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Distribution of Deoxynivalenol and Nivalenol in Milling Fractions from *Fusarium*-Infected Japanese Wheat Cultivars

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ABSTRACT

The fate of the *Fusarium* mycotoxins deoxynivalenol and nivalenol during the milling of Japanese wheat cultivars artificially infected with *Fusarium* was investigated. Grain samples with different mycotoxin concentrations were milled using a laboratory-scale test mill to produce eight fractions: three breaking flours (1B, 2B, and 3B), three reduction flours (1M, 2M, and 3M), wheat bran, and wheat shorts. Patent flour for human consumption was made from the 1B, 2B, 1M, and 2M flours, and low-grade flour was made from 3B and 3M flours. The four resulting samples (patent flour, low-grade flour, bran, and shorts) were analyzed for deoxynivalenol and/or nivalenol with an in-house validated analytical method using high-performance liquid chromatography with UV absorbance detection. In samples with different mycotoxin concentrations, the distribution of those toxins differed among the milling fractions. Grains with a lower level of contamination produced bran and shorts samples with a high relative concentration of nivalenol. A high percentage of nivalenol was found in patent flour, followed by bran. Contrary to the less-contaminated sample, the concentration of nivalenol in moderately contaminated grain was high only in the shorts sample. The highest percentage of deoxynivalenol and nivalenol was observed in the patent flour. The results of this study indicate that the distribution of deoxynivalenol and nivalenol in milled Japanese wheat could be influenced by the contamination level of the original grain, and the milling process is not always effective for removal of toxins from wheat grains.

Wet and temperate weather during and after flowering of grain plants often results in scab or Fusarium head blight, a disease caused by fungi of several *Fusarium* species. Fusarium head blight is a worldwide disease of wheat and other small grain cereals and causes two forms of damage: a reduction in the harvest because of shriveled grains and a threat to food safety via contamination of grains by mycotoxins *(1)*. One of these mycotoxins, deoxynivalenol (DON), which belongs to the type B trichothecenes, is considered to be the most important contaminant of wheat grain worldwide. The intake of DON by farm animals leads to feed refusal, reduced weight gain, and vomiting. Numerous reports have been published on the toxicity of DON in animals, which causes different symptoms depending on the animal species *(3, 15)*.

Another type B trichothecene, nivalenol (NIV), is an analog of DON and often co-occurs with DON (Fig. 1). Currently, regulatory values are set for only DON in many countries because of its worldwide occurrence (including in North America), but no regulatory limits have been placed on NIV contamination. In North America, NIV is not considered as important a hazard as is DON. In contrast, a higher occurrence of NIV than of DON was reported in Europe and Asia *(10, 13)*. The differences in the occurrence of these trichothecene analogs can be derived from the geophysical distribution of *Fusarium* species. In Kyushu (one of the major districts of wheat product in western Japan), *Fusarium asiaticum*, which produces more NIV than DON, is widely distributed *(12)*. Therefore, both DON and NIV are important contaminants in wheat grains in Europe and Asia, including Japan.

Because *Fusarium* is a typical field fungus, numerous field studies have been conducted to forecast the day of flowering based on weather condition, to obtain resistant cultivars by selection, and to screen for more efficient fungicides. With the aim of controlling *Fusarium* mycotoxin contamination, researchers have collected much information on the occurrence and prevention of *Fusarium* contamination in the field *(2)*. However, information about the retention of *Fusarium* mycotoxins after harvest and during processing is very limited and has been focused only on DON. Because most wheat is converted to wheat flour for human consumption, the milling process is a crucial point for controlling the concentrations of both DON and NIV. Most previously obtained data regarding DON retention

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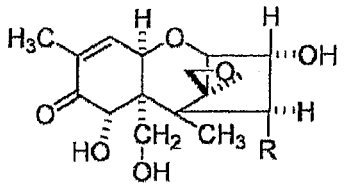

FIGURE 1. *Chemical structures of deoxynivalenol (DON) and nivalenol (NIV).*

during milling of North American and European wheat cultivars suggested that DON contamination was exclusively restricted to the outer layers of grain: bran and shorts *(4, 8, 9, 11, 16, 17, 19)*. However, little information is available on the retention of DON and NIV in Asian wheat cultivars during milling, and no internationally validated simultaneous analytical methods for quantification of DON and NIV in wheat grains and their resulting milling fractions have been described.

In this study, we used a laboratory scale test mill to mill samples of Japanese wheat grains with different DON and NIV concentrations. Milling was followed by the preparation of four types of samples: patent flour (standard grade flour), low-grade flour, wheat bran, and wheat shorts. The DON and NIV concentrations of each sample type were analyzed using an in-house validated analytical method that included high-performance liquid chromatography with UV absorbance detection (HPLC-UV). The effects of milling on the DON and NIV concentrations was determined.

MATERIALS AND METHODS

Wheat samples. Wheat grains of two Japanese wheat cultivars, Chikugoizumi and Minaminokaori, were provided. The grains were planted, and the plants were inoculated with *Fusarium graminearum* and harvested in Kumamoto, Kyushu, Japan in 2008. A mixture of two isolates of *F. graminearum* was used for inoculation: MAFF101551 (a DON-producing type strain) and MAFF240548 (an NIV-producing type strain) (Genebank, National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan). Preparation of macroconidial suspension stock and spray inoculation of the suspension onto wheat plants were performed as described previously *(18)*. Chikugoizumi is a cultivar for noodle making and Minaminokaori is used for bread making. The grains were combined, cleaned, and stored at 4°C until milling.

Milling and flour preparation. Cleaned grains (3 to 5 kg) from the inoculated wheat plants were tempered to 14.5 to 16.0% water content depending on the cultivar. Milling was performed with a laboratory mill (model MLU-202, Bühler AG, Uzwil, Switzerland) equipped with three break rolls and three middling (reduction) rolls to produce eight fractions: 1B, 2B, 3B, 1M, 2M, 3M, bran, and shorts (Fig. 2). Milling conditions such as the spacing of the rolls and milling speed were based on the manufacturer's instructions. Each fraction was collected separately and mixed to homogeneity. Patent flour, which is for human consumption, was made from mixing 1B, 1M, 2B, and 2M fractions, and low-grade flour was made from mixing 3B and 3M fractions. Patent flour, low-grade flour, bran, and shorts were stored at 4°C.

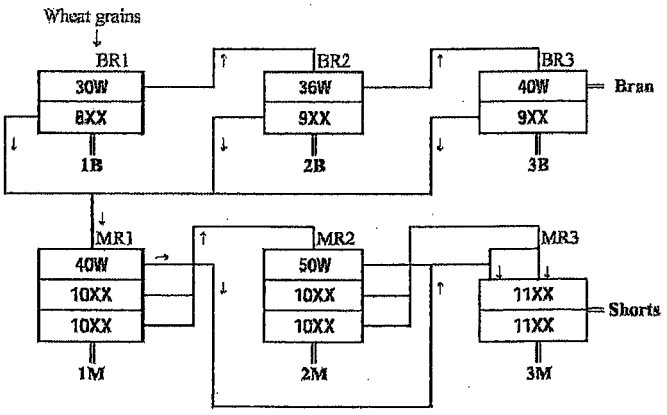

FIGURE 2. *Milling diagram of a Japanese wheat cultivar Chikugoizumi on a Bühler laboratory mill. BR, break roll; MR, middling (reduction) roll.*

Moisture content analysis. Moisture content was analyzed by heating the samples under atmospheric pressure using a standard protocol *(6)*.

Chemicals and standards. Standard DON and NIV were purchased from Wako Co. (Osaka, Japan). All other reagents were of analytical or HPLC grades. Stock solutions of analytes (10 to 50 μg/ml each) were prepared in acetonitrile and diluted to the desired concentration in acetonitrile-methanol-water (1:1:18). DON and NIV stocks and standard working solutions were kept for up to 6 months at 4°C.

Sample extraction and cleanup. Analysis was done in triplicate ($n = 3$) (replicates from weighing and extraction). An accurately weighed 10.0 g of ground whole grain or each milling preparation served as an analytical sample. Each analytical sample was weighed in a 300-ml Erlenmeyer flask with a ground-in stopper, and DON and NIV were extracted with 40 ml of acetonitrile-water (85:15) by shaking for 60 min on a laboratory shaker (TAITEC, Saitama, Japan). Ten milliliters of the supernatant was applied to a MF-T1500 multifunctional column (Showa Denko, Tokyo, Japan) without preconditioning. The first 3 ml of eluate was discarded, and the following 5 ml was collected in a new test tube. A 2.0-ml aliquot of the collected solution was evaporated at 40°C under a gentle flow of nitrogen. The residue was redissolved in 0.5 ml of acetonitrile-methanol-water (1:1:18) and served as the sample solution for direct injection into the HPLC-UV apparatus. When the DON and/or NIV concentration was outside of the calibration curve, the sample was diluted or concentrated appropriately.

Spike and recovery test. Samples in which the DON and/or NIV concentration was below the limit of detection (LOD) were tested in triplicate ($n = 3$) (replicates from weighing and extraction). To spike samples, 0.2, 0.5, or 1.0 ml of standard DON and NIV solution (10 μg/ml each in acetonitrile) was added with shaking for 5 min. Final spiked levels were 0.2, 0.5, or 1.0 mg/kg because of the 1.0-fold dilution factor (40.0/10.0 [at extraction] × 0.5/2.0 [at purification] = 1.0). Five minutes after the addition of standard solution, the extraction solvent was added, and the extraction and clean-up procedures were conducted as described above. Recovery was calculated using the following equation:

$$\text{recovery } (\%) = [(A - B)/S] \times 100$$

where A is the signal of the spiked sample, B is the signal of the blank sample, and S is the spiked level.

TABLE 1. *Recovery of toxins spiked into uncontaminated Chikugoizumi wheat*

Sample	Spiked toxin	Spiked amt (mg/kg)	Recovery (%) Mean	RSD
Grain	DON	0.2	107.0	5.9
	DON	0.5	96.0	3.7
	DON	1.0	100.3	1.2
	NIV	0.2	103.5	4.4
	NIV	0.5	79.5	3.6
	NIV	1.0	80.7	2.2
Patent flour	DON	0.2	107.8	4.9
	DON	0.5	106.5	1.7
	DON	1.0	106.3	1.4
	NIV	0.2	83.2	4.3
	NIV	0.5	87.5	4.9
	NIV	1.0	79.9	2.2
Bran	DON	0.2	103.0	1.3
	DON	0.5	94.1	3.1
	DON	1.0	94.0	2.5
	NIV	0.2	80.0	1.3
	NIV	0.5	73.7	1.5
	NIV	1.0	72.1	1.2
Shorts[a]	DON	0.2	123.0	
	DON	0.5	115.8	
	DON	1.0	100.0	
	NIV	0.2	108.5	
	NIV	0.5	92.2	
	NIV	1.0	77.1	
Low-grade flour[a]	DON	0.2	106.0	
	DON	0.5	93.0	
	DON	1.0	95.7	
	NIV	0.2	98.5	
	NIV	0.5	81.2	
	NIV	1.0	90.1	

[a] RSD was not applicable for shorts and low-grade flour because only one measurement was obtained per spiking level.

TABLE 2. *Recovery of toxins spiked into uncontaminated Minaminokaori wheat*

Sample	Spiked toxin	Spiked amt (mg/kg)	Recovery (%) Mean	RSD
Grain	DON	0.2	95.7	0.6
	DON	0.5	108.8	3.4
	DON	1.0	100.3	1.9
	NIV	0.2	104.3	2.8
	NIV	0.5	91.1	0.8
	NIV	1.0	104.0	0.3
Patent flour	DON	0.2	102.2	1.1
	DON	0.5	95.5	2.1
	DON	1.0	92.8	1.1
	NIV	0.2	82.0	0.6
	NIV	0.5	82.9	0.8
	NIV	1.0	82.4	1.6
Bran	DON	0.2	105.7	6.9
	DON	0.5	97.6	0.7
	DON	1.0	99.1	1.6
	NIV	0.2	102.8	2.0
	NIV	0.5	88.4	0.5
	NIV	1.0	82.7	1.1
Shorts[a]	DON	0.2	120.0	
	DON	0.5	109.2	
	DON	1.0	100.0	
	NIV	0.2	98.5	
	NIV	0.5	84.0	
	NIV	1.0	80.1	
Low-grade flour[a]	DON	0.2	116.5	
	DON	0.5	102.4	
	DON	1.0	98.0	
	NIV	0.2	96.5	
	NIV	0.5	87.0	
	NIV	1.0	84.0	

[a] RSD was not applicable for shorts and low-grade flour because only one measurement was obtained per spiking level.

Apparatus and determination condition for HPLC-UV analysis. An LC1100 series HPLC system connected to a photodiode array detector (Agilent Technologies, Santa Clara, CA) was used, and data were acquired and integrated using ChemStation software (Agilent). Isocratic HPLC conditions were adopted at a mobile phase of methanol-water (1:4). A C_{18} L-column (250 by 4.6 mm inside diameter, 5 μm spherical particle size; CERI, Tokyo, Japan) was used at 40°C, and the injection volume was 5 μl. The detector was set at a wavelength of 220 nm.

Calibration curves were based on the analysis of standard working solutions in the range 0.05 to 1.0 μg/ml (0.05, 0.1, 0.2, 0.5, and 1.0 μg/ml) for DON and NIV. The LOD was defined as the concentration that was three times higher than the standard deviation of the blank signal, i.e., 0.04 mg/kg for DON and 0.03 mg/kg for NIV. The limit of quantification was defined as twice the LOD.

Statistical analysis. For concentration data of DON and NIV, an analysis of variance using the milling fraction as a variable was performed using SPSS software (SPSS, Chicago, IL). Tukey's multiple range test was used to test for significant differences for each variable.

RESULTS AND DISCUSSION

In-house validation of DON and NIV analysis: spike and recovery tests. The recovery of DON and NIV spiked into wheat grains and milling fractions are shown in Tables 1 and 2. Recovery values are shown as the average value of triplicate data with relative standard deviation (RSD) except for shorts and low-grade flour, which could not be obtained in sufficient amounts for triplicate analysis. A tentative limit for wheat grains in Japan has been set only for DON, at 1.1 mg/kg. Therefore, spiking concentrations were set at 0.2, 0.5, and 1.0 mg/kg, which reflect one-fifth, one-half, and the same as the tentative limit, respectively.

The toxin recoveries in Chikugoizumi wheat, a cultivar for noodle making, were 72.1 to 123.0% (Table 1). All recovery percentages, except those for shorts spiked with 0.2 mg/kg DON, were between 70 and 120%, the range generally accepted as an in-house validation of an analytical method for spike and recovery tests. Although the percentage for shorts spiked at 0.2 mg/kg DON was out of the accepted range, the difference was slight (123.0%)

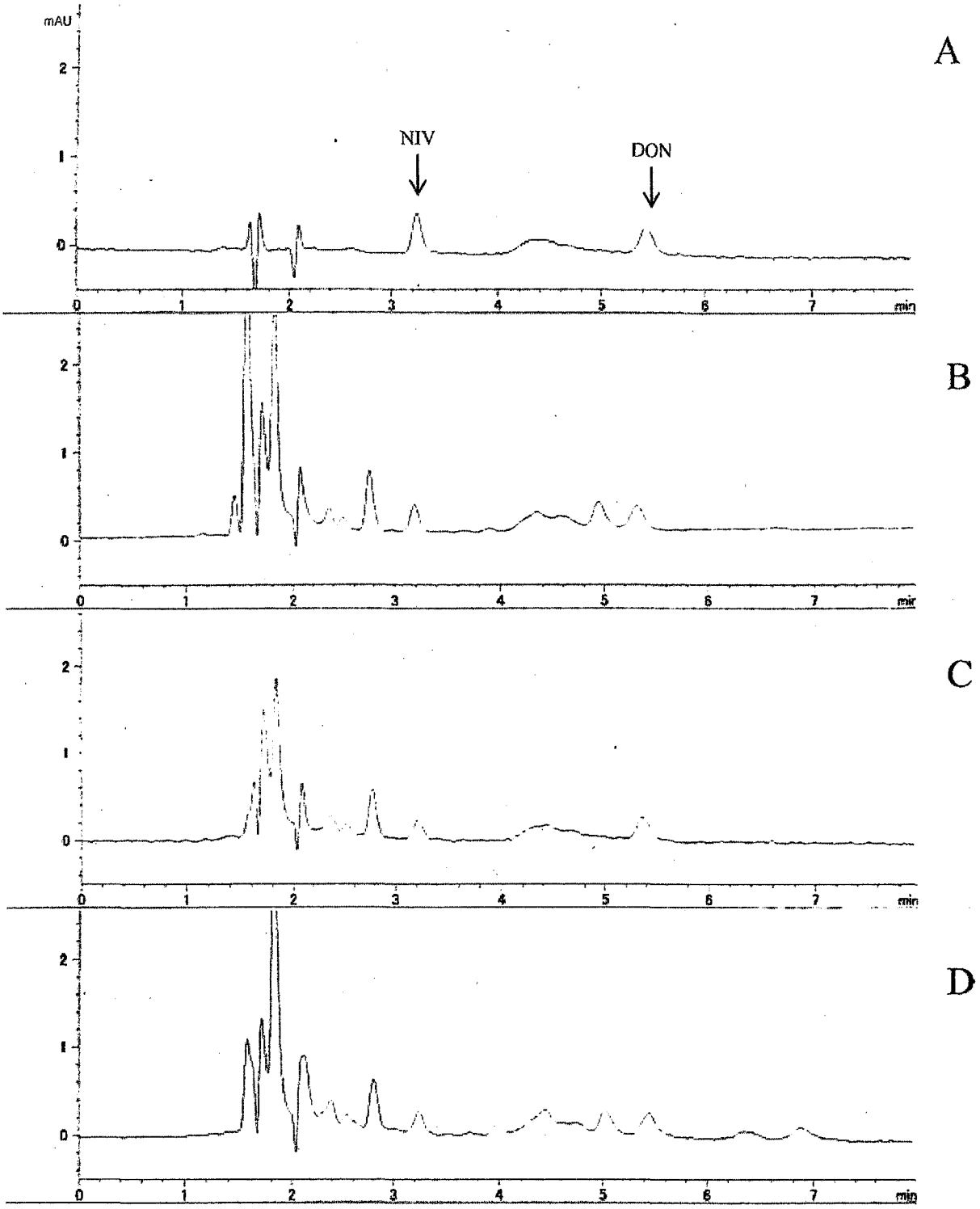

FIGURE 3. *HPLC-UV chromatogram of a Chikugoizumi grain with a medium level of contamination. (A) Standard NIV and DON; (B) extract of original grain; (C) extract of patent flour; (D) extract of bran.*

and it was not considered important for practical purposes. The RSDs of DON recovery in wheat grain from samples spiked at 0.2, 0.5, and 1.0 mg/kg were 5.9, 3.7, and 1.2%, respectively. For NIV in wheat grain, the RSDs of recovery from spiking at 0.2, 0.5, and 1.0 mg/kg were 4.4, 3.6, and 2.2%, respectively. For DON and NIV in patent flour and bran, the recovery values also were stable, with low RSDs.

Table 2 shows the toxin recovery data for Minamino-kaori wheat, a cultivar for bread making. All values were between 80.1 and 120.0%, i.e., within the 70 to 120% acceptable range for in-house validation of the analytical method. The RSDs of DON recovery in wheat grain from samples spiked at 0.2, 0.5, and 1.0 mg/kg were 0.6, 3.4, and 1.9%, respectively. For NIV in wheat grain, the RSDs of recovery for spiking at 0.2, 0.5, and 1.0 mg/kg were 2.8, 0.8, and 0.3%, respectively. As for Chikugoizumi, the recovery values for DON and NIV in patent flour and bran also were stable, with low RSDs.

These results indicate that the simultaneous analytical method for DON and NIV in both cultivars is applicable for

TABLE 3. *Milling properties of Chikugoizumi wheat with a low level of contamination and the distribution of NIV in flours*

Sample	Wt		Concn (mg/kg)[a]	Relative concn (%)[b]	Mass balance (mg/kg)[c]	Distribution (%)
	g	%				
Original grain			0.297 ± 0.0066	100 A		
Patent flour	2,432	67.3	0.132 ± 0.0003	44 B	0.089	34.0
Low-grade flour	109	3.0	0.148 ± 0.0029	50 B	0.004	1.7
Bran	977	27.0	0.541 ± 0.0170	182 C	0.146	56.0
Shorts	94	2.6	0.837 ± 0.0648	282 D	0.022	8.3
Total	3,612	100.0			0.261	100.0

[a] Values are mean ± standard deviation ($n = 3$).
[b] Among sample types, values with different letters are significantly different ($P < 0.05$) by Tukey's multiple range test.
[c] Mass balance: [weight (%) × concentration (mg/kg)].

the practical analysis of wheat grain and milling preparations, including bran (Fig. 3).

Distribution of NIV and DON in the milling fractions of Japanese wheat. In this study, we used a laboratory mill (Bühler model MLU-202; Fig. 2) that contains basic components for milling wheat and mimics industrial scale mills without a purifier; this mill has been widely used in previous studies *(7)*. The concentration of NIV and DON in original grains and in milling fractions ($n = 3$) is shown in Tables 3 through 5 with the yield of each flour fraction.

Tables 3 and 4 describe the results of the wheat samples from the same cultivar, Chikugoizumi, with two different levels of NIV contamination. The sample with a low level of contamination contained NIV at 0.297 mg/kg. The sample with a medium level of contamination contained NIV at 0.722 mg/kg and DON at 0.896 mg/kg; this DON concentration is near to 1.1 mg/kg, the tentative regulatory limit of DON in Japan. No differences in milling properties were observed between the grains with the different toxin concentrations; they had very similar weight percentages for the four preparations, patent flour, low-grade flour, bran, and shorts. In contrast, the distribution of toxin was considerably different among the preparations. The percentage of NIV was twofold higher in patent flour made from grain with a medium level of contamination (65.3%, Table 4) than that in the patent flour made from grain with a low level of contamination (34.0%, Table 3). The concentration of NIV was high in bran and shorts from grain with a low level of contamination, with two- and threefold higher values than in the original grain, respectively (Table 3). For grain with a medium level of contamination, a particularly high concentration of NIV and DON was observed only in shorts (Table 4). In previous studies, high concentrations of DON were detected in the outer layers of grain (bran and shorts) *(4, 7–9, 11, 16, 17, 19)*. Lee et al. *(9)* conducted a milling study using Korean wheat grains, presumably from Asian wheat cultivars, and reported that the concentrations of DON and NIV in bran and shorts were about twofold higher than those in raw wheat. Thus, our result obtained from Chikugoizumi with a low level of contamination is in agreement with the findings reported by Lee et al., indicating a higher percentage of NIV in outer layers of milling fractions (Table 3). However, the result from Chikugoizumi with a medium level of contamination is not in agreement with the previous findings; a higher percentage of NIV was found only in shorts (Table 4).

We analyzed for the first time the change in NIV and DON concentration during milling using an in-house validated simultaneous quantitative method for DON and NIV (Fig. 3). In Chikugoizumi, the portion of NIV in patent flour was 65.3%, which was similar to that for DON (68.8%) (Table 4). Similar percentages of NIV and DON were also found for the other samples (low-grade flour,

TABLE 4. *Milling properties of Chikugoizumi wheat with a medium level of contamination and the distribution of DON and NIV in flours*

Sample	Wt		Concn (mg/kg)[a]		Relative concn (%)[b]		Mass balance (mg/kg)[c]		Distribution (%)	
	g	%	DON	NIV	DON	NIV	DON	NIV	DON	NIV
Original grain			0.896 ± 0.0157	0.722 ± 0.0092	100 A	100 A				
Patent flour	2,556	66.9	0.860 ± 0.0432	0.555 ± 0.0234	96 AB	77 B	0.575	0.371	68.6	65.3
Low-grade flour	134	3.5	0.798 ± 0.0302	0.567 ± 0.0237	89 AB	79 B	0.028	0.020	3.3	3.5
Bran	1,037	27.1	0.725 ± 0.0977	0.562 ± 0.0458	81 B	78 B	0.197	0.152	23.5	26.8
Shorts	95	2.5	1.556 ± 0.0309	1.012 ± 0.0353	174 C	140 C	0.039	0.025	4.6	4.4
Total	3,822	100.0					0.838	0.569	100.0	100.0

[a] Values are mean ± standard deviation ($n = 3$).
[b] Among sample types, values with different letters are significantly different ($P < 0.05$) by Tukey's multiple range test.
[c] Mass balance: [weight (%) × concentration (mg/kg)].

TABLE 5. *Milling properties of Minaminokaori wheat with a low level of contamination and the distribution of NIV in flours*

Sample	Wt		Concn (mg/kg)[a]	Relative concn (%)[b]	Mass balance (mg/kg)[c]	Distribution (%)
	g	%				
Original grain			0.552 ± 0.007	100 A		
Patent flour	2,633	62.3	0.263 ± 0.0015	48 B	0.164	27.3
Low-grade flour	341	8.1	0.341 ± 0.0135	62 C	0.028	4.6
Bran	1,019	24.1	1.400 ± 0.0458	254 D	0.337	56.2
Shorts	234	5.5	1.292 ± 0.0172	234 E	0.072	11.9
Total	4,227	100.0			0.600	100.0

[a] Values are mean ± standard deviation ($n = 3$).
[b] Among sample types, values with different letters are significantly different ($P < 0.05$) by Tukey's multiple range test.
[c] Mass balance: [weight (%) × concentration (mg/kg)].

bran, and shorts). Moreover, the percentage of NIV and DON was almost identical to that the weight percentage, suggesting that these two toxins accumulated in the whole grain homogeneously in grain with a medium level of contamination. In other words, the behavior of NIV during milling is similar to that of DON in Japanese wheat cultivars with a medium level of contamination. However, this distribution of NIV in grain with a medium level of contamination was different from that found in grain with a low level of contamination. Thus, the distribution of NIV and DON in milling fractions seems to be dependent on the contamination level in the original grains. More milling experiments using various Japanese wheat cultivars will provide a basis for setting a regulatory limit for NIV. Some discrepancies also appeared in the mass balance, e.g., the sum of the DON values in milling fractions (0.833) was less than that in the original grain (0.896) (Table 4). We considered this discrepancy to be derived from the accumulated errors of calculation.

Ríos et al. *(14)* recently reported the presence of DON-producing fungi in all the milling fractions examined, including the inner part of the grain, using DNA primers specific for a DON biosynthesis gene in a European durum wheat cultivar with a high level (4.2 mg/kg) of contamination. This observation was consistent with a previous observation based on microscopy *(5)*. The relationship between the accumulation of DON in endosperm and fungal penetration can be clarified with a quantitative method using marker compounds. Further milling experiments will be useful for identifying the threshold DON concentration in the original grain at which DON accumulates in the whole grain. Investigations in this direction should be carried out using artificially and naturally contaminated wheat samples.

The similar NIV concentration also was observed in Minaminokaori grain with a low level of contamination (Table 5). The Chikugoizumi and Minaminokaori Japanese wheat cultivars are distinctly different in their milling and processing properties. In both cultivars with low levels of contamination, higher relative concentrations of NIV were found in bran and shorts (Tables 3 and 5) in agreement with previous findings reported by Lee et al. *(9)*. The higher percentage of NIV was found in the outer layer milling fractions in the Japanese wheat grains with a low level of contamination, independent of the difference between the cultivars (Tables 3 and 5).

The results of this study indicate that the distribution of NIV and DON in milled Japanese wheat fractions differs depending on the level of toxin contamination. When DON and/or NIV concentrations in the original grains come close to 1 mg/kg, the concentration detected in patent flour fractions seems to be comparable to that in the original grain, as indicated by the statistical analysis shown in Tables 3 through 5. Consequently, milling cannot be completely used as an effective treatment for removal of these toxins from wheat grains.

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