JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Effects of Milling and Baking Technologies on Levels of Deoxynivalenol and its Masked Form Deoxynivalenol-3-Glucoside

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ABSTRACT: The co-occurrence of the major *Fusarium* mycotoxin deoxynivalenol (DON) and its conjugate deoxynivalenol-3-glucoside (DON-3-Glc) has been documented in infected wheat. This study reports on the fate of this masked DON within milling and baking technologies for the first time and compares its levels with those of the free parent toxin. The fractionation of DON-3-Glc and DON in milling fractions was similar, tested white flours contained only approximately 60% of their content in unprocessed wheat grains. No substantial changes of both target analytes occurred during the dough preparation process, i.e. kneading, fermentation, and proofing. However, when bakery improvers enzymes mixtures were employed as a dough ingredient, a distinct increase up to 145% of conjugated DON-3-Glc occurred in fermented dough. Some decrease of both DON-3-Glc and DON (10 and 13%, respectively, compared to fermented dough) took place during baking. Thermal degradation products of DON, namely norDON A, B, C, D, and DON-lactone were detected in roasted wheat samples and baked bread samples by means of UPLC-Orbitrap MS. Moreover, thermal degradation products derived from DON-3-Glc were detected and tentatively identified in heat-treated contaminated wheat and bread based on accurate mass measurement performed under the ultrahigh mass resolving power. These products, originating from DON-3-Glc through de-epoxidation and other structural changes in the seskviterpene cycle, were named norDON-3-Glc A, B, C, D, and DON-3-Glc-lactone analogically to DON degradation products. Most of these compounds were located in the crust of experimental breads.

KEYWORDS: mycotoxins, masked mycotoxins, deoxynivalenol, deoxynivalenol-3-glucoside, milling, baking, thermal degradation, ultrahigh performance liquid chromatography, Orbitrap mass spectrometry

INTRODUCTION

Bread and other wheat-based bakery products are important items in the food basket in many countries worldwide. Consumers' exposure to toxic secondary metabolites of fungi belonging to *Fusarium* genus, which may occur in grains used for foodstuffs production, represents a widely discussed food safety concern. DON, a representative of trichothecenes B class, is one of the most common natural contaminant of wheat and other small cereal grains harvested in moderate climate zones. As shown in several studies published until now,[1−9] some reduction of DON and other *Fusarium* toxins content may occur during the processing, e.g. milling. Nevertheless, this mycotoxin is relatively stable under conditions characteristic for other processing steps including those employing elevated temperatures.[1,2] In the recent decade, ubiquitous co-occurrence of DON-3-Glc and DON, with concentration ratios being in the range 0.07−0.29, has been documented in wheat, maize, and other cereals contaminated with DON produced by *Fusarium* pathogens.[10,11] A significant increase of DON and DON-3-Glc, probably due to their release from masked forms or possibly also due to the de novo synthesis from germinating barley grains, was observed during the malting process in our previous studies.[11,12] As documented within follow-up experiments, further increase of DON-3-Glc may take place also during the brewing technology.[12−14] Our preliminary unpublished results also provided evidence on formation of various DON-oligo-glucosides. Unfortunately, pure standards of these DON conjugates are not available at the market yet, thus quantification of their levels cannot be easily performed.

On the basis of available occurrence data obtained within monitoring studies conducted worldwide, as well as with regard to results of toxicological studies, maximum limits have been established by the European Commission[15] for some mycotoxins in cereals and cereal-based food. The value of tolerable daily intake (TDI) values laid down by Scientific Committee on Food (SCF) for DON is 1 μg/kg body weight.[16] Although there is some preliminary evidence on possible release of DON from its conjugated form by action of hydrolytic enzymes catalyzing the cleavage of β-*O*-glucosidic bonds, the bioavailability of DON-3-Glc is not fully understood yet. On this account, its content in a human diet has not been considered in TDI calculation.

To get more knowledge on potential health risks associated with masked mycotoxins such as DON-3-Glc, their fate during technologies employed for cereals processing should be thoroughly recognized. In the current study, we focused for the first

Received: March 12, 2011
Accepted: July 28, 2011
Revised: July 27, 2011
Published: July 28, 2011

time on DON-3-Glc changes (together with DON) during the wheat kernels milling and subsequent bread-making. Milling process belongs to a primary stage of the cereal processing, within which, as indicated above, levels of mycotoxins can be considerably reduced.[1−7] Cleaning of wheat grains may result in DON decrease down to 20−30% (w/w) of the original raw matter content. Milling processes within which the outer layers of grains (bran and shorts) are removed also significantly contributes to decrease the DON amount in the respective milling fractions. This is caused by unequal distribution of these fungi secondary metabolites in kernels.[1−7] Levels of mycotoxins are often correlated with the flour ash content. It is a known fact that the higher levels of inner parts of endosperm are contained in the final flour the lower levels of mycotoxins are detected.[6] In any case, the content of mycotoxins in white flour is significantly reduced by the milling as compared to the input wheat raw material.

It is worth noticing that the data available on the fate of DON within the bread-making process are rather contradictory. The relationship between various baking parameters/baking ingredients and DON concentration levels in the final bread has been recently summarized in a comprehensive way.[3,4,9] While some processing experiments did not result in any decrease of DON levels, in other ones some moderate reduction up to 35−40%, depending on the respective technological conditions, was observed. Processing times and temperatures obviously play an important role.

Although DON is considered to be a relatively thermostable compound, the origination of breakdown products, namely isoDON, norDONs A−F, and DON lactones, was documented not only in model experiments (15 min, 200 °C) but also in some commercial samples such as tortillas, cookies, and/or corn flakes.[17−19] An interesting observation describing increase of DON levels within the fermentation phase of dough preparation and also during the bread baking has been reported very recently.[3] Authors assumed that enzymatic release of DON from its unknown bound forms takes place. The same hypothesis on possible occurrence of masked DON pool has been proposed.[5,9] In any case, there is a general agreement on the need of further investigation in this area.

The aim of this study was to extend the knowledge on the fate of DON and DON-3-Glc within the milling and baking technologies and also learn more about their degradation products, which can emerge as the result of heat-treatment within the baking. Model experiments simulating the process of bread-baking of naturally and artificially infected wheat samples were conducted.

MATERIALS AND METHODS

Standards. Analytical standards of DON (purity 99.4%), isotope-labeled internal standard $^{13}C_{15}$-DON (purity 98.1%), DON-3-Glc (purity 96%), and 3- and 15-acetyl-deoxynivalenol (ADONs) (purity 99.4 and 98.8%, respectively) were purchased from Biopure (Tulln, Austria). Crystalline standards of DON and ADONs were dissolved in acetonitrile (1 mg/mL), and their stock solutions as well as standard of DON-3-Glc were stored at −20 °C. A composite standard solution of all three analytes at concentration 5 μg/mL and also calibration standards (1−500 ng/mL) were stored no longer than 30 days.

Chemicals and Materials. Methanol, acetonitrile (both HPLC grade), and ammonium formate were obtained from Sigma-Aldrich (Taufkirchen, Germany). Deionized water was produced by Milli-Q system (Millipore Corporation, Bedford, MA, USA).

Samples. All together, 15 varieties of wheat harvested in 2009 were grown under the specific organic and conventional conditions intended for the laboratory-scale milling and baking experiments. In total, three wheat cultivars (Merrito, Akteur, and Eurofit) were used in designed experiments. Merrito wheat is classified as B (bread supplementary) bakery class of wheat, Akteur belong to E (elite bakery wheat) class, and Eurofit is A (quality) bakery wheat class. Each of wheat cultivars was grown under various conditions: (i) conventional farming (grains were obtained from three subgroups of field experiments control and treated by fungicides, either Prosaro (active ingredients prokanazol and tebukonazol) or Fandango (active ingredients fluoxastrobin and prokanazol), overall batch of nitrogen 100 kg N/ha divided into two parts regenerative and productive, herbicide application, (ii) organic farming (certified according the IFOAM), and (iii) artificially infected by *Fusarium culmorum* and *Fusarium graminearum* in rate 1:1, inoculums density 10^7/mL, 2 L of suspension per plot (12 m^2). Inoculation was done at the beginning of flowering. The wet content of all samples was in the range 13.3−14.2%.

Milling and Baking Processing Experiments. *Milling.* Wheat samples were milled using a Bühler laboratory mill (Bühler, Switzerland). The day prior to processing, wheat samples were tempered to approximately 14% of moisture (w/w). As the result of milling, white flour samples corresponding to common T 550 baker flour and fine grained bran, were obtained.

Baking. For preparation of one batch of bread was used 300 g flour, 12 g leaven, 3 g fat, 5.1 g salt, 4.5 g saccharose, and distilled water (150−165 mL); the volume of water was used according to the flour bonding power, which was measured at Farinograph instrument (Brabender, Germany). The ingredients were then kneaded and dough was further fermented for 45 min at 30 °C. After this time, the dough was kneaded again and divided into three dough pieces of 80 g each, which were proofed for 50 min at 30 °C. Bread loaves, approximately 55 g each final weight, were baked in an electric laboratory oven for 14 min at 240 °C. For experiments with conjugated DON-3-Glc, 3, 9, and 12 g of two different bakery improvers were added to flour (artificially inoculated Akteur wheat). Bakery improvers (BI1; BI2) were obtained from a commercial bakery company dealing with production, sale, and distribution of improvers. BIs consisted of saccharose, dry whey, malt powder, enzymes, emulsifiers, and antioxidants (the exact composition is proprietary information of the supplier).

Model Heating Experiments with Analytical Standards and Wheat Samples. Analytical standard solutions of DON and DON-3-Glc (100 μL, concentration 10 μg/mL) were placed into glass vials. Regarding wheat, 5 g of samples were used for heat-induced degradation in glass basin. Standards as well as wheat samples were kept in the laboratory dryer for 30 min and tempered at 160 °C. Thermal degradants of DON and DON-3-Glc were tested separately in bread crumb and crust (crust comprised 9% of baked bread).

Analysis of Mycotoxins. *Extraction and Preconcentration.* The representative samples (6.25 g) of wheat grains, milling and/or baking intermediates (samples of dough were lyophylized prior to extraction, concentration of mycotoxins was than calculated on the wet weight of sample), were homogenized and then extracted with 25 mL of acetonitrile−water mixture (84:16, v/v) for 60 min using an automatic shaker (IKA Laboratortechnik, Germany). An aliquot of crude extract (4 mL) was evaporated to dryness, redissolved in 1 mL of methanol−water mixture (1:1, v/v), and subsequently passed through a 0.2 μm microfilter prior to LC-MS analysis. All of samples were processed in two parallel repetitions, and discussed results represent mean values calculated from corresponding measurements.

Heat-treated samples (5 g) of wheat and bread samples were extracted with 20 mL of distilled water, centrifuged at 11000 rpm for 5 min, and finally passed through the 0.2 μm microfilter.

UPLC-Orbitrap MS Method for Mycotoxins and Thermal Degradants Analysis. For separation of analytes as well as for qualitative

Table 1. Levels (μg/kg) and Relative Molar Ratios (mol %) of DON and DON-3-Glc in Wheat Samples and Milling Fractions

		wheat			white flour			bran		
		μg/kg		mol %	μg/kg		mol %	μg/kg		mol %
wheat growing	wheat variety	DON	DON-3-Glc	DON-3-Glc/DON	DON	DON-3-Glc	DON-3-Glc/DON	DON	DON-3-Glc	DON-3-Glc/DON
conventional control	Meritto	182	64	22.7	135	49	23.2	392	60	9.8
	Akteur	198	32	10.4	149	28	12	410	44	6.9
	Eurofit	154	23	9.7	63	13	13.7	178	38	13.8
conventional + Prosaro	Meritto	176	26	9.4	131	15	7.4	201	35	11.3
	Akteur	183	32	11.2	105	19	11.7	326	55	10.9
	Eurofit	82	12	9.7	48	8	10.3	92	24	16.7
conventional + Fandango	Meritto	90	25	17.8	62	15	15.8	197	38	12.4
	Akteur	123	27	14.3	83	19	14.4	252	44	11.3
	Eurofit	125	16	8.3	67	13	12.2	144	30	13.3
organic	Meritto	253	44	11.2	118	30	16.5	313	60	12.5
	Akteur	354	57	10.3	182	37	13.1	444	73	10.5
	Eurofit	191	26	8.9	106	16	9.5	230	44	12.4
artificial inoculation	Meritto	1700	167	6.3	1049	118	7.2	2169	393	11.7
	Akteur	1586	224	9.1	729	160	14.2	3964	558	9.1
	Eurofit	1053	102	6.3	556	98	11.3	1549	235	9.8

determination of DON and DON-3-Glc thermal degradants, an Accela UPLC liquid chromatograph (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a single stage Orbitrap mass spectrometer Exactive (Thermo Fisher Scientific, Bremen, Germany) were used. The analytical column used was a 100 mm × 2.1 mm i.d., 1.8 μm, Acquity UPLC HSS T3 (Waters, Milford, MA, USA), temperature controlled at 40 °C. The mobile phase consisted of 5 mM ammonium formate in water (A) and methanol (B). The flow rate was 300 μL/min, injection volume was 5 and 20 μL for technological samples and heat-treated samples, respectively. Linear gradient elution was performed as follows: start with 5% B, linear increase to 50% B in 6 min, for next 4 min another linear increase to 95% B, maintained for 15 min, switching to 5% B at 15.1 min, and column equilibration for 3 min before the next injection. APCI source operating in negative mode was used for ionization of analytes in technological samples. Operation parameters of MS Exactive were established as follows: discharge current, 5 μA; vaporizer temperature, 250 °C; spray voltage, 4 kV; heater temperature, 150 °C; sheath gas, 35 arbitrary units; aux gas, 10 arbitrary units; capillary temperature, 250 °C. Other source parameters were automatically tuned for maximal intensity of particular analytes in the respective retention time window.

Heated electrospray interface HESI-II (Thermo Fisher Scientific, Bremen, Germany), operated either in positive or negative mode, was used for ionization of DON and DON-3-Glc thermal degradation products. Optimized parameters were as follows: sheath and aux gas flow was 35 and 10 arbitrary units, respectively, capillary temperature was held at 250 °C, the heater temperature was 250 °C. The capillary voltage and spray voltage was: +60 and +4 in positive mode, and −50 V and −3.1 kV in negative ionization mode. The mass spectra were acquired the mass range m/z 120−2000 at resolving power setting of 100000 full width at half-maximum (fwhm) and acquisition rate of 1 spectrum/s.

Validation, Quality Control. For validation of the method, blank samples of all examined matrices (wheat, flour, dough, bread) were used and artificially spiked at levels 25 and 100 μg/kg ($n = 5$). Matrix-matched calibration standards (in case of DON isotope-labeled standard was added) of all matrices were used for calibration purposes; correlation curves of these standards were linear within the working range 1−500 μg/kg for all analytes. Low limits of quantitation (LOQ), not higher than 5 μg/kg, were obtained for all matrices, as well as acceptable recoveries (ranging from 74% to 91%) with repeatabilities below 5.6%, were obtained for all of examined analytes/matrix combinations.

The analytical method used for samples examination was accredited (according to ISO 17025) for cereals. As a part of external quality control procedures, the trueness of generated data was demonstrated through periodical participation in the Food Analysis Performance Assessment Scheme (FAPAS) organized by The Food and Environment Research Agency (FERA, York, UK). Without any exceptions, z-scores values corresponding to reported DON concentration levels were always in the range from −1 to +1.

RESULTS AND DISCUSSION

The co-occurrence and, in many cases, considerable increase (as compared to initial barley) of levels of DON and also its masked form DON-3-Glc in malt and beer have been documented quite recently.[12−14] Regarding other food technologies such as bread-baking, no information on changes of masked mycotoxin DON-3-Glc has been reported yet. We believe that experimental data presented and discussed below will contribute to fill in this gap.

Deoxynivalenol and Masked DON-3-Glc in Wheat and Milling Fractions. The overview of parent DON and its conjugate DON-3-Glc levels in wheat and fractions obtained within the milling experiments is shown in Table 1. Without any exception, all the wheat samples contained these two natural contaminants; nevertheless, in none of them DON exceeded

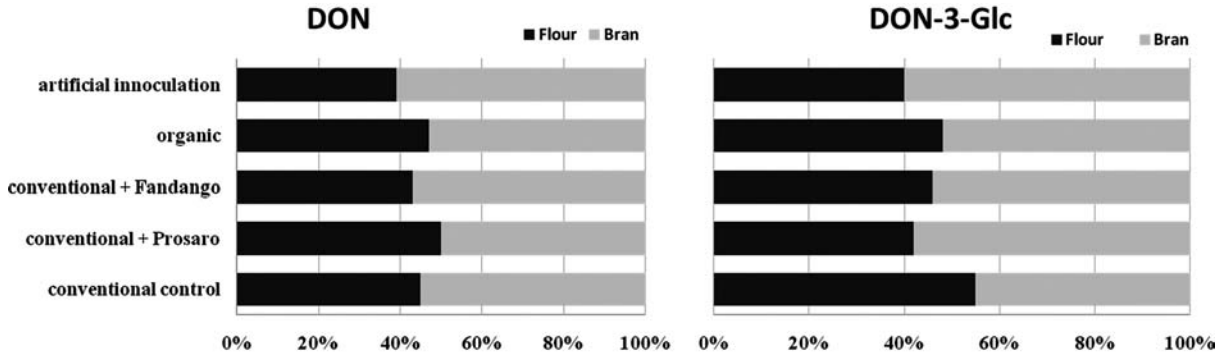

Figure 1. Distribution of DON and DON-3-Glc (mean values for naturally contaminated samples) within wheat milling fractions: white flour and bran. Data are based on particular fractions weights and sample contamination (100% = total contamination of processed grains).

regulation levels (1250 μg/kg, EC 1126/2007), the maximum concentration detected in examined samples was 354 μg/kg. The amount of DON bound in DON-3-Glc is given in mol % [(M(DON)/M(DON3-Glc) × (c(DON3-Glc)/c(DON)] × 100, values are also summarized in Table 1. The mean mol % of DON-3-Glc concentration relative to DON concentration was 12.0 (maximum 22.7). In line with our expectation, grains obtained from wheat artificially infected in field by *Fusarium* species contained significantly higher levels of DON, interestingly, the mean mol % of DON-3-Glc was lower, only 7.2 (maximum value 9.1 mol %) what might indicate a limited glycosylation capacity in heavily infected plants. In addition to glucose conjugate, also acetylated-DONs (ADONs) were present in grains, nevertheless, their concentration were in average as low as 5 μg/kg, therefore, they were not monitored in follow-up experiments.

Table 1 also illustrates the effectiveness of two alternative approaches of fungicide treatment in terms of grains contamination reduction. The usage of Prosaro did not result in significant reduction of DON levels, while in Fandango treated wheat, the contamination was lower (28% compared to control). The highest levels of DON among naturally infected wheat were determined in grains from organic farming (approximately 149% of conventional control and 236% of Fandango treated samples). In our previous study (unpublished results), it was observed that higher levels of *Fusarium* toxins occurred in most of samples from organic crop compared to conventional ones. Supposing fungicides, which may induce elevated toxins levels, were not used (e.g., strobilurins); this observation is in line with several other similar studies.[20] The differences in overall DON concentration, which were observed among three wheat tested varieties, were not too significant and rather higher contamination was found in samples of variety Akteur.

In Table 1, the concentrations of DON and DON-3-Glc in milling fractions obtained by the laboratory scale processing are summarized. Generally, the reduction of DON-3-Glc level in white flour occurred similarly to DON (the decrease of the later toxin corresponds to results of other earlier studies).[1−7] However, the distribution pattern between main milling fractions, bran and white flour, was not identical within the set of tested wheat. The graphs presented in Figure 1, illustrate that with exception of conventionally grown control wheat, most of the DON-3-Glc amount was located in bran. In addition to that type of sample, the most pronounced difference between DON and DON-3-Glc distribution was observed in grains treated with Prosaro. While the ratio of DON amounts contained in bran and flour was 50:50, in the case of DON-3-Glc, this value was 58:42.

Bread Baking Process. Contrary to the milling process, the knowledge on distribution, occurrence, and fate of *Fusarium* mycotoxins within baking processes is very limited. Among others, the reason might be some difficulties encountered when applying analytical procedures validated only for grains/milling fraction to analysis of dough or baked product. It is worth to noticing that the nature of these matrices is due to added water/fat, reduced pH, and/or changes in matrix composition/texture induced by heat treatment, etc., rather different. On this account, when using method not optimized for respective matrix, biased results might be obtained. Another difficulty to get general conclusion on the impact of baking on DON levels are widely ranging recipes/baking practices employed for the bread-making. In our study, 15 flours characterized in Table 1 were used for laboratory baking, aimed at monitoring of DON and DON-3-Glc content across the overall process, i.e. not only baked bread, but also samples of intermediates, namely freshly kneaded dough, dough after 1 h fermentation, and final proofed dough, were analyzed. The results are summarized in Table 2. The graph shown in Figure 2 illustrates general trends that were found for all naturally contaminated samples: DON levels were not significantly influenced by the dough fermentation process, only slight increase of DON (approximately 5%) occurred, as compared to its concentration in starting flours. Some decrease in concentration levels (up to 13% in an average) could have been observed in case of DON-3-Glc after the dough kneading. Nevertheless, further dough fermentation resulted in its slight increase (up to 8%). The final phase of baking in oven (240 °C for 14 min) led to some reduction of both analytes. These trends are in line with a comprehensive study[17,18] concerned with thermal degradation of DON under baking conditions. As documented in experiments presented below, the same explanation can be applied also for DON-3-Glc levels drop.

The Impact of Bakery Improvers on DON and Conjugated DON-3-Glc Levels. As mentioned in several recent papers concerned with masked *Fusarium* mycotoxins,[21] it is assumed that within field infection, part of mycotoxins is incorporated into plants and there modified. Most often, secondary fungal metabolites are converted to more polar substances that are further stored in vacuoles or conjugated to biopolymers, e.g. cell wall components. Interestingly, the recent studies of DON changes during malting have shown that its increase is accompanied by an intensive DON-3-Glc release, which might be, at least partly, associated with the action of enzymes. On the basis of this

Table 2. Levels (μg/kg) and Relative Molar Ratios (mol %) of DON and DON-3-Glc in Intermediate Dough Samples and Bread

		kneaded dough			fermented dough			proofed dough			bread		
		μg/kg		mol %	μg/kg		mol %	μg/kg		mol %	μg/kg		mol %
wheat growing	wheat variety	DON	DON-3-Glc	DON-3-Glc/DON	DON	DON-3-Glc	DON-3-Glc/DON	DON	DON-3-Glc	DON-3-Glc/DON	DON	DON-3-Glc	DON-3-Glc/DON
conventional control	Meritto	182	29	10.3	184	32	11.4	174	27	9.9	80	25	20.2
	Akteur	94	22	15.4	93	23	15.7	99	24	16.0	45	12	17.2
	Eurofit	59	10	11.0	40	8	12.8	40	7	11.4	23	7	19.9
conventional + Prosaro	Meritto	75	11	9.5	122	24	12.8	71	13	12.2	87	15	11.2
	Akteur	65	12	11.8	59	11	11.7	66	13	12.7	32	8	16.8
	Eurofit	43	8	11.9	38	6	10.3	29	5	10.5	15	4	18.0
conventional + Fandango	Meritto	65	11	10.7	62	10	9.9	79	13	10.7	42	12	19.0
	Akteur	57	15	16.6	54	15	18.0	65	15	15.0	77	16	13.5
	Eurofit	48	7	9.6	60	9	9.6	63	10	10.2	39	10	16.2
organic	Meritto	74	30	25.7	71	23	20.6	83	25	19.7	95	28	18.8
	Akteur	109	24	14.0	94	22	15.1	98	25	16.6	187	31	10.8
	Eurofit	118	18	9.9	115	16	9.0	112	15	8.6	77	14	11.8
artificial inoculation	Meritto	758	95	8.1	705	102	9.4	773	133	11.1	821	114	9.0
	Akteur[a]	561	73	8.4	550	79	9.3	547	87	10.3	624	81	8.4
	Eurofit	720	105	9.4	668	101	9.7	724	131	11.7	687	103	9.7
bakery improvers (BI)	addition of BI (%)	DON	DON-3-Glc	DON-3-Glc/DON	DON	DON-3-Glc	DON-3-Glc/DON	DON	DON-3-Glc	DON-3-Glc/DON	DON	DON-3-Glc	DON-3-Glc/DON
white flour	0	561	73	8.4	550	79	9.3	547	87	10.3	624	81	8.4
BI 1	1	547	99	11.7	584	116	12.8	580	121	13.5	786	123	10.1
	3	590	106	11.6	578	106	11.8	629	117	12.0	698	111	10.3
	5	591	101	11.0	474	69	9.4	588	106	11.6	677	102	9.7
BI 2	1	598	109	11.8	531	116	14.1	510	137	17.4	770	120	10.1
	3	526	105	12.9	517	110	13.8	502	117	15.0	709	107	9.8
	5	518	95	11.8	528	98	12.0	542	109	13.0	756	102	8.7

[a] White flour used for experiments with BI.

dx.doi.org/10.1021/jf202428f | J. Agric. Food Chem. 2011, 59, 9303–9312

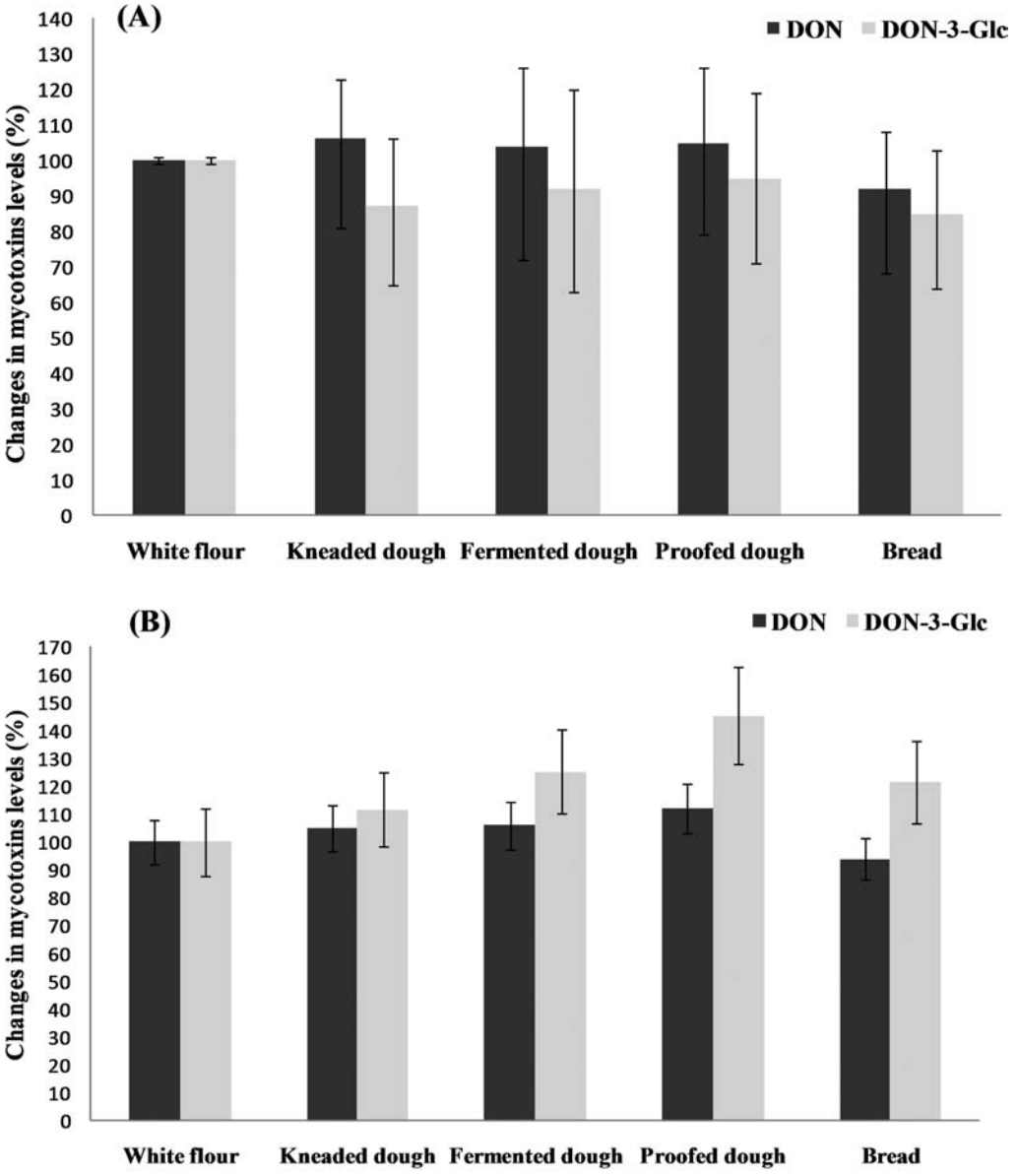

Figure 2. Transfer of DON and DON-3-Glc from flour to final bread. (A) General trends for naturally infected samples obtained from conventional and organic farming; (B) general trends for artificially infected Akteur flour with bakery improvers addition.

assumption, we hypothesized whether the addition of so-called bread improvers (enzyme mixtures commonly used in the bread making for better dough rheological parameters and quality of bakery products) influence DON and DON-3-Glc levels. In a series of experiments 1%, 3%, and 5% (w/w) of two different bakery improvers (BI1, BI2) were added to flour. The obtained results are summarized in Table 2 and illustrated in Figures 2 and 3. While the addition of bread improvers did not result in significant changes in DON content during fermentation process, a significant increase of conjugated DON-3-Glc (145% in average) was observed in proofed dough containing enzyme-based preparation. Although some decrease of DON-3-Glc in bread occurred due to thermal degradation, the total content in bread was higher, 120% of that in product prepared from the same but unfortified flour. Unfortunately, the specific composition of bread improvers was not available, the only information we obtained was that the glycolytic enzymes were contained. Considering the fact that DON content was not influenced (bond between DON and glucose unit in DON-3-Glc remained intact), splitting of α-glycosidic bonds between DON-3-Glc and cell polysaccharides probably occurred. It should be noted that the release of DON-3-Glc within brewing and baking technologies initiated a follow-up study which documented that, in addition to this DON-monoglucoside, also DON-oligo-glucosides are contained in fermented intermediates as well as in final products such, bread and malt/beer (unpublished results).

DON and DON-3-Glc Thermal Degradation during Baking. To get more detailed insight into DON and DON-3-Glc changes under heat processing conditions, we conducted a series of experiments aimed at explanation of a drop of both analytes that was observed in the final phase of baking process (Figure 2). Regarding DON, we presumed, based on study published earlier, [17,18] that norDONs A−F and/or DON lactones might be the main degradation products. To confirm this assumption, a

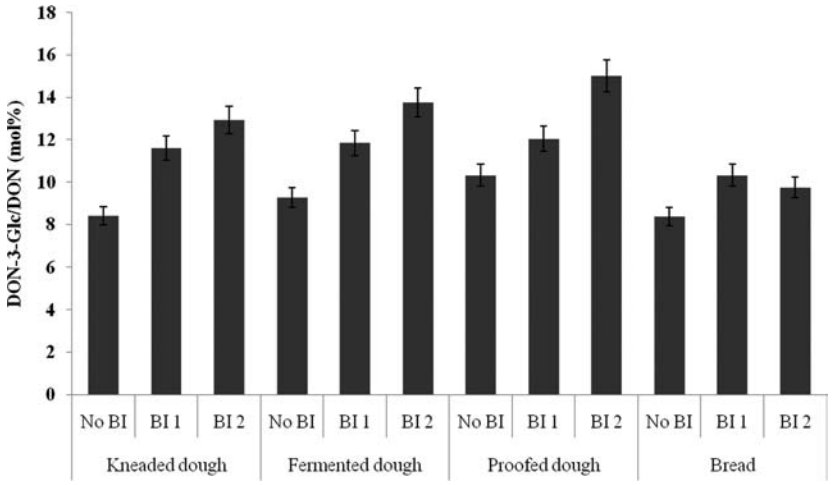

Figure 3. Changes in relative molar ratios of DON-3-Glc/DON within baking experiments with and without addition of bakery improvers: 3% addition of BI1 and BI2 to flour, Akteur, artificial inoculation.

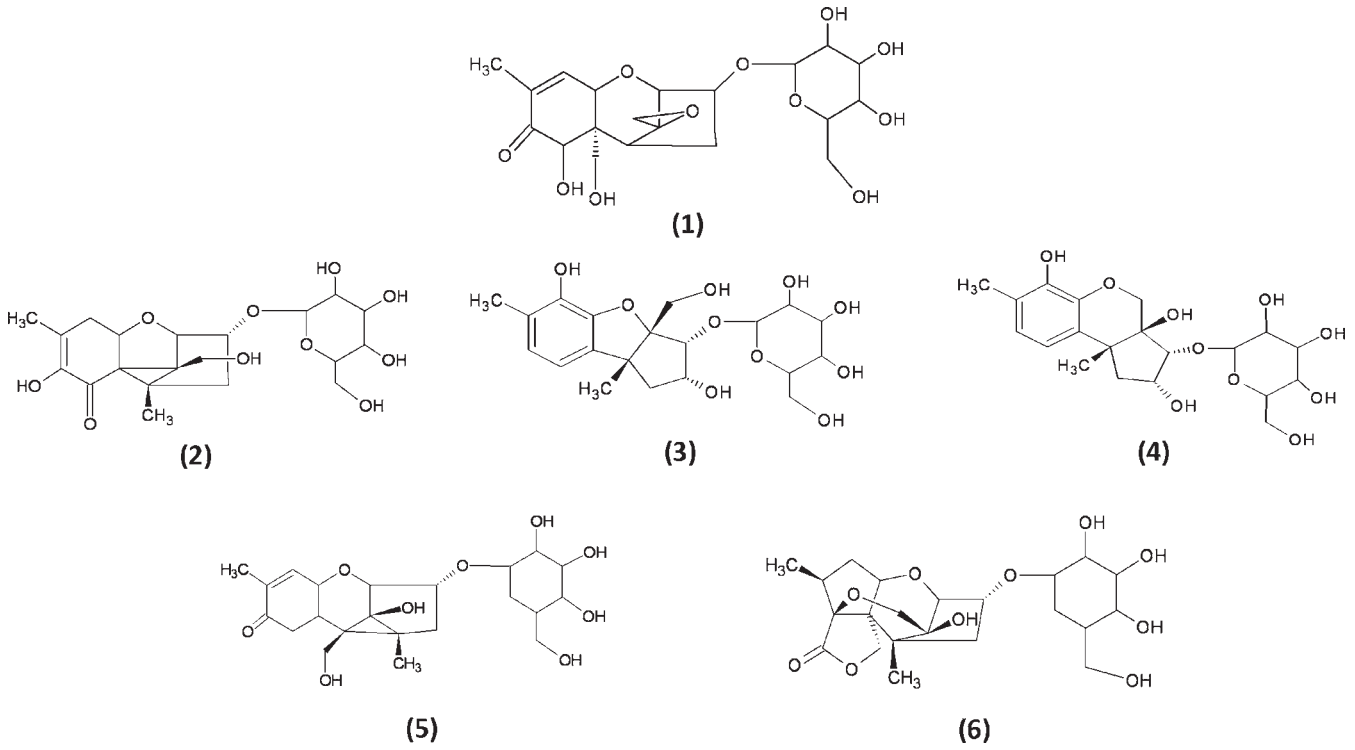

Figure 4. Structures of DON-3-Glc (**1**) and its thermal degradation products norDON-3-Glc A (**2**), norDON-3-Glc B (**3**), norDON-3-Glc C (**4**), norDON-3-Glc D (**5**), and DON-3-Glc lactone (**6**).

range of degradation experiments was conducted. On the basis of extraction of exact masses and considering LC elution order reported in the quoted study, all conceivable norDONs and DON-lactone (Figure 5) were detected and tentatively identified in model samples obtained by the thermal degradation (160 °C, 30 min) of DON analytical standard.

Only some of these compounds, presumably norDON A, B, and C, were found in thermally treated contaminated wheat and bread prepared thereof. As expected, DON thermodegradation products occurred only in bread crust that is exposed to higher temperatures; in crumb, where temperature during baking typically does not exceeded 85 °C, no changes of DON took place. Figure 5 shows UPLC-Orbitrap S chromatogram obtained by analysis of the bread crust extract. The reduction of DON content in this part was estimated to be 10%. Because of the lack of the commercial analytical standards, we were unable to accurately quantitate the content of degradation products.

In the next series of similar experiments, we focused on DON-3-Glc thermal degradation, which has never been studied before. In Figure 6, there are shown UPLC-Orbitrap MS chromatograms of thermal degradation products of DON-3-Glc, which were obtained from analytical standard subjected to heating at 160 °C for 30 min. Accurate masses of the most intense peaks together with estimated elemental formulas of respective compounds are

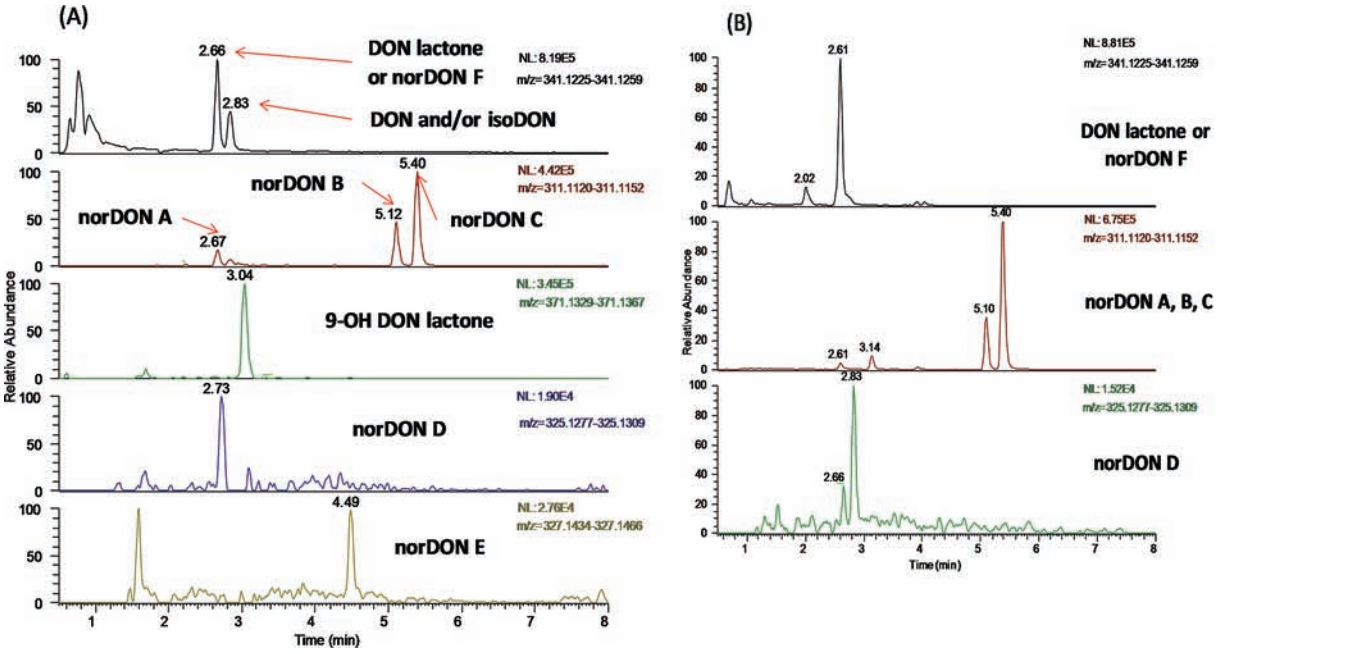

Figure 5. UPLC-(HESI-)Orbitrap MS chromatogram of DON thermal degradants in: (A) heated analytical standard and (B) real bread sample (only chromatograms of positive degradants are shown), resolving power 100000 fwhm, extraction window 10 ppm.

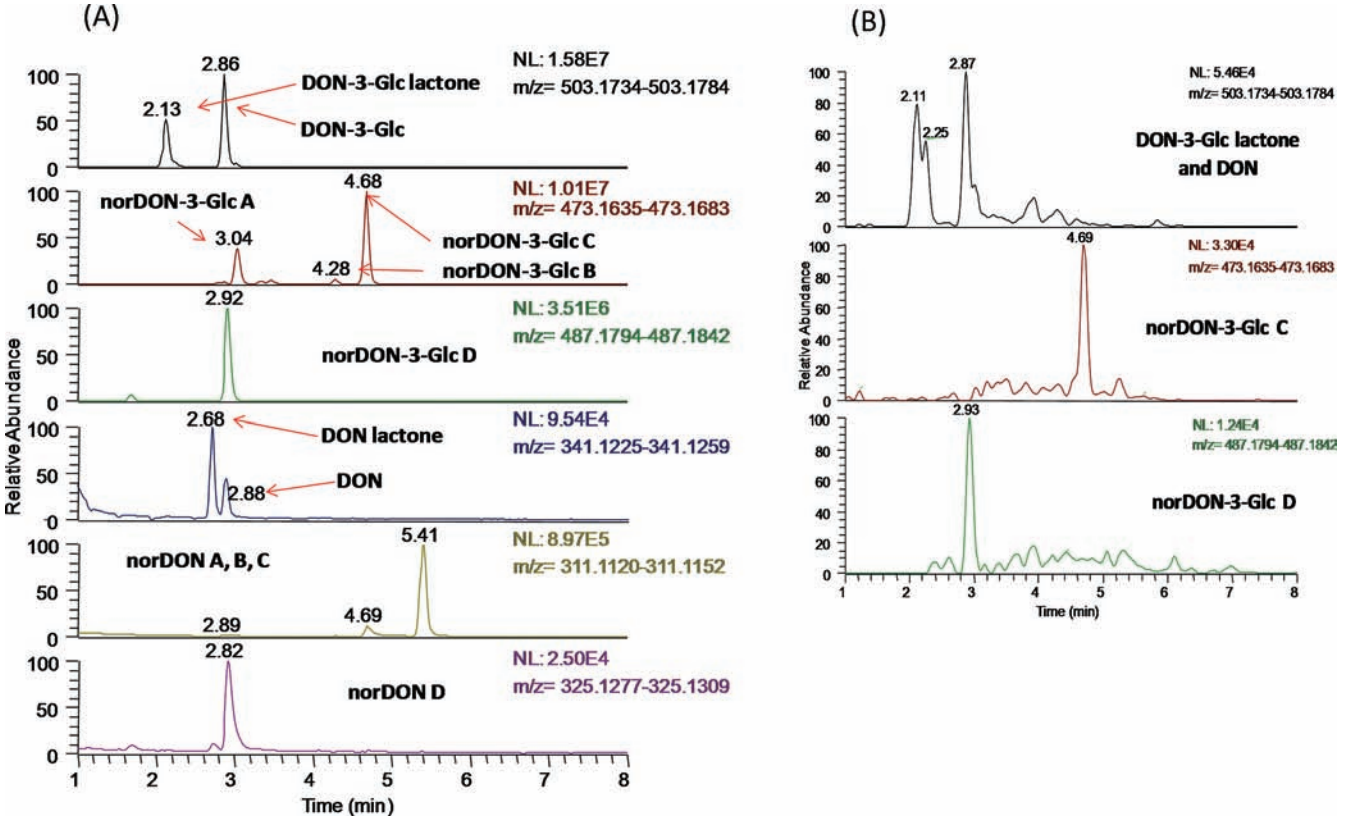

Figure 6. UPLC-(HESI-)Orbitrap MS chromatogram of DON-3-Glc thermal degradants in: (A) heated analytical standard and (B) bread sample (only chromatograms of positive degradants are shown), resolving power 100000 fwhm, extraction window 10 ppm.

summarized in Table 3. In Figure 4, the overview of proposed DON-3-Glc thermal degradation products is presented. It is noteworthy that these structures were proposed based on previous results.[18] Signals of the same compounds were detected also in heat-treated samples of contaminated wheat. In total, five compounds named norDON-3-Glc A, B, C, and D and DON-3-Glc

Table 3. Overview of the Most Intense Ions and Their Mass Errors (in Bold) and other Confirmation of DON and DON-3-Glc Degradants Identified by OrbitrapMS

	retention time (min)	elemental formula	M	$[M - H]^{-a}$	$[M + Cl]^{-a}$	$[M + HCOO]^{-a}$	mass error[b] (ppm)
DON Degradants							
DON	2.83	C15H20O6	296.1259	295.1187	331.0954	**341.1242**	**1.307**
DON lactone	2.66	C15H20O6	296.1259	295.1187	331.0954	**341.1242**	**2.154**
norDON A, B, C	2.67/5.12/5.40	C14H18O5	266.1154	265.1081	301.0848	**311.1136**	**2.091**
norDON D	2.73	C15H20O5	280.1310	279.1238	315.1005	**325.1293**	**3.170**
nor DON E	4.49	C15H22O5	282.1467	281.1395	317.1161	**327.1449**	**3.245**
norDON F	2.83	C15H20O6	296.1259	295.1187	331.0954	**341.1242**	**3.549**
9-OH DON lactone	3.04	C16H22O7	326.1365	325.1293	361.1060	**371.1348**	**3.479**
DON-3-Glc Degradants							
DON-3-Glc	2.85	C21H30O11	458.1788	457.1715	493.1482	**503.1770**	**0.443**
DON-3-Glc lactone	2.13	C21H30O11	458.1788	457.1715	493.1482	**503.1770**	**0.562**
norDON-3-Glc A, B, C	3.02/4.27/4.66	C20H28O10	428.1682	427.1610	463.1377	**473.1665**	**0.544**
norDON-3-Glc D	2.89	C21H30O10	442.1838	441.1766	477.1533	**487.1821**	**1.780**
DON lactone	2.66	C15H20O6	296.1259	295.1187	331.0954	**341.1242**	**2.099**
norDON A, B, C	2.67/5.12/5.40	C14H18O5	266.1154	265.1081	301.0848	**311.1136**	**3.506**
norDON D	2.73	C15H20O5	280.1310	279.1238	315.1005	**325.1293**	**2.893**

[a] Exact masses calculated for respective ion types. [b] Values calculated for experimental m/z of detected $[M + HCOO]^-$ ions.

lactone were also later identified in the samples of tested bread. In addition to DON-3-Glc degradation products, also DON degradation products (again mainly norDON A, B, and C) were detected in the model solution of heat-treated DON-3-Glc. This finding indicates that bond between DON and glucose unit can be cleaved during heat-treatment.

A wide range of laboratory-scale experiments were carried out to monitor the fate of not only DON but also DON-3-Glc within the milling and bread making processes. Analyses of wheat milling fractions showed that DON and DON-3-Glc are fractionated into white flour and bran in a similar way, down to 62% and 60%, respectively, compared to their content in unprocessed grains. In the final bread, contents of DON and DON-3-Glc were 87% and 90%, respectively, as compared to their amount in starting white flour. The decrease occurred mainly in crust (8% of bread, w/w) in which highest temperature was achieved. The addition of glycolytic enzyme mixtures (bakery improvers) during dough making resulted in DON-3-Glc increase, up to 145% of its content in flour taken for bread making. It is assumed that this was due to the release of the key DON conjugate from bonded forms to a starch-based matrix. DON levels were not influenced by the presence of bakery improvers which typically possess only α-glycolytic activity. In other words, splitting of β-glycosidic bond in DON-3-Glc does not seem to be probable. A wide range of DON and DON-3-Glc thermo degradants was identified by UPLC-Orbitrap MS, both in heated model samples and real-life breads. The products of DON-3-Glc thermal breakdown products norDON-3-Glc A, B, C, D and DON-3-Glc lactone were characterized for the very first time. In addition to these glucose containing degradants, also norDON A, B, C, and D, typical for DON thermodegradation, originated from DON-3-Glc.

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Funding Sources

This research was financially support by Ministry of Education, Youth and Sports of the Czech Republic as a part of national project NPV II 2B08049 and NPV II QI111B154.

ABBREVIATIONS USED

ADONs, Sum of 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol; APCI, atmospheric pressure chemical ionization; BI, bakery improver; DON, deoxynivalenol; DON-3-Glc, deoxynivalenol-3-ss-D-glucopyranoside, DON-3-glucoside; EC, European Commission; FAPAS, Food Analysis Performance Assessment Scheme; fwhm, full width at half-maximum; HESI, heated electrospray; HPLC, high performance liquid chromatography; LOQ, limit of quantitation; MS, mass spectrometry; RPM, rotations per minute; SCF, scientific committee on food; TDI, tolerable daily intake; UPLC, ultra performance liquid chromatography

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