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Masked Mycotoxins: Determination of a Deoxynivalenol Glucoside in Artificially and Naturally Contaminated Wheat by Liquid Chromatography−Tandem Mass Spectrometry

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Conjugated mycotoxins, in which the toxin is usually bound to a more polar substance like glucose, are referred to as masked mycotoxins, as these substances escape routine detection methods but can release their toxic precursors after hydrolysis. This is the first report on the natural occurrence of a glucoside of deoxynivalenol (DON) in *Fusarium*-infected wheat and maize. To obtain appropriate standards, we chemically synthesized deoxynivalenol-3-β-D-glucopyranoside (DON-3-glucoside) and deoxynivalenol-15-β-D-glucopyranoside (DON-15-glucoside). The synthesis products were characterized by liquid chromatography−tandem mass spectrometry. The DON-glucosides showed different collision-induced dissociation (CID) fragmentation behaviors and could therefore be distinguished. Wheat plants were either treated with DON ($n = 52$) or with *Fusarium* spp. ($n = 4$) at anthesis, and after harvest, wheat ears were analyzed for DON and DON-glucosides. All 56 treated wheat samples contained DON and a DON-glucoside with the same retention time, molecular mass, and CID fragmentation behavior as the synthetic DON-3-glucoside. Moreover, the DON-glucoside was also found in two out of three analyzed naturally DON-contaminated maize and in five out of five naturally contaminated wheat samples, in a range from 4 to 12% of the DON concentration. To further confirm the identity of the DON-glucoside, the compound was isolated from wheat extracts and characterized as DON-3-glucoside with NMR. The results of this study indicate the importance to consider both DON and DON-3-glucoside with regard to food and feed safety.

KEYWORDS: Deoxynivalenol-3-β-D-glucopyranoside; conjugated mycotoxins; masked mycotoxins; LC-MS/MS; wheat; maize

INTRODUCTION

Mycotoxins are poisonous, low molecular weight secondary metabolites of molds. The topic of conjugated or masked mycotoxins first caught attention in the mid-1980s because in some cases of mycotoxicoses, clinical observations in animals did not correlate with the low mycotoxin content determined in the corresponding feed. The unexpected high toxicity could for instance be attributed to the occurrence of undetected, conjugated forms of mycotoxins that hydrolyze to the precursor toxins in the digestive tracts of animals (*1*). It was shown that plants can reduce the toxicity of mycotoxins either by chemical modification and/or by inclusion into the plant matrix (*2*). This detoxification process includes the conjugation of mycotoxins to polar substances such as sugars, amino acids, or sulfate (*3*) and subsequent storage of the conjugates in vacuoles. So far, the natural occurrence of a zearalenone glucoside in wheat has been reported (*4*). High-performance liquid chromatography (HPLC) combined with tandem mass spectrometry (MS/MS) offers a powerful tool for identification and characterization of mycotoxin conjugates. Derivatives can be identified due to their molecular mass and their collision-induced dissociation (CID) fragmentation behavior. Plant extracts can be screened for unknown mycotoxin conjugates with different MS/MS techniques. If standards are available, analytical methods can be developed and known conjugates can be quantified.

The trichothecene mycotoxin deoxynivalenol (DON) is often found in contaminated cereals and used as a guide substance in

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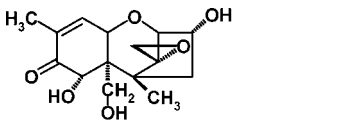
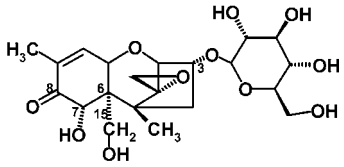


DON, MW: 296 amu



DON-3-glucoside, MW: 458 amu

Figure 1. Chemical structures of DON and DON-3-glucoside.

the determination of mycotoxins produced by *Fusarium* spp. Analytical methods for the determination of DON and other trichothecenes have been reviewed (*5*, *6*). In 1983, Miller et al. (*7*) showed that the DON concentration of *Fusarium graminearum*-infected wheat reached a maximum and then declined until harvest. Young et al. (*8*) reported a year later that the DON content of yeast fermented food products was higher than that of the contaminated flour used for their production. Since these early studies, it has been speculated that a DON conjugate might exist, arising from plant metabolism. Conjugated mycotoxins, however, escape routine analysis for several reasons. As these substances are more polar than the precursor toxins, they are hard to extract with the usual solvents and/or get lost in the cleanup process. Moreover, standards for these substances are not commercially available. Savard (*9*) was the first to chemically synthesize glucose and fatty acids conjugates of DON. Sewald et al. (*10*) identified deoxynivalenol-3-β-D-glucopyranoside (DON-3-glucoside) as the main DON metabolite after treatment of maize cell suspension cultures with DON. They characterized the compound with different NMR techniques. Recently, we could identify a UDP-glucosyltransferase from the model plant *Arabidopis thaliana*, which is able to form deoxynivalenol-3-glucoside (*11*). This study was undertaken to determine whether deoxynivalenol-3-glucoside can be produced in significant amounts in artificially and naturally contaminated cereals.

MATERIALS AND METHODS

Synthesis of DON Conjugates. DON-3-glucoside (**Figure 1**) and deoxynivalenol-15-β-D-glucopyranoside (DON-15-glucoside) were synthesized in a two-step reaction from 1-β-bromo-1-deoxy-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose and 15-acetyl-DON (15ADON) or 3-acetyl-DON (3ADON) (*12*) after a modified protocol of Savard (*9*). DON-3-glucoside was further purified in small-scale with HPLC, at 25 °C using methanol/water (10/90, v/v) on a 100 mm × 4.6 mm i.d., 3 μm RP-18 Aquasil column (Thermo-Keystone, Waltham, MA) and used as an analytical standard. All organic solvents were purchased from J. T. Baker (Deventer, The Netherlands). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

The concentration of DON-3-glucoside in the standard solution was estimated from measurement of the UV absorbance at 220 nm, assuming the same molar extinction coefficients for DON and DON-3-glucoside.

Treatment of Wheat with DON or *Fusarium* Strains. Wheat ears from 52 different cultivars were treated with DON (1 mg per ear) at anthesis (unpublished data). Alternatively, wheat ears were spray-inoculated with either *F. graminearum* or *Fusarium culmorum* (n = 4) at anthesis as described by Buerstmayr et al. (*13*). Whole ears were harvested after ripening and ground immediately before extraction.

Collection of Naturally Contaminated Samples. Five naturally contaminated wheat and three naturally contaminated maize samples, originating from Austria, Germany, and Slovakia, were chosen from a pool of cereal samples, which had been analyzed for their trichothecene content by our mycotoxin routine analysis laboratory. The ground cereal kernels had been stored in paper bags at room temperature in the dark until analysis. The samples were selected according to their DON concentrations in order to cover a range from about 0.1 to 5 mg/kg of DON.

Sample Preparation and Cleanup. A 10.00 ± 0.01 g amount of naturally contaminated ground wheat or maize kernels or 5.00 ± 0.01 g of whole wheat ears for treated or inoculated wheat samples were extracted for 90 min at 180 rpm on an orbital shaker with 40 mL of acetonitrile/water (84/16, v/v). The extracts were filtered and pressed through MycoSep 230 columns (Romer Labs Inc., Union, MO), and 4 mL of the cleaned extract was evaporated to dryness. The residue was dissolved in 1 mL of methanol/water (10/90, v/v), passed through 0.45 μm membrane filters, and analyzed by LC-MS/MS.

LC-MS/MS Measurements. LC-MS/MS analyses were performed with a QTrap-LC-MS/MS System (Applied Biosystems, Foster City, CA), equipped with an atmospheric pressure chemical ionization (APCI) source and a 1100 Series HPLC System (Agilent, Waldbronn, Germany), including a 1100 Series diode array detector. Chromatographic separation of DON and DON-3-glucoside was achieved at 22 °C using methanol/water (15/85, v/v) on an 100 mm × 4.6 mm i.d., 3 μm RP-18 Aquasil column (Thermo-Keystone). The injection volume was 50 μL, while the flow rate was 0.8 mL/min. The APCI source was operated at 450 °C in negative ionization mode. Further MS parameters were as follows: curtain gas, 35 psi (241 kPa); nebulizer gas (GS1), 60 psi (414 kPa); auxiliary gas (GS2), 15 psi (103 kPa); corona discharge needle current, −2 μA; collision-activated dissociation gas, 6 (arbitrary units); multiple reaction monitoring (MRM); dwell time, 25 ms; and pause between mass ranges 5 ms. Product ion spectra were gained at a collision energy (CE) of −30 eV and a declustering potential of −46 V. MRM transitions of m/z 457.3 to 295.3 for DON-3-glucoside and m/z 295.3 to 265.3 for DON were used. For quantification, the MRM mode and the UV signal at 220 nm were used, while enhanced product ion (EPI) and MS/MS/MS (data not shown) scans were used to gain structural information about the molecules. For the quantitative determination of 3ADON and 15ADON by LC-MS/MS, the LC and MS parameters were chosen as described by Berthiller et al. (*14*).

Purification of DON-3-glucoside from Contaminated Wheat Ears and NMR Confirmation. DON-3-glucoside was isolated and purified from extracts of wheat ears treated with DON using silica gel normal phase MPLC and semipreparative reversed phase HPLC. Therefore, acetonitrile/water extracts of the DON treated wheat samples (in total: 150 g wheat ears) were pooled. Silica gel 60 (63−200 μm particle size) (Merck KGaA, Darmstadt, Germany) was added to the liquid until a slurry was formed, which was than evaporated to dryness under reduced pressure on a rotary evaporator. The dried silica gel was then packed on top of a 230 mm silica gel 60 column (40−63 μm particle size) (Merck KGaA) and washed with ethyl acetate on a MPLC system from Büchi Labortechnik AG (Flawil, Switzerland), to elute most of the low polar substances. Afterwards, DON-3-glucoside was eluted from the column with ethyl acetate/methanol (4/1, v/v). Fractions of 5 mL were collected and analyzed by thin-layer chromatography using ethyl acetate/methanol (3/1, v/v) and $AlCl_3$ (15% in ethanol) (Sigma-Aldrich, St. Louis, MO) as developing reagents. DON-3-glucoside containing fractions was pooled, evaporated to dryness under reduced pressure, and dissolved in methanol. The normal phase chromatography was repeated once, before reversed phase chromatography purification on a PrepLC semipreparative HPLC (Waters, Milford, MA) equipped with a UV detector. The sample was dissolved in acetonitrile/water (5/95, v/v) and purified over a 250 mm × 15 mm i.d., 10 μm, Jupiter C-18 semipreparative column from Phenomenex (Torrance, CA). The flow was set to 4 mL/min, and the temperature was set to 30 °C. Gradient elution was performed starting from acetonitrile/water (5/95, v/v) and reaching acetonitrile/water (95/5, v/v) in 20 min. Afterwards, the column was washed for 10 min with 100% acetonitrile, followed by a 10 min conditioning step at the initial conditions. The UV detector was operated at 230 nm. Fractions containing DON-3-glucoside were pooled. Subsequently, the dried residue (8.8 mg) was dissolved in deuterated

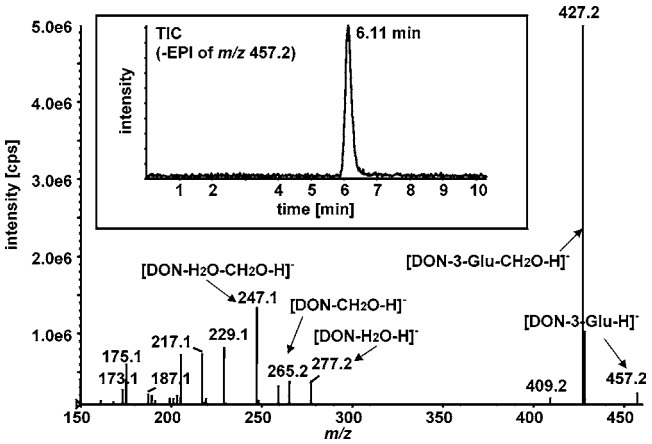


Figure 2. MS/MS (EPI) spectrum and total ion chromatogram (TIC) of synthesized and purified DON-3-glucoside (*m*/*z* 457.2), APCI negative ion mode, CE (–30 eV).

methanol and further characterized by ^{1}H NMR and ^{13}C NMR on a 600 MHz Inova NMR instrument (Varian, Palo Alto, CA).

RESULTS AND DISCUSSION

Characterization of Synthetic DON Glucosides with LC-MS/MS. Although the reactions gave rise to a mixture of compounds, with *m*/*z* values in the full scan mass spectra corresponding to the expected molecular mass of the DON glucosides (458 amu) and showing only slightly different retention times, it was possible to distinguish the chemically synthesized DON-3-glucoside and DON-15-glucoside due to the loss of H_2CO (−30 amu) from the $-CH_2OH$ group attached to the C-6 position of the DON-3-glucoside. The EPI spectrum of DON-3-glucoside therefore showed an intense peak at *m*/*z* 427 (**Figure 2**). In the case of the DON-15-glucoside, cleavage of the bond between C-6 and C-15 resulted in the loss of the whole sugar moiety (−192 amu, corresponding to the fragment $[DON-CH_2O-H]-$ at *m*/*z* 265), and no peak at *m*/*z* 427 was observed as described earlier (*11*). Moreover, the formation of synthesis byproducts and a CID fragmentation pathway of DON-3-glucoside has been described elsewhere (*15*). Unambiguous structural confirmation of the purified metabolite was achieved using ^{1}H, ^{13}C, COSY, and ROESY NMR techniques (data not shown) and is in agreement with both our LC-MS/MS results and the NMR results of Savard (*9*) and Sewald et al. (*10*).

Occurrence of DON-3-glucoside in DON-Treated or *Fusarium*-Inoculated Wheat. The occurrence of DON-glucosides in wheat was investigated by LC-MS/MS measurements. After grinding and extraction with acetonitrile/water, the raw extracts were purified using MycoSep 230 columns. These columns have been developed for the cleanup of polar mycotoxins such as nivalenol and will be commercially available shortly. The application of MycoSep 230 cleanup columns showed an average recovery of 60 ± 8% for DON-3-glucoside, estimated after spiking of DON-3-glucoside to noncontaminated sample extracts [0.15 mg/L, 57.9% (n = 2); 0.3 mg/L, 57.8% (n = 2); 0.6 mg/L, 58.4% (n = 2); 1.2 mg/L, 64.1% (n = 2)]. For DON, the application of these columns resulted in an average recovery of 80 ± 8%. Recoveries for individual spiking levels were as follows: 0.15 mg/L, 77.5% (n = 2); 0.3 mg/L, 80.2% (n = 2); 0.6 mg/L, 81.7% (n = 2); and 1.2 mg/L, 80.4% (n = 2).

In all analyzed wheat samples that had been treated with DON (n = 52), DON-3-glucoside was detected along with DON. The DON concentrations in whole wheat ears were in the range from

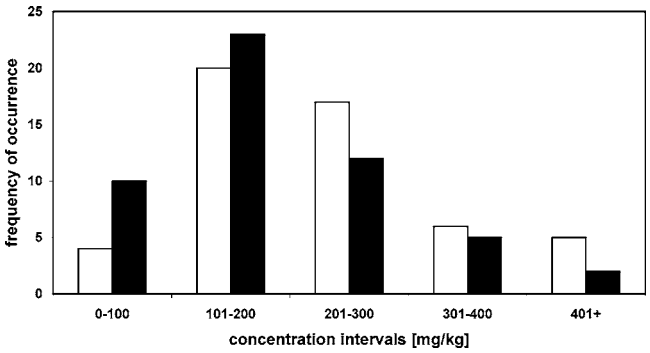


Figure 3. Frequency of different concentration intervals of DON (white bars) and DON-3-glucoside (black bars) DON-treated wheat samples. The total number of samples was 52.

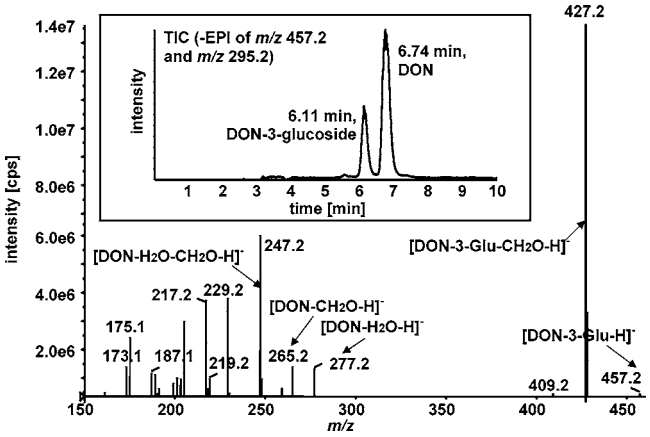


Figure 4. MS/MS (EPI) spectrum of the LC peak at 6.11 min and total ion chromatogram (TIC) of wheat extract (*m*/*z* 457.2 and *m*/*z* 295.2) after purification with MycoSep 230 column, APCI negative ion mode, CE (–30 eV).

77 to 574 mg/kg. DON-3-glucoside was detected in a range between 81 and 455 mg/kg. In 22 out of 52 samples, the DON-3-glucoside concentration was higher than the concentration of nonderivatized DON. On average, the frequencies of different concentration intervals approximately showed the same distribution for both compounds (**Figure 3**).

Moreover, field inoculation of wheat with *F. graminearum* or *F. culmorum* at anthesis, which were further cultivated under natural conditions, also resulted in the formation of DON-3-glucoside, along with DON. 3ADON and 15ADON were also produced by both fungi. An EPI chromatogram of *Fusarium*-inoculated wheat with the precursor masses 457.2 (corresponding to $[DON\text{-}3\text{-}glucoside-H]^-$) and 295.2 (corresponding to $[DON-H]^-$) showed two peaks, corresponding to DON-3-glucoside (6.11 min) and DON (6.74 min) (**Figure 4**). The MS/MS spectrum of the peak at 6.11 min corresponded well with the synthetically prepared DON-3-glucoside standard, showing the characteristic cleavage of "CH_2O" (−30 amu) thus confirming the identity of the DON-3-glucoside. The concentrations of DON, 3ADON, 15ADON, and DON-3-glucoside were determined in four different wheat samples with respect to the whole ears as described above. The DON-3-glucoside content ranged from approximately 2 to 10 mg/kg, while the DON content was in the range of about 6−74 mg/kg (n = 4) in the *Fusarium* spp.-inoculated samples. 3ADON and 15ADON could also be detected in each of the field-inoculated samples, showing concentrations between 0.3 and 11.1 mg/kg for 3ADON and 0.5 and 5.5 mg/kg for 15ADON. For the field-inoculated

Table 1. Concentrations of DON and DON-3-glucoside in Artificially ($n = 4$) and Naturally Contaminated Cereals ($n = 5$ for Wheat, $n = 3$ for Maize), Each Group Sorted by DON Concentration

sample	DON (mg/kg)	DON-3-glucoside (mg/kg)	3ADON (mg/kg)	15ADON (mg/kg)[a]	ratio DON-3-glucoside/DON
art. cont. wheat #1	5.5	1.6	0.9	0.5	0.29
art. cont. wheat #2	11.2	2.2	0.3	0.5	0.20
art. cont. wheat #3	61.8	8.8	1.8	5.5	0.14
art. cont. wheat #4	74.0	10.0	11.1	4.7	0.14
wheat #1	0.54	0.05	<0.01	<0.01	0.09
wheat #2	1.08	0.06	<0.01	<0.01	0.06
wheat #3	1.34	0.16	0.02	<0.01	0.12
wheat #4	1.54	0.09	0.02	<0.01	0.06
wheat #5	5.08	0.20	0.05	0.02	0.04
maize #1	0.09	<0.02	<0.01	<0.01	
maize #2	0.66	0.07	<0.01	0.10	0.11
maize #3	0.68	0.07	0.01	<0.01	0.10

[a] Concentration values for 15ADON were calculated as the difference between the sum of 3ADON and 15ADON and the concentration of 3ADON, as described in ref *14*.

samples, the concentration levels of DON-3-glucoside generally were higher than for the acetylated DON species (**Table 1**).

In addition, minor extents of a substance with a MS signal at *m*/*z* 499.4, corresponding to 15-acetyl-DON-3-glucoside, and presumed molecular fragmentation behavior of 15-acetyl-DON-3-glucoside, was detected in a fraction after MPLC purification of the DON-treated wheat (data not shown).

Natural Occurrence of DON-3-glucoside in Cereals. The DON, 3ADON, 15ADON, and DON-3-glucoside contents of five wheat and three maize samples, naturally infected with *Fusarium* spp., were determined according to the procedures described above. In this case, however, just the ground kernels and not the whole ears were analyzed. The DON concentrations obtained by LC-MS/MS were in the range from 0.09 to 5.1 mg/kg and were in good agreement with the results of our validated in-house GC-ECD method (*16*). DON-3-glucoside was detected in seven out of eight DON-contaminated samples at concentrations between 0.05 and 0.20 mg/kg. In addition to the MRM measurements, the identity of DON-3-glucoside was confirmed by full scan MS/MS experiments in the EPI mode. For the five wheat samples, the average relative concentration of DON-3-glucoside was 7% as compared to the concentration of DON, while for the two maize samples the average relative DON-3-glucoside concentration was about 10%. Interestingly, for each of the naturally contaminated samples, which had been investigated in this study, the DON-3-glucoside concentration was higher than for the acetylated DON derivatives (**Table 1**). This finding also underlines the potential significance of DON-3-glucoside when assessing the total amount of DON in cereals. Recovery of the glucoside was checked for these naturally contaminated wheat and maize samples by spiking the raw sample extracts with DON-3-glucoside standard before cleanup. The average recovery of 59 ± 11% ($n = 16$) was in good agreement with the behavior of the cleanup columns. No significant ion suppression effect of the MS signal was observed during the LC-MS/MS measurements. This allowed the method detection limits (MDL) directly to be estimated from the signal intensities of the standard solutions. A signal-to-noise ratio of 3:1 was chosen as MDL. For DON-3-glucoside, a MDL of about 0.012 mg/L in purified sample extracts, corresponding to about 0.020 mg/kg in contaminated cereals, was achieved, while for DON a MDL of about 0.005 mg/L corresponding to about 0.006 mg/kg cereal sample was calculated. The MDL for 3ADON and 15ADON was about 0.010 mg/kg (*14*).

As part of their metabolism, plants are capable of transforming phytotoxins into conjugated forms. DON-3-glucoside was identified and characterized by LC-MS/MS and NMR experiments. For the first time, DON-3-glucoside was detected in maize and wheat. A suitable cleanup for the simultaneous purification of DON-3-glucoside and DON from wheat extracts was found with MycoSep 230 columns. Both compounds could be determined in one LC-MS/MS run. Each of the investigated 52 DON-treated wheat samples did contain DON-3-glucoside with the average DON-3-glucoside concentration being in the same range as that of DON. For the four *Fusarium*-inoculated wheat ear samples, the concentration of DON-3-glucoside ranged from 1.6 to 10 mg/kg, corresponding to 14−29% relative to the respective DON concentration. Moreover, DON-3-glucoside was detected in seven out of eight naturally DON-contaminated maize and wheat samples at concentrations between 0.05 and 0.20 mg/kg, according to 4−12% relative to the respective DON concentration. As it is expected that DON-3-glucoside can be cleaved easily under acidic or suitable enzymatic conditions, at present, the total DON content in cereals might be underestimated. The present study might also be of some interest for the ongoing discussion within the European Community of setting legally binding maximum tolerated limits for DON in cereals, especially as this substance seems to occur in levels comparable to or even higher than the levels of 3ADON or 15ADON in cereals. To prove the significance of DON-3-glucoside with respect to its contribution to the total mycotoxin content, the LC-MS/MS method presented will be further optimized, validated, and applied in a survey to study the occurrence of this masked mycotoxin in contaminated cereals.

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