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Abstract

Deoxynivalenol-3- β -D-glucoside (D3G), a phase II plant metabolite of the mycotoxin deoxynivalenol (DON), occurs in naturally *Fusarium* contaminated cereals. In order to investigate the frequency of occurrence as well as relative and absolute concentrations of D3G in naturally infected cereals, 23 wheat samples originating from fields in Austria, Germany and Slovakia as well as 54 maize samples from Austrian fields were analyzed for DON and D3G by LC-MS/MS. Both analytes were detected in all the 77 field samples. DON was found at levels from 42 to 4130 ng/g (977 \pm 1000 ng/g on average). The D3G concentrations in all cereal samples were in the range from 10 to 1070 ng/g (216 \pm 253 ng/g on average), corresponding to about 5 to 46 mol% of their DON concentrations (15 \pm 8 mol% on average).

Keywords

LC/MS; cereals; mycotoxins - *Fusarium*

Introduction

D3G (structure shown in Figure 1) can be formed by *Fusarium* infected plants as a response to the production of the mycotoxin DON by the fungi. The existence of a plant conjugate of DON - which might be responsible for partial *Fusarium* head blight disease of wheat - was proposed in the mid-eighties (Miller et al., 1983; Young et al., 1984). It was assumed that the conjugate might be D3G, which therefore was chemically synthesized (Savard, 1991). D3G was indeed formed by DON treated maize cell suspension cultures (Sewald et al., 1992). We first detected D3G in naturally contaminated wheat and maize (Berthiller et al., 2005). Recently, D3G was also found in naturally contaminated barley as well as in malt and beer made thereof (Lancova et al., 2008).

Glucosylation, a reaction occurring in phase II metabolism of plants, represents a possibility to detoxify xenobiotics (reviewed in Lim and Bowles, 2004). The detoxification potential of *Arabidopsis thaliana* (Poppenberger et al., 2003) towards DON has been linked to the UDP-glucosyltransferase UGT73C5, which converts DON to D3G. The importance of this conversion for *Fusarium* resistance of wheat (Lemmens et al., 2005) has been shown and the quantitative trait locus (QTL) *Qfhs.ndsu-3BS* has been made responsible for this reaction. However, the fate of D3G after digestion by animals is still largely unknown. Preliminary data from our group indicate that certain intestinal bacteria are able to cleave D3G, converting it back to DON (manuscript in preparation). Therefore, D3G should be regarded as potentially hazardous for human and animal health.

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6 In this work we present occurrence data for D3G along with DON in wheat and maize,
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8 obtained by analysis using an LC-MS/MS method (Sulyok et al., 2006). Seventy-seven
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10 naturally infected field samples from fields in Central Europe were analyzed for their
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12 DON and D3G content to obtain a more comprehensive picture of the co-occurrence of
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14 these two compounds.
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17 18 19 20 **Materials and Methods**

21 22 *Chemicals*

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24 D3G was isolated from DON treated wheat ears (Berthiller et al., 2005). The identity of
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26 the substance was confirmed by NMR (data not shown). DON was obtained from
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28 Biopure Referenzsubstanzen GmbH (Tulln, Austria) as calibrant in acetonitrile solution.
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30 HPLC grade methanol and acetonitrile were purchased from J.T. Baker (Deventer, The
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32 Netherlands). MS grade ammonium acetate and per analysis grade acetic acid were
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34 supplied by Sigma-Aldrich (St. Louis, MO, USA). Water for HPLC was purified with a
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36 Millipore Milli-Q plus system (Molsheim, France) after reverse osmosis.
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43 44 *Cereal samples*

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46 Twenty-three wheat samples from the harvest of 2005 originated from different fields in
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48 Austria, Germany and Slovakia. Fifty-four maize samples from the harvest of 2006
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50 originated from different fields in southern Austria. For each cereal, at least 10
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52 incremental samples of 100 g each were taken and combined, according to (EC
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3 401/2006). All samples were ground to a fine powder and stored in paper bags at room
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5 temperature until analysis.
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8 9 10 ***Sample preparation***

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12 Sample preparation was carried out according to Sulyok et al. (2006) with slight
13 modifications. In brief, 5.00±0.01 g of the ground cereals were extracted with 20 mL
14 acetonitrile/water/acetic acid (79/20/1, v/v/v) for 90 min on a rotary shaker. The filtered
15 raw extracts were diluted 1+1 with acetonitrile/water/acetic acid (20/79/1, v/v/v) and
16 measured with LC-MS/MS without any further clean-up. If an analyte concentration
17 exceeded 1000 ng/mL (above the linear range of the method), the raw extract was diluted
18 1+9 with the same solvent mixture and the sample was re-analyzed by LC-MS/MS.
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32 ***LC-MS/MS instrumentation and methodology***

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34 An 1100 Series HPLC System (Agilent, Waldbronn, Germany) in conjunction with a
35 QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, USA) equipped with
36 a TurboV-ESI source were used for analysis. A multianalyte method was used for
37 analysis in the negative ion mode (Sulyok et al., 2006). A methanol/acetic acid (1%)/
38 aqueous ammonium acetate (5 mM) linear gradient (from 10 to 97% methanol) was
39 applied for separation on a Phenomenex Gemini-C18 column (4.6 x 150 mm, 5µm). The
40 [M+CH₃COO]⁻ acetate adducts of the analytes (*m/z* 355.1 for DON, *m/z* 517.3 for D3G)
41 were taken as precursor masses. Declustering potentials of -40V for DON and -50V for
42 D3G were chosen. Three product ions each were selected in the third quadrupole (DON:
43 *m/z* 59.1 (collision energy, CE=-40eV), *m/z* 295.1 (CE=-16eV), *m/z* 265.0 (CE=-16eV);
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3 D3G: m/z 427.1 (CE=-30eV), m/z 59.1 (CE=-85eV), m/z 457.1 (CE=-20eV)). The most
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5 abundant transitions were used for quantification, the other two as qualifiers. Standards
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7 were diluted separately from their stock solutions with pure water to final concentrations
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9 of 10, 30, 50, 100, 300, 500 and 1000 ng/mL. After external calibration, 1/x weighted
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11 linear calibration functions were generated with Analyst version 1.4.1 (Applied
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13 Biosystems).
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20 In order to evaluate the apparent recoveries of DON and D3G, the toxins were spiked
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22 separately to ground blank wheat and maize at concentrations of 50 ng/g, 100 ng/g, 200
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24 ng/g, 400 ng/g and 800 ng/g (n=3 each). After extraction and 1+1 dilution, the samples
25
26 were measured as described. Recovery and repeatability (RSD) were calculated from
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28 linear regression curves using Validata (Wegscheider et al., 1999). Limits of detection
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30 (LOD) and quantification (LOQ) were calculated, based on signal to noise ratios of 3/1
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32 and 10/1, respectively.
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39 **Results and discussion**

40 Due to a slightly modified sample preparation procedure (larger sample weight and a
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42 lower dilution factor for the maize samples) to the original method, the recoveries of
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44 DON and D3G in both matrices were determined again. Total (apparent) recoveries of
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46 95% for DON and 71% for D3G were obtained for wheat. These values are in complete
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48 conformity with the values from the original method (Sulyok et al., 2006). For maize,
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50 recoveries of 106% for DON and 130% for D3G were evaluated. These values are also in
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52 good agreement to the original method (with 1+1 diluted raw maize extracts).
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3 Interestingly, the higher apparent recovery for D3G in maize compared to wheat is
4 caused by a strong matrix enhancement effect in maize, while the extraction recovery is
5 similar for both cereals (Sulyok et al., 2006). For both matrices and both substances the
6 recoveries were constant across the entire range of concentrations. All concentrations for
7 DON and D3G in cereals have been corrected by the obtained recovery factors.
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17 Despite similar retention times (around 7.6 min) for DON and D3G, no cross-talk was
18 obtained even at high concentrations, confirming the selectivity of the used method.
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22 LOQs of about 10 ng/g have been achieved for D3G in both matrices. For DON, the LOQ
23 in wheat was 20 ng/g, in maize 40 ng/g. Relative standard deviations (n=15 for each
24 matrix) were around or below 10% (Table 1).
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32 Twenty-three naturally contaminated wheat samples were analyzed for their
33 concentration of DON and D3G as described above. The results are shown in Table 2,
34 sorted by increasing DON concentrations. Minimum, maximum and average (mean)
35 concentrations are shown in bold letters in the table. DON was found in all measured
36 wheat samples, ranging from 203 to 4130 ng/g. D3G was also detected in all 23 wheat
37 samples at concentrations between 76 and 1070 ng/g. As the molecular mass of D3G (458
38 g/mol) is higher than the molecular mass of DON (296 g/mol), the D3G/DON ratios are
39 given in mol% in Table 2. The concentrations of D3G in individual wheat samples ranged
40 from 7 mol% to 29 mol% relative to their DON concentrations. In average, 17 ± 5 mol%
41 of the DON concentration in wheat was found as D3G.
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3 Fifty-four naturally contaminated maize samples from fields in the southern part of
4 Austria (Styria) were tested for DON and D3G. Again, DON was found to be present in
5 all samples, with concentrations ranging from 42 to 3680 ng/g. D3G also was detected in
6 all maize samples at concentrations between 10 and 763 ng/g. The concentrations of D3G
7 in individual maize samples ranged from 5 mol% to 46 mol% relative to their DON
8 concentrations. On average, 14 ± 8 mol% of the DON concentration was found as D3G in
9 maize.
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22 Due to the use of a multi-analyte method, other mycotoxins have been detected as well,
23 but were not explicitly quantified. Nonetheless, 43 maize samples (80%) contained 15-
24 acetyl-DON, 37 maize samples (69%) contained zearalenone, 19 maize samples (35%)
25 contained nivalenol, 18 maize samples (33%) contained moniliformin and 6 maize
26 samples (11%) contained 3-acetyl-DON above their respective detection limits. Similarly,
27 16 wheat samples (70%) contained zearalenone, 15 wheat samples (65%) contained 15-
28 acetyl-DON, 13 wheat samples (56%) contained moniliformin, 7 wheat samples (30%)
29 contained nivalenol, 7 wheat samples (30%) contained 3-acetyl-DON.
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43 **Conclusion**

44 Besides the parent mycotoxins, their plant metabolites might also be hazardous for
45 animals and men. Such conjugated or masked mycotoxins can also be easily analyzed
46 with LC-MS methods if appropriate standards are available. To stimulate research efforts
47 in the area of masked mycotoxins, D3G has been made available to the scientific
48 community by the authors. The consistent occurrence of D3G in all tested wheat and
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3 maize samples confirms the presence of conjugated forms of mycotoxins along with their
4 mycotoxin precursors in cereals and the underestimation of the total DON load. Based on
5 the findings of the present study, consumers might currently be exposed to an additional
6 DON load of up to +50% in certain cases arising solely from D3G (assuming complete
7 hydrolysis of D3G to DON during digestion). DON enhances the virulence of *Fusarium*
8 *graminearum* on wheat, while it seemingly has little effect on virulence on barley or
9 maize (Jansen et al., 2005). The increased use of wheat cultivars which carry a major
10 QTL for increased *Fusarium* resistance and increased ability to conjugate DON
11 (Lemmens et al., 2005) may lead to a higher D3G/DON ratio in the future. In this case
12 the contribution of D3G to overall DON toxicity may no longer be negligible. We
13 therefore conclude that in the future, D3G should be monitored in cereals along with
14 DON, which currently is the only regulated trichothecene in Europe (EC 1126/2007).
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37 the Austrian Science Fund (FWF) for financial support. We also thank Dr. Karl Mayer of
38 the Styrian Chamber of Agriculture for the provision of the maize samples.
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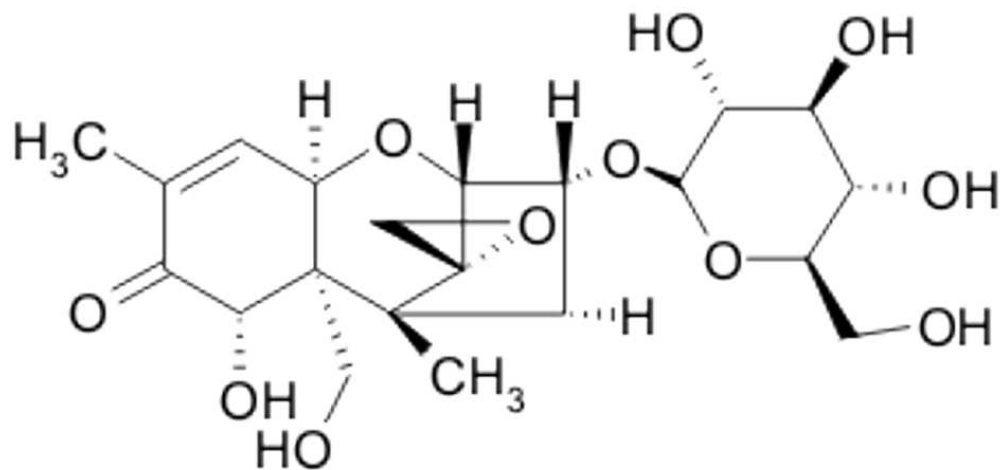
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30 **Figure Captions**

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33 Figure 1: Structure of DON-3- β -D-glucoside
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38 Table 1: Method performance
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40 Table 2: Concentrations of DON and DON-3- β -D-glucoside in naturally
41 contaminated cereals sorted by increasing DON concentration.
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45 Extreme and average values are shown bold.
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Structure of DON-3-β-D-glucoside

Analyte	Matrix	LOD (ng/g)	LOQ (ng/g)	Recovery (%)	RSD _r (%, n=15)	Accreditation Yes/No
DON	Wheat	8	20	95	5.7	No
DON	Maize	16	40	106	7.5	No
D3G	Wheat	4	10	71	9.7	No
D3G	Maize	4	10	130	10.2	No

	DON	D3G	D3G/DON		DON	D3G	D3G/DON
	[ng/g]	[ng/g]	[mol%]		[ng/g]	[ng/g]	[mol%]
wheat_01	203	76	24	maize_16	238	25	7
wheat_02	239	92	25	maize_17	245	59	16
wheat_03	311	84	18	maize_18	255	24	6
wheat_04	349	82	15	maize_19	259	44	11
wheat_05	386	92	15	maize_20	273	61	14
wheat_06	422	114	18	maize_21	282	71	16
wheat_07	518	110	14	maize_22	287	58	13
wheat_08	628	125	13	maize_23	327	41	8
wheat_09	725	124	11	maize_24	357	57	10
wheat_10	807	134	11	maize_25	420	75	12
wheat_11	831	221	17	maize_26	453	320	46
wheat_12	893	207	15	maize_27	466	64	9
wheat_13	985	110	7	maize_28	521	48	6
wheat_14	1300	347	17	maize_29	593	123	13
wheat_15	1360	601	29	maize_30	604	56	6
wheat_16	2210	551	16	maize_31	635	145	15
wheat_17	2500	670	17	maize_32	650	66	7
wheat_18	2570	645	16	maize_33	695	113	11
wheat_19	2850	827	19	maize_34	732	108	10
wheat_20	3080	899	19	maize_35	737	141	12
wheat_21	3230	781	16	maize_36	830	188	15
wheat_22	4030	1070	17	maize_37	883	90	7
wheat_23	4130	1070	17	maize_38	981	154	10
wheat				maize_39	1020	295	19
average	1500	393	17	maize_40	1140	342	19
				maize_41	1140	278	16
maize_01	42	10	15	maize_42	1170	254	14
maize_02	46	25	36	maize_43	1170	170	9
maize_03	54	12	14	maize_44	1230	194	10
maize_04	65	27	27	maize_45	1280	193	10
maize_05	103	43	27	maize_46	1290	223	11
maize_06	115	61	34	maize_47	1450	194	9
maize_07	134	43	21	maize_48	1510	122	5
maize_08	135	39	19	maize_49	1560	340	14
maize_09	142	46	21	maize_50	1640	218	9
maize_10	143	53	24	maize_51	2090	462	14
maize_11	174	28	10	maize_52	2830	482	11

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4	maize_12	178	35	13	maize_53	2850	391	9
5	maize_13	186	14	5	maize_54	3680	763	13
6	maize_14	189	81	28	maize			
7	maize_15	200	27	9	average	753	141	14
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