Fate of Fusarenon-X in Broilers and Ducks

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ABSTRACT In order to investigate the comparative fates and dispositions of fusarenon-X (FX) in broilers and ducks, FX was administered i.v. or orally (p.o.) to broilers and ducks. The FX and its metabolite (nivalenol, NIV) were determined in plasma and excreta using gas chromatography-mass spectrometry. The plasma concentrations of FX were determined up to 180 and 120 min in broilers and ducks, respectively, after i.v. and p.o. administration. The NIV was eliminated more slowly than its parent compound. The FX disposition fit an open 2-compartment pharmacokinetic model in broilers and ducks. The elimination half-life ($t_{1/2\beta}$) of FX was longer in ducks than in broilers. The elimination rate constant (kel) was higher in broilers than in ducks, whereas the oral bioavailability of FX was higher in ducks than in broilers. The gas chromatography-mass spectrometry profile in plasma showed that a large proportion of FX was recovered as NIV after administration of FX in both broilers and ducks. In vitro incubation of liver microsomal and cytosolic fractions with FX demonstrated that the liver and kidney are capable of the FX-to-NIV conversion. Thus, this study demonstrated that FX is absorbed more efficiently in ducks than in broilers, whereas it is eliminated more slowly in ducks than in broiler chickens. Consequently, the toxicity would have more serious consequences in ducks rather than broilers.

Key words: fate, fusarenon-X, nivalenol, broiler, duck

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INTRODUCTION

Fusarenon-X (**FX**; 3, 7, 15-trihydroxy-4-acetoxy-12, 13 epoxytrichothec-9-e-8-on) is one of the 12, 13-epoxytrichothecenes mainly produced by *Fusarium crookwellense*, which naturally occurs in agricultural commodities such as wheat and barley (IARC, 1993). The FX and nivalenol (**NIV**) have been reported to induce adverse health effects, particularly apoptosis, in organs containing actively dividing cells such as the small intestine, thymus, spleen, bone marrow, testes, reticulocytes, and mitogen-stimulated human lymphocytes, as observed in other trichothecenes (Ohta et al., 1978; Forsell and Pestka, 1985; Miura et al., 1998; Poapolathep et al., 2002). In general, limited pharmacokinetic data are available for trichothecene mycotoxins in animals, especially for FX. Although FX has been observed to occur frequently with deoxynivalenol (**DON**; 3, 7, 15-trihydroxy-12, 13-epoxytrichothec-9-e-8-on) in agricultural products (Yoshizawa, 1983; Miller et al., 1991), the fate of FX in animal bodies has not been studied as extensively as DON. It is well known that species differences affect the fate of drugs and chemicals in animals (Walker, 1980). In our previous investigation, we demonstrated that FX given orally is rapidly converted to NIV in mice (Poapolathep et al., 2003, 2004). However, our previous findings in mice cannot be directly extrapolated to other animal species.

To gain insight into the mechanism underlying the toxicity of fusarenon-X between broilers and ducks, we studied the toxicokinetic properties and the metabolites in excreta of FX in broilers and ducks. The metabolites of these toxins appearing in liver and kidney postmitochondrial fractions were also studied.

MATERIALS AND METHODS

Standards and Chemicals

The FX and NIV were purchased from Sigma Chemical Co. (St Louis, MO). Scirpentriol, *N*-trimethylsilylimidazole, *N*,*O*-Bis(trimethylsilyl)acetamide were purchased from Wako Chemical Co. (Tokyo, Japan). Trimethylchlorosilane was purchased from GL Sciences Inc., Tokyo, Ja-

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pan. Other reagents and chemicals of analytical grade were purchased from Sigma Chemical Co.

Animals

Six 4-wk-old broiler chickens (average weight: 1.35 ± 0.17 kg) and ducks (average weight: 1.16 ± 0.14 kg) were purchased from Animal Farm, Nakornpathom Province, Thailand. The experimental animals were housed in animal cages at the Laboratory Animal Facility, Faculty of Veterinary Medicine, Kasetsart University and acclimatized to the environment for 1 wk. The animals were fed with a commercial diet and water ad libitum throughout the experiments. All experimental procedures carried out on the animals were approved by the Animal Ethics Research Committee of Faculty of Veterinary Medicine, Kasetsart University.

Experimental Design In Vivo Study

To obtain the fundamental toxicokinetic data of FX, 6 broilers or 6 ducks at 5 wk of age were divided into 2 groups (n = 3). Each group was administered i.v. or orally (**p.o.**) with FX at a dosage of 2.2 mg/kg of BW. The dosage was based on our previous studies. Blood samples were taken from brachial (wing) veins using heparinized syringes just before and at 5, 10, 20, 30, 60, 120, 180, 240, and 600 min following administration. Plasma were separated by centrifugation (1,968 × *g*) for 15 min. Excreta was collected up to 6 h after the toxin was given. All the plasma and excreta samples were frozen at –20 C° until analysis.

Metabolism of FX to NIV In Vitro

A female duck and both sexes of broiler chickens were killed with pentobarbitotone sodium at a dosage of 40 mg/kg of BW by intravenous administration. The blood was taken from wing vein with a heparinized syringe, and red blood cells and plasma were separated by centrifugation at 1,968 × *g* for 15 min. The livers and kidneys were immediately removed, frozen in liquid nitrogen and stored at –80°C until used. Postmitochondrial fractions were prepared by the previous method (Bammler et al., 2000; Esaki and Kumagai, 2002). The red blood cells, plasma, and postmitochondrial fractions of liver and kidney were incubated with shaking (60 cycles/min) with 10 μg of FX at 37°C for 45 min.

Extraction and Clean-up

Plasma, excreta, red blood cells, and microsomal and cytosolic fractions of the liver were extracted in the 3 mL of acetonitrile (**ACN**)-water (3:1). Ammonium sulfate was added to the mixture (Tanaka et al., 2001; Poapolathep et al., 2003), and then the ACN fraction was separated by centrifugation at 1,968 × *g* for 15 min. Extraction was repeated 2 additional times. The parent and metabolites in the ACN fraction were purified with a Sep-pak silica cartridge (Waters Corp., Milford, MS) as described previously (Poapolathep et al., 2003). The elute was evaporated to dryness under a nitrogen stream at 40 C° on a heating block. The residue was derivatized with trimethylsilylating agents according to the method of Tanaka et al. (2000) and then analyzed by gas chromatography-mass spectrometry (**GC-MS**). To evaluate recovery, 1 mL of plasma or excreta was added the FX and NIV standard solution. The spiked samples were then analyzed as described in the extraction procedure. The average (±SD) recoveries of FX in plasma and excreta were 83.21 ± 3.14, 93.37 ± 2.87% and 84.59 ± 3.99, 96.49 ± 2.81% in broilers and ducks, respectively. The average (±SD) recoveries of NIV were 72.57 ± 4.99%, 79.73 ± 5.63% and 73.36 ± 3.31%, 75.89 ± 2.55% in broilers and ducks, respectively.

GC-MS

The GC-MS system was composed of GC-MS (MS-Agilent 5973N GC-MS system, Agilent Technology, Palo Alto, CA) equipped with a capillary column (DB-5, 30 m × 0.25 mm I.D., 0.25 μm df, Agilent Technology). The column conditions, flow rate, and mass spectrometry conditions were the same as described by Tanaka et al. (2000). The detection limit of this method was 1 ng/mL. All samples were subjected to GC-MS with scirpentriol as an internal standard.

Calculation of Toxicokinetic Parameters

The toxicokinetic characteristics of FX in broilers and ducks were described by a 2-compartment pharmacokinetic model using the PK Solution 2.0 Program (http://www.summitPK.com), where Kel was the elimination rate constant, F the oral bioavailability, AUC the area under the curve, $t_{1/2\beta}$ the elimination half-life, $t_{1/2\alpha}$ the distribution half-life, Cl the body clearance, and K_{12}; K_{21} the micro-rate constants.

The oral bioavailability (F) was calculated using the equation

$$(\%)F_{(p.o.)} = (AUC_{p.o.})/(AUC_{i.v.}) \times 100.$$

Statistical Analysis

Plasma concentration curves of FX and NIV were shown as mean (±SD) of 3 broilers and ducks. Pharmacokinetic parameters were shown as mean (±SD). Statistical analysis was generally done according to Student's *t*-test. When individual differences were large, Welch's *t*-test was performed. A value of $P < 0.05$ was judged to be significant and $P < 0.01$ to be highly significant.

RESULTS

Toxin Excretion

The GC-MS analysis of excreta from broiler chickens and ducks treated with FX revealed excretion of both NIV and FX (data not shown).

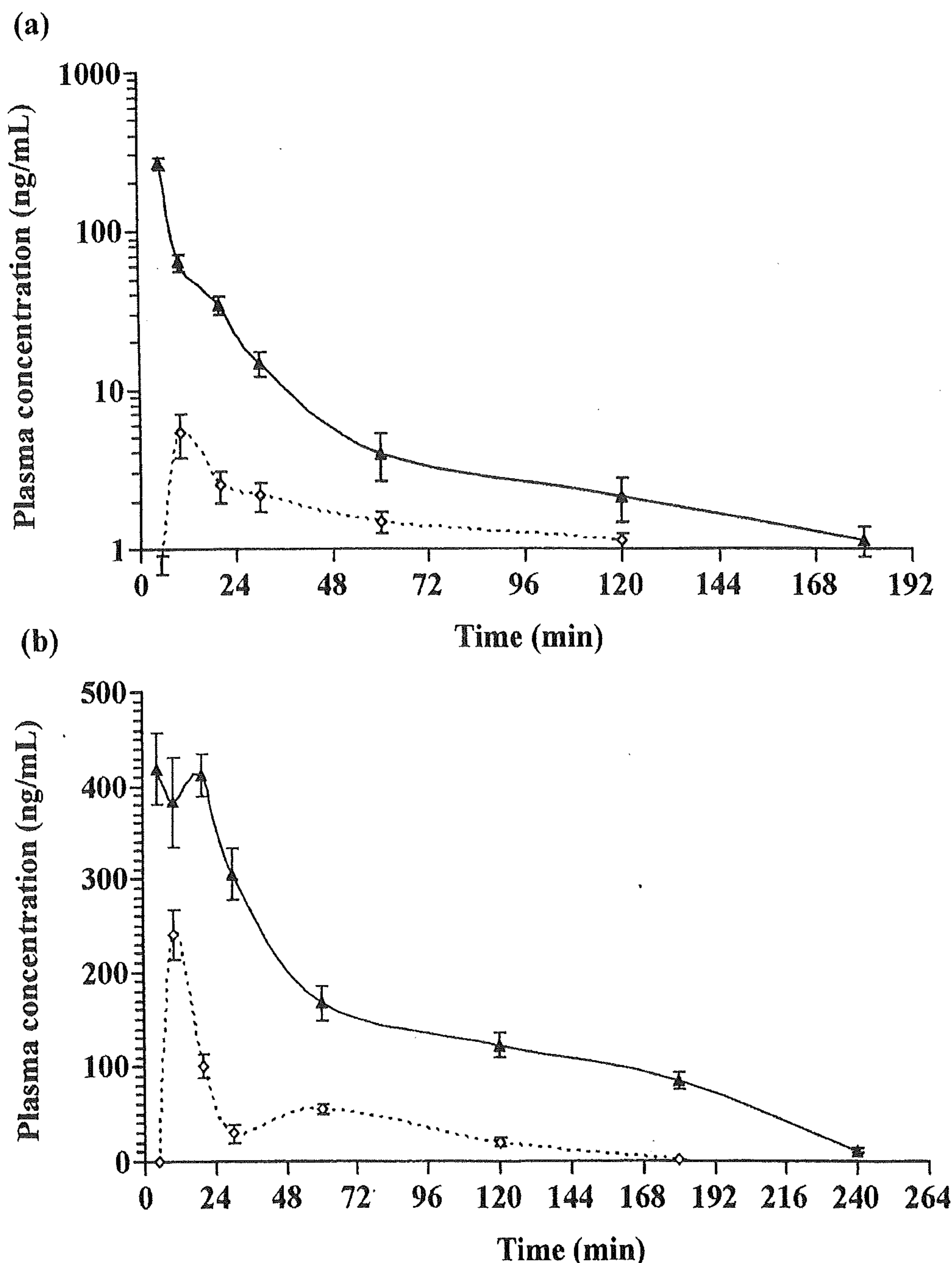

Figure 1. Mean (±SD) plasma levels (ng/mL) of fusarenon-X (FX; a) and nivalenol (NIV; b) after i.v. and oral (p.o.) administration in broilers (▲, i.v.; ◇, p.o.).

Plasma Concentration and Pharmacokinetic Parameters

Figures 1 and 2 show the plasma concentration-time plot of FX and NIV in broilers and ducks following i.v. and p.o. administration. Pharmacokinetic parameters were calculated from the FX plasma concentration following i.v. administration. The elimination half-life ($t_{1/2\beta}$) was longer in ducks than in broilers. The oral bioavailability (F) was slightly higher in ducks than in broilers (Table 1). The values of body clearance (Cl), elimination rate constant (Kel) and distribution half-life ($t_{1/2\alpha}$) were greater in broilers than in ducks but the micro-rate constant (k_{12}) and area under the curves (AUC) for both FX and NIV were higher in ducks than in broilers (Table 1). A large proportion of NIV peaks were detected in plasma after i.v. and p.o. administration (Figures 1 and 2).

In Vitro Conversion of FX to NIV in Liver Microsomal and Cytosolic Fractions

To study the tissue capable of the conversion of FX to NIV in broiler chickens and ducks, FX was incubated

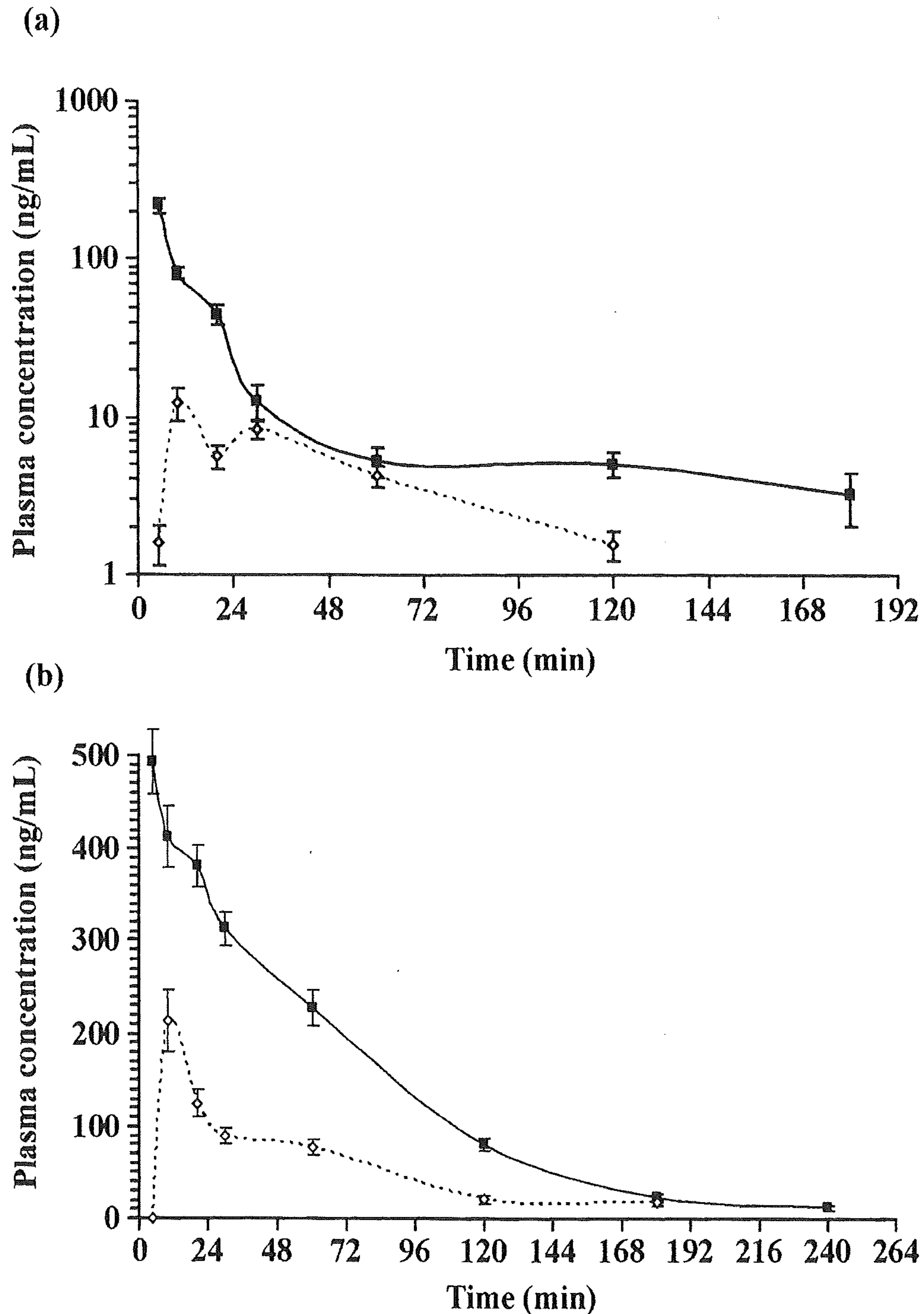

Figure 2. Mean (±SD) plasma levels (ng/mL) of fusarenon-X (FX; a) and nivalenol (NIV; b) after i.v. and oral (p.o.) administration in ducks (■, i.v.; ◇, p.o.).

with liver and kidney postmitochondrial fractions, red blood cell and plasma, and the amount of NIV formed was determined. The FX to NIV conversion was noted clearly in the liver and kidney, the highest activity being in the liver in ducks (98.95%), but in the kidney in broiler chickens (94.39%). The FX to NIV conversion by broiler liver was 70.12% and that by duck kidney was 94.32%. The FX to NIV conversion in broiler plasma and red blood cells were 5.45 and 8.06%, respectively, whereas that in duck plasma and red blood cell were 1.3 and 9.92%, respectively.

DISCUSSION

The present study used GC-MS to analyze FX and its active metabolite NIV in plasma after i.v. and p.o. administration in broilers and ducks. The FX plasma level in broilers and ducks was detected up to 120 and 180 min,

Table 1. Toxicokinetic parameters of fusarenon-X (FX) after administration (2.2 mg/kg of BW) in broilers and ducks

Toxicokinetic parameters (units)	Broilers	Ducks	*P*-value
i.v.			
$t_{1/2\alpha}$[1] (min)	34.6 ± 4.8	18.1 ± 2.9	<0.01
$t_{1/2\beta}$[2] (min)	73.2 ± 6.2	133.8 ± 3.6	<0.01
K_{12}[3] (min^{-1})	0.002 ± 0.001	0.015 ± 0.005	<0.05
K_{21}[3] (min^{-1})	0.015 ± 0.005	0.018 ± 0.004	0.46
Kel[4] (h^{-1})	0.54 ± 0.13	0.30 ± 0.07	<0.05
Cl[5] (mL/min-kg)	882.2 ± 85.6	573.7 ± 71.3	<0.01
AUC_{FX}[6] (i.v., ng-min/mL)	2,328 ± 201	3,129 ± 323	<0.05
AUC_{NIV}[7] (i.v., ng-min/mL)	24,081 ± 712	34,150 ± 998	<0.01
p.o.			
F[8] (%)	9.8 ± 1.1	19.46 ± 2.85	<0.01
AUC_{FX} (p.o., ng-min/mL)	229 ± 54	609 ± 87	<0.01
AUC_{NIV} (p.o., ng-min/mL)	7,159 ± 444	10,681 ± 613	<0.01

[1]$t_{1/2\alpha}$ = distribution half-life.
[2]$t_{1/2\beta}$ = elimination half-life.
[3]K_{12}, K_{21} = micro-rate constants.
[4]Kel = elimination rate constant.
[5]Cl = body clearance.
[6]AUC = area under the curve.
[7]NIV = nivalenol.
[8]F = oral bioavailability.

respectively. The NIV was found in plasma at 10 min in broilers and ducks orally given with FX, indicating FX was absorbed and metabolized very rapidly presumably in the liver and kidney. These results corresponded well with our previous research, showing FX was rapidly converted to NIV in plasma of mice orally given with FX. We also found that ^{3}H-FX was also metabolized to ^{3}H-NIV in liver (85%) and kidney (28.5%) at 15 min in vitro experiment (Poapolathep et al., 2003). The oral bioavailability of FX was higher in ducks than broilers. The elimination half-life ($t_{1/2\beta}$) of FX was also longer in ducks than in broilers. The value of body clearance (Cl) and elimination rate constant (kel) were higher in broilers than that in ducks. The GC-MS profile of excreta showed a large proportion of NIV after administration of FX in both broilers and ducks. These findings clarify that FX is absorbed more efficiently in ducks than in broilers, but the FX is more rapidly excreted from broilers than ducks almost all in the NIV form. The results also corresponded well to our previous investigation of FX in mice (Poapolathep et al., 2003). In addition, the area under the curve of NIV (AUC_{NIV}) was higher in ducks than that in broilers after i.v. and p.o., respectively. This may reflect that more efficient conversion in ducks than in broilers.

The in vitro study of FX metabolism indicates that the liver and kidney are capable for the FX to NIV conversion. Consistent with this, the liver and kidney have also been observed to be major organs for FX to NIV conversion in vitro in mice, rat, and rabbit (Ohta et al., 1978; Poapolathep et al., 2003).

In conclusion, the results demonstrated that FX is absorbed from the gastrointestinal tract more efficiently in ducks than in broilers, followed by its rapid conversion to NIV probably by the liver and kidney. In addition, it appears that AUC data represent the length of time the compounds were detectable, higher AUC numbers longer tissue tenures in ducks, indicating less efficient excretion and enhanced opportunity for damage. Therefore, the toxicity would be greater in ducks than in broiler chickens.

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Research Note

Detection of Deoxynivalenol Contamination in Wheat Products in Thailand

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ABSTRACT

A total of ninety samples in three kinds of wheat products (30 noodle, 30 bread, and 30 cereal samples) were collected from the supermarkets in Bangkok, Thailand, from February to April 2007. The occurrence of deoxynivalenol (DON) contamination in wheat products was investigated using high-performance liquid chromatography equipped with a UV light detector. The extraction method was performed using a multifunctional cleanup column. The limit of quantification was 0.10 $\mu g \cdot g^{-1}$ from the range obtained in a linear calibration. The survey found almost 94% of the DON-contaminated samples below 1 $\mu g \cdot g^{-1}$, which corresponds to the U.S. Food and Drug Administration advisory level. DON was detected in 18.9% (17 of 90) of all samples, in 6.67% (2 of 30) and 16.67% (5 of 30) of noodle and bread samples at levels from 0.17 to 0.35 and 0.14 to 1.13 $\mu g \cdot g^{-1}$, respectively, while it was in 33.33% (10 of 30) of cereal samples at levels from 0.13 to 0.39 $\mu g \cdot g^{-1}$. The results suggest that the exposure to DON from the consumption of wheat products, especially noodles, bread, and cereal, is at a very low risk level.

Deoxynivalenol (DON) is a trichothecene mycotoxin produced by several plant pathogenic fungi, of which *Fusarium graminearum* and *Fusarium culmorum* are the most important sources. It is known to frequently contaminate a variety of foodstuffs, including wheat, maize, barley, oats, and rice *(1, 2, 4, 6, 9, 10, 12, 13, 18, 21–23)*. DON is the most abundant of the trichothecenes and has been found in high concentrations in cereal crops in the United States and Europe *(7)*. DON contamination has been associated with various adverse effects such as feed refusal, vomiting, and immunotoxic effects in animals and humans *(11, 18, 19)*. Human food poisoning has been reported to be caused by ingestion of DON *(18)*. Symptoms described include abdominal pain or a feeling of fullness in the abdomen, dizziness, headache, throat irritation, nausea, vomiting, diarrhea, and blood in the stool *(5, 15)*. The consumption of wheat products by the human population raises the risk of exposure to DON; studies on exposure to mycotoxins via the diet have been limited, particularly in Thailand.

The Joint FAO–WHO Expert Committee on Food Additives has established the provisional maximum tolerable daily intake for humans at 1 μg of DON per kg of body weight per day. The U.S. Food and Drug Administration has established its advisory level for DON in finished wheat products for human consumption at 1 $\mu g \cdot g^{-1}$.

In order to investigate the situation of DON contamination in foods in Thailand in relation to the recommended guidelines for assuring food safety, we surveyed DON contamination in noodles, bread, and cereals retailed in Thailand.

MATERIALS AND METHODS

Standard and reagents. DON standard was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetonitrile and methanol were high-performance liquid chromatography (HPLC) grade. Purified water was produced using a Milli-Q water purification system from Millipore (Bedford, Mass.). The multifunctional columns were MultiSep 227 columns (Romer Labs, Inc., Union, Mo.).

Sample extraction and cleanup. A total of 90 samples (30 instant noodles, 30 crackers or breads, and 30 cereals), mainly composed of wheat, were randomly collected from five supermarkets in Bangkok from February to April 2007, a season of relatively high humidity and temperature. The extraction and cleanup methods of DON in wheat products were carried out in accordance with previous publications *(3, 14, 17, 20)*. In short, 50 g of homogenized sample was extracted with 200 ml of acetonitrile-water (85:15 [vol/vol]) by shaking for 30 min. After filtration with a glass microfiber filter (Whatman GF/B, Whatman International, Ltd., Maidstone, Kent, UK), 10 ml of the filtrate was applied to a multifunctional cleanup column, without preconditioning. The first 3 ml of the eluate was discarded, and the next 4 ml was collected and then evaporated to dryness under a nitrogen stream. The dry residue was redissolved in 1 ml of acetonitrile-methanol-water (5:5:90 [vol/vol/vol]). After passing through

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TABLE 1. *The levels of DON contamination in noodles, bread, and cereals in Bangkok, Thailand*

Type of wheat product	Quantifiable samples/total samples (%)	Mean contamination levels of quantifiable samples ($\mu g \cdot g^{-1}$)	Range ($\mu g \cdot g^{-1}$)
Noodles	2/30 (6.67)	0.26	0.17–0.35
Breads	5/30 (16.67)	0.37	0.14–1.13
Cereals	10/30 (33.33)	0.24	0.13–0.39

an Acrodisc syringe filter (0.45-μm pore size; Pall Life Sciences, Ann Arbor, Mich.), the sample was subjected to HPLC.

Fortification procedure. For evaluating recovery, 1 ml of the DON standard solution was added to 50 g of blank samples to yield final DON concentrations of 0.1, 0.25, 0.5, 1.0, and 2 $\mu g \cdot g^{-1}$. The spiked samples were subjected to extraction and cleanup as described above. The average (±standard deviation) recovery was 81.2% ± 2.2%, 80.4% ± 2.8%, and 80.1% ± 3.3% in noodle, bread, and cereal samples, respectively.

HPLC conditions. The analytical method was performed in accordance with previous publications *(14, 17)*. The eluted samples (20 μl) were injected into a model 09 autosampler (SIC Co., Tokyo, Japan) with a Pegasil ODS HPLC column (5 μm, 4.6 by 250 mm; Senshu Scientific Co., Tokyo, Japan). The HPLC column was maintained at a temperature of 40°C, using a column oven. The acetonitrile-methanol-water (5:5:90 [vol/vol/vol]) mobile phase was delivered at a flow rate of 1 ml/min, using a PU-2080 HPLC pump (Jasco, Tokyo, Japan). Separation was monitored with a UV detector (Jasco) set at a wavelength of 220 nm. A DON peak appeared approximately at 16 min after injection. The limit of quantification was 0.1 $\mu g \cdot g^{-1}$.

RESULTS AND DISCUSSION

The results of DON levels of contamination in three kinds of wheat product samples are shown in Table 1. DON was quantifiable in 18.9% (17 of 90) of all collected samples, in 6.67% (2 of 30) of noodle samples at levels from 0.17 to 0.35 $\mu g \cdot g^{-1}$, and in 16.67% (5 of 30) of bread samples at levels from 0.14 to 1.13 $\mu g \cdot g^{-1}$. Cereals were contaminated more frequently. DON being quantifiable in 33.33% (10 of 30) at levels from 0.13 to 0.39 $\mu g \cdot g^{-1}$. The majority of quantifiable samples (94%) were contaminated with levels less than 1 $\mu g \cdot g^{-1}$.

The daily intake *(8)* and estimated exposure to DON from noodles and bread by the Thai population were calculated by averaging the values of DON concentration in quantifiable samples and the assumed values summarized in Table 2. In upper estimated DON exposure, actual values below the limit of quantification were assumed to be half the limit of quantification (0.05 $\mu g \cdot g^{-1}$), whereas the values were assumed to be 0 in lower estimated exposure. The maximal estimated DON exposure by the Thai population in Bangkok (3- to 6-year-olds) were 0.0038 and 0.0111 μg of body weight per day from noodles and bread, respectively. Sugita-Konishi et al. *(16)* reported that boiling reduced DON concentration and its cytotoxicity by approximately 30%. The average value of estimated exposure to DON in the noodles and bread by the people residing in Bangkok was below the provisional maximum tolerable daily intake value established by the Joint FAO–WHO Expert Committee on Food Additives for humans of 1 μg of DON per kg of body weight per day. This study was conducted in a limited number of samples from Bangkok, but the sample products are distributed widely and consumed by a large population in Thailand.

In conclusion, this study suggests that the risk of DON exposure via wheat products (as food) appears very low in urban areas of Thailand because DON contamination in wheat products, particularly noodles, bread, and cereals, marketed in Bangkok, Thailand, is at a low level.

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TABLE 2. *The daily intake of noodles and bread, and estimated exposure to DON via the noodles and bread, by the Thai population in Bangkok*[a]

	Noodles			Bread		
Age (yr)	DI (g/person/day)	Lower estimated exposure (μg/kg BW/day)	Upper estimated exposure (μg/kg BW/day)	DI (g/person/day)	Lower estimated exposure (μg/kg BW/day)	Upper estimated exposure (μg/kg BW/day)
3–6	0.94	0.0010	0.0038	1.73	0.0066	0.0111
6–9	1.17	0.0008	0.0031	2.05	0.0052	0.0088
9–16	1.61	0.0007	0.0026	2.05	0.0031	0.0052
16–19	1.81	0.0007	0.0026	1.64	0.0023	0.0038
19–35	1.90	0.0006	0.0023	1.32	0.0015	0.0025
35–65	0.96	0.0003	0.0011	1.17	0.0012	0.0021
>65	0.33	0.0001	0.0004	1.01	0.0010	0.0017

[a] DI, daily intake; BW, body weight.

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Aflatoxin and ochratoxin A contamination of retail foods and intake of these mycotoxins in Japan

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A survey was undertaken of aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2), ochratoxin A (OTA), and fumonisin B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) contamination of various retail foods in Japan during 2004–05. The mycotoxins were analysed by high-performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS) or high-performance thin-layer chromatography (HPTLC). Aflatoxins (AFs) were detected in ten of 21 peanut butter and in 22 of 44 bitter chocolate samples; the highest level of AFB_1, $2.59\,\mu g\,kg^{-1}$, was found in peanut butter. Aflatoxin contamination was not observed in corn products ($n = 55$), corn ($n = 110$), peanuts ($n = 120$), buckwheat flour ($n = 23$), dried buckwheat noodles ($n = 59$), rice ($n = 83$) or sesame oil ($n = 20$). OTA was detected in 120 out of 192 samples of oatmeal, wheat flour, rye, buckwheat flour, raw coffee, roasted coffee, raisin, beer, wine and bitter chocolate, but not in rice or corn products. OTA levels in the positive samples were below $13\,\mu g\,kg^{-1}$. AFs and OTA intakes through the consumption of foods containing cacao were estimated using the data for mycotoxin contamination in bitter chocolate and those for the consumption of foods containing cacao in Japan.

Keywords: aflatoxins; ochratoxin A; retailed food; intake; Japan; exposure assessment; surveillance

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Introduction

Mycotoxin contamination of agricultural products causes a significant threat to human health and enormous economic losses. Among the numerous mycotoxins that have been identified, aflatoxins (AFs) and ochratoxin A (OTA) are of high priority for control because of their frequent and worldwide contamination of agricultural products.

AFs are potent hepatotoxic and hepatocarcinogenic compounds produced by *Aspergillus* spp. Human hepatic cancer and acute fatal diseases, notably hepatitis, have occurred in association with consumption of heavily contaminated foods in Asia and Africa (IARC 1993). Peanuts, tree nuts, spices, corn, rice, cottonseed, dry fruits and copra are frequently contaminated with AFs (Joint FAO/WHO Expert Committee on Food Additives 1998). OTA is produced by *Penicillium verrucosum* and various species of *Aspergillus*, and is regarded to be a causal agent of endemic nephropathy and urinary tract tumours in the Balkans (Joint FAO/WHO Expert Committee on Food Additives 2001). A variety of commodities are contaminated with OTA and relatively high contamination levels are found in corn, rye and coffee (Joint FAO/WHO Expert Committee on Food Additives 2001).

Many countries have set regulatory levels for AFs and OA in foods to minimize the consumption of foods considered to be potentially hazardous because of these mycotoxins (Van Egmond and Jonker 2004). To collect data that is required for setting appropriate regulatory levels for AFs and OTA in foods, we conducted a survey of contamination in various foods collected from retail markets in Japan in 2004–05. Bitter chocolate was frequently contaminated with AFs and we therefore used the bitter chocolate data to estimate AFs and OTA intake by the Japanese population.

Materials and methods

Sampling

Two samples of rice harvested during the 2003 fiscal year (1 April 1 2003–31 March 2004) and rice and wheat samples harvested during the 2004 fiscal year

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(1 April 1 2004–31 March 2005) were supplied by the Ministry of Agriculture, Forestry and Fisheries. All other samples were purchased in a random manner from local supermarkets and small retail shops located throughout Japan from the summer of 2004 to the winter of 2005. The samples were stored at 4°C until analysis.

Reagents

Aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), OTA standards were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Methanol and acetonitrile were HPLC grade. Water was purified in a Milli-Q system (Millipore Co., Bedford, MA, USA). All other reagents were of the highest analytical grade available.

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Standard solutions

AFB_1, AFB_2, AFG_1, AFG_2 were dissolved in toluene–acetonitrile (9 : 1), and OTA was dissolved in toluene–acetic acid (99 : 1). The mycotoxin concentration of these original solutions was measured according to AOAC International (Truckness 2000). A stock solution containing the four AFs (4 $\mu g\,ml^{-1}$ each) and an OTA stock solution (1 $\mu g\,ml^{-1}$) were prepared by diluting the original AFs and OTA solutions with acetonitrile or toluene–acetic acid (99 : 1), respectively.

Extraction and analysis of AFs

Samples were analysed at five independent laboratories for AFs using AOAC Official Method 999.07 (Stroka et al. 2000). A total of 25 g of the sample in finely ground form, except for peanut butter and sesame seed oil, were shaken with 100 ml of methanol–water (8 : 2) and 5 g of sodium chloride for 30 min and the extract was filtered through Whatman No. 4 paper (Whatman International Ltd, Maidstone, UK). The filtered solution (10 ml) was diluted to 50 ml with phosphate-buffered saline, pH 7.4 (PBS) and filtered through a Whatman 934AH glass microfibre filter, and 25 ml was loaded onto an AflaTest P column (Vicam, Watertown, MA, USA) at a flow rate of 1 drop per second. After washing the column with 10 ml of PBS followed by 10 ml of purified water, the column was dried by pushing air through it with a syringe. AFs were then eluted with 3 ml of acetonitrile into a silanized amber vial (4.0 ml, Supelco, Bellefonte, PA, USA). The eluate was evaporated to dryness under a gentle stream of nitrogen at 40°C. For trifluoroacetic acid (TFA) derivatization, the residue was treated with 0.1 ml of TFA for 15 min, and 0.9 ml of injection solvent (acetonitrile–water = 1 : 9) was added to the mixture. For photochemical reactor (PCR) derivatization, the residue was dissolved in 1.0 ml of injection solvent and injected into the HPLC system.

Typical HPLC consisted of an LC-10AD pump, a SIL-10A auto-injector (100 µl loop), a CTO-10AC column oven, a RF-10AXL fluorescence detector (excitation = 360 nm; emission = 450 nm), DGU-3A degasser, a CBM-10A communication bus module and a Class LC-10 chromatography data system (Simadzu, Kyoto, Japan). The analytical column (Inertsil ODS-3V, 4.6 × 250 mm, 5 µm; GL Sciences, Inc., Tokyo, Japan) was kept at 45°C. The mobile phase was acetonitrile–methanol–water (1 : 3 : 6) at a flow rate of 1.0 $ml\,min^{-1}$ for TFA derivatization, or acetonitrile–methanol–water (2 : 3 : 5) at a flow rate of 0.7 $ml\,min^{-1}$ for PCR derivatization. In the latter case, the PCR (Photochemical Reactor for Enhanced Detection: short wave UV lamp, knitted Teflon tube, 0.25 mm × 20 m, Aura Industry Inc., New York, NY, USA) was set between the analytical column and the detector. Quantification of aflatoxins in the samples was performed by comparing their peak heights to the relevant calibration curve.

For peanut butter and sesame seed oil samples, the method by Kamimura et al. (1995) was used for analysis. Briefly, 10 or 20 g of sample was extracted with 50 or 100 ml of chloroform. The extract was loaded onto a florisil column and the AFs eluted with acetone–water (99 : 1). The eluate was evaporated to dryness under reduced pressure, and the residue was dissolved in 200 µl of chloroform. A 20-µl portion was evaporated to dryness and derivatized with TFA for quantitative analysis by HPLC as described above.

Extraction and analysis of OTA

The samples were analysed for OTA at five independent laboratories using a standardized method (Sugita-Konishi et al. 2006). Each sample, other than raisins, beer and wine, was thoroughly mixed and ground to a fine powder. Raisins were slurried with water in the ratio of 5 parts raisins to 4 parts water (w/w) to form a homogenous paste. Beer was degassed by ultrasonic bath for 30 min. Extraction solvents were as follows; methanol–water (8 : 2) and 5 g of sodium chloride for corn, corn products, rice, oatmeal and buckwheat flour; acetonitrile–water (6 : 4) for wheat and rye flour; and methanol–1% sodium bicarbonate (7 : 3) for coffee and raisins. Sample (25 or 45 g for raisin paste) were shaken in 100 ml (80 ml for raisin paste) of extraction solvent for 30 min. The extract was filtered through Whatman No. 4 paper. The filtered solution (10 ml) was diluted to 50 ml (100 ml for coffee sample) with PBS, or with PBS-0.01% Tween 20 (PBS-Tween) for coffee and raisin samples, and filtered through a Whatman 934AH glass microfibre filter. The filtered solution (25 or 40 ml for coffee sample) was loaded

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onto an OchraTest column (Vicam, Watertown, MA, USA) at a flow rate of 1 drop per second. After washing the column with 10 ml of PBS (PBS-Tween for coffee and raisin samples) followed by 10 ml of purified water, OTA was eluted with 3 ml of acetonitrile. For beer and wine, 10 g of the sample solution was diluted with 10 ml of 1% polyethylene glycol 8000 in 5% sodium bicarbonate (pH 7.1–7.2), and filtered through a Whatman 934AH glass microfibre filter. Filtrate (10 ml) was loaded onto OchraTest (Vicam) at a flow rate of 1 drop per second. The column was washed with 2.5% sodium chloride–0.5% sodium bicarbonate (pH 8.3) followed by 10 ml of purified water. OTA was then eluted with 3 ml (1 ml × 3) of methanol–acetic acid (99 : 1) into a silanized amber vial. The eluate was evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was dissolved in 1.0 ml of injection solvent (acetonitrile–water–acetic acid = 30 : 70 : 1) and injected into the HPLC system. The analytical column (Inertsil ODS-3V, 4.6 × 250 mm, 5 μm; GL Sciences, Inc., Tokyo, Japan) was kept at 45°C and the mobile phase was acetonitrile–water–acetic acid (55 : 43 : 2) at a flow rate of 1.0 ml min^{-1}. As for AF, OTA was quantified by comparing peak heights to those of a relevant calibration curve.

Confirmation of identity

The identity of AFB_1 and AFG_1 in the sample was confirmed by quenching their fluorescence in reversed-phase HPLC. AFB_1 and AFG_1 were detected as AFB_{2a} and AFG_{2a}, respectively, by pre-column derivatization with TFA or in-line post-column derivatization for PCR as used in this survey. The disappearance of the AFB_{2a} and AFG_{2a} peaks by non-TFA derivatization confirmed the original presence of AFB_1 or AFG_1 in the sample. Decreased peak heights of AFB_1 or AFG_1 with the photo chemical reaction ultra violet lamp off confirmed the original presence of AFB_1 or AFG_1. In peanut butter and sesame oil, the positive samples detected by HPLC analysis were spotted onto silica gel plate (Merck KGaA, Darmstadt, Germany) for HPTLC.

The confirmation of OTA was performed by OTA-methyl ester formation as follows (Levi et al. 1975). A portion of the purified sample solution or standard solution was evaporated to dryness and 500 μl of boron trifluoride methanol complex solution was added to the residue. The mixture was heated at 60°C for 10 min, evaporated to dryness and the residue dissolved in injection solvent for injection onto HPLC. The identity of OTA in the sample was confirmed by the presence of an OTA-methyl ester peak at a delayed retention time along with the simultaneous disappearance of the OTA peak.

AFs and OTA intake through cacao consumption

AFs and OTA intakes were estimated based on cacao intake and mycotoxin contamination data. Cacao intake based on the 2005 National Health and Nutrition Survey (2006) of the consumption of major foods containing cacao such as solid covering chocolate, solid milk chocolate, liquid cocoa and liquid milk cocoa. The distribution of cacao consumption in the 1–6, 7–14, 15–19 or over 19 years age groups was simulated (100 million iterations) by fitting log-normal distribution to the consumption data for each food, and then by multiplying food consumption by the cacao content of each food. The mycotoxin concentration of bitter chocolate was converted to mycotoxin concentration in cacao from cacao content in the chocolate. A simulation was carried out to get a distribution of mycotoxin concentration in cacao, assuming a log-normal distribution of data for the positive samples, and a uniform distribution for the samples that were below the limit of quantitation (LOQ) level (0.1 μg kg^{-1}). Finally, the distribution of mycotoxin intake in each age group was simulated (10 million iterations) using the distributions of cacao consumption and mycotoxin concentration of the cacao. Simulation was conducted using Cristal Ball (Kozo Keikaku Engineering, Inc., Tokyo, Japan).

The risk of cancer in the population caused by AFs intake through cacao consumption was calculated from the estimated AFs intake using a formula developed by the FAO/WHO Joint Expert Committee on Food Additives (1998): 0.3 or 0.01 cancers $year^{-1}$ ng^{-1} aflatoxin kg^{-1} body weight day^{-1} in 100,000 carrier or non-carrier of hepatitis B and C populations. In the calculation, 127 million and 3 million were used for the total population and the number of hepatitis B or C carriers in Japan, respectively. For OTA, ratio of OTA intake through cacao consumption to the provisional tolerable weekly intake (PTWI) (100 ng kg^{-1} body weight $week^{-1}$) proposed by JECFA was calculated.

Results and discussion

The recoveries of AFs from the matrices analysed in this study ranged from 65.0% to 103.7% for AFB_1, from 68.7% to 113.7% for AFB_2, from 64.5% to 102.5% for AFG_1, and from 70.2% to 101.2% for AFG_2 ($n = 2–3$, each matrix). OTA recoveries ranged from 60.4% to 107.9%. The LOQ was calculated by the signal-to-noise (S/N) ratio to be more than 10.

AFs were detected in ten of 21 peanut butter samples, and in 22 of 42 bitter chocolate samples (Table 1). Six and two of the ten positive peanut butter samples were imported and domestic products, respectively. The origin of the other two positive peanut butter products is unknown. All four AFs

Table 1. Aflatoxin contamination in positive samples.

Commodity	Number of analysed samples	Number of positive samples*	Contamination levels ($\mu g\ kg^{-1}$) in positive samples, mean (range)			
			AFB_1	AFB_2	AFG_1	AFG_2
Peanut butter	21	10	1.07 (0.17–2.59)	0.27 (0.16–0.52)	0.4 (0.17–0.81)	0.21 (0.12–0.46)
Bitter chocolate	42	22	0.18 (0.11–0.60)	n.d.	n.d.	n.d.

Notes: *Greater than the limit of quantitation (LOQ) (0.1 $\mu g\ kg^{-1}$). n.d., Not detected.

(AFB_1, AFB_2, AFG_1 and AFG_2) were found in four positive samples, AFB_1 and AFB_2 were detected in three, and AFB_1 only was found in the other three. All positive bitter chocolate samples were produced in Japan, and only AFB_1 was detected.

However, AFs were not detected in ten raw corn, 50 frozen or canned corn, 50 rice, 30 whole peanut, 30 shelled peanut, ten peanut flour, twelve buckwheat flour, 39 dried buckwheat noodle, ten unpopped popcorn, 20 cornflakes, ten sesame oil, and ten corn grits samples.

OTA was detected in oatmeal, wheat flour, rye, buckwheat flour, raw coffee, roasted coffee, raisins, beer and wine, but not in rice, corn flakes, corn grits, popcorn, canned or frozen corn, and bitter chocolate (Table 2). Contamination was frequently noted in raisins, beer, rye, wine and bitter chocolate: all positive rye and raisin samples were imported products, while positive beer, buckwheat flour and wine samples were products of both imported and domestic origin. The positive bitter chocolate samples were produced in Japan. OTA levels of higher than 1 $\mu g\ kg^{-1}$ have frequently been observed in wine (Joint FAO/WHO Expert Committee on Food Additives 2001; Lombaert et al. 2004), but the levels noted in this study were below 0.8 $\mu g\ kg^{-1}$. OTA was not detected in 30 frozen or canned corn, five popcorn grain, 20 cornflake, five corn grits or 50 rice samples.

Reports of OTA contamination in buckwheat are very limited. A survey conducted in Germany from 1995 to 1998 showed that only three of 14 buckwheat samples were contaminated with more than 0.01 $\mu g\ kg^{-1}$ and the highest concentration found was 12.1 $\mu g\ kg^{-1}$ (Joint FAO/WHO Expert Committee on Food Additives 2001). In the present study, buckwheat flour was very frequently contaminated with OTA, although at levels that were lower than those reported from Germany. This indicates the importance of broadening the survey of OTA contamination of domestic and imported buckwheat in Japan over successive years. OTA contamination levels in the other positive samples were similar to or lower than those reported by many European and American countries (Biffi et al. 2004; Joint FAO/WHO Expert Committee on Food Additives 1998; Lombaert et al. 2004; Ng et al. 2004; Rosa et al. 2004; Tabata et al. 1993).

The sources of all the materials used in the food samples processed domestically were not known. However, the rice and raw corn in these processed foods were confirmed to be domestic products. AFs have been detected in imported rice in Japan (Lipigorngoson et al. 2003), but not in domestic rice until now, as confirmed by Tabata et al. (1993) and in this study. AFs contamination was observed in peanut butter; the maximum levels were 2.59, 0.52, 0.81 and 0.46 $\mu g\ kg^{-1}$ for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. Taken together with the findings that AFs contamination in peanut butter retailed from 1988 to 1992 in Japan (Tabata et al. 1993) was lower than those noted in this study, it was concluded that some peanut butter products distributed in Japan may continuously be contaminated with low levels of AFs. The survey in the Republic of Cyprus indicated that 21 of 74 peanut butter samples were positive with the maximum reported level of AFB_1 being 73 $\mu g\ kg^{-1}$ (Ioannou-Kakouri et al. 1999). Siame et al. (1998) reported that the average AFs level in peanut butter was 23 $\mu g\ kg^{-1}$ in Botswana. In Sudan, heavy contamination of peanut butter with AFs has been regarded to be a risk factor of hepatic cell cancer (Omer et al. 2001). Although the risk to human health of the low levels of AFs in peanut butter noted in this study may be very low, control measures to reduce AFs contamination further will be needed.

Based on data of cacao consumption and those of AFs or OTA concentration in cacao, the intake of AFs or OTA through consumption of cacao was estimated by age group and shown as intake ($ng\ kg^{-1}$ body weight day^{-1}) at 95, 99 or 100 percentiles (Tables 3 and 4). Estimated AFs intake was greater in the young age groups than in the senior age groups. The maximum level was 1.530 $ng\ kg^{-1}$ body weight day^{-1} at 1–6 years of age at the 100th percentile, when LOQ was assumed to be 0.05 $\mu g\ kg^{-1}$. The population risk of cancer caused by this level of AFs intake was calculated to be 0.00040 cancers/year/10^6 people at the 99th percentile in 1–6 years group ($0.01 \times 0.026\ ng\ kg^{-1} day^{-1} \times 98\% + 0.3 \times 0.026\ ng\ kg^{-1} day^{-1} \times 2\%$).

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Table 2. Ochratoxin A (OTA) contamination in retail foods.

Commodity	Number of analysed samples	Number of positive samples	LOQ ($\mu g\,kg^{-1}$)	Contamination levels of OTA ($\mu g\,g^{-1}$) in positive samples, mean (range)
Rye	10	9	0.1	1.05 (0.28–1.59)
Wheat flour	50	28	0.1	0.09 (0.10–0.48)
Oat meal	20	10	0.05	0.09 (0.06–0.18)
Buckwheat flour	10	8	0.1	0.51 (0.16–1.79)
Raisins	11	10	0.04	1.73 (0.02–12.5)
Wine	10	8	0.004	0.26 (0.02–0.72)
Beer	20	14	0.01	0.019 (0.010–0.054)
Green beans	11	3	0.1	0.45 (0.14–0.76)
Roast coffee	9	3	0.1	0.22 (0.11–0.33)
Bitter chocolate	41	27	0.1	0.35 (0.10–0.94)

Table 3. Estimated AFs intake ($ng\,kg^{-1}$ body weight day^{-1}).

Age group	95%	99%	100%
Uniform[a]			
1–6 years	0.00647943	0.02666246	1.43461050
7–14 years	0.00519781	0.02031575	1.64973305
15–19 years	0.00416538	0.01741264	1.10132707
> 19 years	0.00064606	0.00581051	0.40526561
LOQ[b]			
1–6 years	0.00723121	0.02706037	1.52939886
7–14 years	0.00571573	0.02042507	1.06746737
15–19 years	0.00470295	0.01750246	0.68623998
> 19 years	0.00098557	0.00594889	0.40166620

Notes: [a]A uniform distribution (=50 $ng\,kg^{-1}$) was assumed for samples that were below the limit of quantitation (LOQ). [b]An LOQ (=100 $ng\,kg^{-1}$) was assumed for the samples that were below the LOQ.

Table 4. Estimated ochratoxin A (OTA) intake ($ng\,kg^{-1}$ body weight day^{-1}).

Age group	95%	99%	100%
Uniform[a]			
1–6 years	0.01127981	0.04112623	1.65398846
7–14 years	0.00915522	0.03063379	1.54575986
15–19 years	0.00748142	0.02649262	1.24825494
> 19 years	0.00114861	0.00949159	0.45068869
LOQ[b]			
1–6 years	0.01162600	0.04122850	1.44919900
7–14 years	0.00934272	0.03071149	0.88425257
15–19 years	0.00771151	0.02660415	1.45312934
> 19 years	0.00147937	0.00952279	0.56560195

Notes: [a]A uniform distribution (=50 $ng\,kg^{-1}$) was assumed for the samples that were below LOQ. [b]LOQ (=100 $ng\,kg^{-1}$) was assumed for the samples that were below LOQ.

Estimated OTA intake also tended to be greater in the younger age groups. The maximum level was 1.653 $ng\,kg^{-1}$ body weight day^{-1} at 1–6 years of age at the 100th percentile, when LOQ was assumed to take uniform distribution. This level was 11.58% of the PTWI proposed by the Joint FAO/WHO Expert Committee on Food Additives.

This study estimated OTA and AFs intake through consumption of foods containing cacao. A survey of mycotoxin contamination in a variety of foods and the estimation of total intake of mycotoxins in Japan are now in progress.

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A reduced rate of deoxynivalenol and nivalenol during bread production from wheat flour in Japan

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Summary

Deoxynivalenol (DON) and nivalenol (NIV), which are produced by *Fusarium* fungi as secondary metabolite, are among the mycotoxins known as trichothecenes and they naturally occur in cereal grains of bread making wheat. In this study, contamination levels of these mycotoxins in various wheat flour samples containing domestic flour used for the mass production of bread and related products were collected and analyzed in Japan. Samples of flour and bread were collected from nine prefectures, and their trichothecene levels were measured by a validated High Performance Liquid Chromatography-Mass Spectrometry system. The average concentrations of DON and NIV in flour samples collected were 31.3 ± 28.9 and 8.5 ± 3.7 μg/kg, whereas those in bread samples were 8.6 ± 5.1 μg/kg and 3.4 ± 2.0 μg/kg, respectively. These results suggest that the percentage of DON and NIV remaining after converting flour into bread using industrial equipment and baking yeast were estimated as approximately 74.4 and 65.8 %, respectively.

Key words : deoxynivalenol, nivalenol, attenuation, domestic flour, bread

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Introduction

Trichothecene mycotoxins which are produced by *Fusarium* molds are found in cereals such as wheat, maize, barley, rice and oats [1]. Deoxynivalenol (DON) is a trichothecene mycotoxin and found in corn, barley, wheat and feedstuff worldwide, including European Union, the United States and Japan [2]. In addition, the occurrence of nivalenol (NIV) which is also trichothecene mycotoxins is frequently observed in some countries including Japan [3]. These trichothecene mycotoxins possess a hazard to human health when they are taken through the ingestion of contaminated cereals and related products. It has been reported that the toxic effects of trichothecene mycotoxins include immunosuppression, diarrhea and body growth inhibition [4].

Recently, the consumption of domestic wheat for baking has been increasing in Japan because the bulk price of foreign wheat supplied by the Japanese government to the market has been raised

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due to a lower supply of imported wheat. Moreover, Japanese consumers are showing the tendency to prefer domestic foods to imported foods which may sometimes pose unexpected risks to human health.

The aim of this study is to estimate the degradation degree of DON and NIV during bread production using flour containing domestic wheat in Japan. Therefore, the contamination levels of DON and NIV were measured in flour made of imported and domestic wheat supplied to the baking industry, and the concentrations of these trichothecenes in bread were also analyzed.

Materials and Methods

Sampling From June 2007 to July 2007, samples of wheat flour (n=12) used in the baking industry were collected from flour milling companies or bread baker located in nine prefectures (A – I) in Japan. All flour was mixtures of both domestic and imported flour. Samples of bread (n=35) produced from the flour explained above were simultaneously obtained from bread bakers. These samples were placed in plastic bags and brought to the laboratory for analysis of DON and NIV. All samples were stored at -80 °C until processing and analysis.

Analytical Methodology DON and NIV in samples were analyzed according to the method of Sugita-Konishi, *et al.* [5]. 25 g of flour and homogenized bread were extracted with 100 mL of an extraction solution (acetonitrile:water, 85:15) by shaking for 30 min. The extraction solution was centrifuged briefly and the obtained supernatant was cleaned by passing it through a multi functional column (Autoprep® MF-T 1500, Showa Denko, Tokyo, Japan). The first elution of 4 mL was discarded and the following elution of 4 mL was collected. The elution solvent was removed under a nitrogen steam and the sample was immediately dissolved in 1 mL of 10 mmol/L ammonium acetate-methanol (90:10).

The DON and NIV concentration were determined using a High Performance Liquid Chromatography-Mass Spectrometry (LC-MS) system consisted of Shimadzu Model LC-2010C$_{HT}$ liquid chromatograph system (Shimadzu, Kyoto, Japan) including a degassing unit, a binary gradient pump, an auto-injector, a column oven and Shimadzu LCMS-2010A mass spectrometer. Separation was performed isocratically at a flow rate of 0.2 mL/min using 10 mmol/L ammonium acetate-methanol (90:10) on Inertsil® ODS-SP column (150 mm × 2.1 mm i.d., 5 µm; GL Sciences, Tokyo, Japan) held at 40 °C. MS experiments were performed in the APCI negative ionization mode. The probe voltage, the probe temperature, the nebulizer gas, the drying gas, the CDL voltage, the CDL temperature, the block heater temperature, the Q-array DC and the Q-array RF were set at 4.5 V, 400 °C, 2.5 L/min, 0.02 MPa, 5 V, 250 °C, 200 °C, 40 V and 150 V, respectively. For the quantitative analysis of both mycotoxins, selected-ion monitoring was used and the following ions were detected.

DON (*Mr* 296) *m/z* 355.1

NIV (*Mr* 312) *m/z* 371.1

The limit of quantitation of the assay was 5.0 µg/kg for flour and 1.0 µg/kg for bread, respectively.

Calculation of the percentage of DON or NIV remaining after making bread It has been reported that 437 g of bread with water is produced when 300 g of flour with water is used in Japan (personal communication). If other starches except wheat were not contained in all the bread samples, contents of trichothecenes in bread would eventually be diluted as ration of 300:437.

Therefore, contents of trichothecenes in the flour utilized in bread production were calculated using the ration of 437:300 (approximately 1.46), and then the percentage of DON or NIV remaining after making bread was assumed by the following equation:
% DON or NIV remaining = [(1.46 × toxin containing bread (μg/kg))/toxin containing flour (μg/kg)] × 100.

Results and Discussion

The contamination level of DON in flour and bread was measured using the LC-MS system (Table 1). The DON level in flour was 31.3 ± 28.9 μg/kg in this study. The difference in the contamination level of DON among areas might be due to that in the rate of domestic flour contained in materials for baking bread. The DON contamination level in bread was estimated at 8.6 ± 5.1 μg/kg in this study. On the other hand, the contamination level of NIV was 8.5 ± 3.7 μg/kg for flour and 3.4 ± 2.0 μg/kg for bread (Table 2). These results indicated that the concentration of DON in flour and bread is higher than that of NIV as reported by Sugita-Konishi *et al.* (unpublished observation). No samples showed DON levels above the Japanese temporary limit (1,100 μg/kg).

According to these results, the percentages of DON and NIV remaining after converting flour into bread were estimated approximately 74.4 and 65.8 %, respectively. Although baking process has been carried out at temperatures in the range of 160 to 220 °C for approximately 30 min in traditional bakeries, it is reported that DON is stable under these conditions [6]. Therefore, some steps in making bread except baking might mainly contribute to reduce DON and NIV in flour. On the other hand, it was also observed that the concentrations of DON (Cb-5, Db-1, 2, 3, 4, 5, Fb-1) and NIV (Bb-1, 2, 3, Db-2, 4, Eb-6, Fb-1, Hb-2, 3) in bread increased through the process of making bread (Table1, 2). Young *et al.* observed the increase of DON levels in bread making involved biological processes [7]. Consistent with this report, these results indicated that the precursors of these toxins might be converted into each toxin during fermentation.

This report demonstrated for the first time that there is an attenuation of DON and NIV contamination levels during the industrial bread producing process in Japan. A previous analysis demonstrated a reduction in DON levels of 2.9 % when bread is baked at home (Kumagai *et al.*, unpublished data). This might result from differences in the processes of yeast fermentation and baking, as Samar *et al.* reported that DON reduction during bread-making is caused by these processes [8]. The present results indicated an attenuation of the contamination levels of both DON and NIV during the bulk fermentation baking process, thus showing important information regarding the risk analysis for these toxins in bread. Moreover, the observed contamination levels of DON and NIV in bread supplied to the Japanese market indicate a need of conducting comprehensive risk assessments of both toxins. The more toxicological profiles of NIV which are less reported than those of DON would be required to estimate the hazardous risk of NIV.

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Table 1. Concentration of DON in flour and bread, and the estimated percentage of DON remaining in bread

Area	Sample		DON (μg/kg)			% DON remaining
	Flour	Bread	Flour	Bread	Flour content** in bread	
A	Af-1	Ab-1	51.3	15.1	22.0	42.9
	Af-2	Ab-2	27.1	3.9	5.7	21.0
	Af-1	Ab-3	51.3	20.3	29.5	57.6
	Af-2	Ab-4	27.1	8.0	11.7	43.2
	Af-1	Ab-5	51.3	10.7	15.6	30.4
	Af-2	Ab-6	27.1	8.5	12.3	45.5
B	Bf-1	Bb-1	14.0	4.6	6.7	47.7
	Bf-1	Bb-2	14.0	4.0	5.8	41.8
	Bf-1	Bb-3	14.0	2.0	2.9	20.6
C	Cf-1	Cb-1	30.0	5.7	8.2	27.5
	Cf-1	Cb-2	30.0	9.5	13.8	46.1
	Cf-1	Cb-3	30.0	13.5	19.6	65.5
	Cf-1	Cb-4	30.0	8.7	12.6	42.1
	Cf-1	Cb-5	30.0	25.2	36.7	122.5
D	Df-1	Db-1	5.0*	12.2	17.8	355.1
	Df-1	Db-2	5.0*	8.1	11.8	236.7
	Df-1	Db-3	5.0*	6.1	8.8	176.7
	Df-1	Db-4	5.0*	10.9	15.8	316.5
	Df-1	Db-5	5.0*	7.1	10.3	206.5
E	Ef-1	Eb-1	21.0	1.9	2.8	13.5
	Ef-1	Eb-2	21.0	3.5	5.1	24.4
	Ef-2	Eb-3	31.6	6.0	8.7	27.6
	Ef-1	Eb-4	21.0	8.8	12.8	61.0
	Ef-1	Eb-5	21.0	5.4	7.9	37.7
	Ef-1	Eb-6	21.0	12.4	18.0	85.9
	Ef-1	Eb-7	21.0	4.8	7.0	33.2
	Ef-1	Eb-8	21.0	3.0	4.4	20.8
F	Ff-1	Fb-1	5.2	4.7	6.8	131.1
G	Gf-1	Gb-1	34.4	11.9	17.3	50.4
	Gf-2	Gb-2	28.3	12.1	17.6	62.1
	Gf-1	Gb-3	34.4	2.9	4.2	12.2
H	Hf-1	Hb-1	113.2	7.8	11.3	10.0
	Hf-1	Hb-2	113.2	14.3	20.8	18.4
	Hf-1	Hb-3	113.2	10.4	15.1	13.4
I	If-1	Ib-1	14.8	5.9	8.7	58.5
Number	12	35	12	35	35	35
Mean			31.3	8.6	12.5	74.4
Standard Deviation			28.9	5.1	7.4	84.8

* Its concentration was below the LOQ of the method for the analysis of DON in flour (5.0 μg/kg), and considered value of the LOQ for calculation.

** This value was assumed by the equation [1.46 × DON containing bread (μg/kg)] bacause 437 g of bread is produced by using 300 g of flour in the case of using only wheat starch.

Table 2. Concentration of NIV in flour and bread, and the estimated percentage of NIV remaining in bread

Area	Sample		NIV (μg/kg)			% NIV remaining
	Flour	Bread	Flour	Bread	Flour content*** in bread	
A	Af-1	Ab-1	12.5	4.7	6.9	54.9
	Af-2	Ab-2	9.6	5.0	7.3	75.9
	Af-1	Ab-3	12.5	1.7	2.4	19.3
	Af-2	Ab-4	9.6	1.5	2.2	22.5
	Af-1	Ab-5	12.5	2.0	3.0	23.6
	Af-2	Ab-6	9.6	2.2	3.3	33.9
B	Bf-1	Bb-1	5.0 *	5.0	7.3	145.7
	Bf-1	Bb-2	5.0 *	5.0	7.3	145.7
	Bf-1	Bb-3	5.0 *	5.0	7.3	145.7
C	Cf-1	Cb-1	10.9	1.0	1.5	13.9
	Cf-1	Cb-2	10.9	3.6	5.3	48.4
	Cf-1	Cb-3	10.9	2.9	4.3	39.1
	Cf-1	Cb-4	10.9	3.2	4.7	42.8
	Cf-1	Cb-5	10.9	4.9	7.2	65.6
D	Df-1	Db-1	5.0 *	1.0 **	1.5	29.1
	Df-1	Db-2	5.0 *	5.0	7.3	145.7
	Df-1	Db-3	5.0 *	1.7	2.5	50.2
	Df-1	Db-4	5.0 *	5.0	7.3	145.7
	Df-1	Db-5	5.0 *	1.0 **	1.5	29.1
E	Ef-1	Eb-1	11.8	5.0	7.3	61.7
	Ef-1	Eb-2	11.8	5.0	7.3	61.7
	Ef-2	Eb-3	15.9	2.6	3.8	24.0
	Ef-1	Eb-4	11.8	2.9	4.2	35.8
	Ef-1	Eb-5	11.8	2.5	3.6	30.3
	Ef-1	Eb-6	11.8	8.5	12.4	105.4
	Ef-1	Eb-7	11.8	1.1	1.6	13.7
	Ef-1	Eb-8	11.8	8.0	11.7	98.8
F	Ff-1	Fb-1	5.0 *	5.0	7.3	145.7
G	Gf-1	Gb-1	9.0	1.3	1.8	20.6
	Gf-2	Gb-2	6.9	2.8	4.0	58.4
	Gf-1	Gb-3	9.0	1.2	1.8	19.8
H	Hf-1	Hb-1	5.0 *	1.0 **	1.5	29.1
	Hf-1	Hb-2	5.0 *	5.0	7.3	145.7
	Hf-1	Hb-3	5.0 *	5.0	7.3	145.7
I	If-1	Ib-1	5.0 *	1.0 **	1.5	29.1
Number	12	35	12	35	35	35
Mean			8.5	3.4	5.0	65.8
Standard Deviation			3.7	2.0	2.9	49.0

* Its concentration was below the LOQ of the method for the analysis of NIV in flour (5.0 μg/kg), and considered value of the LOQ for calculation.

** Its consentration was below the LOQ of the method for the analysis of NIV in bread (1.0 μg/kg), and considered value of the LOQ for calcuration.

*** This value was assumed by the equation [1.46 × NIV containing bread (μg/kg)] because 437 g of bread is produced by using 300 g of flour in the case of using only wheat starch.