495-502. 2013.

H. 知的所有権の取得状況

１．特許取得　　なし

２．実用新案登録　　なし

３．その他　なし

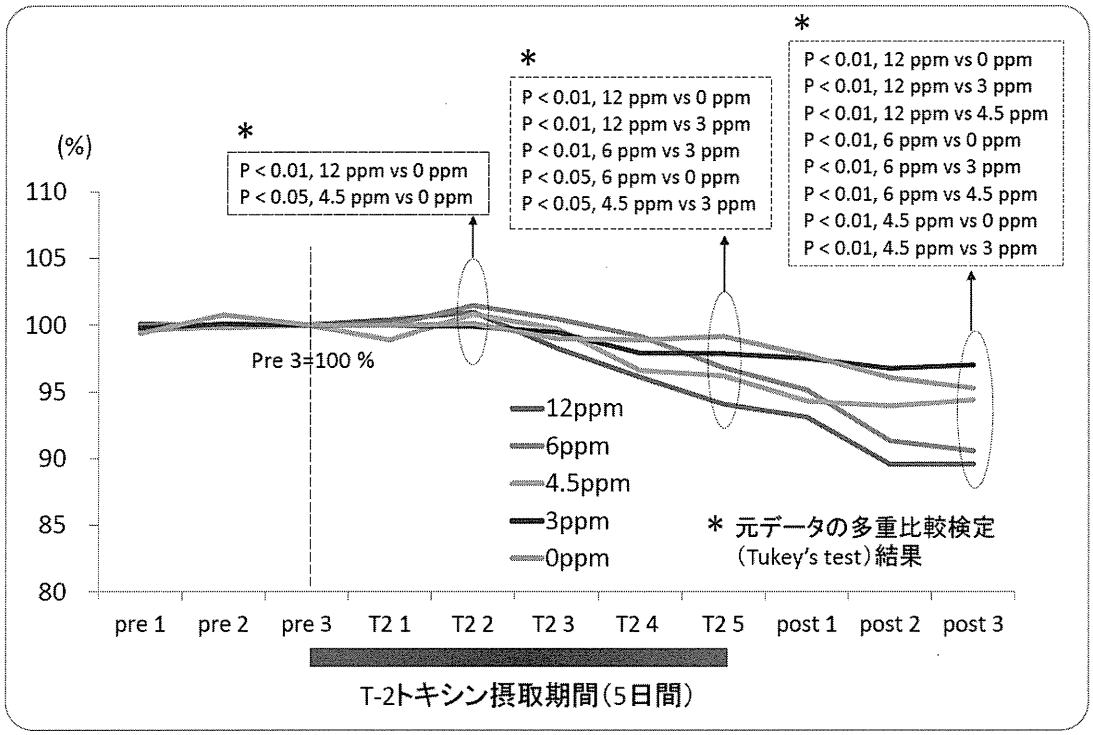

図1．実験を行ったすべての濃度のT-2トキシン群における心拍数の経時的変化（T-2トキシン摂取前日を100％で表示）

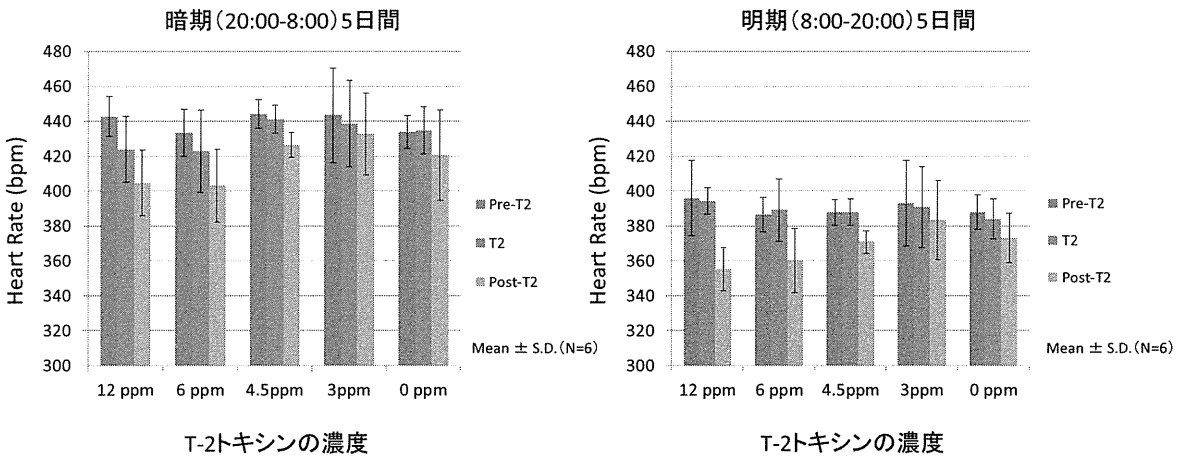

図2．各濃度のT-2トキシン群における暗期（左）および明期（右）の心拍数
各濃度の棒グラフにおいてT-2トキシン摂取前（左）、摂取期間中（中央）、摂取終了後（右）を示す。

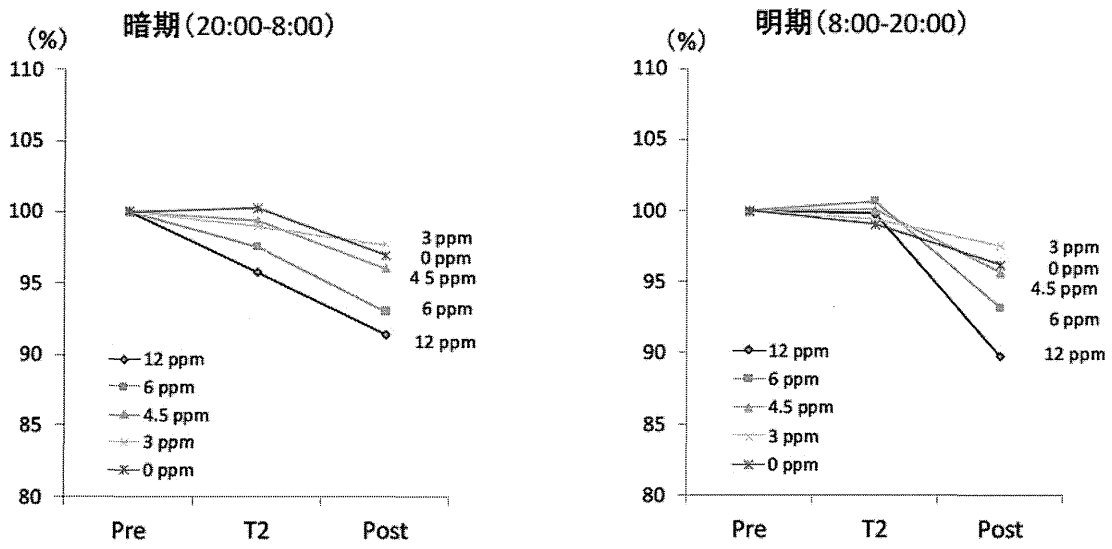

図3．各濃度のT-2トキシン摂取群における暗期（左）および明期（右）における全期間の心拍数の変化　T-2トキシン摂取前の平均値（n=6）を100％として表示。

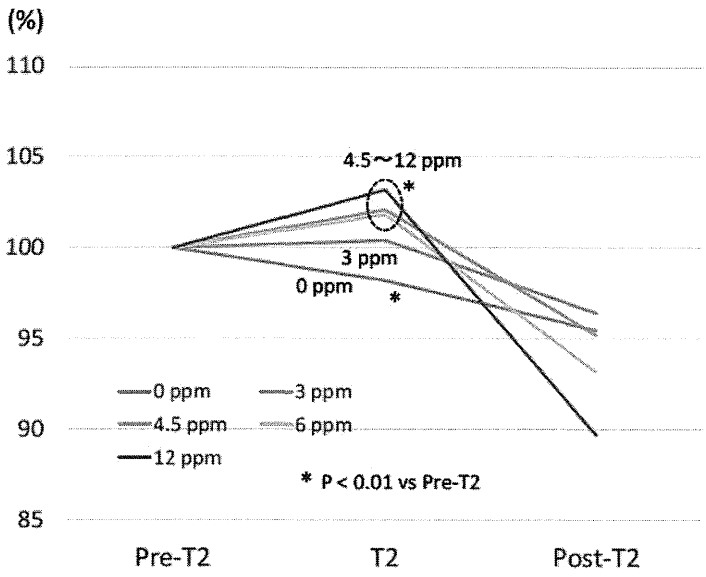

図4．T-2トキシン摂取開始から2日間の明期における平均心拍数の比較
有意差検定（Tukey's test）は元データに基づく

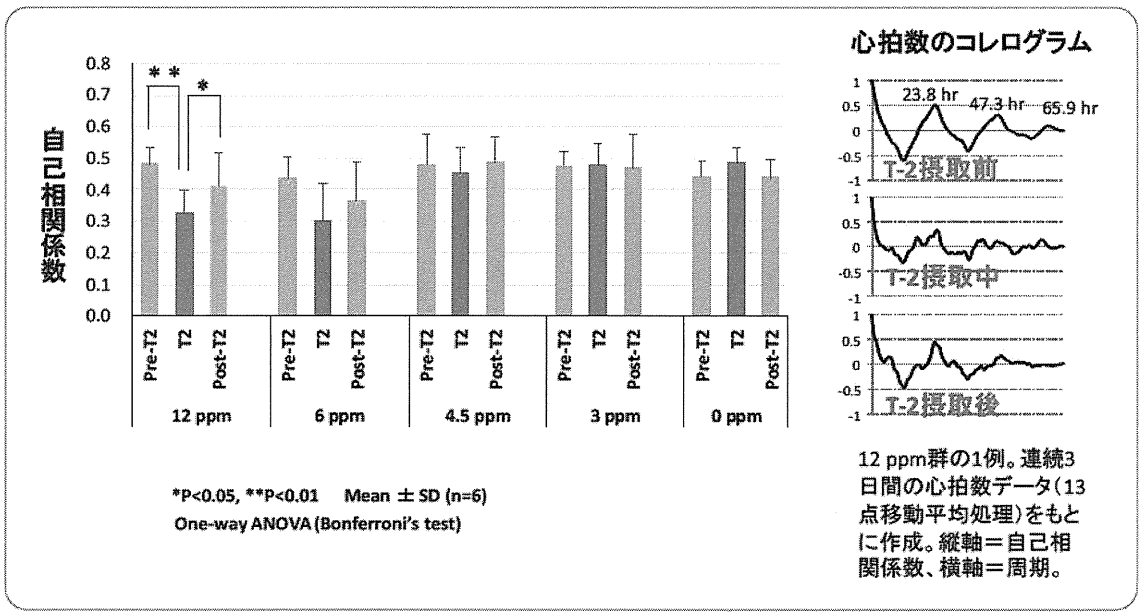

図５．各濃度のT-2 トキシン摂取群における心拍数日周リズムの自己相関係数
自己相関係数はT-2 トキシン摂取前、摂取期間中、摂取終了後のそれぞれ３日間から求めた。

表１．T-2 トキシン摂取群における心拍数の自己相関係数（A）および周期（B）の変化

(A)

		Auto-correlation Coefficient														
Positive peak of auto-correlation	Rat	12 ppm T-2 toxin			6 ppm T-2 toxin			4.5 ppm T-2 toxin			3 ppm T-2 toxin			0 ppm-T2 toxin (Control)		
		Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2
Peak 1 (2π)	1	0.45	—	0.22	0.51	0.31	0.14	0.56	0.44	0.51	0.55	0.56	0.55	0.43	0.50	0.53
	2	0.50	0.33	0.43	0.43	0.20	0.41	0.49	0.51	0.52	0.43	0.48	0.39	0.50	0.49	0.44
	3	0.53	—	0.44	0.47	0.17	0.48	0.31	0.36	0.42	0.46	0.39	0.49	0.36	0.46	0.41
	4	0.44	0.23	0.38	0.48	0.35	0.45	0.48	0.41	0.39	0.48	0.43	0.27	0.48	0.57	0.45
	5	0.54	0.36	0.56	0.39	—	0.32	0.44	—	—	0.51	0.57	0.56	0.47	0.47	0.46
	6	0.43	0.38	0.42	0.33	0.47	0.38	0.59	0.56	0.59	0.41	0.45	0.56	0.42	0.42	0.35
Mean		0.48	0.33	0.41	0.43	0.30	0.37	0.48	0.45	0.49	0.47	0.48	0.47	0.44	0.48	0.44
SD		0.05	0.07	0.11	0.07	0.12	0.12	0.10	0.08	0.08	0.05	0.07	0.11	0.05	0.05	0.06

—: No obvious peak

(B)

		Periodicity (hour)														
Positive peak of auto-correlation	Rat	12 ppm T-2 toxin			6 ppm T-2 toxin			4.5 ppm T-2 toxin			3 ppm T-2 toxin			0 ppm-T2 toxin (Control)		
		Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2
Peak 1 (2π)	1	24.50	—	23.75	24.25	24.50	23.75	24.00	23.67	23.42	23.83	23.83	24	23.75	24.00	24.12
	2	23.75	24.42	23.75	24.00	21.00	23.67	23.92	23.58	24.17	24.25	23.25	23.58	24.08	23.83	23.33
	3	23.83	—	24.25	24.33	25.25	23.92	24.58	24.42	24.00	23.08	21.75	24.3	23.92	24.17	25.00
	4	23.67	23.83	24.33	23.50	22.75	24.33	24.00	23.58	23.33	23.58	23.25	22.42	24.00	23.67	24.00
	5	24.00	23.08	23.58	24.00	—	24.42	24.42	—	—	24.08	23.83	23.5	23.92	23.83	24.25
	6	24.41	23.83	24.33	24.33	24.25	23.58	24.00	23.92	24.00	24.17	24	24	24.33	23.42	23.83
Mean		24.03	23.79	24.00	24.07	23.55	23.95	24.15	23.83	23.78	23.83	23.32	23.63	24.00	23.82	24.09
SD		0.35	0.55	0.34	0.32	1.69	0.35	0.28	0.35	0.38	0.40	0.76	0.61	0.20	0.26	0.55

—: No obvious peak

表２．心拍数の周期性に異常が認められた個体数

	T-2トキシンの濃度				
	0 ppm	3 ppm	4.5 ppm	6 ppm	12 ppm
*自己相関係数	0/6(0%)	0/6(0%)	1/6(17 %)	4/6(67 %)	5/6 (83 %)
**日周リズムの周期	0/6(0%)	0/6(0%)	1/6(17 %)	1/6(17 %)	2/6 (33 %)

*Pre-T2 の値に比べて自己相関係数が 30 %以上の低下を示した個体数

**日周リズムが消失した個体

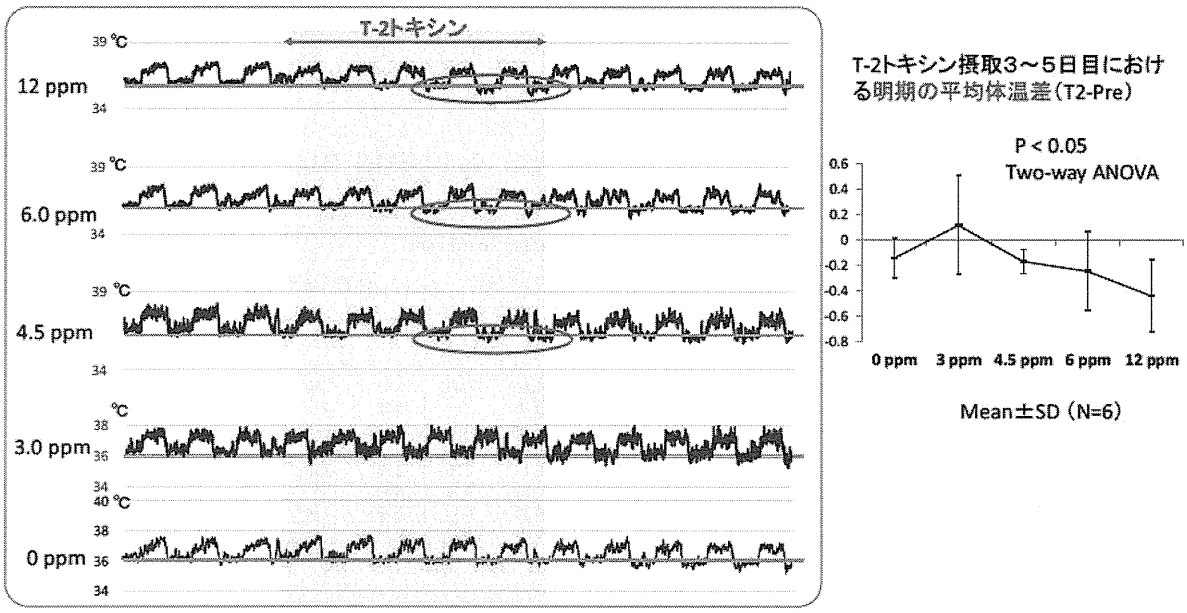

図 6. 各濃度の T-2 トキシン摂取群における体温リズム
ラット 6 匹の平均で示す。

表 3. T-2 トキシン摂取群における体温の自己相関係数（A）および周期（B）の変化

(A)

		Auto-correlation Coefficient														
Positive peak	Rat	12 ppm-T2 toxin			6 ppm-T2 toxin			4.5 ppm-T2 toxin			3 ppm-T2 toxin			0 ppm-T2 toxin (Control)		
		Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2
Peak 1 (2π)	1	0.61	0.39	0.24	0.64	0.48	0.11	0.60	0.58	0.62	0.52	0.53	0.58	0.55	0.62	0.51
	2	0.61	0.44	0.55	0.62	0.41	0.54	0.60	0.58	0.48	0.48	0.42	0.34	0.48	0.50	0.37
	3	0.60	0.41	0.46	0.61	0.40	0.52	0.50	0.56	0.51	0.50	0.50	0.52	0.40	0.59	0.59
	4	0.57	0.52	0.48	0.54	0.50	0.40	0.56	0.57	0.53	0.50	0.28	0.34	0.54	0.62	0.55
	5	0.51	0.55	0.49	0.53	0.38	0.52	0.59	0.04	0.00	0.46	0.48	0.55	0.53	0.58	0.54
	6	0.49	0.54	0.51	0.56	0.60	0.50	0.62	0.55	0.43	0.50	0.38	0.42	0.52	0.57	0.51
Mean		0.57	0.47	0.46	0.58	0.46	0.43	0.58	0.48	0.43	0.49	0.43	0.46	0.50	0.58	0.51
SD		0.05	0.07	0.11	0.04	0.08	0.16	0.04	0.22	0.22	0.02	0.09	0.11	0.05	0.04	0.08

(B)

		Periodicity (hour)														
Positive peak	Rat	12 ppm-T2 toxin			6 ppm-T2 toxin			4.5 ppm-T2 toxin			3 ppm-T2 toxin			0 ppm-T2 toxin (Control)		
		Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2
Peak 1 (2π)	1	24.17	23.58	23.92	23.83	23.92	23.08	23.83	23.83	23.50	23.83	23.83	24.08	23.92	23.75	24.17
	2	23.92	24.08	23.92	23.92	23.83	23.58	23.83	23.42	23.83	24.58	23.08	23.42	23.92	23.92	23.17
	3	23.83	24.08	22.83	24.08	23.00	24.08	22.58	23.75	23.83	24.25	23.92	23.75	23.67	23.83	24.08
	4	23.67	23.58	23.92	24.17	24.00	24.33	24.08	23.42	23.50	24.00	23.75	23.92	24.00	23.75	23.83
	5	23.92	23.67	23.67	24.17	24.33	24.33	23.92	22.83	—	23.90	24.00	24.25	23.92	24.00	24.08
	6	23.75	23.83	24.08	24.08	23.83	24.58	23.58	24.33	22.75	24.00	23.30	24.00	24.08	23.92	23.83
Mean		23.88	23.81	23.72	24.04	23.82	24.00	23.64	23.60	23.48	24.09	23.65	23.90	23.92	23.86	23.86
SD		0.17	0.23	0.46	0.14	0.44	0.56	0.54	0.50	0.44	0.28	0.37	0.29	0.14	0.10	0.37

表 4. T-2 トキシン摂取群における運動量の自己相関係数（A）および周期（B）の変化

(A)

		Auto-correlation Coefficient														
Positive peak of auto-correlation	Rat	12 ppm-T2 toxin			6 ppm-T2 toxin			4.5 ppm-T2 toxin			3 ppm-T2 toxin			0 ppm-T2 toxin (Control)		
		Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2
Peak 1 (2π)	1	0.17	0.28	0.06	0.48	0.21	0.27	0.32	0.43	0.44	0.42	0.50	0.41	0.38	0.38	0.28
	2	0.44	0.22	0.16	0.17	—	—	0.16	0.19	–	0.32	0.29	0.16	0.21	0.29	0.25
	3	0.29	0.18	0.26	0.32	0.21	0.25	0.25	0.39	0.54	0.13	0.27	0.38	0.23	0.33	0.26
	4	0.43	0.35	0.44	0.39	0.28	—	0.22	0.36	0.38	0.27	0.30	0.37	0.22	0.15	0.26
	5	0.26	0.19	0.31	0.17	0.28	0.39	0.38	0.47	0.35	0.36	0.36	0.36	0.38	0.37	0.29
	6	0.39	0.29	0.43	0.23	0.28	0.32	0.39	0.45	0.53	0.18	–	0.26	0.31	0.25	—
Mean		0.33	0.27	0.28	0.29	0.25	0.31	0.29	0.38	0.45	0.28	0.34	0.32	0.29	0.29	0.27
SD		0.11	0.07	0.15	0.13	0.04	0.06	0.09	0.10	0.09	0.11	0.09	0.09	0.08	0.09	0.02

—: no obvious peak

(B)

		Periodicity (hour)														
Positive peak of auto-correlation	Rat	12 ppm-T2 toxin			6 ppm-T2 toxin			4.5 ppm-T2 toxin			3 ppm-T2 toxin			0 ppm-T2 toxin (Control)		
		Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2	Pre-T2	T2	Post-T2
Peak 1 (2π)	1	21.58	23.00	24.92	24.25	23.25	3.42	23.92	24.50	22.80	23.9	23.8	23.8	23.83	23.92	24.33
	2	22.92	9.75	7.50	23.08	—	—	24.67	24.00	–	25.1	23.8	21.8	23.83	23.83	24.25
	3	23.58	15.67	7.58	24.25	24.92	24.42	23.58	23.75	24.70	17.8	21.5	23.8	23.67	23.25	23.67
	4	24.25	23.83	23.83	24.33	4.75	—	23.75	24.08	24.50	26.2	23.5	24.0	23.92	23.58	25.25
	5	24.17	23.58	23.92	23.58	25.75	24.75	24.17	23.67	22.90	26.0	24.0	22.8	23.42	23.67	24.33
	6	24.33	4.75	24.50	24.33	23.67	24.33	23.10	23.25	22.90	30.2	–	23.9	24.00	23.83	—
Mean		23.47	15.48	20.95	23.97	20.47	19.23	23.87	23.88	23.56	24.86	23.3	23.33	23.78	23.68	24.37
SD		1.07	8.13	7.49	0.52	8.84	10.54	0.53	0.42	0.95	4.04	1.02	0.90	0.21	0.24	0.57

—: no obvious periodicity

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

(関連参考図書)
Mycotoxin in Risk Management in Animal Production (Special Edition World Nutrition Forum 2012), Edited by Eva Maria Binder. （「マイコトキシン入門書」畜産におけるカビ毒対策. 作田庄平、局　博一、小西良子（日本語版監修）、高橋有希子（日本語版編集）2015.

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yoshinari T, Sakuda S, Furihata K, Furusawa H, Ohnishi T, Sugita-Konishi Y, Ishizaki N, Terajima J.	Structural determination of a nivalenol glucoside and development of an analytical method for the simultaneous determination of nivalenol and deoxynivalenol, and their glucosides, in wheat.	J. Agric. Food Chem.	62	1174-1180	2014
Yoshinari T, Takeuchi H,Aoyama K, Taniguchi M, Hashiguchi S, Kai S, Ogiso M, Sato T, Akiyama Y, Nakajima M,Tabata S, Tanaka T, Ishikuro E, Sugita-Konishi Y.	Occurrence of four Fusarium mycotoxins, deoxynivalenol, zearalenone, T-2 toxin, and HT-2 toxin, in wheat, barley, and Japanese retail food	J. Food Prot.	77	1940-1946	2014
Yoshinari T, Ohashi H,Abe R, Kaigome R, Ohkawa H, Sugita-Konishi Y.	Development of a rapid method for the quantitative determination of deoxy- nivalenol using Quenchbody.	Anal Chim Acta.	12:888	126-130	2015
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Tanaka T, Abe H, Kimura M, Onda N, Mizukami S, Yoshida T, Shibutani M.	Developmental exposure to T-2 toxin reversibly affects postnatal hippocampal neurogenesis and reduces neural stem cells and progenitor cells in mice.	Arch Toxicol.		In press	2016

Tanaka T, Mizukami S, Hasegawa-Baba Y, Onda N, Sugita-Konishi Y, Yoshida T, Shibutani M.	Developmental exposure of aflatoxin B1 reversibly affects hippocampal neurogenesistargeting late-stage neural progenitor cellsthrough suppression of cholinergic signaling in rats.	Toxicology	336	59-69	2015
Ngampongsa S, Hanafusa M, Ando K, Ito K, Kuwahara M, Yamamoto Y, Yamashita M, Tsuru Y,Tsubone H	Toxic effects of T-2 toxin and deoxynivalenol on the mitochondrial electron transport system of cardiomyocytes in rats.	J Toxicol Sci.	38	495-502	2013

Ⅲ. 研究成果の刊行物・別刷

Journal of Food Protection, Vol. 77, No. 11, 2014, Pages 1940–1946
doi:10.4315/0362-028X.JFP-14-185

Occurrence of Four *Fusarium* Mycotoxins, Deoxynivalenol, Zearalenone, T-2 Toxin, and HT-2 Toxin, in Wheat, Barley, and Japanese Retail Food

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MS 14-185: Received 24 April 2014/Accepted 19 June 2014

ABSTRACT

A survey of the contamination of wheat, barley, and Japanese retail food by four *Fusarium* mycotoxins, deoxynivalenol (DON), zearalenone (ZEN), T-2 toxin (T-2), and HT-2 toxin (HT-2), was performed between 2010 and 2012. A method for the simultaneous determination of the four mycotoxins by liquid chromatography–tandem mass spectrometry was validated by a small-scale interlaboratory study using two spiked wheat samples (DON was spiked at 20 and 100 μg/kg and ZEN, T-2, and HT-2 at 6 and 20 μg/kg in the respective samples). The recovery of the four mycotoxins ranged from 77.3 to 107.2%. A total of 557 samples of 10 different commodities were analyzed over 3 years by this validated method. Both T-2 and HT-2 were detected in wheat, wheat flour, barley, Job's tears products, beer, corn grits, azuki beans, soybeans, and rice with mixed grains. Only T-2 toxin was detected in sesame seeds. The highest concentrations of T-2 toxin (48.4 μg/kg) and HT-2 toxin (85.0 μg/kg) were present in azuki beans and wheat, respectively. DON was frequently detected in wheat, wheat flour, beer, and corn grits. The contamination level of wheat was below the provisional standard in Japan (1,100 μg/kg). The maximum contamination level of DON was present in a sample of a Job's tears product (1,093 μg/kg). ZEN was frequently detected in Job's tears products, corn grits, azuki beans, rice with mixed grains, and sesame seeds. A sample of a Job's tears product presented the highest ZEN contamination (153 μg/kg). These results indicate that continuous monitoring by multiple laboratories is effective and necessary due to the percentage of positive samples detected.

Fusarium is a fungal genus frequently found in crops, and its members produce a number of different mycotoxins, such as trichothecenes, zearalenone (ZEN), and fumonisins *(5)*. The natural occurrence of these mycotoxins is frequent in a wide variety of agricultural products, such as corn, wheat, and barley, and is of great concern to animal and human health *(11, 12, 17, 18)*. Trichothecenes can be divided into type A and type B depending on their structures. Deoxynivalenol (DON), a type B trichothecene, is the most common trichothecene found in crops and is produced primarily by *Fusarium graminearum* and *Fusarium culmorum*. T-2 toxin (T-2) and HT-2 toxin (HT-2) are type A trichothecenes produced by *Fusarium sporotrichioides (2)*. The toxicity of these trichothecenes has been well studied in animal models. Acute oral exposure causes vomiting, diarrhea, and gastroenteritis, whereas chronic effects at low dietary concentrations include growth retardation, reduced food consumption, and immunosuppression *(8)*. ZEN is an estrogenic mycotoxin biosynthesized by a variety of *Fusarium* fungi, including *F. graminearum, F. culmorum,* and *F. crookwellense (7)*. ZEN has low acute toxicity, but studies in various animals have shown that ZEN has estrogenic and anabolic activities. Its major effects are on reproduction, including reproductive organs and their function, leading to hyperestrogenism *(21)*.

The Joint Food and Agriculture–World Health Organization Expert Committee on Food Additives (JECFA) established the provisional maximum tolerable daily intake (PMTDI) of DON for humans at 1 μg/day/kg of body weight based on a reduction of body weight gain. JECFA also allocated a PMTDI of 0.5 μg/day/kg of body weight for ZEN and a group PMTDI of 0.06 μg/day/kg of body weight for T-2 and HT-2, when assessed alone or in combination

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TABLE 1. *LC-MS/MS analysis parameters for each mycotoxin*

Parameter	DON	ZEN	T-2	HT-2
Source polarity	Negative	Negative	Positive	Positive
Source temp (°C)	500	500	500	500
Ionization voltage (V)	−4,500	−4,500	5,000	5,000
Curtain gas (lb/in^2)	10	10	10	10
Nebulizer gas (lb/in^2)	70	70	70	70
Collision-activated dissociation gas (arbitrary units)	3	3	3	3
Precursor ion (*m/z*)	295 $[M-H]^-$	317 $[M-H]^-$	484 $[M+NH_4]^+$	442 $[M+NH_4]^+$
Declustering potential (V)	−35	−70	36	26
Target ion (*m/z*) (collision energy [eV])	265 (−12)	131 (−36)	305 (21)	263 (21)

(8, 9). To reduce the intake of DON, some countries have established regulatory limits or guidance concentrations for DON in foods and feeds. The U.S. Food and Drug Administration has set an upper limit of 1 ppm of DON in finished wheat products. In the European Union, the maximum limits are set between 200 μg/kg for processed cereal–based foods and baby foods for infants and young children and 1,750 μg/kg for unprocessed durum wheat and oats and unprocessed maize. In Japan, a provisional standard of 1,100 μg/kg of DON in wheat grain was set in 2002. Unlike the case for DON, only a few countries set a regulatory limit for T-2, HT-2, and ZEN, although JECFA has evaluated them. In order to adopt appropriate regulation for these mycotoxins, surveillance data collected by a validated analytical method are essential. The occurrence of these mycotoxins in foods has been well studied in Europe *(6)*, but relatively little information has come from Asia.

Many analytical methods using gas chromatography combined with mass spectrometry (MS), high-performance liquid chromatography (LC) with fluorescence, LC combined with MS (LC-MS), or LC combined with tandem MS (LC-MS/MS) have been developed for the determination of *Fusarium* mycotoxins *(3, 10, 14, 15, 19)*. In these methods, toxins were extracted from samples by an organic solvent, such as methanol or acetonitrile, and cleanup was performed using a multifunctional column or an immunoaffinity column. Recently, our group developed a method for the simultaneous determination of DON, ZEN, T-2, and HT-2 in wheat by LC-MS/MS coupled with immunoaffinity extraction *(14)*.

In this study, we first validated the LC-MS/MS method by a small-scale collaborative study in eight laboratories. When evaluating the potential risks of chemical contaminants in food, WHO recommends validation of the analytical method for surveillance *(20)*. Next, surveillance for contamination levels of DON, ZEN, T-2, and HT-2 in wheat, barley, and Japanese retail food was performed by the validated method.

MATERIALS AND METHODS

Chemicals and samples. Solid crystals of DON, ZEN, T-2, and HT-2 were purchased from Sigma-Aldrich (St. Louis, MO). Each compound was dissolved in acetonitrile (50 μg/ml each), and the solution was stored at −20°C. Portions of the four stock solutions were mixed and diluted with acetonitrile to make two concentrations of mixed spiked solutions (solution 1 was 1.2 μg/ml each of ZEN, T-2, and HT-2 and 4.0 μg/ml of DON, and solution 2 was 6.0 μg/ml each of ZEN, T-2, and HT-2 and 20 μg/ml of DON). LC-MS–grade methanol, acetonitrile, and water and reagent-grade ammonium acetate were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Phosphate-buffered saline (PBS) was prepared by dissolving commercial tablets (DS Pharma Biomedical, Osaka, Japan) in distilled water. The immunoaffinity column, a DZT MS-PREP column, was purchased from R-Biopharm Rhone Ltd. (Glasgow, UK). The DON-negative wheat (<0.1 mg/kg for DON) was purchased from Trilogy Analytical Laboratory, Inc. (Washington, MO). Wheat and barley samples from the United States, Canada, and Australia were supplied by an import inspection agency in 2010 and 2012. The samples of wheat flour, Job's tears (*Coix lachryma-jobi*) products, beer, corn grits, azuki beans (*Vigna angularis*), soybeans (*Glycine max*), rice with mixed grains, and sesame seeds (*Sesamum indicum*) were purchased from local supermarkets and small retail shops in Japan from the summer of 2010 to the winter of 2012. The sampling plan was not statistically valid, but samples were collected randomly. The country of origin of all azuki bean samples was Japan. Of other foods, on average, 32% were domestic, 30% were imported, and 41% were of unknown origin.

Design of the interlaboratory study. To validate the method, a study was carried out using a blank sample and two spiked samples of wheat. The study involved eight laboratories in Japan. Each participant in the study received the DON-negative wheat and the three mixed spiking solutions (solution 1 and solution 2, described above in "Chemicals and samples," and pure acetonitrile) for the fortification test. The toxin concentrations in the mixed spiked solutions were not revealed to the participants.

Fortification procedure. To evaluate recovery, 125 μl of each mixed spiking solution was added to a 25.0-g amount of the DON-negative wheat (final concentrations were as follows: spike 1, 0 μg/kg each of DON, ZEN, T-2, and HT-2; spike 2, 30 μg/kg DON and 6 μg/kg each of ZEN, T-2, and HT-2; and spike 3, 100 μg/kg DON and 30 μg/kg each of ZEN, T-2, and HT-2), and the spiked samples were kept at room temperature. After 1 h, toxins were extracted from the spiked samples and quantified according to the protocol defined in the next section.

Protocol used by participants. The method used in this study is based on our previous report *(14)*. Briefly, 25.0 g of sample was extracted with 100 ml of methanol-water (75:25), followed by shaking for 30 min. The extract was transferred to a 50-ml centrifuge tube and centrifuged at 1,410 × *g* for 10 min. Aliquots of 10 ml of the supernatant were diluted with 40 ml of

TABLE 2. *Results of the interlaboratory study on the determination of DON and ZEN by LC-MS/MS in wheat*

	Recovery of analytes at different spiking levels											
	DON						ZEN					
Laboratory	0 μg/kg		20 μg/kg		100 μg/kg		0 μg/kg		6 μg/kg		30 μg/kg	
A	5.4	5.5	25.9	24.6	105.3	107.4	ND[a]	ND	4.6	4.7	22.1	22.6
B	5.7	6.0	25.0	22.4	99.4	104.0	ND	ND	3.2	4.3	14.3	16.1
C	6.0	5.4	25.5	24.1	94.8	98.8	0.07	0.07[b]	4.3	4.1	21.4	22.5
D	9.7	8.7	25.2	23.8	85.4	93.6	ND	ND	5.2	5.3	26.6	27.8
E	13.5	12.1	31.0	33.2	116.4	99.8	ND	ND	8.1	8.6	31.2	28.4
F	7.8	8.2	23.9	27.0	99.5	110.3	0.4	ND	4.3	4.9	24.3	24.2
G	5.5	6.4	23.0	22.2	80.9	113.2	0.5	0.2	4.4	4.3	21.5	24.3
H	8.4	8.0	27.4	26.7	103.1	106.8	ND	ND	4.5	4.6	21.7	21.9
Mean (μg/kg)	7.7		25.7		101.2				5.0		23.2	
Mean recovery (%)			90.2		93.5				82.6		77.3	
RSDr (%)			5.2		9.8				6.9		5.0	
RSDR (%)			11.9		9.8				29.2		18.8	
HorRat			0.5		0.4				1.3		0.9	

[a] ND, not detected (less than LOD).
[b] Underlining indicates a value between LOD and LOQ.

PBS. After the diluted extract was filtered through a glass microfiber filter, 5 ml of the filtrate was applied to the DZT MS-PREP immunoaffinity column. The column was washed with 10 ml of distilled water, and toxins were eluted using 2 ml of methanol. The eluate was dried under nitrogen at 40°C. The residue was dissolved in 0.5 ml of aqueous 10 mmol/liter ammonium acetate–methanol (90:10). After centrifugation (10,000 × *g* for 5 min), the sample was subjected to LC-MS/MS analysis. When the measured value of the toxin in the sample fell out of the range of the standard curve, the sample solution was diluted 10 times with aqueous 10 mmol/liter ammonium acetate–methanol (90:10).

LC-MS/MS conditions. Chromatographic separation was achieved at 40°C with an Inertsil ODS-4 column (3.0 by 50 mm, 2-μm particle size; GL Sciences, Inc., Tokyo, Japan). The injection volume was 10 μl, and the flow rate was set to 0.2 ml/min. The mobile phase was a binary gradient of solvent A (10 mmol/liter ammonium acetate in water) and solvent B (methanol) programmed as follows: at 0 min, 5% B; at 8 min, 90% B; and at 14 min, 90% B. Electrospray ionization in the positive and negative mode was used. The selected reaction monitoring mode of LC-MS/MS and all other conditions were set by each laboratory.

Statistics. The relative standard deviations for repeatability (RSDr) and reproducibility (RSDR) and the Horwitz ratio (HorRat) value, which is the ratio of the RSDR to the predicted RSDR, were obtained according to the AOAC guidelines *(1)*. The predicted RSDR was calculated according to the Thompson report *(16)*. The criteria for analytical methods mentioned in Commission Regulation (EC) No 401/2006 were used for evaluation of these parameters *(4)*.

TABLE 3. *Results of the interlaboratory study on the determination of T-2 and HT-2 by LC-MS/MS in wheat*

	Recovery of analytes at different spiking levels											
	T-2						HT-2					
Laboratory	0 μg/kg		6 μg/kg		30 μg/kg		0 μg/kg		6 μg/kg		30 μg/kg	
A	ND[a]	ND	5.4	6.0	26.1	28.7	ND	ND	6.5	6.5	30.0	30.8
B	ND	ND	5.1	5.3	31.6	29.4	ND	ND	6.2	6.1	29.2	29.8
C	ND	ND	5.0	5.3	30.0	27.3	ND	ND	6.4	6.0	31.1	31.7
D	ND	ND	5.6	5.4	25.4	29.2	ND	ND	7.0	6.1	29.0	29.2
E	ND	ND	10.1	9.9	35.2	32.8	ND	ND	9.8	9.5	35.6	29.4
F	ND	ND	5.6	5.8	26.3	27.8	ND	ND	5.7	5.4	27.4	28.6
G	0.1[b]	ND	3.9	4.5	29.9	32.8	ND	ND	4.4	4.8	26.7	30.0
H	ND	ND	4.5	4.9	24.9	24.6	ND	ND	6.3	6.2	32.0	31.2
Mean (μg/kg)			5.8		28.9				6.4		30.1	
Mean recovery (%)			96.2		96.2				107.2		100.3	
RSDr (%)			4.3		6.1				4.3		6.0	
RSDR (%)			31.2		11.0				22.7		6.9	
HorRat			1.4		0.5				1.0		0.3	

[a] ND, not detected (less than LOD).
[b] Underlining indicates a value between LOD and LOQ.

TABLE 4. *Criteria for the analytical methods mentioned in Commission Regulation (EC) No 401/2006*

	Criteria for analytes, including spiking levels							
	DON		ZEN		T-2		HT-2	
Parameter	>100–≤500 μg/kg	>500 μg/kg	≤50 μg/kg	>50 μg/kg	50–250 μg/kg	>250 μg/kg	100–200 μg/kg	>200 μg/kg
Recovery (%)	60–110	70–120	60–120	70–120	60–130	60–130	60–130	60–130
RSDr (%)	≤20	≤20	≤40	≤25	≤40	≤30	≤40	≤30
RSDR (%)	≤40	≤40	≤50	≤40	≤60	≤50	≤60	≤50

Survey of DON, ZEN, T-2, and HT-2. Mycotoxins in each sample (except for beer) were analyzed by the method described above in "Protocol used by participants" at nine independent laboratories, including the participants in the small-scale collaborative study. For beer, after degassing in an ultrasonic bath for 30 min, 2.5 g of sample was applied directly to the immunoaffinity column. A recovery test was performed in each laboratory before analyzing the samples. Table 1 shows an example of the LC-MS/MS conditions used in a laboratory. The limit of detection (LOD) and the limit of quantification (LOQ) were obtained as signal-to-noise ratios of 3:1 and 10:1, respectively, by using the standard solution of each mycotoxin.

RESULTS AND DISCUSSION

Small-scale interlaboratory study. The results of the small-scale interlaboratory study are shown in Table 2 (DON and ZEN) and Table 3 (T-2 and HT-2). All laboratories detected DON in the blank sample (spiked level, 0 μg/kg), and the mean value was 7.7 μg/kg. Three laboratories detected ZEN and one laboratory detected T-2 in the blank sample. To calculate recovery, the mean value of the blank sample was subtracted from each measured value for each laboratory. The recovery, RSDr, RSDR, and HorRat values for the four mycotoxins were in the ranges of 77.3 to 107.2%, 4.3 to 9.8%, 6.9 to 31.2%, and 0.3 to 1.4, respectively. The criteria for analytical methods mentioned in Commission Regulation (EC) No 401/2006 are shown in Table 4. Although the spiked levels of DON, T-2, and HT-2 in this study were lower than those mentioned in the EC criteria, the values obtained from this study were in good agreement with those criteria. A range of 0.5 to 1.5 for the HorRat value is acceptable according to AOAC International *(1)*. The HorRat values for spiked levels of 100 μg/kg for DON and 30 μg/kg for HT-2 were less than 0.5, but no problems were recognized throughout this interlaboratory study. These results indicate that the method was validated for determination of DON, ZEN, T-2, and HT-2 in wheat.

Occurrence of DON, ZEN, T-2, and HT-2 in wheat, barley, and Japanese retail food. The method validated by the small-scale interlaboratory study was used in a survey for mycotoxins in wheat, barley, and Japanese retail food. The application of the method to the analysis of *Fusarium* mycotoxins in barley and retail food was confirmed by a recovery test. The recoveries of each standard mycotoxin spiked to the individual commodity are shown in Table 5. The recoveries of DON, ZEN, T-2, and HT-2 were in the ranges of 88.1 to 108.9%, 58.8 to 111.1%, 72.8 to 102.6%, and 86.9 to 109.2%, respectively. Only the recovery of ZEN from sesame seeds was less than 60%. This result shows that the method is applicable to many kinds of foods. The analytical method validated in this study enabled a survey targeting a wide range of samples.

TABLE 5. *Recovery of DON, ZEN, T-2, and HT-2 from each spiked individual matrix*

	Concn (μg/kg) in spiked sample			Mean recovery ± SD (%)			
Commodity	DON	T-2, HT-2, ZEN	No. of samples	DON	ZEN	T-2	HT-2
Barley	20	6	6	103.3 ± 7.1	96.8 ± 10.1	91.2 ± 7.4	106.5 ± 1.7
	100	20	6	95.6 ± 5.8	88.8 ± 10.0	92.9 ± 9.5	98.7 ± 2.8
Wheat flour	20	6	3	107.1 ± 8.9	87.2 ± 2.9	92.6 ± 5.6	100.4 ± 7.2
	100	20	3	100.4 ± 3.7	83.9 ± 0.9	91.5 ± 6.1	105.9 ± 7.1
Job's tears products	5	5	3	93.9 ± 15.5	92.8 ± 10.6	98.0 ± 2.4	103.9 ± 5.9
	200	50	3	92.7 ± 5.0	72.6 ± 1.4	102.6 ± 3.2	105.3 ± 4.1
Beer	5	5	3	88.1 ± 2.2	102.2 ± 1.9	88.0 ± 2.0	86.9 ± 2.6
	200	50	3	89.2 ± 2.1	90.9 ± 2.1	94.8 ± 0.8	88.4 ± 0.5
Corn grits	5	5	3	98.0 ± 1.2	88.5 ± 1.3	96.9 ± 1.3	99.6 ± 3.2
	200	50	3	100.2 ± 1.2	78.7 ± 1.7	93.1 ± 0.6	93.2 ± 2.1
Azuki beans	5	5	3	100.1 ± 10.0	101.5 ± 6.0	101.2 ± 6.4	100.7 ± 9.0
	200	50	3	95.8 ± 3.9	87.0 ± 10.7	94.6 ± 12.2	99.3 ± 5.9
Soybeans	5	5	3	100.1 ± 34.3	74.1 ± 1.4	88.6 ± 0.7	101.9 ± 8.7
	200	50	3	88.5 ± 7.9	98.0 ± 3.7	86.6 ± 1.9	95.1 ± 2.9
Rice with mixed grains	5	5	3	108.9 ± 3.5	110.7 ± 3.4	83.1 ± 4.9	105.8 ± 2.6
	200	50	3	97.2 ± 3.6	111.1 ± 1.6	86.7 ± 2.9	109.2 ± 2.4
Sesame seeds	5	5	3	97.6 ± 6.5	65.5 ± 0.2	72.8 ± 7.0	91.2 ± 4.1
	200	50	3	88.6 ± 1.5	58.8 ± 4.6	73.9 ± 12.0	89.6 ± 5.3

TABLE 6. *Natural DON and ZEN in wheat, barley, and retail food*

Commodity	No. of samples analyzed	DON				ZEN			
		LOQ (μg/kg)	No. (%) of positives	Mean of positives (μg/kg)	Maximum (μg/kg)	LOQ (μg/kg)	No. (%) of positives	Mean of positives (μg/kg)	Maximum (μg/kg)
Wheat	150	8	90 (60.0)	102	654	0.7	8 (5.3)	20.5	151
Wheat flour	50	3	44 (88.0)	71.8	789	0.2	9 (18.0)	1.2	3.3
Barley	41	6	18 (43.9)	90.2	579	0.2	4 (9.8)	9.0	27.1
Job's tears products	60	4	26 (43.3)	192	1,093	0.4	39 (65.0)	17.5	153
Beer	30	0.5	28 (93.3)	2.8	22.1	0.05	0 (0)		
Corn grits	60	8	41 (68.3)	62.5	170	0.4	42 (70.0)	7.9	32.2
Azuki beans	40	2	15 (37.5)	12.2	38.2	0.4	29 (72.5)	44.6	125
Soybeans	36	10	0 (0)			3	0 (0)		
Rice with mixed grains	60	5	24 (40.0)	18.1	59.4	0.7	50 (83.3)	4.3	39.3
Sesame seeds	30	2	0 (0)			0.2	20 (66.7)	4.5	21.3

Table 6 shows the results of the survey, including the number of samples, LOQ, the number and percentage of positives, and the mean and maximum concentration per commodity. A positive means the sample was above LOQ. A total of 557 samples were analyzed over 3 years. DON was prevalently detected in the samples of wheat (60.0%), wheat flour (88.0%), beer (93.3%), and corn grits (68.3%). The mean concentrations exceeded 100 μg/kg for wheat (102 μg/kg) and Job's tears products (192 μg/kg). The maximum contamination level was present in a sample of a Job's tears product (1,093 μg/kg). The contamination levels of wheat were under the provisional standard in Japan (1.1 mg/kg). DON was not detected in soybeans and sesame seeds.

ZEN was frequently detected in samples of Job's tears products (65.0%), corn grits (70.0%), azuki beans (72.5%), rice with mixed grains (83.3%), and sesame seeds (66.7%) (Table 6). The mean ZEN concentration of azuki beans was the highest in the survey (44.6 μg/kg). A sample of a Job's tears product had the highest ZEN contamination (153 μg/kg). The second highest level of ZEN was found in a wheat sample (151 μg/kg). ZEN was not detected in beer or soybeans. Worldwide contamination of ZEN in cereal grains has been reported previously, but information about the occurrence of ZEN in Job's tears products, azuki beans, and sesame seeds is rare. Our results suggest that these foods could also be contributors to dietary exposure to ZEN.

T-2 was detected in more than 50% of the samples of beer (56.7%) and azuki beans (70.0%) (Table 7). The mean T-2 levels of azuki beans (11.1 μg/kg), Job's tears products (9.1 μg/kg), and corn grits (3.0 μg/kg) were the three highest values, and the maximum contamination level was present in an azuki bean sample (48.4 μg/kg). As for wheat and barley, there were no samples containing more than 10 μg/kg of T-2.

HT-2 was detected in 70% of the azuki bean samples, but the detection levels of the other samples were less than 20% (Table 7). The mean HT-2 concentrations of wheat (13.3 μg/kg), azuki beans (9.9 μg/kg), and Job's tears products (8.6 μg/kg) were the three highest values. The maximum contamination level was present in the wheat sample (85.0 μg/kg).

Contamination of T-2 and HT-2 in grains and processed foods has been reported. A large-scale survey was performed in European countries between 2005 and 2010. In that survey, totals of 17,683 analytical results for T-2 toxin and 16,536 for HT-2 toxin were obtained, and results below the LOD or LOQ accounted for 77% for T-2 and 65% for HT-2 *(6)*. A Russian group monitored *Fusarium* toxins in wheat, barley, oats, rye, and maize between 2005 and 2010. Their results demonstrated the presence of T-2 toxin in 14% and HT-2 toxin in 17% of all samples *(17)*. Our results showed that contamination of T-2 and HT-2 in crops also occurred in the pan-Pacific zone, including Japan, Canada, and the United States. The contamination levels in our results were equal to or lower than those in previously reported studies.

In this survey, T-2 and HT-2 were detected in amounts higher than the LOQ in all retail food tested. Especially, the

TABLE 7. *Natural T-2 and HT-2 in wheat, barley, and retail food*

Commodity	No. of samples analyzed	T-2				HT-2			
		LOQ (μg/kg)	No. (%) of positives	Mean of positives (μg/kg)	Maximum (μg/kg)	LOQ (μg/kg)	No. (%) of positives	Mean of positives (μg/kg)	Maximum (μg/kg)
Wheat	150	0.6	9 (6.0)	2.8	8.4	2	20 (14.0)	13.3	85.0
Wheat flour	50	0.3	5 (10.0)	0.6	1.0	1	6 (12.0)	2.7	3.4
Barley	41	0.4	4 (9.8)	1.7	4.0	1	8 (19.5)	6.4	21.4
Job's tears products	60	0.7	13 (21.7)	9.1	44.3	3	8 (13.3)	8.6	21.5
Beer	30	0.03	17 (56.7)	0.06	0.2	0.4	2 (6.7)	0.6	0.6
Corn grits	60	0.6	12 (20.0)	3.0	25.8	2	2 (3.3)	12.6	23.1
Azuki beans	40	0.1	28 (70.0)	11.1	48.4	0.3	28 (70.0)	9.9	45.7
Soybeans	36	0.2	1 (2.8)		1.4	0.8	1 (2.8)		3.1
Rice with mixed grains	60	0.2	12 (20.0)	0.6	1.7	0.5	10 (16.7)	1.1	2.3
Sesame seeds	30	0.1	1 (3.3)		0.1	0.3	0 (0)		

contamination levels of T-2 in Job's tears products and azuki beans were higher than those of wheat and barley. Aflatoxin contamination in Job's tears products is frequent *(13),* but little information exists about T-2 and HT-2 contamination in these products. In Japan, Job's tears products are used in cereal foods and traditional herbal medicine, while azuki beans are the material of confectionaries. Since these foods are routinely consumed by broad age groups, mycotoxin contamination in them is a great concern for human health. In order to assess the exposure to *Fusarium* toxins in Japan, a continuous survey is required in order to obtain detailed data about *Fusarium* toxin levels not only in cereals but also in foods which are unique to Japan.

ACKNOWLEDGMENT

This work was supported by a research grant from the Ministry of Health, Labor and Welfare of Japan.

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Structural Determination of a Nivalenol Glucoside and Development of an Analytical Method for the Simultaneous Determination of Nivalenol and Deoxynivalenol, and Their Glucosides, in Wheat

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Supporting Information

ABSTRACT: Trichothecene mycotoxins such as nivalenol and deoxynivalenol frequently contaminate foodstuffs. Recently, several trichothecene glucosides have been found in trichothecene-contaminated foods, and information about their chemistry, toxicity, and occurrence is required. In this study, a glucoside of nivalenol was isolated from nivalenol-contaminated wheat and was identified as nivalenol-3-*O*-*β*-D-glucopyranoside. Analytical methods using a multifunctional column or an immunoaffinity column have been developed for the simultaneous determination of nivalenol, nivalenol-3-*O*-*β*-D-glucopyranoside, deoxynivalenol, and deoxynivalenol-3-*O*-*β*-D-glucopyranoside in wheat. The methods were validated in a single laboratory, and recovery from wheat samples spiked at four levels ranged between 86.4 and 103.5% for the immunoaffinity column cleanup. These mycotoxins in contaminated wheat samples were quantitated by the validated method. Nivalenol-3-*O*-*β*-D-glucopyranoside was detected in the nivalenol-contaminated wheat, and the percentage of nivalenol-3-*O*-*β*-D-glucopyranoside to nivalenol ranged from 12 to 27%. This result indicates that the analytical method developed in this study is useful for obtaining data concerning the state and level of food contamination by nivalenol, deoxynivalenol, and their glucosides.

KEYWORDS: *nivalenol, glucoside, wheat*

INTRODUCTION

Trichothecenes are a large group of mycotoxins produced by various species of *Fusarium, Myrothecium, Trichoderma,* and *Trichothecium*. They are divided into four types (A–D) on the basis of their structures. The majority of trichothecenes found in cereals belong to type A, including T-2 toxin and HT-2 toxin, and type B, including deoxynivalenol (**1**), 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and nivalenol (**2**).[1] Deoxynivalenol and nivalenol, which are mainly produced by *Fusarium graminearum* and *Fusarium culmorum,* are frequently detected in agricultural staples such as wheat, barley, and maize.[2−4] Adverse effects of these compounds have been shown by in vitro and in vivo experiments.[5−7] Acute oral exposure causes vomiting, nausea, diarrhea, and gastroenteritis, whereas chronic effects at low dietary concentrations include growth retardation, reduced food consumption, and immunosuppression. Contamination of agricultural products with these compounds has caused several outbreaks of intoxications in humans and animals.[8] Acute human mycotoxicoses have occurred in Asian countries such as Japan, China, and India. The outbreak in India was associated with the consumption of bread made from mold-damaged wheat. Some trichothecenes, including deoxynivalenol, nivalenol, and T-2 toxin, were contaminated in the wheat.

To reduce the daily intake of deoxynivalenol, many countries have established regulatory limits or guidance concentrations for deoxynivalenol in foods and feeds. In Japan, a provisional regulatory limit of 1.1 mg/kg was set for deoxynivalenol in wheat in 2002. In the European Union (EU), the maximum limits are set between 200 μg/kg for processed cereal-based foods and baby foods for infants and young children and 1750 μg/kg for unprocessed durum wheat and oats and unprocessed maize. On the other hand, no country regulates nivalenol to date. However, the Food Safety Commission in Japan assessed the risk of nivalenol in 2010 and established a tolerable daily intake (TDI) for nivalenol of 0.4 μg/kg body weight per day based on decreased white blood cell counts observed in a 90 day rat study.[9] Subsequently, the European Food Safety Authority (EFSA) provided a scientific opinion on the risks for nivalenol and established a TDI of 1.2 μg/kg body weight per day.[10] Consequently, concern about the risk of nivalenol has also risen worldwide.

Recently, a new group of mycotoxin derivatives has been detected in naturally contaminated cereals. As it is difficult to detect them by conventional analytical methods, they have been termed "masked mycotoxins".[11] A glucoside derivative of deoxynivalenol, deoxynivalenol-3-*O*-*β*-D-glucopyranoside (**3**), was isolated from deoxynivalenol-treated wheat and characterized by NMR.[12] It is known that some plants infected with DON-producing fungi are capable of transforming the

Received: October 29, 2013
Revised: January 14, 2014
Accepted: January 16, 2014
Published: January 16, 2014

toxin.[13,14] Because deoxynivalenol-3-*O*-*β*-D-glucopyranoside inhibited protein synthesis of wheat ribosomes in vitro much more weakly than deoxynivalenol, it appeared that glycosylation of deoxynivalenol is a detoxification process in plants.[15]

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2010 evaluated the health risks of 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and deoxynivalenol-3-*O*-*β*-D-glucopyranoside as well as deoxynivalenol. The Committee decided to set a group provisional maximum tolerable daily intake (PMTDI) at 1.0 mg/kg of body weight per day for deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol, but deoxynivalenol-3-*O*-*β*-D-glucopyranoside was not included in the group because data on the toxicity and occurrence were insufficient at that time.[16] However, recently, the occurrence of deoxynivalenol-3-*O*-*β*-D-glucopyranoside in various foods has been reported since its analytical standard became commercially available.[17−19] In addition, deoxynivalenol can be released from deoxynivalenol-3-*O*-*β*-D-glucopyranoside by some human intestinal bacteria.[20,21] On the basis of these backgrounds, not only deoxynivalenol acetates but also deoxynivalenol-3-*O*-*β*-D-glucopyranoside are considered to pose potential risks to humans.

In addition to deoxynivalenol-3-*O*-*β*-D-glucopyranoside, some masked trichothecene mycotoxins, such as glucosides of nivalenol, T-2 toxin, and HT-2 toxin, were detected in artificially contaminated cereals by MS analysis.[22−24] Because there is a possibility that these compounds exert their toxic effects after the release of aglycone during digestion, information about the occurrence of the glucosides in food products is important for conducting risk assessment of the parent mycotoxins. However, the lack of commercially available standards prevents the quantitative analysis of the glucosides.

The aims of this study were two-fold. The first was to isolate a glucoside of nivalenol from naturally contaminated wheat and to determine the chemical structure by NMR analysis. Information about a glucoside of nivalenol is as important as that for deoxynivalenol-3-*O*-*β*-D-glucopyranoside because nivalenol is frequently detected in various cereals. Second, methods for the simultaneous determination of nivalenol, deoxynivalenol, and their glucosides in wheat were developed to verify their occurrence.

MATERIALS AND METHODS

Chemicals. Solid crystals of deoxynivalenol and nivalenol and a standard solution of deoxynivalenol-3-*O*-*β*-D-glucopyranoside (50 ng/mL in acetonitrile) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Liquid chromatography grade acetonitrile and water and other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

Q-TOF LC-MS Conditions. The LC system consisted of an Agilent 1200 series (Agilent Technologies, Palo Alto, CA, USA). The LC conditions were as follows: mobile phase, 10 mM ammonium acetate/acetonitrile; hold at 5% acetonitrile for 3 min, linear gradient of 5−90% acetonitrile for 10 min, hold at 90% acetonitrile for 6 min, linear gradient of 90−5% acetonitrile for 1 min, followed by equilibration at 5% acetonitrile for 20 min before the next injection. The flow rate was 0.2 mL/min, and the column used was a 150 mm × 2.1 mm i.d., 3 μm, InertSustain C18 (GL Sciences Inc., Tokyo, Japan). The column oven was held at 40 °C, and the autosampler tray was maintained at 4 °C. The HPLC system was connected to an Agilent 6530 Q-TOF mass spectrometer. An electrospray ionization (ESI) interface with Agilent Jet Stream Technology in the negative mode was used. The instrument was calibrated in the high-resolution mode (4 GHz) with a standard mass range (m/z < 3200). Reference masses at m/z 119.0363 and 980.0164 were continually introduced along with the LC stream for accurate mass calibration. The drying gas (nitrogen) temperature was set at 325 °C, drying gas flow at 10 L/min, nebulizer pressure at 30 psi, and capillary voltage (Vcap) at 3500 V. A centroid datum within the mass range m/z 100−1000 was acquired at a 1 spectrum/s rate with a MassHunter workstation (Agilent). Peak identification was performed using Qualitative Analysis software version B.04.00 (Agilent).

Purification of Nivalenol-3-*O*-*β*-D-glucopyranoside from Naturally Contaminated Wheat. Naturally contaminated ground wheat (12 kg) was extracted with 48 L of acetonitrile/water (85:15, v/v). Extraction was performed on a horizontal shaker for 120 min at 100 rpm. The filtrate was collected by vacuum filtration using an A-3S aspirator (Tokyo Rikakikai Co., Tokyo, Japan), 1.2 kg of silica gel 60 (63−200 mm particle size) (Merck KGaA, Darmstadt, Germany) was added to the filtrate, and the mixture was dried by removing the solvent on a rotary evaporator. Fifty grams of the dried silica gel was packed in a column (50 cm × 3 cm i.d.) and eluted with *n*-hexane (400 mL), ethyl acetate (400 mL), and ethyl acetate/methanol (80:20, v/v, 600 mL), successively. This process was repeated until all of the dried silica gel had been treated. The ethyl acetate/methanol fraction containing nivalenol-3-*O*-*β*-D-glucopyranoside was evaporated to dryness, and the residue was suspended in water. The suspension was extracted twice with ethyl acetate. The remaining water layer was evaporated to dryness, and the residue (40 g yield) was suspended in 400 mL of water. After centrifugation (3000*g*, 10 min), 2 mL of the supernatant was subjected to an HF Mega BE-C18 (5 mg) cartridge (Agilent), which was preequilibrated with 20 mL of water containing 10 mM ammonium acetate. The column was washed with 20 mL of water containing 10 mM ammonium acetate, and nivalenol-3-*O*-*β*-D-glucopyranoside was eluted from the cartridge with 20 mL of 10% acetonitrile in water containing 10 mM ammonium acetate. This process was repeated until all of the supernatant had been treated. The eluate was evaporated to dryness, and the residue (3.6 g yield) was subjected to an LC-20A series HPLC system (Shimadzu Corp., Kyoto, Japan). The column used was a 250 mm × 10 mm i.d., 5 μm, Inertsil ODS-3 (GL Sciences). Isocratic elution with 5% acetonitrile in water at a flow rate of 4.0 mL/min with detection at 220 nm was used to obtain fraction A (18.5−21 min retention time, 100 mg yield). Fraction A was purified by reverse-phase HPLC equipped with the same column. Isocratic elution with water containing 0.1% trifluoroacetic acid from 0 to 5 min and then gradient elution of 0−5% acetonitrile in 0.1% trifluoroacetic acid from 5 to 50 min at a flow rate of 4.0 mL/min with detection at 220 nm was used to obtain fraction B (45 min retention time, 16.9 mg yield). Fraction B was purified by reverse-phase HPLC equipped with the same column. Isocratic elution with water from 0 to 2 min and then gradient elution of 0−4% acetonitrile in water from 2 to 74 min at a flow rate of 4.0 mL/min with detection at 220 nm was used to obtain nivalenol-3-*O*-*β*-D-glucopyranoside (65 min retention time, 9.2 mg (19%) yield): $[\alpha]_D^{20}$ −5.5 (*c* 0.25, H_2O); HRESI-TOF/MS m/z 474.1743 (calcd for $C_{21}H_{30}O_{12}$, 474.1737); ^1H and ^{13}C NMR, see Table 1.

D-Glucose Oxidase Digestion. Five hundred micrograms of nivalenol-3-*O*-*β*-D-glucopyranoside was mixed with 4 M HCl (1 mL) and then hydrolyzed in a sealed test tube at 100 °C for 4 h. The reaction solution was dried on a rotary evaporator and lyophilized for 12 h. The resulting residue was dissolved in 400 μL of water and mixed with 50 μL of a 0.5 M potassium phosphate buffer (pH 6.0) and 100 U of D-glucose oxidase (Sigma-Aldrich) in 50 μL of water. The reaction solution was incubated at 35 °C for 1 h, and the solution was directly infused into the LC-MS. D-Glucose oxidase digestion resulted in the disappearance of the signal corresponding to glucose (m/z 179 $[M - H]^-$), which originated from nivalenol-3-*O*-*β*-D-glucopyranoside, and the appearance of a new signal corresponding to gluconolactone (m/z 177 $[M - H]^-$).

Analytical Method with the Multifunctional Column. Twenty-five grams of the wheat sample was extracted with 200 mL of acetonitrile/water (85:15, v/v). The extraction was performed on a horizontal shaker for 30 min at 180 rpm, and then the extract was filtered through a filter paper (Toyo Roshi Kaisha, Tokyo, Japan). A portion (10 mL) of the filtered extract was transferred into an InertSep VRA-3 multifunctional column (GL Sciences). The first 4 mL of eluate

dx.doi.org/10.1021/jf4048644 | J. Agric. Food Chem. 2014, 62, 1174−1180

Table 1. NMR Spectroscopic Data for Nivalenol-3-*O*-β-D-glucopyranoside

position	δ_C[a]	δ_H[a] (J in Hz)	HMBC[b]
2	80.2	3.94, d (4.4)	4, 5, 11, 12
3	86.7	4.33, dd (4.4, 3.7)	4, 1′
4	78.7	4.64, d (3.7)	3, 5, 6, 12
5	49.8		
6	54.5		
7	74.4	4.88, s	5, 6, 8, 11, 15
8	202.7		
9	138.5		
10	137.8	6.67, d (5.1)	6, 8, 11, 16
11	71.4	4.63, d (5.1)	7, 10, 12, 15
12	66.2		
13	47.0	3.15, d (5.0)	2, 5, 12
		3.19, d (5.0)	
14	8.2	1.05, s	4, 5, 6, 12
15	60.8	3.82, d (12.7)	5, 6, 7, 11
		3.85, d (12.7)	
16	15.4	1.82, s	8, 9, 10
1′	103.3	4.74, d (7.8)	3, 2′, 3′
2′	73.8	3.38, dd (9.3, 7.8)	1′, 3′
3′	76.7	3.53, dd (9.3, 8.0)	2′
4′	70.3	3.46	
5′	76.9	3.47	
6′	61.4	3.74, dd (12.0, 4.6)	
		3.89, d (12.0)	

[a]150 MHz (δ_C) and 500 MHz (δ_H) in D_2O. Dioxane δ_C 67.5 and DSS δ_H 0.00 were used as external standards. [b]From protons stated to the indicated carbon.

was discarded, and the next 4 mL of eluate was collected and evaporated to dryness. The residue was dissolved in 1 mL of 10% acetonitrile in water.

Analytical Method with the Immunoaffinity Column. Twenty-five grams of the wheat sample was extracted with 100 mL of distilled water and then shaken for 30 min. The extract was transferred to a 50 mL centrifuge tube and centrifuged at 1410*g* for 10 min. Aliquots of 10 mL of the supernatant were diluted with 50 mL of phosphate-buffered saline (PBS). After the diluted extract was filtered through a glass fiber filter GA-55 (Toyo Roshi Kaisha), 6 mL of the filtrate (equivalent to 0.25 g sample matrix) was applied to a DON-NIV WB immunoaffinity column (VICAM, Milford, MA, USA). The immunoaffinity column had a capacity of approximately 1 μg of nivalenol, deoxynivalenol, and their glucosides, respectively. When the toxins in the highly contaminated wheat samples (no. 1, 3, 4, and 5 in Table 3) were analyzed, the amount of the filtrate applied to the column was decreased to 1 mL (equivalent to 0.042 g of sample matrix). The column was washed with 10 mL of PBS followed by a wash with 10 mL of distilled water. Toxins were eluted using 0.5 mL of methanol followed by 1.5 mL of acetonitrile. The eluate was dried under nitrogen at 40 °C. The residue was dissolved in 0.25 mL of 10% acetonitrile in water.

LC-MS/MS Conditions. LC-MS/MS analyses were performed with a 3200 Q TRAP LC-MS/MS system (AB Sciex, Foster City, CA, USA) equipped with an ESI source and an LC-20A series high-performance LC system (Shimadzu Corp.). The column used was a 150 mm × 2.1 mm i.d., 3 μm, Inertsil ODS-3 (GL Sciences). Chromatographic separation was achieved at 40 °C, using a gradient elution of 5−73% acetonitrile in water from 0 to 8 min and then isocratic elution of 90% acetonitrile in water from 8 to 10 min at a flow rate of 0.2 mL/min. The sample was centrifuged at 10000*g* for 5 min before analysis. The injection volume was 10 μL. The ESI source was operated at 400 °C in the negative ionization mode. Other MS parameters were as follows: curtain gas at 10 psi, nebulizer gas (GS1) at 50 psi, turbo heater gas (GS2) at 50 psi, collision-activated dissociation gas at 5 (arbitrary units), multiple reaction monitoring, dwell time of 250 ms, and a 5 ms pause between mass ranges. The following multiple reaction monitoring transitions were used: nivalenol, 371 $[M + CH_3COO]^-$ to 281 (collision energy, −18 eV); nivalenol-3-*O*-β-D-glucopyranoside, 533 $[M + CH_3COO]^-$ to 263 (collision energy, −28 eV); deoxynivalenol, 295 $[M - H]^-$ to 265 (collision energy, −12 eV); deoxynivalenol-3-*O*-β-D-glucopyranoside, 517 $[M + CH_3COO]^-$ to 427 (collision energy, −24 eV).

Method Validation. The method was validated with deoxynivalenol-negative wheat (Trilogy Analytical Laboratory Inc., Washington, MO, USA) spiked at four concentrations, 10, 50, 250, and 1000 μg/kg, with six replicates for each analyte. When using the DON-NIV WB immunoaffinity column, the amount of the filtrate applied to the column was 6 mL for the samples spiked at 10, 50, and 250 μg/kg and 1 mL for the sample spiked at 1000 μg/kg. Detected concentrations were calculated on a peak area basis using Analyst version 1.5.1 software (AB Sciex). Limits of detection (LODs) and limits of quantification (LOQs) were calculated on the basis of signal-to-noise ratios of 3:1 and 10:1, respectively.

RESULTS AND DISCUSSION

Detection of a Nivalenol Glycoside in Naturally Contaminated Wheat. Naturally contaminated wheat was extracted with 85% acetonitrile in water, and the extract was analyzed by Q-TOF LC-MS. Compounds having estimated molecular formulas of $C_{15}H_{20}O_7$ and $C_{21}H_{30}O_{12}$ (compound **4**) were observed at retention times of 7.5 and 8.0 min, respectively. The former compound was identified as nivalenol. The molecular formula of **4** was larger than that of nivalenol by $C_6H_{10}O_5$. As a result of MS/MS fragmentation analysis, common fragment ions (m/z 191, 217, 233, 281) were found

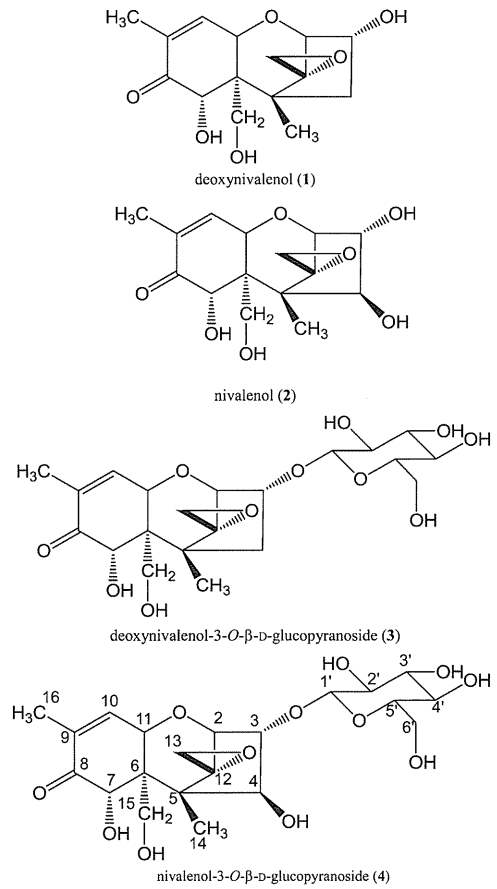

Figure 1. Structures of deoxynivalenol, nivalenol, and their glucosides.

dx.doi.org/10.1021/jf4048644 | J. Agric. Food Chem. 2014, 62, 1174−1180