS experiments, we also had to decide on the optimal analyte ionization mode. Whereas in our routine mycotoxin method, atmospheric pressure chemical ionization (APCI) is preferred for sensitive detection of deoxynivalenol and deoxynivalenol-3-glucoside,[23] in this case, poor detectability for larger molecules of deoxynivalenol di-, tri-, and tetraglucosides was encountered. Therefore, electrospray ionization (ESI), a more universal ionization technique, was employed. The presence of deoxynivalenol di-, tri-, and tetraglucosides was demonstrated on the basis of exact mass measurement and elemental composition calculation. The orbitrap was able to routinely achieve a mass resolving power as high as 65000 fwhm at the m/z of deoxynivalenol oligoglycosides, with calculated maximum mass errors not higher than 3 ppm. In addition to the $[M - H]^-$ molecular ions, the formic and chloride adducts were identified under negative ionization mode conditions. The presence of deoxynivalenol oligoglucosides could be further confirmed in positive ionization mode, with intensive $[M + H]^+$ ions present in the mass spectrum.

More than one isomer of deoxynivalenol di- and triglucosides occurred in malt and beer as depicted in Figure 3. Moreover, their relative intensity patterns in malt and beer were fairly different. To learn more about deoxynivalenol oligoglucoside structures, that is, to determine to which position(s) in deoxynivalenol molecule the sugar moiety is bound, the in-source fragmentation of deoxynivalenol glycosides was performed. The information on MS fragmentation patterns of various deoxynivalenol glycosides published in earlier scientific studies was of key importance for generic interpretation of mass spectra obtained within our experiments. As suggested by Dall'Asta et al.,[9] the binding position of the sugar unit in the deoxynivalenol molecule distinctly influences the stability of the molecular ion, thus leading to different fragmentation products. This phenomenon had already been clearly demonstrated on synthetically prepared deoxynivalenol-3-glucoside, deoxynivalenol-7-glucoside, and deoxynivalenol-15-glucoside. During fragmentation of deoxynivalenol-3-glucoside, cleavage of the bond between C-6 and C-15 resulted in the formation of an intense peak at m/z 427 corresponding to $[M - CH_2O - H]^-$. To the contrary, in the case of deoxynivalenol-15-glucoside, cleavage of this bond led to the additional loss of the sugar moiety to form the $[M - CH_2O - \text{glucose} - H]^-$ ion at m/z 265.[1] When the glucose unit was bound at the C-7 position, the fragmentation process led to the formation of the $[M - \text{glucose} - H]^-$ ion at m/z 295, and no signal was observed at m/z 427.[9] This generic approach was employed for interpretation of deoxynivalenol oligoglucoside mass spectra. As a result of their screening after the in-source fragmentation, fragments at m/z 589.2138, 751.2666, and 913.3194 corresponding to $[M - CH_2O -$

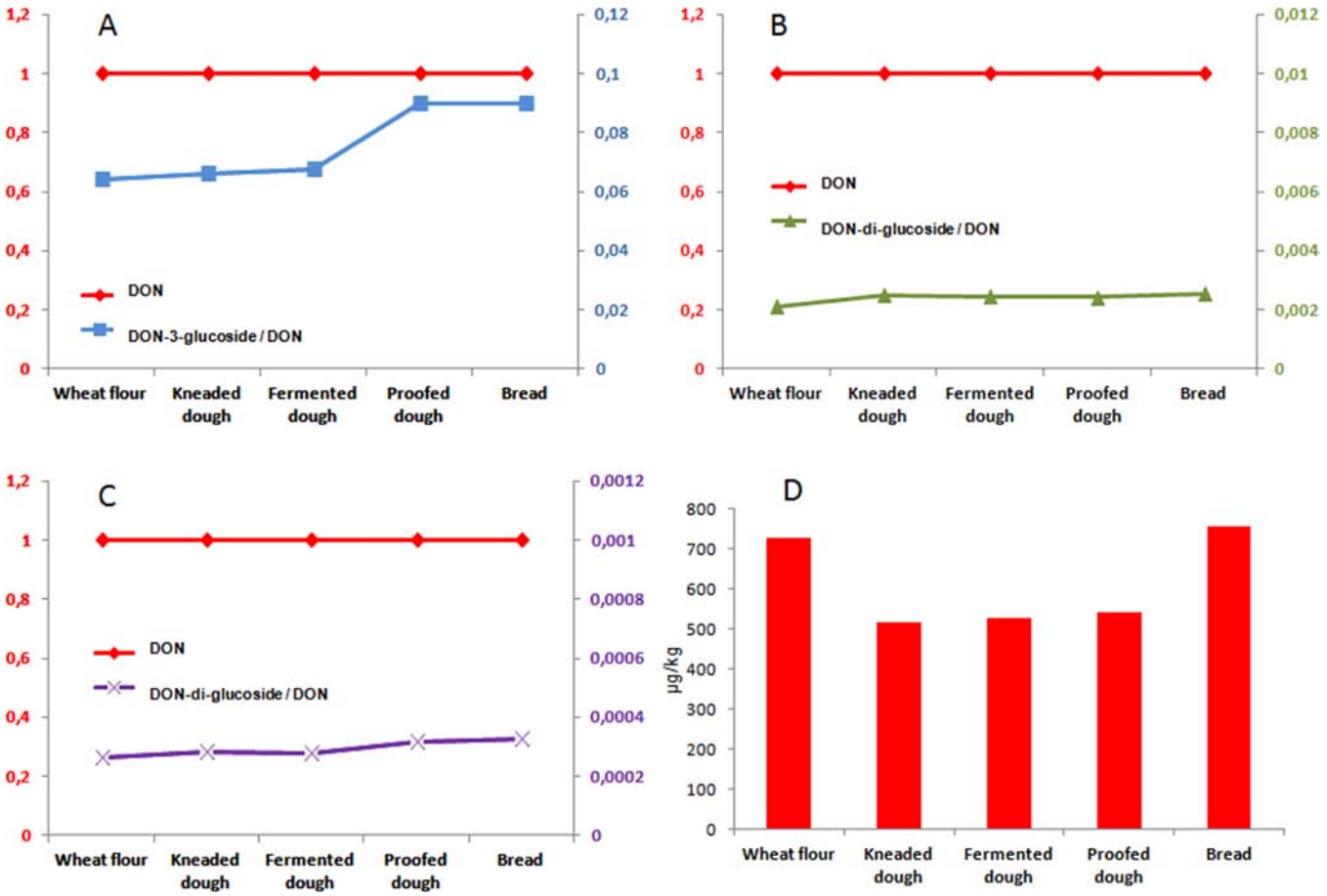

Figure 6. (A–C) Relative changes of deoxynivalenol glycosides content during baking technology, deoxynivalenol, left *y*-axis, deoxynivalenol glycosides, right *y*-axis; (D) deoxynivalenol concentration during baking technology. Experiments were done in three repetitions; the RSDs for deoxynivalenol and deoxynivalenol-3-glucoside concentrations in the processing intermediates were up to 14.3 and 16.5%, respectively.

H]$^-$ ions of deoxynivalenol di-, -tri-, and tetraglucosides were found at 6.6, 7.6, and 8.2 min retention times, respectively. These results can support the suggestion that the sugar moiety is bound on C-3 of the deoxynivalenol molecule, that is, the most likely structures are deoxynivalenol-3-diglucoside (Figure 1, **3**), deoxynivalenol-3-triglucoside (Figure 1, **4**), and deoxynivalenol-3-tetraglucoside (Figure 1, **5**).

Enzymatic Treatment; Investigation of Glycosidic Bonds. For investigation of the type of glycosidic bonds (α vs β) present in the deoxynivalenol oligoglucoside molecules, specific enzymatic hydrolysis reactions were tested. Deoxynivalenol glycoside concentrate containing 685 and 524 ng/mL of deoxynivalenol and deoxynivalenol-3-glucoside, respectively, prepared from the malt extract according to the procedure described above, was treated. Brewing enzyme preparations containing the heat-stable α-amylase, amyloglucosidase, and β-glucosidase were used for this purpose. As shown in Figure 4 illustrating the changes of deoxynivalenol and its glucosides during hydrolysis of malt extract by particular enzymes, rather different trends were observed. When α-amylase and amyloglucosidase were employed, levels of deoxynivalenol-3-glucoside slowly increased during the initial 6 h, from the starting concentration of 524 ng/mL to 579 and 682 ng/mL for α-amylase and amyloglucosidase, respectively. Then, its content slightly declined after 16 h to 561 and 644 ng/mL, respectively. At the same time, also a slight increase of deoxynivalenol was noted for both enzymes, from the initial 685 ng/mL to the final 736 and 815 ng/mL for α-amylase and amyloglucosidase, respectively. The successive decreases of di- and triglucosides occurred presumably due to hydrolysis of α-glycosidic bonds between glucose units.

Fairly different dynamics of deoxynivalenol conjugates and deoxynivalenol concentrations took place when fungal β-glucosidase was applied. A significant drop of deoxynivalenol-3-glucoside by 428 ng/mL occurred (decrease from the starting concentration of 524 ng/mL to 96 ng/mL during 22 h). Also, other deoxynivalenol glycosides showed the decreasing trend. Interestingly, the decline in deoxynivalenol glycosides content was accompanied by a proportional increase of free deoxynivalenol by 311 ng/mL, from 685 to 996 ng/mL during 22 h. This was obviously caused by its release from the bound forms, predominantly from deoxynivalenol-3-glucoside after hydrolysis of the β-glycosidic bond. A value of 428 ng/mL of deoxynivalenol-3-glucoside equals 278 ng/mL of deoxynivalenol after consideration of their molar ratio, which nicely corresponded with the deoxynivalenol increase of 311 ng/mL. The rest should be a result of the contribution from deoxynivalenol oligoglucosides.

Deoxynivalenol Glycosides during Food-Processing Technologies. The changes of deoxynivalenol glycosides during a model semiscale malt and beer production from the artificially *Fusarium* infected Bojos barley are shown in Figure 5A. The amount of deoxynivalenol-3-glucoside, in particular technological intermediates, was expressed as its molar ratio to

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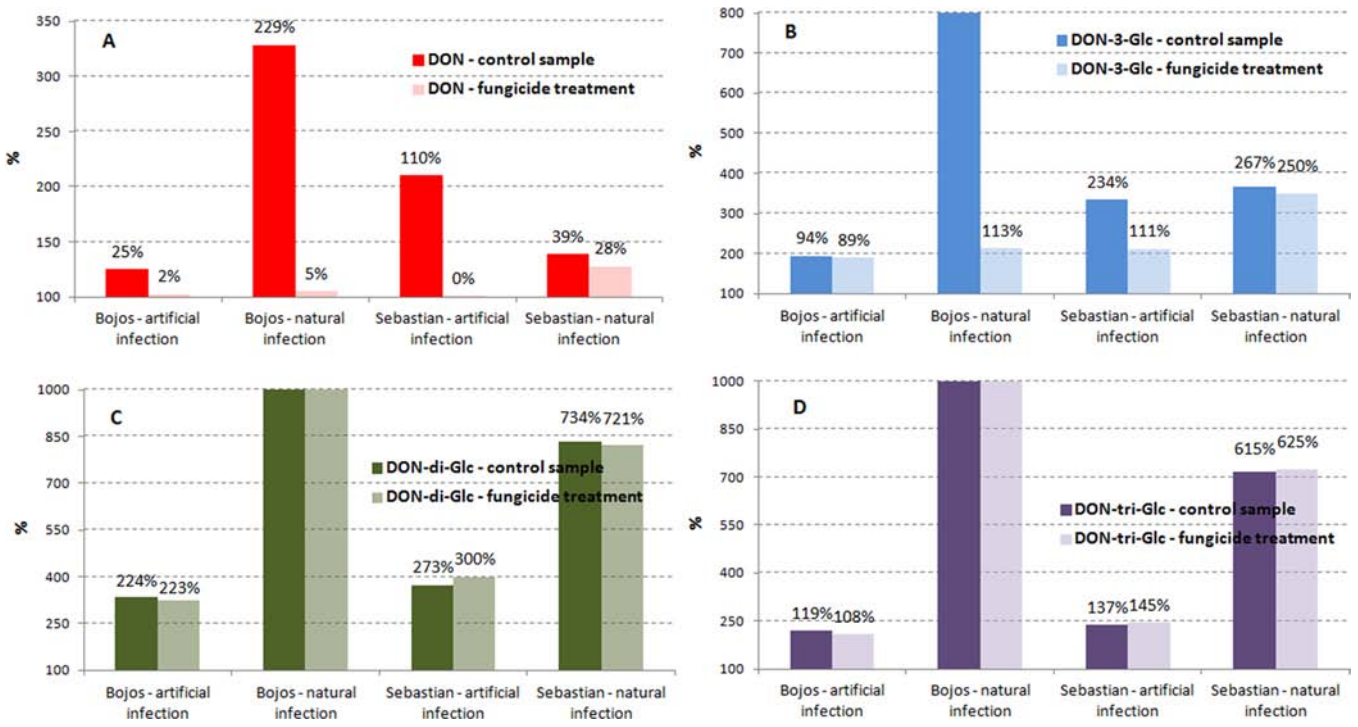

Figure 7. Relative increase of deoxynivalenol (A) and its glycosides (B–D) content in the green malt (their responses in starting barley = 100%); comparison of fungicide treated barley and a control variant. Experiments were carried out in three repetitions; the RSDs of the obtained deoxynivalenol and deoxynivalenol-3-glucoside concentrations were 21.6 and 17.8%, respectively.

Table 2. Occurrence of Deoxynivalenol and Its Conjugated Forms in Real Malt and Beer Samples

	μg/kg (μg/L)		peak area ratio	
sample	deoxynivalenol	deoxynivalenol-3-glucoside	deoxynivalenol diglucoside[a]/deoxynivalenol	deoxynivalenol triglucoside[a]/deoxynivalenol
malt 1	36.5	127.1	0.19	0.004
malt 2	12.6	32.7	nd[b]	nd
malt 3	75	115	0.11	0.001
malt 4	8.9	12.9	nd	nd
malt 5	136	157	0.08	0.007
malt 6	139	186	0.06	0.009
beer 1	14.0	25.0	0.31	0.056
beer 2	12.0	22.4	0.25	0.045
beer 3	13.3	21.2	0.18	0.032
beer 4	12.3	26.4	0.16	0.029
beer 5	14.4	33.4	0.19	0.046
beer 6	11.5	27.4	0.22	0.031
beer 7	5.6	10.1	nd	nd
beer 8	37.1	58.6	0.26	0.024
beer 9	62.2	82.1	0.28	0.038
beer 10	41.6	63.2	0.19	0.024
beer 11	6.4	15.9	0.01	0.012
beer 12	7.8	6.9	nd	nd
beer 13	20.0	36.5	0.28	0.014
beer 14	9.7	6.0	nd	nd
beer 15	45.5	79.7	0.31	0.042

[a]Sum of isomers. [b]nd, not detected.

deoxynivalenol. In the case of deoxynivalenol oligoglucosides, the deoxynivalenol conjugate to deoxynivalenol peak area ratio was used due to the lack of their analytical standards. The figure implies that the steepest growth of deoxynivalenol conjugates was determined during the germination of barley, and further, during sweet wort production, called malt mashing. The common feature of both of these procedures is abundant hydrolytic enzymatic activity, causing the release of deoxynivalenol conjugates from starch and dextrins. The dynamics of concentrations of the reference deoxynivalenol in malting and brewing intermediates are presented in Figure 5B,C. These figures depict the increase in deoxynivalenol concentration during malting and its rather significant decrease during brewing. The reason is just the dilution during the beer

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Table 3. Occurrence of Deoxynivalenol and Its Conjugated Forms in Commercial Baked Goods

	μg/kg		peak area ratio	
sample	deoxynivalenol	deoxynivalenol-3-glucoside	deoxynivalenol diglucoside[a]/deoxynivalenol	deoxynivalenol triglucoside[a]/deoxynivalenol
wheat baguette 1	157	30	0.0091	0.0009
wheat baguette 2	135	23	0.0080	0.0008
wheat baguette 3	350	25	0.0063	0.0005
wheat baguette 4	347	28	0.0062	0.0005
wheat baguette 5	148	31	nd[b]	nd
wheat baguette 6	120	28	nd	nd
wheat−rye baguette 1	431	33	0.0065	0.0006
wheat−rye baguette 2	43	16	nd	nd
BIO wheat flakes	347	46	0.0086	nd
multicereal baguette 1	264	19	0.0079	0.0006
multicereal baguette 2	351	28	0.0071	0.0007
multicereal baguette 3	349	28	0.0076	0.0008
multicereal baguette 4	296	21	0.0046	nd
multicereal baguette 5	131	6	nd	nd
sunflower baguette	399	31	0.0045	0.0003

[a]sum of isomers. [b]nd, not detected.

production technology. As a result of the percentage balance, its absolute amount grew, due to its release from the masked forms.

A similar experiment was performed for baking technology with artificially *Fusarium*-infected Akteur variety wheat flour. As shown in Figure 6, the deoxynivalenol glycosides to deoxynivalenol ratios slightly increase during dough proofing, which is probably caused by the enzymatic activity after the bakery improver's application. However, in comparison with the malting and brewing technologies, their growth was not substantial.

Cause of Deoxynivalenol Glycosides Formation during Malting and Brewing. Figure 5 depicts a substantial increase of both deoxynivalenol and its glycosides levels that occurred within the germination of barley during malting. Considering approximately 45% moisture in swollen grains and a temperature of 14−22 °C, it is obvious that the *Fusarium* fungus growth and de novo deoxynivalenol production are highly probable.[24] Unfortunately, real-time PCR that would confirm the fungal biomass growth was not available. For deeper insight into deoxynivalenol and its conjugates changes, germination of two batches of variously contaminated barley varieties was performed. Results obtained are illustrated in Figure 7. Destruction of *Fusarium* spores by the fungicide treatment significantly lowered the occurrence of deoxynivalenol in the green malt. The trend in deoxynivalenol-3-glucoside changes was not so unequivocal; the only pronounced decrease of this deoxynivalenol conjugate occurred in naturally infected Bojos barley. With regard to deoxynivalenol oligoglucosides, no significant impact of fungicide treatment on their concentration changes occurred in any germination experiments. Practically the same increase of their signals took place in treated/control pairs of sample. Interestingly, relatively higher increases of deoxynivalenol di- and triglucosides were observed in naturally infected green malt. With regard to the origin of deoxynivalenol-3-glucoside and corresponding oligoglucosides, either the glycosidation of deoxynivalenol by glucose and oligosaccharides created during the starch hydrolysis, as proposed by Maul et al.,[13] or the enzyme-catalyzed release from the "masked mycotoxins pool", that is, deoxynivalenol bound to starchy grains inside the barley kernels, could occur. In any case, the extent of deoxynivalenol conjugates origin depends both on the particular barley variety and on the mechanism of *Fusarium* infection.

The relative increase of deoxynivalenol glycosides during mashing of malt (Figure 5) within the brewing technology can be explained in a similar way, that is, by their release from glucosylated oligosaccharides originated within the barley germination when malting.

Deoxynivalenol Glycosides in Naturally Infected Samples. To gain some knowledge on deoxynivalenol oligoglucoside occurrence in naturally contaminated samples, a small screening survey was conducted. The set consisting of 6 malt, 15 beer, and 15 bakery samples obtained from the Czech retail market was analyzed. As a result, the presence of deoxynivalenol oligoglucosides was identified in 67% of malts, 80% of beers, and 73% of bakery products. The deoxynivalenol and deoxynivalenol-3-glucoside concentrations, as well as the specific deoxynivalenol oligoglucosides to deoxynivalenol ratios, are presented in Tables 2 and 3. Generally, the mean values of deoxynivalenol diglucoside to deoxynivalenol and deoxynivalenol triglucoside to deoxynivalenol ratios in malt and beer samples were 0.19 and 0.026, respectively. Rather lower values of deoxynivalenol oligoglucosides to deoxynivalenol ratios were detected in bakery products, <0.009 for all tested samples.

Consumption of cereal-based diet might be associated with the masked *Fusarium* mycotoxin intake. Within this study, several novel findings on higher deoxynivalenol conjugates have been obtained. For effective isolation of deoxynivalenol conjugates, immunoaffinity-based approaches are very much desirable. For their separation/detection, UHPLC-orbitrapMS strategy employing HILIC-based chromatography coupled with ultrahigh-resolution mass spectrometry represents the best option. Occurrence of isomers of deoxynivalenol oligoglucosides has been reported for the first time in real-life samples, mainly in malts and beers, but also in other wheat- and barley-based breadstuffs obtained from the consumer's market. For their quantitation, pure standards should become commercially available. With regard to the deoxynivalenol oligoglucoside structure, the sugar moiety was proven to be bound to the C-3 position of deoxynivalenol molecule, mostly by the β-glycosidic bond. Between the particular glucose units of the saccharidic

part of molecule, both α-1,4 and α-1,6 glycosidic bonds are present. Nevertheless, for exact structure elucidation, complementary spectroscopic techniques such as nuclear magnetic resonance (NMR) would be needed.

Various mechanisms might be responsible for an increase of deoxynivalenol and its conjugates during malting. On the basis of experiments with fungicide application on the barley grains before germination, it can be assumed that additional deoxynivalenol originates due to its de novo synthesis during the germination phase, which is favorable for fungus growth. Origination of deoxynivalenol glycosides, both mono- and oligo-, might include both the glycosylation of deoxynivalenol by glucose and oligosaccharides after enzymatic hydrolysis of starch and their release from a "masked mycotoxins pool". The dynamics of deoxynivalenol conjugates origination obviously depends on many factors. Besides the processing conditions, the extent and conditions of barley infection and respective variety (morphology, resistance against FHB, etc.) are probably also very important. It should be noted that a TDI of 1 μg/kg bw has been established for deoxynivalenol by SCF based on the consideration of dietary exposure to the free form only. Supposing at least part of deoxynivalenol is in vivo released from its conjugates, underestimation of dietary intake may occur. This study might contribute to reassessment of current regulation of maximum limits for deoxynivalenol in cereals and cereal-based products.

■ ASSOCIATED CONTENT

Supporting Information

Figures S-1−S-122: possible structures of deoxynivalenol glycosides. Table S-1: summary of calculated exact masses of deoxynivalenol and its glycosides, together with their conceivable diagnostic fragments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; ELISA, enzyme-linked immunosorbent assays; ESI, electrospray ionization; FHB, *Fusarium* head blight; HILIC, hydrophilic interaction liquid chromatography; IAC, immunoaffinity column; TDI, tolerable daily intake; TOF-MS, time-of-flight mass spectrometry; UHPLC-orbitrapMS, ultrahigh-performance liquid chromatography coupled with orbitrap mass spectrometry.

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