— Regular Article —

A Survey of the Occurrence of *Fusarium* Mycotoxins in Biscuits in Japan by Using LC/MS

Hiroki Tanaka,*, [a,1] Yoshiko Sugita-Konishi,[a] Masahiko Takino,[b] Toshitsugu Tanaka,[c] Akira Toriba,[d] and Kazuichi Hayakawa[d]

[a]National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan, [b]Agilent Technologies Japan, Limited, Hachioji Site, 9–1 Takakura-cho, Hachioji-shi, Tokyo 192–8501, Japan, [c]Kobe Institute of Health, 4–6 Minatojima-Nakamachi, Chuo-ku, Kobe 650–0046, Japan, and [d]Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920–1192, Japan

(Received January 4, 2010; Accepted January 18, 2010; Published online January 22, 2010)

By adopting a rapid and sensitive method for simultaneous detection of nivalenol (NIV), deoxynivalenol (DON), fusarenon-X (FX), 3-acetyl deoxinivalenol (3ADON), HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalenone (ZEN), the natural occurrence of these mycotoxins in biscuits made of wheat (201 samples) in Japan was surveyed. Samples were analyzed by LC/MS with atmospheric pressure photo ionization (APPI). Further confirmation was performed by liquid chromatography/time of flight mass spectrometry (LC/TOFMS). The average contamination of each *Fusarium* mycotoxin was 3.1, 23, 0.7, 0.1 and 4.2 ng/g for NIV, DON, HT-2, T-2 and ZEN, respectively. Multiple toxins were observed in 120 samples while FX and 3ADON were not detected. The incidence of these toxins was 41% for NIV, 98% for DON, 19% for HT-2, 11% for T-2 and 2% for ZEN. There were no significant differences in the concentration and incidence between conventional biscuits made of wheat and biscuits made of wheat for infants. This is the first report concerning the presence of NIV, DON, HT-2, T-2 and ZEN in biscuits in Japan.

Key words —— *Fusarium* mycotoxin, contamination survey, LC/MS, LC/time of flight mass spectrometry, biscuit, Japan

IINTRODUCTION

Trichothecene mycotoxins (TRs), such as nivalenol (NIV), deoxynivalenol (DON), fusarenon-X (FX), 3-acetyl deoxynivalenol (3ADON), HT-2 toxin (HT-2) and T-2 toxin (T-2), belong to the secondary toxic metabolites produced by various filamentous fungi, such as *Fusarium graminearum*, *F. culmorum* and *F. sporotrichioides*.

TRs exhibit a potent inhibitory activity toward protein and DNA syntheses in eukaryotic cells, and this biological activity is closely related to their high lethality to animals, cellular damage to actively dividing cells, potent suppression of immunoresponses[1,2] and inhibition of protein synthesis.[3,4]

Zearalenone (ZEN) is an estrogenic metabolite produced by *Fusarium* species such as *F. graminearum*, *F. culmorum* and *F. crookwellense* (*F. cerealis*), and causes hyperestrogenism in livestock.[5,6]

Co-contamination of *Fusarium* mycotoxins (TRs and ZEN) occurs worldwide in agricultural commodities and consumption of these has caused several outbreaks of intoxication in human and animal populations.[7–12] With the development of highly sensitive and simultaneous analytical methods, many reports regarding the co-contamination of *Fusarium* mycotoxins in processed cereal foods have been reported in Europe and North America,[13–15] but relatively little work is available for Asian countries despite these depending heavily on imported wheat and wheat-derived products. Thus, an accurate determination of processed food contaminated with these toxins is an urgent need for

[1]Present address: Research Center, Suntory Business Expert Limited, 1–1–1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618–8503, Japan

*To whom correspondence should be addressed: National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan. Tel.: +81-3-3700-1141; Fax: +81-3-3700-9527; E-mail: hiroki.t@poem.ocn.ne.jp

food supply.

The purpose of this study was to simultaneously determine of TRs and ZEN based on a method established in a previous study.[16] Furthermore, by using this method, we determined the incidence and concentration of *Fusarium* mycotoxins in biscuits made of wheat in Japan for the first time and to use this information to evaluate the potential health risk to Japanese consumers.

MATERIALS AND METHODS

Samples —— A total of 201 samples of biscuits made of wheat were purchased from random local retail shops throughout Japan between the summer 2004 and summer 2006, including 110 of infant food, 39 imported, 17 domestic and the remaining of unknown origin. All samples were stored at 4°C until analysis.

Chemicals and Reagents —— The standards for NIV, DON, FX, 3ADON, HT-2, T-2 and ZEN were obtained from Sigma-Aldrich Japan (Tokyo, Japan). HPLC grade acetonitrile, HPLC grade methanol and reagent grade ammonium acetate were obtained from Wako Chemical (Osaka, Japan). Water was purified with a Milli-Q system (Millipore, Tokyo, Japan). MultiSep #226 columns (Romer Labs, Inc., Union, MO, U.S.A.) were purchased from Showa Denko Limited (Tokyo, Japan). All other reagents were of the highest analytical grade available.

LC/MS —— The LC/MS was performed using a Shimadzu Model LC-2010C$_{HT}$ liquid chromatograph system (Shimadzu, Kyoto, Japan) including a degassing unit, a binary gradient pump, an autoinjector, a column oven and a Shimadzu LCMS-2010A mass spectrometer with atmospheric pressure photo ionization (APPI) capabilities. Liquid chromatography (LC) separation was performed on a 150 mm × 2.0 mm inside diameter (I.D.) column packed with a 5 μm Shimadzu Shim-pack VP-ODS. The LC mobile phase was a mixture of aqueous 10 mM ammonium acetate (A) and methanol (B). The initial gradient condition was 90% A and 10% B, and was equilibrated for 5 min. Then, solvent B was changed linearly to 100% in 20 min, and was held for 10 min. The flow rate was set at 0.1 ml/min. Further, acetone was added after the diode array detector at a flow rate of 60 μl/min via a tee by an isocratic pump (Agilent Technologies, Waldbronn, Germany). The column temperature was maintained at 40°C and the injection volume was 10 μl. MS experiments were performed in the APPI mode. Nitrogen as the nebulizer gas in the ion source was generated from pressurized air by a SLP-07-S2 (ANEST IWATA Co., Yokohama, Japan). The following analytical conditions for APPI were optimized by using an analytical column with the mixture standard of 7 *Fusarium* mycotoxins at 100 ng/ml. The probe voltage, probe temperature, nebulizer gas, drying gas, curved desolvation line (CDL) voltage, CDL temperature, block heater temperature, Q-array direct current (DC) and Q-array radio frequency (RF) were set at 0 V, 200°C, 2.5 l/min, 0 MPa, 5 V, 150°C, 150°C, 5 V and 150 V, respectively. Acetone was used as the dopant solvent. The quantitative analysis of each *Fusarium* mycotoxin was carried out using the selected ion monitoring (SIM) mode of each base ion peak at m/z 371 (NIV), 355 (DON), 413 (FX), 397 (3ADON), 483 (HT-2) and 317 (ZEN) in the negative mode and m/z 484 (T-2) in the positive mode, respectively (Table 1).

LC/Time of Flight Mass Spectrometry (TOF-MS) —— The LC/TOFMS instrument and condition for analysis of *Fusarium* mycotoxins were reported in an earlier paper.[17]

Table 1. SIM for the Analysis of Target Analytes

Mycotoxins	M.W.[a]	Ionization mode					
		APPI		APCI		ESI	
NIV	312	371	$[M+CH_3COO]^-$	371	$[M+CH_3COO]^-$	371	$[M+CH_3COO]^-$
DON	296	355	$[M+CH_3COO]^-$	355	$[M+CH_3COO]^-$	355	$[M+CH_3COO]^-$
FX	354	413	$[M+CH_3COO]^-$	413	$[M+CH_3COO]^-$	413	$[M+CH_3COO]^-$
3ADON	338	397	$[M+CH_3COO]^-$	339	$[M+H]^+$	397	$[M+CH_3COO]^-$
HT-2	424	483	$[M+CH_3COO]^-$	483	$[M+CH_3COO]^-$	483	$[M+CH_3COO]^-$
T-2	466	484	$[M+NH_4]^+$	484	$[M+NH_4]^+$	484	$[M+NH_4]^+$
ZEN	318	317	$[M]^-$	317	$[M]^-$	317	$[M]^-$

a) M.W. ; Molecular weight.

Preparation of Standard Solution and Samples —— The mixture of TRs and ZEN standard solutions (10 μg/ml) for stock and fortification experiments were dissolved in acetonitrile and stored at 4°C in the dark until use. For preparation of a mixed working standard solution, an appropriate amount of individual stock standard solution was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 1 ml of aqueous 10 mM ammonium acetate/methanol (90/10). For fortification experiments, 50 μl of the stock standard solution was spiked into 10 g of blank samples (= 50 ng/g) before extraction. Three replicates for each level were prepared.

Sample extraction and cleanup was carried out as follows. Ten grams of each sample was weighed in a 100 ml Erlenmeyer flask, suspended in 40 ml acetonitrile/water (85/15) and shaken for 30 min. The mixed solution was centrifuged for 5 min at 1410 *g*, and then 10 ml of the supernatant were applied to a MultiSep #226 cartridge column for the cleanup. After discarding the first 3 ml of elutant, the next 2 ml were collected and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 1.0 ml of aqueous 10 mM ammonium acetate/methanol (90/10).

The analysis of TRs and ZEN was carried out using primary screening with LC/MS, and confirmed with LC/TOFMS.

RESULTS AND DISCUSSION

Comparison of Ionization Techniques for LC/MS Analysis

Ionization methods were compared: APPI, electrospray ionization (ESI) and atmospheric chemical ionization (APCI) modes. The SIM of each *Fusarium* mycotoxin was evaluated in the scan mode (m/z 100–500) by direct injection of each *Fusarium* mycotoxin standard (1 μg/ml) without the use of an analytical column. The most sensitive ion differed depending on the mycotoxin, ionization, polarity, and ammonium adduct ion ($[M+NH_4]^+$) and acetic acid adduct ion ($[M+CH_3COO]^-$) (Table 1). In order to achieve maximum sensitivity, the instrument detection limits (IDLs) were calculated when the peak to peak signal-to-noise (S/N) ratio was 3 by injecting 10 μl of a mixture standard solution of TRs and ZEN. As shown in Table 2, the APPI mode provided the optimum intensity for NIV, DON and ZEN. Although the ESI mode showed the strongest intensity of 3ADON, the effect of a sample matrix is possibly high.[16] A recent report describes the optimization of NIV and DON detection using the APPI mode;[16] therefore, we used the APPI mode to analyze the 7 *Fusarium* mycotoxins in this study.

Table 2. Instrument Detection Limits of *Fusarium* Mycotoxins by LC/MS

Mycotoxins	Instrument detection limits[a] (pg)		
	APPI	APCI	ESI
NIV	1.8	2.5	2.4
DON	2.5	4.5	8.5
FX	7.7	6.3	7.7
3ADON	2.1	8.3	1.0
HT-2	6.8	5.0	14
T-2	4.3	6.8	6.5
ZEN	1.3	4.3	1.7

a) Instrument detection limits defined as S/N ratio = 3. Injection volume of the standard solution was 10 μl.

Matrix effects are a major problem for mycotoxin quantification by LC/MS. The sample matrix may either enhance or suppress the ionization of mycotoxins; however, the effects vary from sample to sample ultimately affecting the quantitative performance of the LC/MS system. To evaluate matrix effects, the concentration of 7 *Fusarium* mycotoxins ranging from 1 to 500 ng/ml in solvent standard and spiked biscuit samples were analyzed. The calibration curves for both conditions showed good linearity with correlation coefficients (r^2) above 0.999. However, the slope of the linearity curve for ZEN in the spiked biscuit matrix standard was about 89% lower than the solvent standard (data not shown). These results indicate that ZEN showed a matrix effect of ion suppression.

To evaluate recoveries, the proposed method was applied to the analysis of biscuit samples spiked with known concentrations of *Fusarium* mycotoxins. Samples were spiked at a final concentration of 50 ng/g for all 7 *Fusarium* mycotoxins. Quantification was carried out by the solvent standard and biscuit matrix matched standard. The mean recovery of each *Fusarium* mycotoxin in spiked samples ranged from 28 to 121% in the solvent standard and from 83 to 120% in the matrix matched standard (Table 3). At the same concentration, the experiment was repeatable (n = 3) with relative standard deviations (RSDs) ranging from 3.4 to 13% in solvent standard and from 1.0 to 11% in matrix matched standard (Table 3). Under these conditions, ZEN showed a large matrix effect on ion suppression, because ZEN is different structure to have the macrolide ring

Table 3. Recoveries, Limits of Detection and Limits of Quantification of *Fusarium* Mycotoxins in Spiked Biscuits by LC/MS

Mycotoxins	Standard	Recovery[a] (%)	RSD[a] (%)	Limit of detection[b] (ng/g)	Limit of quantification[b] (ng/g)
NIV	Solvent	94	3.4	1.4	4.7
	Matrix	83	3.8		
DON	Solvent	105	6.1	0.9	3.0
	Matrix	104	1.0		
FX	Solvent	121	6.2	1.2	4.1
	Matrix	120	4.2		
3ADON	Solvent	116	4.8	1.3	4.2
	Matrix	112	4.9		
HT-2	Solvent	115	11	0.6	2.0
	Matrix	115	11		
T-2	Solvent	116	9.2	0.1	0.3
	Matrix	113	8.9		
ZEN	Solvent	28	13	4.2	14
	Matrix	97	5.4		

a) Recoveries and RSDs were calculated on the basis of three replicates at 50 ng/g. *b*) Limits of detection and limits of quantification calculated by the biscuit matrix matched standard defined as S/N ratio = 3 and 10, respectively.

Table 4. Occurrence of *Fusarium* Mycotoxins in Biscuits

Mycotoxins	Positive samples[a] (Incidence, %)	Range (ng/g)	Mean[b] (ng/g)
NIV	83 (41)	1.4– 35	3.1
DON	196 (98)	0.9–791	23
HT-2	38 (19)	0.6– 20	0.7
T-2	22 (11)	0.1– 6.0	0.1
ZEN	4 (2)	4.2– 4.4	4.2

a) Number of samples analyzed = 201. *b*) When the number of samples of under the limit of quantification is > 60%, the concentration was calculated as follows. The value less than the limit of detection was calculated as the detection limit. The value between the limit of detection and the limit of quantification was calculated as the limit of quantification. When the number of samples of under the limit of quantification is < 60%, the concentration was calculated as follows. The value under the limit of quantification was calculated as 1/2 of the limit of detection.

and is a low polar compound when compared with the six other TRs, and is thus considered susceptible to matrix effects; therefore, the biscuit matrix matched standard was used for the quantitation of all 7 *Fusarium* mycotoxins throughout this study.

The limits of detection (LODs) and limits of quantification (LOQs) of the TRs and ZEN in biscuits were determined by the signal corresponding to three times and ten times the background noise on each SIM chromatogram, respectively. The LODs and LOQs of each *Fusarium* mycotoxin ranged from 0.1 to 4.2 ng/g and from 0.3 to 14 ng/g, respectively (Table 3).

Analysis of TRs and ZEN in Biscuits Based on Wheat

The developed method was applied in the quantitation of *Fusarium* mycotoxins in biscuits made of wheat. The level of occurrence of *Fusarium* mycotoxins in biscuits is represented in Table 4 and typical chromatograms of standard mixture and extracts from naturally contaminated biscuit shown in Fig. 1. *Fusarium* mycotoxins were quantified in 196 out of the 201 samples with DON being the most commonly detected *Fusarium* mycotoxin in this study. It was found in 98% of all samples at concentrations between 0.9 and 791 ng/g (mean = 23 ng/g). In this study, 120 samples were co-contaminated with two or more *Fusarium* mycotoxins. With the exception of two samples (NIV/HT-2 and HT-2/T-2), all samples with multiple toxins also contained DON. The co-contamination of two or more toxins detected in this study were found in 120 (incidence = 60%) samples. In samples containing two toxins, DON was present in combination with each of NIV, HT-2 and ZEN, and both NIV and DON occurred as the most frequent combination in 77 (incidence = 38%) samples. Of these, 99, 16, and 5

Table 5. Occurrence of *Fusarium* Mycotoxins in Biscuits for Infants

Mycotoxins	Positive samples[a] (Incidence, %)	Range (ng/g)	Mean[b] (ng/g)
NIV	37 (34)	1.4– 35	3.0
DON	98 (89)	0.9–177	17
HT-2	25 (23)	0.6– 11	1.4
T-2	13 (12)	0.1– 6.0	0.4
ZEN	1 (1)	4.2	—

a) Number of samples analyzed = 110. *b*) When the number of samples of under the limit of quantification is > 60%, the concentration was calculated as follows. The value less than the limit of detection was calculated as the detection limit. The value between the limit of detection and the limit of quantification was calculated as the limit of quantification. When the number of samples of under the limit of quantification is < 60%, the concentration was calculated as follows. The value under the limit of quantification was calculated as 1/2 of the limit of detection.

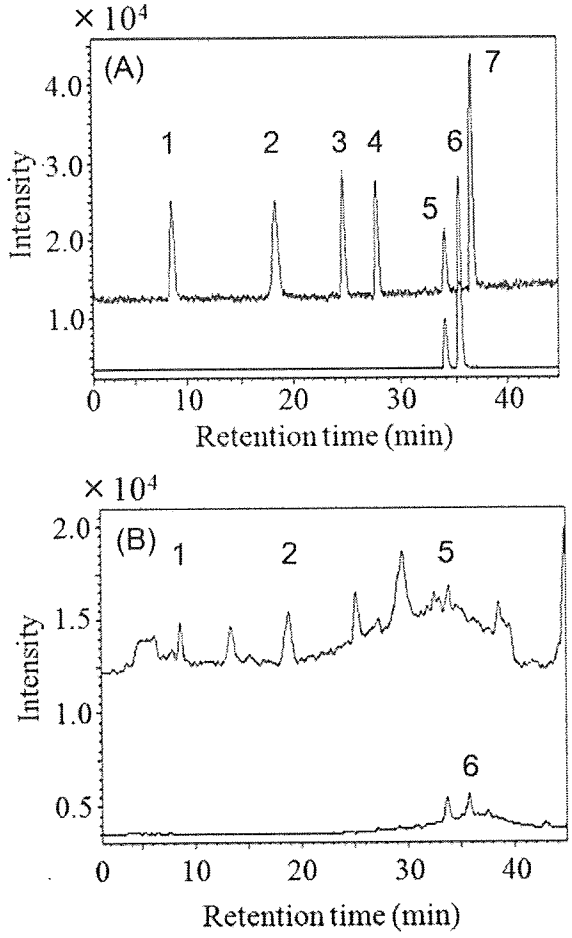

Fig. 1. Total Ion Chromatograms of (A) *Fusarium* Mycotoxins Standard Mixture Solution at 50 ng/ml and (B) Naturally Contaminated Biscuit

(A) 1, NIV; 2, DON; 3, FX; 4, 3ADON; 5, HT-2; 6, T-2; 7, ZEN. (B) 1, NIV (13 ng/g); 2, DON (22 ng/g); 5, HT-2 (4 ng/g); 6, T-2 (1 ng/g).

samples contained 2, 3, and 4 toxins, respectively. Triple toxin combinations were DON/HT-2/T-2 and NIV/DON/ZEN while a combination of four toxins was NIV/DON/HT-2/T-2 (Fig. 1). In infant biscuits the concentration of DON ranged from 0.9 to 177 ng/g (mean = 17 ng/g, Table 5).

NIV was found in 41% of the samples, ranging from 1.4 to 35 ng/g (mean = 3.1 ng/g, Table 4).

HT-2 and T-2 were detected in 38 (incidence = 19%) and 22 (incidence = 11%) samples, with a maximum concentration of 20 ng/g (mean = 0.7 ng/g) and 6.0 ng/g (mean = 0.1 ng/g), respectively (Table 4). Both HT-2 and T-2 were observed in infant products and occurred simultaneously in one sample. Since the simultaneous occurrence of HT-2 and T-2 is rare in Japan, we recommend continuation of this surveillance monitoring program.

The incidence (2%) and highest concentration (4.4 ng/g) of ZEN in the samples were lower than those of the other six *Fusarium* mycotoxins (Table 4).

In contrast, none of the samples analyzed were found to contain FX and 3ADON above the LODs.

Biselli and Hummert reported the co-occurrence of DON and T-2 in wheat-based products in Europe.[13] They showed that DON contamination was high (maximum level = 2350 ng/g), but the concentration of T-2 was lower than that in our study (maximum level = 0.95 ng/g). Schollenberger *et al.*[18] showed that NIV, DON, HT-2, T-2 and ZEN occurred in foodstuffs marketed in Germany with DON the most commonly observed. The incidence of HT-2 was higher than that of T-2, differences depending on the kind of sample, similarly to the incidence of NIV and ZEN. These results were similar to our present study. Recently, there have been some reports in which NIV, DON, HT-2 and ZEN were detected in infant products and conventional wheat products.[14,15,19,20] Compared with these reports the maximum DON and HT-2 concentrations were similar to our study of infant products. Although the occurrence of NIV and ZEN was confirmed in these reports, that of T-2 was not, and on

this point it was different from our study of infant products.

As shown in Tables 4 and 5, there were no great differences in the incidence and concentration between conventional biscuits and biscuits for infants. However, the risk will be greater for infants due to body weight and intake when compared to adults.

It is difficult to assess the exposure of *Fusarium* mycotoxins under the present study because the general information data regarding real consumption of biscuits by Japanese is limited. Therefore, continuous surveillance for contamination levels and an exposure assessment will be required to avoid an unexpected risk of exposure to high concentrations of mycotoxins from now on.

In conclusion, we are the first to report the presence of NIV, DON, HT-2, T-2 and ZEN in biscuits marketed in Japan. It is suggested from our study that biscuits are co-contaminated with several *Fusarium* mycotoxins; therefore, there is a need for continuous monitoring in order to evaluate their risk to consumers. In addition, it should be taken into account that the risk of *Fusarium* mycotoxins may be increased by co-occurrence with other mycotoxins, as shown in this study. It is important to continue accumulating the occurrence data of mycotoxins from food products in order to develop appropriate risk assessment tools with the goal of reducing contamination levels in marketed commodities.

REFERENCES

1) Ueno, Y. (1980) Trichothecene mycotoxins: mycology, chemistry and toxicology. In *Advances in Nutritional Research*, Vol.3 (Draper, H. H., Ed.), Plenum, New York, pp.301–353.
2) Ueno, Y. (1983) General toxicology. In *Trichothecenes: Chemical, Biological and Toxicological Aspects* (Ueno, Y., Ed.), Elsevier, Amsterdam, pp.135–146.
3) Shifrin, V. I. and Anderson, P. (1999) Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. *J. Biol. Chem.*, **274**, 13985–13992.
4) Yang, G. H., Jarvis, B. B., Chung, Y. J. and Pestka, J. J. (2000) Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK, and SAPK/JNK activation. *Toxicol. Appl. Pharmacol.*, **164**, 149–160.
5) Miller, J. D. and Trenholm, H. L. (Eds.) (1994) *Mycotoxins in Grain-Compounds Other Than Aflatoxin*, Eagan Press, St. Paul.
6) Mirocha, C. J., Pathre, S. V. and Christensen, M. (1977) In *Mycotoxins in Human and Animal Health* (Rodricks, J. V., Hesseltine, C. W. and Mehlman, M. A., Eds.), Pathotox, IL, p.345.
7) World Health Organization (WHO) (1990) Selected Mycotoxins: Ochratoxins, Trichothecens, Ergot. *Environmental Health Criteria 105*, WHO, Geneva.
8) Müller, H.-M., Reimann, J., Schumacher, U. and Schwadorf, K. (1998) Natural occurrence of *Fusarium* toxins in oats harvested during five years in an area of southwest Germany. *Food Addit. Contam.*, **15**, 801–806.
9) Tanaka, T., Hasegawa, A., Yamamoto, S., Lee, U.-S., Sugiura, Y. and Ueno, Y. (1988) Worldwide contamination of cereals by the *Fusarium* mycotoxins nivalenol, deoxynivalenol, and zearalenone. 1. Survey of 19 countries. *J. Agric. Food Chem.*, **36**, 979–983.
10) Tanaka, T., Yamamoto, S., Hasegawa, A., Aoki, N., Besling, J. R., Sugiura, Y. and Ueno, Y. (1990) Asurvey of the natural occurrence of *Fusarium* mycotoxins, deoxynivalenol, nivalenol and zearalenone, in cereals harvested in the Netherlands. *Mycopathologia*, **110**, 19–22.
11) Yoshizawa, T. (1991) In *Mycotoxins and Animal Foods* (Smith, J. E. and Henderson, R. S., Eds.), CRC Press, Boca Raton, FL, p. 301.
12) Yuwai, K. E., Rao, K. S., Singh, K., Tanaka, T. and Ueno, Y. (1994) Occurrence of nivalenol, deoxynivalenol, and zearalenone in imported cereals in Papua, New Guinea. *Nat. Toxins*, **2**, 19–21.
13) Biselli, S. and Hummert, C. (2005) Development of a multicomponent method for *Fusarium* toxins using LC-MS/MS and its application during a survey for the content of T-2 toxin and deoxynivalenol in various feed and food samples. *Food Addit. Contam.*, **22**, 752–760.
14) Lombaert, G. A., Pellaers, P., Roscoe, V., Mankotia, M., Neil, R. and Scott, P. M. (2003) Mycotoxins in infant cereals foods from the Canadian retail market. *Food Addit. Contam.*, **20**, 494–504.
15) Schollenberger, M., Suchy, S., Jara, H. T., Drochner, W. and Muller, H.-M. (1999) A survey of *Fusarium* toxins in cereal-based foods marketed in an area of southwest Germany. *Mycopathologia*, **147**, 49–57.
16) Tanaka, H., Takino, M., Sugita-Konishi, Y., Tanaka, T., Toriba, A. and Hayakawa, K. (2009) Determination of nivalenol and deoxynivalenol by liquid chromatography/atmospheric pressure photo ionizationmass spectrometry. *Rapid Commun. Mass Spectrom.*, **23**, 3119–3124.

17) Tanaka, H., Takino, M., Sugita-Konishi, Y. and Tanaka, T. (2006) Development of a liquid chromatography/time-of-flight mass spectrometric method for the simultaneous determination of trichothecenes, zearalenone and aflatoxins in foodstuffs. *Rapid Commun. Mass Spectrom.*, **20**, 1422–1428.
18) Schollenberger, M., Müller, H.-M., Rüfle, M., Suchy, S., Planck, S. and Drochner, W. (2005) Survey of Fusarium toxins in foodstuffs of plant origin marketed in Germany. *Int. J. Food Microbiol.*, **97**, 317–326.
19) Cirillo, T., Ritieni, A., Galvano, F. and Amodio-Cocchieri, R. (2003) Natural co-occurrence of deoxynivalenol and fumonisins B1 and B2 in Italian marketed foodstuffs. *Food Addit. Contam.*, **20**, 566–571.
20) Food Standards Agency (2003) Survey of Retail Cereal Products for Trichothecenes and Zearalenone. *Food Survey Information Sheet*, No.35/03, http://www.food.gov.uk/multimedia/pdfs/35cereal.pdf

Food Additives and Contaminants
Vol. 27, No. 3, March 2010, 365–372

Taylor & Francis
Taylor & Francis Group

Exposure to aflatoxins in Japan: risk assessment for aflatoxin B_1

Y. Sugita-Konishi[a]*, T. Sato[b], S. Saito[b], M. Nakajima[c], S. Tabata[d], T. Tanaka[e], H. Norizuki[f], Y. Itoh[a], S. Kai[g], K. Sugiyama[a], Y. Kamata[a], N. Yoshiike[h] and S. Kumagai[i]

[a]*National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan;* [b]*Kitasato University, 1-15-1, Kitasato, Sagamihara, Kanagawa 228-8555, Japan;* [c]*Nagoya City Public Health Research Institute, 1-11, Hagiyama-cho, Mizuho-ku, Nagoya 467-8615, Japan;* [d]*Tokyo Metropolitan Institute of Public Health, 3-24-1, Hyakunin-cho, Shinjuku-ku, Tokyo 169-0073, Japan;* [e]*Kobe Institute of Health, 4-6, Minatojima-nakamachi, Chuo-ku, Kobe 650-0046, Japan;* [f]*Japan Grain Inspection Association, 2-17-3, Arai, Ichikawa-shi, Chiba 272-0144, Japan;* [g]*Kanagawa Prefectural Institute of Public Health:1-3-1, Shimomachiya, Chigasaki, Kanagawa 253-0087, Japan;* [h]*National Institute of Health and Nutrition, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8636, Japan;* [i]*The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8581, Japan*

(*Received 31 January 2009; final version received 8 September 2009*)

The intake of total aflatoxins (AFT) and aflatoxin B_1 (AFB_1) from food in Japan was estimated from AFT and AFB_1 concentration and frequency data in 24 foods (884 samples) from a 3-year retail market survey from the summer of 2004 to the winter of 2006, and by food consumption data from the National Health and Nutrition Survey performed in 2005. The AFT and AFB_1 survey revealed that peanut, peanut products, cocoa, chocolate, pistachio, white pepper, red pepper, almond, job's tears, buckwheat and corn grits are considered to be contributors of AFT (or AFB_1) intake in Japan (maximum AFB_1 (AFT) levels ranged from 0.21 to 28.0 $\mu g\,kg^{-1}$ (from 0.21 to 9.0 $\mu g\,kg^{-1}$)) in AFT-contaminated food. A probabilistic approach using the Monte Carlo method was carried out to simulate an estimate of the AFT (or AFB_1) intake distributions in each age group in Japan. In this study, AFB_1 intake ranged from 0.003 to 0.004 $ng\,kg^{-1}$ body weight day^{-1} (from lower to upper limits), and the potential risk for cancer using a formula devised by the Joint Food and Agricultural Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) was estimated at 0.00004–0.00005 person/year/100,000 persons, even though this was in the higher levels (95.0th percentile) of the consumer population. The results suggest that the current dietary intake of AFB_1 in Japan has no appreciable effect on health.

Keywords: risk assessment; survey; aflatoxins; cereals; peanuts; processed foods

Introduction

Total aflatoxins (AFTs) produced by *Aspergillus* spp. are potent hepatotoxic, mutagenic, immunosuppressive, and carcinogenic toxins (International Agency for Research on Cancer (IARC) 1993). As for hepatocarinogenicity, AFT is classified as a human carcinogen by the International Agency for Research of Cancer (IARC 2002). In 1997, the Joint Food and Agricultural Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) concluded that an estimate of the carcinogenic potency of AFT and its potential risk is related with the intake of AFT (JECFA 1998). It is generally accepted that peanuts, tree nuts, spices, corn, rice, cottonseed, dry fruits, cocoa and copra are contributors to AFT contamination around the world (JECFA 1998). In developing countries, AFT is associated with acute toxicity more than chronic toxicity, but in developed countries, including Japan, chronic toxicity such as hepato-carcinogenicity has increased as a serious problem in food safety.

Since in practice it is difficult to achieve zero tolerance with AFT contamination in commodities, it is important to reduce the exposure to AFT through dietary food to prevent the risk of cancer. Therefore, many countries have set regulatory limits for AFB_1 and AFT in foods to avoid consumption of AFT-contaminated foods based on scientific evidence obtained from risk assessment (FAO 2004). The Codex Alimentarius Commission, Joint FAO/WHO Food Standards Program adopted a maximum level of 15 $\mu g\,kg^{-1}$ for AFT for unprocessed peanuts and tree nuts and 10 $\mu g\,kg^{-1}$ for ready-to-eat tree nuts (JECFA 2008). In the European Union, the maximum limits of AFB_1 and AFT are 2 and 4 $\mu g\,kg^{-1}$, respectively (European Commission 2006). In Japan, the present regulation limit is 10 $\mu g\,kg^{-1}$ of AFB_1 in all foods. In a previous paper (Sugita-Konishi et al. 2006),

*Corresponding author. Email: ykonishi@nihs.go.jp

ISSN 0265-203X print/ISSN 1464-5122 online

DOI: 10.1080/19440040903317497
http://www.informaworld.com

we reported a 1-year survey conducted from the summer of 2004 to the spring of 2005 in which corn products, corn, peanuts, peanut products, buckwheat flour, dried buckwheat noodles, rice, and sesame oil obtained from Japanese retail foods were analysed. Among these foods, AFT was detected only in peanut butter samples (ten positive in 21 samples) and the highest concentration of AFB_1 was 2.6 $\mu g\,kg^{-1}$. However, since the level of AFT contamination in food should be influenced by meteorological phenomena, we continued the survey of AFB_1 and AFT contamination in various foods collected from retail markets in Japan for a further 2 years.

In this study, based on the data of a 3-year survey, we estimated the daily intake of AFT and AFB_1 by a Japanese population in each age group and assessed the risk of liver cancer by AFB_1 intake in all age groups.

Materials and methods

Chemicals and reagents

Acetonitrile, methanol and chloroform were supplied by Wako Pure Chemical Co. (Tokyo, Japan). For high-performance liquid chromatography (HPLC), HPLC-grade reagents were used. Aflatoxin B_1, B_2, G_1 and G_2 for standard and for the spiked test were purchased from Sigma Chemical Co. (St Louis, MO, USA). The concentration was determined according to AOAC International Official Methods of Analysis. An immunoaffinity column (IAC), the AflaTest P column (Vicam, Watertown, MA, USA), was used in the 2004 survey and an AFLAPREP (R-Biopharm-Rhone Ltd, Darmstadt, Germany) in the 2005 and the 2006 surveys.

Samples

Rice originating from domestic samples was harvested in the 2004–2006 fiscal year except for two samples of rice (2003 fiscal year), which were supplied from the Ministry of Agriculture, Forestry and Fisheries of Japan. Other foods (23 foods) beside rice and wheat were selected from a list of typical AFT-contaminated foods (Weidenbörner 2001). All other food samples were purchased at local retail shops in Tokyo, Nagoya and Kobe and in small retail shops in all parts of Japan in a random manner at more than 0.5 kg or 0.5 litres of each sample from the summer of 2004 to the winter of 2006.

AFT determination

The analytical method used was the same as that reported previously (Sugita-Konishi et al. 2006). Briefly, 25 g of sample (in finely ground form), except for cocoa, chocolate, beer, peanut butter and sesame seed oil, were extracted with 100 ml of methanol–water (8 : 2) and 5 g of NaCl by shaking for 30 min. For cocoa and chocolate, 60% acetonitrile was used for extraction. Beer was diluted two-fold with phosphate-buffered saline. Clean-up was performed by IAC according to the method described previously (Sugita-Konishi et al. 2006) for all foods except for peanut butter and sesame seed oil. AFT was analysed by an HPLC-fluorescence detector (excitation, 360 nm; emission, 450 nm) by using pre-column-trifluoroacetic acid derivatization or post-column-photochemical reactor derivatization (Waltking and Wilson 2006).

As for peanut butter and sesame seed oil samples, the method of Kamimura et al. (1985) was used for analysis. Briefly, a 10 or 20 g sample was extracted with 50 or 100 ml of chloroform. The extract was loaded on Florisil columns, from which AFT were eluted with an acetone–water (99 : 1) solution. After evaporation of the solvent under reduced pressure, the residue was dissolved in 200 µl of chloroform. A 20-µl portion was dried and derivatized with Trifluoro acetic acid (TFA) for quantitative analysis by HPLC under the same conditions as described above.

Method validation

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the signal-to-noise ratio as more than 3 : 1 and 10 : 1, respectively. Recoveries were ascertained by spiking with AFB_1, AFB_2, AFG_1 and AFG_2. The concentrations of AFB and AFT in each food were not corrected by recovery rate.

AFB_1 (or AFT) intake simulation

The daily intake of AFB_1 (AFT) was estimated with the concentration of AFB_1 (AFT) and the amount of AFB_1 (AFT) consumed in the AFB_1 (AFT)-contaminated foods. The food consumption data originated from a database of the National Health and Nutrition Survey conducted in 2005 of consumption over 2 consecutive days from 17 827 individuals ranging in age of: 1–6, 7–14, 15–19, and greater than 20 years. The National Health and Nutrition Survey breaks the broad category 'food' into more detailed items according to their food code. For example, chocolate is broken into solid-covered chocolate, solid milk chocolate, liquid cocoa, liquid milk chocolate, and pure chocolate. Buckwheat is broken into buckwheat flour and buckwheat noodles. The distribution of the consumption of these foods in the 1–6, 7–14, 15–19, or over 20 years age groups was simulated by fitting the log-normal distribution to consumption data of each food and then by multiplying the distribution of the

consumption of these foods with the database of each food product. On the other hand, assuming a log-normal distribution for an AFB_1 (AFT)-detected samples, the simulations were done by two kinds of concentration data, namely lower and upper bounds, according to the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) (1995) instruction. The lower-bound simulation is calculated on the supposition that the value under the LOD is regarded as zero while the value under the LOQ is regarded as the LOD, that of the upper bound is the value under the LOD regarded as the LOD while the value under the LOQ is regarded as the LOQ. The distributions of AFB_1 (AFT) intake in each age group were simulated using the distribution of food consumption and the distribution of AFB_1 (AFT) concentration in food. To estimate the risk of cancer in Japan, AFB_1 intake at all ages was simulated by multiplying with weighting coefficients depending on the population ratio of each age group (zero years old: 0.9%, 1–6 years: 5.4%, 7–14 years: 7.5%, 15–19 years: 5.1%; and over 20 years: 81.1%).

The population risk of cancer caused by AFB_1 intake through food was calculated using a quantitative risk assessment of AFB_1 performed by JECFA (1998). Due to the synergistic hepato-carcinogenic effects of AFB_1 and hepatitis B virus infection, two potencies are advocated. In hepatitis B surface antigen-positive individuals, the potency is 0.3 cancers per year ng^{-1} AFB_1 kg^{-1} body weight (bw) day^{-1} in a 100,000 population. In hepatitis B surface antigen-negative individuals, the potency is 0.01 cancers per year ng^{-1} AFB_1 kg^{-1} bw day^{-1} in 100,000 populations. In this assessment, a total population of 127.7 million individuals and 1.4 million hepatitis B surface antigen-positive individuals in Japan were used.

Statistical analysis

Recovery values are expressed as the mean ± residual standard deviation (SD) for more than $n = 3$. To simulate the contamination levels of AFB_1 and AFT in foods and the consumption of foods in which AFT was detected, the percentile exposure of AFB_1 assessed was based on a Monte Carlo simulation by Crystal Ball 2000 (Japanese version, Kozokeikaku Kenkyujo Ltd, Tokyo, Japan) with an iteration number of 10,000,000.

Results and discussion

Three-year survey of AFB_1 (AFT) in retail foods in Japan

The recoveries of AFB_1, AFB_2, AFG_1, AFG_2, limit of detection (LOD) and limit of quantitative (LOQ) in the individual matrix were analysed in this study (data not shown). The recovery in foods surveyed in this study, except for AFB_1, AFG_1 and AFG_2 in sweet corn, ranged from 70% to 120%. In sweet corn, recoveries of AFB_1 AFG_1 and AFG_2 were relative low (55.0–63.4%).

The LOD and LOQ in individual foods ranged from 0.001 to 0.1 $\mu g\,kg^{-1}$ and from 0.005 to 0.2 $\mu g\,kg^{-1}$, respectively. Throughout this study, although two different brands of IAC were used, there was no difference in the recovery rates of LOD and LOQ following HPLC analysis. These results indicated that the methods used in this study are satisfactory for the survey.

Results of the 3-year survey in Japan are shown in Table 1. In 24 selected foods (884 samples), AFB_1 (AFT) was detected in eleven foods, including peanut, peanut butter, cocoa, chocolate, pistachio, almond, white pepper, red pepper, job's tears, buckwheat and corn grits. These foods are considered to contribute to exposure to AFB_1 (AFT) in Japan. On the other hand, levels that were below the respective LODs were detected in sesame oil, rice, popcorn, sweets containing peanut, corn flakes, raw corn, sweet corn, buckwheat noodle, rice cake, beer, cinnamon, black pepper, or dried figs.

The percentage of samples below the LOD of AFT in individual foods ranged from 20.0% to 100.0% (Table 2). Chocolate, cocoa and white pepper, job's tears and peanut butter showed less than 70%. AFB_1, AFB_2, AFG_1 and AFG_2 were all detected in peanut, peanut butter, spices, chocolate, job's tears and almond. Only AFB_1 and AFB_2 were detected in red pepper and pistachio (data not shown). The maximum levels of AFT and AFB_1 in these foods ranged from 0.21 to 28.0 $\mu g\,kg^{-1}$ and from 0.21 to 9.00 $\mu g\,kg^{-1}$, respectively. Surprisingly, 28.0 $\mu g\,kg^{-1}$ of AFT were detected in the peanut sample, in which there was only one positive sample within 150 samples. Because AFB_1 concentration in the peanut was lower than the Japanese regulation and since Japan has no regulation for AFT, these peanuts could pass through custom's inspection despite the high concentration of AFG_1 (data not shown).

Though a single-year survey in Japan performed in 2006 showed that peanut butter was the only food contributor for AFT exposure described in a previous report (Sugita-Konishi et al. 2006), a further 2-year survey, or in total a 3-year survey, revealed that ten other foods were contaminated with AFB_1 (AFT). Furthermore, the contamination level of a mycotoxin is affected by climate changes, and the length of the survey would be recommended to be at least another 2–3 years to perform a more adequate survey.

AFT (AFB_1) intake estimates

Table 2 lists AFB_1 (AFT)-contaminated food categorized by the National Nutrition Surveillance system and

Table 1. A 3-year survey of aflatoxin (AFT) and aflatoxin B_1 (AFB_1) and means of these toxins concentrations in various foods in Japan.

Year: Food	Number of samples				$n <$ LOD (%)	Maximum level of AFB_1 ($\mu g\,kg^{-1}$)	Maximum level of AFT ($\mu g\,kg^{-1}$)	AFB_1 mean ($\mu g\,kg^{-1}$) of all samples		AFT mean ($\mu g\,kg^{-1}$) of all samples	
	2004	2005	2006	Total				Lower bound	Upper bound	Lower bound	Upper bound
Peanut	60	60	30	150	149 (99.3)	4.88	28.0	0.03	0.08	0.19	0.29
Peanut butter	21	20	21	62	41 (66.7)	2.59	3.92	0.30	0.34	0.45	0.52
Cocoa	–	–	11	11	3 (27.3)	0.60	0.85	0.24	0.25	0.29	0.32
Chocolate	–	40	24	64	30 (46.9)	0.88	0.21	0.15	0.17	0.18	0.23
Pistachio	–	–	5	5	4 (80.0)	0.38	0.38	0.08	0.12	0.08	0.16
White pepper	–	–	5	5	1 (20.0)	0.30	0.50	0.16	0.18	0.24	0.30
Red pepper	–	–	6	6	5 (83.3)	1.00	1.00	0.20	0.28	0.20	0.36
Almond	–	–	24	24	18 (75.0)	0.89	1.06	0.09	0.13	0.11	0.18
Job's tears	–	–	17	17	11 (64.7)	9.00	9.71	0.86	0.90	0.98	1.04
Buckwheat flour	12	11	6	28	26 (92.9)	0.81	0.99	0.04	0.08	0.04	0.14
Corn grits	10	10	10	30	28 (93.3)	0.21	0.21	0.01	0.06	0.01	0.11

Notes: Lower bound: results below the limit of detection (LOD) are regarded as zero.
Upper bound: results below the LOD are regarded as the LOD and those between the LOD and the limit of quantification (LOQ) are regarded as the LOQ.

Table 2. Number of consumers in each aflatoxin (AFT)-detected food in different age groups in Japan.

Food surveyed in the study	Code of foods in National Nutrition Surveillance	Age group (number of people)				
		1–6 years old (788)	7–14 years old (1359)	15–19 years old (948)	More than 20 years old (14,732)	All ages (17,827)
Chocolate	Covering chocolate	26	69	51	161	307
	Milk chocolate	65	125	94	697	981
	Pure chocolate	11	14	11	83	119
	Total chocolate	153	258	177	1222	1810
Buckwheat	Buckwheat flour	0	0	0	14	14
	Buckwheat noodle	28	40	16	768	852
Cocoa	Milk cocoa	70	85	36	346	537
Almond	Almond	7	54	2	104	167
Job's tears	Job's tears tea	0	0	0	42	42
Peanut butter	Peanut butter	16	31	6	130	183
Peanut	Peanut	18	47	7	277	349
Red pepper	Red pepper	3	7	11	175	196
Pistachio	Pistachio	1	1	1	15	18
White pepper	White pepper	27	63	49	746	885

Note: These data were obtained from a database of the National Health and Nutrition Survey conducted in 2005 of consumption over 2 consecutive days from 17827 individuals.

consumer number of different age groups in individual foods. These data show the frequency of consumption in individual foods, indicating that a relatively high number of people consumed total chocolate (1810/17, 827 people), milk chocolate (981/17, 827 people), buckwheat noodle (852/17, 827 people), white pepper (885/17, 827 people) and cocoa (537/17, 827 people) among AFT-contaminated foods.

Based on these data and consumption values, the simulation of food intake in individual foods in each age group was performed (Table 3). Throughout all age groups, the intake of buckwheat noodle was highest among other contributed foods. However, the AFT (AFB_1)-contaminated level and frequency in buckwheat noodle was quite low. Even though the AFT (AFB_1)-contaminated level and frequency in buckwheat noodle was assumed to be the same as that in buckwheat flour because buckwheat flour is the raw material of noodles, this food would not be the main contributor of aflatoxin intake. In addition,

white pepper would not be the main contributor due to the low amount eaten.

The Anderson–Daring (AD) test value is used as an indicator of whether or not the distribution of food intake is suitable for exposure assessment. Usually, a value less than 1.5 shows good distribution (Anderson and Darling 1954). In this study, the AD values obtained from the distribution of most contributed foods were under 1.5. In cases where the AD value was over than 1.5, there is the possibility that a significant discrepancy occurred between real data and the simulated distribution. However, the estimated difference in intake of buckwheat noodle in this study (Table 3) would be less than 1% at the 95th percentile in respondents over 20 years of age (data not shown). Also, with this simulation, the exposure to AFB_1 and AFT from buckwheat noodle was estimated to be very few. Thus, the discrepancy that occurred from high AD values in the simulation for buckwheat noodle could be neglected. Therefore, the high AD values in the simulations are not really a serious problem in countries where AFT contamination is extremely low, such as in Japan.

From data shown in Tables 1 and 3, chocolate, peanut butter and cocoa seemed to be the main contributors to AFT exposure in Japan. Since chocolate and cocoa are by-products of cacao, the contamination level in cacao was calculated by AFB_1 (AFT)-concentration and by the cacao concentration in cocoa and chocolate. The mean, standard deviation and AD values in AFB_1 intake of cacao were estimated to be 0.38, 0.31 and 0.77 ng kg^{-1} bw day^{-1}, respectively, while the same values for peanut butter were estimated to be 0.75, 1.03 and 0.56 ng kg^{-1} bw day^{-1}, respectively.

Table 4 shows the estimated intake of AFB_1 (AFT) in each age group in Japan. At less than the 90th percentile, the value could not be expressed because of an extreme amount. At the 95th percentile, AFB_1 intake in each age group was estimated from 0.003 ng kg^{-1} bw day^{-1} (lower bound in greater than 20 year olds) to 0.013 ng kg^{-1} bw day^{-1} (upper bound in 1–6 year olds). AFT intake was estimated to be from 0.003 ng kg^{-1} bw day^{-1} (lower bound in greater than 20 year olds) to 0.014 ng kg^{-1} bw day^{-1} (upper bound in 1–6 year olds). The results indicate that AFB_1 was the main compound in the exposure to AFT among other aflatoxins.

The European Union estimated that dietary exposure to AFT ranged from 0.93 to 2.45 ng kg^{-1} bw day^{-1} for lower bound to upper bound (Leblanc et al. 2005; European Food Safety Authority (EFSA) 2007). In the United States, exposure was estimated at 2.7 ng kg^{-1} bw day^{-1} (JECFA 2008). AFT intakes in these countries correspond to the 99.9th percentile population in Japan. In Africa, dietary exposure to AFT was 3.5–14.8 ng kg^{-1} bw day^{-1} in Kenya, 11.4–158.6 ng kg^{-1} bw day^{-1} in Swaziland, 38.6–183.7 ng kg^{-1} bw day^{-1} in Mozambique, 16.5 ng kg^{-1} bw day^{-1} in South Africa, and 4–115 ng kg^{-1} bw day^{-1} in Gambia (Williams et al. 2004). In Ghana, the exposure from peanut was estimated to be 9.9–99.2 ng kg^{-1} bw day^{-1}. According to a recent report (Shephard 2008), the contamination level and consumption of maize was 20 μg kg^{-1} and 400 g day^{-1} per person, respectively, and exposure to AFT from maize was estimated at 133 ng kg^{-1} bw day^{-1} in Kenya. On the other hand, data of the contamination level (7.9 μg kg^{-1}; Hudson et al. 1992) and consumption of rice (103 g day^{-1} per person) indicated that induced exposure from rice was 14 ng kg^{-1} bw day^{-1} in Gambia. These data indicate that maize is a popular principal diet, more than rice, in some African countries and a serious contributor of AFT exposure in these countries.

In Asian countries, in contrast, dietary exposure was estimated at 11.7–2027.0 ng kg^{-1} bw day^{-1} in Guangxi province, China, and 6.5–53 ng kg^{-1} bw day^{-1} in Thailand (Williams et al. 2004). The contributor of AFT exposure in these Asian countries is not clear, although rice is likely to be a main contributor of AFT exposure.

An interesting example was shown from the intake data of AFB_1 in South Korea. When taking into account domestic rice in which high levels of AFB_1 were detected (Park et al. 2005), the estimated dose of exposure to AFB_1 ranged from 1.19 to 5.79 ng kg^{-1} bw day^{-1} (Park et al. 2004). On the other hand, when taking into account the recent survey data of foods purchased from retailed shops in which there was no AFB_1 contamination in rice, the estimated exposure dose was dropped down to 0.642 ng kg^{-1} bw day^{-1} (Ok et al. 2007). The difference of exposure dose to AFB_1 in Korea demonstrated that the AFB_1 level in rice is a serious problem in countries where people have rice as their principal diet. In Japan as well as Korea, rice is capable of becoming a serious contributor, but there was no AFB_1 (AFT) contamination throughout our 3-year survey. Even though AFB_1 (AFT) was not currently detected from Japanese rice, it is very important to regulate and monitor AFT contamination in principal diet foods to prevent the exposure to AFB_1 and AFT.

According to JECFA's (2008) evaluation, intakes estimates ranged from 0.56 to 11 ng kg^{-1} bw day^{-1} for AFT and from 0.82 to 22.7 ng kg^{-1} bw day^{-1} for AFB_1 when no samples were excluded. When excluding commodities contaminated with more than 10 or 20 μg kg^{-1} of AFT, intake estimates ranged from 0.3 to 6.7 ng kg^{-1} bw day^{-1} for AFB_1. Compared with other countries, it is clear that AFB_1 intake estimates in Japan are quite low under the present regulation.

Table 3. Simulation data of food intakes in different age groups.

Age group	Food category	Mean of food intake (g kg^{-1} bw day^{-1})	Standard deviation (SD)	AD
1–6 years old	Covering chocolate	0.12	0.11	0.40
	Milk chocolate	0.13	0.15	0.77
	Pure chocolate	n.a.	n.a.	n.a.
	Milk chocolate	0.03	0.02	0.23
	Drink (pure plus cocoa)	0.03	0.02	0.35
	Solid (covering chocolate plus milk)	0.13	0.14	0.42
	Almond	0.36	1.01	1.31
	Peanut butter	0.82	0.72	0.64
	Peanut	0.29	2.36	1.10
	Red pepper	0.01	0.01	0.91
	Pistachio	0.35	0.51	0.34
	Buckwheat noodle	6.94	5.6	0.46
	White pepper	0.01	0.01	1.51
7–14 years old	Covering chocolate	0.09	0.08	0.85
	Milk chocolate	0.13	0.15	0.77
	Pure chocolate	n.a.	n.a.	n.a.
	Milk chocolate	0.03	0.02	0.73
	Drink (pure plus cocoa)	0.03	0.02	0.56
	Solid (covering chocolate plus milk)	0.08	0.09	0.47
	Almond	0.1	0.53	4.22
	Peanut butter	0.37	0.24	0.45
	Peanut	0.26	3.44	3.93
	Red pepper	0.01	0.01	0.91
	Pistachio	0.35	0.51	0.34
	Buckwheat noodle	4.72	3.75	0.69
	White pepper	0.01	0.01	1.47
15–19 years old	Covering chocolate	0.07	0.06	1.06
	Milk chocolate	0.05	0.05	1.03
	Pure chocolate	n.a.	n.a.	n.a.
	Milk chocolate	0.02	0.02	0.43
	Drink (pure plus cocoa)	0.02	0.03	0.43
	Solid (covering chocolate plus milk)	0.06	0.06	1.95
	Almond	0.10	0.53	4.22
	Peanut butter	0.37	0.24	0.45
	Peanut	0.26	3.44	3.93
	Red pepper	0.01	0.01	0.91
	Pistachio	0.35	0.51	0.34
	Buckwheat noodle	2.37	1.39	0.59
	White pepper	0.003	0.004	6.66
More than 20 years old	Covering chocolate	0.05	0.04	1.64
	Milk chocolate	0.04	0.04	2.4
	Pure chocolate	0.01	0.01	0.61
	Milk chocolate	0.02	0.03	1.41
	Drink (pure plus cocoa)	0.02	0.03	1.07
	Solid (covering chocolate plus milk)	0.04	0.04	3.50
	Almond	0.16	0.3	2.7
	Job's tears	0.11	0.34	0.87
	Peanut butter	0.24	0.22	0.67
	Peanut	0.49	0.77	7.31
	Red pepper	0.01	0.01	1.13
	Pistachio	0.35	0.51	0.34
	Buckwheat flour	0.01	0.01	0.59
	Buckwheat noodle	2.64	1.56	6.51
	White pepper	0.004	0.007	79.84

Note: AD, Anderson–Daring test; n.a., not analysed.

Table 4. Estimated intakes of total aflatoxin (AFT) and aflatoxin B_1 (AFB_1) in each age in Japan (ng kg^{-1} bw day^{-1}).

Each age	Percentile	50th	80th	90th	95th	99th	99.5th	99.9th
1–6 years old, upper bound	AFT	0	0.000	0.004	0.014	0.443	1.332	6.051
	AFB_1	0	0.001	0.004	0.013	0.280	0.908	4.369
1–6 years old, lower bound	AFT	0	0.000	0.003	0.013	0.401	1.270	5.905
	AFB_1	0	0.001	0.005	0.014	0.288	0.909	4.369
7–14 years old, upper bound	AFT	0	0.000	0.004	0.012	0.222	0.641	3.286
	AFB_1	0	0.001	0.004	0.010	0.141	0.415	2.313
7–14 years old, lower bound	AFT	0	0.000	0.003	0.011	0.203	0.601	3.097
	AFB_1	0	0.001	0.004	0.012	0.142	0.415	2.313
15–19 years old, upper bound	AFT	0	0.000	0.002	0.007	0.040	0.118	1.335
	AFB_1	0	0.000	0.002	0.006	0.029	0.069	0.831
15–19 years old, lower bound	AFT	0	0.000	0.002	0.006	0.037	0.096	1.118
	AFB_1	0	0.001	0.003	0.006	0.032	0.074	0.832
More than 20, upper bound	AFT	0	0.000	0.000	0.003	0.060	0.411	2.736
	AFB_1	0	0.000	0.001	0.003	0.038	0.288	1.989
More than 20, lower bound	AFT	0	0.000	0.000	0.003	0.049	0.368	2.567
	AFB_1	0	0.000	0.001	0.003	0.043	0.289	1.989

Notes: Lower bound: results below the limit of detection (LOD) are regarded as zero.
Upper bound: results below the LOD are regarded as the LOD and those between the LOD and the limit of quantification (LOQ) are regarded as the LOQ.
Maximum level of AFB_1 in Japan is 10 μg kg^{-1} for all foods.

Table 5. Estimated intake of AFB_1 and cancer risk in all ages in Japan.

Percentile	Estimated intake of AFB_1 (ng kg^{-1} bw day^{-1})[a]		Estimation of cancer risk (cancers/year/100,000 persons)[b]	
	Lower bound	Upper bound	Lower bound	Upper bound
90th	0.001	0.001	0.00001	0.00001
95th	0.003	0.004	0.00004	0.00005
99.9th	2.063	2.063	0.00059	0.00067

Notes: [a]Lower bound: results below the limit of detection (LOD) are regarded as zero.
Upper bound: results below the LOD are regarded as the LOD and those between the LOD and the limit of quantification (LOQ) are regarded as the LOQ.
Maximum level of AFB_1 in Japan is 10 μg kg^{-1} for all foods.
[b]Risk was calculated according to the formula reported by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (1998).
The estimated cancer risk (cancers/year/100,000 persons) = AFB_1 intake ng kg^{-1} bw day^{-1} × $\{0.01\ (1-p)+0.3p\}$.
Assuming $p = 0.01$ (Japanese population = 127.7 million, HVB (Hepatitis B Virus) carrier = 1.4 million).

Risk of AFB_1 for cancer in Japan

To evaluate the potential cancer risk of AFB_1 to Japanese consuming these foods, the AFB_1 intake in all age groups and the population risk of AFB_1 for cancer were estimated (Table 5). AFB_1 intake in all age groups was estimated at 0.001 ng kg^{-1} bw day^{-1} (lower and upper) at the 90th percentile and 2.063 ng kg^{-1} bw day^{-1} (lower and upper) at the 99th percentile. Risk was obtained as the product of the intake data of AFB_1 and an average potency figure arrived at from the individual potencies of hepatitis B surface antigen-positive and -negative groups evaluated by JECFA (1998). In Japan, the hepatitis B prevalence rate is assumed to be 1% of the entire population. At the 90th percentile, the population risk was 0.00001 cancer cases/year per 100,000 population at the lower and upper bound even though at the 99.9th percentile (high level consumer group), 0.00059 and 0.00067 cancer/year per 100,000 populations at lower bound and upper bound, respectively, were observed.

In conclusion, exposure assessment based on a 3-year survey indicated that the population risk of AFB_1 for hepato-carcinogenicity is extremely low in Japan.

Acknowledgements

This work was supported by Health and Labour Sciences Research Grants (Research on Food Safety) of the Ministry of Health, Labour and Welfare of Japan.

References

Anderson TW, Darling DA. 1954. A test of goodness of fit. J Am Stat Assoc. 49:765–769.

European Commission 2006. Commission Regulation (EC) No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs. Off J Eur Union. L364:5–24.

European Food Safety Authority (EFSA). 2007. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. EFSA J. 446:1–127.

Food and Agriculture Organization of the United Nations (FAO). 2004. Worldwide regulations for mycotoxins in food and feed in 2003. Food and Nutrition Paper No. 81. Rome (Italy): FAO.

Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food)-EUROS. 1995. Reliable evaluation of low-level contamination of food. Second Workshop. GEM/Food-EUROS; 26–27 May; Kulmbach, Germany.

Hudson GJ, Wild CP, Zarba A, Groopman JD. 1992. Aflatoxins isolated by immunoaffinity chromatography from foods consumed in The Gambia, West Africa. J Nat Toxins. 1(2):100–105.

International Agency for Research on Cancer (IARC). 1993. WHO IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Aflatoxins. 56:245–395.

International Agency for Research on Cancer (IARC). 2002. WHO IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Aflatoxins. 82:1–556.

Joint Food and Agricultural Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA). 1998. Forty-ninth Meeting of the Joint FAO/WHO Expert Committee on Food Additives. Safety evaluation of certain food additives and contaminants in food: aflatoxins. Food Additives Series No. 40. Geneva (Switzerland): WHO. p. 359–469.

Joint Food and Agricultural Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA). 2008. Sixty-eighth Meeting of the Joint FAO/WHO Expert Committee on Food Additives. Safety evaluation of certain food additives and contaminants. Food Additives Series No. 59. Geneva (Switzerland): WHO. p. 330–332.

Kamimura H, Nishijima M, Yasuda K, Ushiyama H, Tabata S, Matsumoto S, Nishima T. 1985. Simple, rapid cleanup method for analysis of aflatoxins and comparison with various methods. J Assoc Off Anal Chem. 68(3): 458–461.

Leblanc JC, Tard A, Volatier JL, Verger P. 2005. Estimated dietary exposure to principal food mycotoxins from the first French Total Diet Study. Food Addit Contam. 22(7):652–672.

Ok HE, Kim HJ, Shim WB, Lee H, Chung DH, Chun HS. 2007. Natural occurrence of aflatoxin B_1 in marketed foods and risk estimates of dietary exposure in Koreans. J Food Protect. 70(12):2824–2828.

Park JW, Choi SY, Hwang HJ, Kim YE. 2005. Fungal mycoflora and mycotoxins in Korean polished rice destined for humans. Int J Food Microbiol. 103(3):305–314.

Park JW, Kim EK, Kim YB. 2004. Estimation of the daily exposure of Koreans to aflatoxin B1 through food consumption. Food Addit Contam A Chem Anal Control Expo Risk Assess. 21(1):70–75.

Shephard GS. 2008. Risk assessment of aflatoxins in food in Africa. Food Addit Contam A Chem Anal Control Expo Risk Assess. 25(10):1246–1256.

Sugita-Konishi Y, Nakajima M, Tabata S, Ishikuro E, Tanaka T, Norizuki H, Itoh Y, Aoyama K, Fujita K, Kai S, et al. 2006. Occurrence of aflatoxins, ochratoxin A, and fumonisins in retail foods in Japan. J Food Prot. 69(6):1365–1370.

Waltking AE, Wilson D. 2006. Liquid chromatographic analysis of aflatoxin using post-column photochemical derivatization: collaborative study. J AOAC Int. 89:678–692.

Weidenbörner M. 2001. Encyclopedia of food mycotoxins. Berlin (Germany): Springer Science + Business Media.

Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. 2004. Human aflatoxincosis in developing countries: a review of toxicology, exposure, potential health consequences and interventions. Am J Clin Nutr. 80(5):1106–1122.

Journal of Food Protection, Vol. 73, No. 2, 2010, Pages 344–352

Four-Year Surveillance for Ochratoxin A and Fumonisins in Retail Foods in Japan

KOJI AOYAMA,[1]* MASAHIRO NAKAJIMA,[2] SETSUKO TABATA,[3] EIICHI ISHIKURO,[4] TOSHITSUGU TANAKA,[5] HIROKO NORIZUKI,[6] YOSHINORI ITOH,[7] KAZUHIRO FUJITA,[4] SHIGEMI KAI,[8] TORU TSUTSUMI,[6] MASANORI TAKAHASHI,[9] HIROKI TANAKA,[10] SEIICHIRO IIZUKA,[4] MOTOKI OGISO,[4] MAMORU MAEDA,[11] SHIGEAKI YAMAGUCHI,[11] KEI-ICHI SUGIYAMA,[7] YOSHIKO SUGITA-KONISHI,[7] AND SUSUMU KUMAGAI[12]

[1]*Food and Agricultural Materials Inspection Center, Sendai Regional Center, 1-3-15, Gorin, Miyagino-ku, Sendai 983-0842, Japan;* [2]*Nagoya City Public Health Research Institute, 1-11, Hagiyama-cho, Mizuho-ku, Nagoya 467-8615, Japan;* [3]*Tokyo Metropolitan Institute of Public Health, 3-24-1, Hyakunin-cho, Shinjuku-ku, Tokyo 169-0073, Japan;* [4]*Japan Food Research Laboratories, 52-1, Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062, Japan;* [5]*Kobe Institute of Health, 4-6, Minatojimanakamachi, Chuo-ku, Kobe 650-0046, Japan;* [6]*Japan Grain Inspection Association, 2-17-3, Arai, Ichikawa-shi, Chiba 272-0144, Japan;* [7]*National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan;* [8]*Kanagawa Prefectural Institute of Public Health, 1-3-1, Shimomachiya, Chigasaki-shi, Kanagawa 253-0087, Japan;* [9]*All Nippon Checkers Corporation, 3-4, Hatoba-cho, Chuo-ku, Kobe 650-0042, Japan;* [10]*Suntory Business Expert Limited, 1-1-1, Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan;* [11]*Japan Frozen Foods Inspection Corporation, 2-4-6, Shibadaimon, Minato-ku, Tokyo 105-0012, Japan; and* [12]*University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8581, Japan*

MS 09-308: Received 22 July 2009/Accepted 3 October 2009

ABSTRACT

Between 2004 and 2007 we examined foods from Japanese retail shops for contamination with ochratoxin A (OTA) and fumonisins B_1, B_2, and B_3. A total of 1,358 samples of 27 different products were examined for OTA, and 831 samples of 16 different products were examined for fumonisins. The limits of quantification ranged from 0.01 to 0.5 μg/kg for OTA and 2 to 10 μg/kg for the fumonisins. OTA was detected in amounts higher than limits of quantification in wheat flour, pasta, oatmeal, rye, buckwheat flour and dried buckwheat noodles, raisins, wine, beer, coffee beans and coffee products, chocolate, cocoa, and coriander. OTA was found in more than 90% of the samples of instant coffee and cocoa, and the highest concentration of OTA, 12.5 μg/kg, was detected in raisins. The concentration of OTA in oatmeal, rye, raisins, wine, and roasted coffee beans varied remarkably from year to year. Fumonisins were detected in frozen and canned corn, popcorn grain, corn grits, cornflakes, corn soups, corn snacks, beer, soybeans, millet, and asparagus. The highest concentrations of fumonisins B_1, B_2, and B_3 were detected in corn grits (1,670, 597, and 281 μg/kg, respectively). All of the samples of corn grits were contaminated with fumonisins, and more than 80% of the samples of popcorn grain and corn snacks contained fumonisins. OTA and fumonisins were detected in several food products in Japan; however, although Japan has not set regulatory levels for these mycotoxins, their concentrations were relatively low.

Mycotoxins are secondary metabolites produced by fungi; they cause adverse health effects in humans and animals. Ochratoxin A (OTA) and fumonisins have been detected in a number of food products around the world. OTA is produced by *Penicillium* species, a fungus that occurs in cool climates, and by several *Aspergillus* species, which occur in tropical and subtropical regions *(12)*; OTA contamination is therefore a serious problem in international trade. In humans and other animals, OTA targets the kidneys; some researchers hypothesize that OTA is involved in endemic nephropathy and urinary tract tumors in the Balkans *(12)*. Fumonisins are produced by several species of *Fusarium* fungi that frequently contaminate grain *(13)*. In animals such as swine, fumonisins cause hepatotoxic and pulmonary edema *(13)*. In humans, high levels of fumonisins increase the risk of esophageal cancer *(5, 37, 44)*, and recent studies have identified a link between the ingestion of fumonisins and neural tube defects *(18)*.

* Author for correspondence. Tel: 81-22-295-4212; Fax: 81-22-295-0446; E-mail: kouji_aoyama@nm.famic.go.jp.

Although research has demonstrated that OTA and fumonisins are carcinogenic in animals, insufficient evidence in humans *(9–11)* has led the International Agency for Research on Cancer (IARC) to classify OTA and fumonisins as possible human carcinogens (group 2B). The Food and Agriculture Organization and World Health Organization (FAO/WHO) suggest that mycotoxin contamination is a significant cause of foodborne illness and chronic disease, including cancer. The FAO/WHO and Codex Alimentarius establish a maximum level for each mycotoxin based on risk assessment and recommend good agricultural practices, with monitoring of the food chain in order to reduce that risk. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has set the provisional tolerable weekly intake of OTA at 100 ng/kg of body weight per week and the provisional maximum tolerable daily intake of fumonisins at 2 μg/kg of body weight per day. Recently, Codex Alimentarius set the maximum level of OTA in cereals at 5 μg/kg based on JECFA's intake evaluations *(6)*. The maximum level for fumonisins is expected to be set in the near future.

We have previously reported the results of a 1-year (2004) surveillance for OTA and fumonisins *(36)*. In the current study, to determine the average levels of mycotoxin contamination, we additionally examined various foods collected from Japanese retail markets between fiscal year 2005 (from April 2005 to March 2006) and fiscal year 2007. This article presents the results of the survey for 4 years (from fiscal year 2004 to fiscal year 2007).

MATERIALS AND METHODS

Samples. Samples were selected based on the 2004 survey *(36)*, although some food products were added in later years based on published reports and other domestic surveys. The rice and wheat samples that were analyzed between 2004 and 2006 were supplied by the Ministry of Agriculture, Forestry and Fisheries. In 2007, these commodities were purchased in the same manner as all the other samples. All other samples were purchased randomly from local supermarkets and small retail shops in major cities such as Tokyo, Osaka, Nagoya, Kobe, Saitama, Sendai, and Chiba in Japan between the summer of 2004 and the winter of 2007. Although samples were not purchased redundantly in a year, some of the samples were purchased again in a different year. For example, a sample of pasta was not purchased twice in 2006, but it was purchased again in 2007. All samples of polished rice, rice crackers, coffee beverages, and dried bonito were domestic products, and all of the coriander and dried figs were imported products. Of other foods surveyed for OTA, on average, 26% were domestic, 46% were imported, and 28% were of unknown origin; for fumonisins, on average, 29% were domestic, 30% were imported, and 41% were of unknown origin. The samples were stored at 4°C until analyzed.

Reagents. OTA standard was purchased from Sigma-Aldrich Co. (St. Louis, MO). Fumonisin B_1 (FB_1) and FB_2 were purchased from Calbiochem (San Diego, CA), and FB_3 was purchased from PROMEC, Medical Research Council (Tygerberg, South Africa). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Kanto Chemical Co., Inc. (Tokyo, Japan) by each institute. Methanol and acetonitrile were of high-performance liquid chromatography (HPLC) grade. All other reagents were of the highest analytical grade available.

Standard solutions and validation of detection methods. A stock standard solution of OTA (40 μg/ml) was prepared in toluene–acetic acid (99:1), and its concentration was determined according to the AOAC International method *(40)*. An OTA intermediate solution (1 μg/ml) was prepared by dilution with toluene–acetic acid (99:1), and working standard solutions were prepared by diluting with acetonitrile–water–acetic acid (30:70:1). A fumonisin mixed-stock solution (20 μg/ml for each toxin) and working standard solutions were prepared by dilution with acetonitrile-water (1:1).

To determine a recovery rate, all samples were spiked with OTA to obtain final concentrations of 0.01 to 5 μg/kg and with fumonisins to obtain final concentrations of 2 to 1,000 μg/kg. The recoveries were determined each year. The limit of quantification (LOQ) was determined from the height of the signal peak that corresponded to ten times that of the background noise.

Extraction and analysis of OTA. Each sample (except for the raisins, dried figs, cacao products, coriander, and beverages) was thoroughly mixed and ground to a fine powder. Raisins and dried figs were slurred with water (five parts sample by weight to four parts water by weight) to form a homogenous paste. The beer was degassed in an ultrasonic bath for 30 min. All samples were cleaned up with the immunoaffinity column (IAC). The presence of OTA was confirmed by OTA–methyl ester formation as described in our previous article *(36)*.

Our previous report describes the method of analysis used for samples collected in the 2004 fiscal year *(36)*. In summary, methanol-water (8:2) and 5 g of sodium chloride were used as extraction solvents for the corn, corn products, rice, oatmeal, and buckwheat flour; acetonitrile-water (6:4) was used for the wheat and rye flour; and methanol–1% sodium bicarbonate (7:3) was used for the coffee beans and raisins. Twenty-five grams of sample (or 45 g of raisin paste) was extracted in 100 ml (80 ml for the raisin paste) of extraction solvent by shaking for 30 min. The extract was diluted with phosphate-buffered saline (PBS, pH 7.4) or with PBS plus 0.01% Tween 20 (PBS-Tween). A volume of diluted solution equivalent to 1 g of sample was cleaned up with the IAC (OchraTest, Vicam, Watertown, MA). For beer and wine, 10 g of sample solution was diluted with 10 ml of 1% polyethylene glycol 8000 plus 5% sodium bicarbonate, and 10 ml of the diluted solution was cleaned up with the IAC. The purified extract was dissolved in 1.0 ml of acetonitrile–water–acetic acid (30:70:1) and analyzed by HPLC with fluorescence detector. The analytical column (Inertsil ODS-3V, inside diameter: 250 by 4.6 mm, 5 μm; GL Sciences, Tokyo, Japan) was kept at 45°C with a mobile phase of acetonitrile–water–acetic acid (55:43:2) at a flow rate of 1.0 ml/min. The fluorescence detector was set to an excitation wavelength of 333 nm and an emission wavelength of 460 nm. One hundred microliters of standard OTA solutions for HPLC or of test sample solution was injected into the HPLC system. The calibration curve was prepared by plotting the peak height against the concentrations of the OTA standards.

For the samples collected between 2005 and 2007, the extraction protocol for grains, raisins, and coffee beans was modified slightly following a change in the brand of IAC. For grains and their products, 50 g of finely ground sample was extracted in 200 ml of acetonitrile-water (6:4) by homogenizing for 3 min. The extract was filtered through no. 4 filter paper (Whatman, Clifton, NJ). Eight milliliters of filtered solution was diluted to 100 ml with PBS and filtered through a Whatman 934AH glass microfiber filter. Fifty milliliters of filtrate was loaded onto an IAC (Ochraprep, R-Biopharm Rhône Ltd., Glasgow, Scotland) at one drop per second. The column was washed with PBS and then with a 10 mmol/liter ammonium acetate aqueous solution. OTA was eluted into the silanized amber vial with three washes (1 ml each time) of methanol–acetic acid (98:2). The eluate was evaporated until dry and the residue was dissolved in 1.0 ml of acetonitrile–water–acetic acid (30:70:1) and injected into the HPLC system. The HPLC conditions were the same as in earlier analyses *(36)*.

For raisins, cacao products, and dried figs, 90 g of raisin or dried fig paste (and 50 g of each cacao product) was extracted in 160 ml (or 200 ml for cacao products) of methanol–1% sodium bicarbonate (7:3) by homogenizing for 3 min. The extract was filtered, and 10 ml of filtrate was diluted to 50 ml with PBS-Tween. The diluted solution was filtered through the glass microfiber filter. Twenty milliliters of filtrate was loaded onto the IAC at one drop per second. The samples were then analyzed by the same method used for grains in fiscal years 2005 to 2007.

For coffee beans and their products (except for beverages), 50 g of sample was extracted in 200 ml of 1% sodium bicarbonate aqueous solution by homogenizing for 3 min. The extract was filtered, and 50 ml of filtrate was diluted to 100 ml with PBS-Tween. The diluted solution was filtered through a glass microfiber filter. Forty milliliters of filtrate was loaded onto the IAC at one drop per second. Again, the samples were analyzed according to the method used for grains in fiscal years 2005 to 2007.

For coriander, a sample of 2.5 g was extracted in 100 ml of methanol–1% sodium bicarbonate (7:3) by homogenizing for 3 min. Five milliliters of filtered extract and 2 ml of methanol were mixed and diluted to 50 ml with PBS. The samples were then analyzed according to the method used for coffee beans in fiscal years 2005 to 2007.

For beverages, 20 g of sample solution was diluted with 20 ml of 1% polyethylene glycol 8000 plus 5% sodium bicarbonate and filtered through a glass microfiber filter. Twenty milliliters of filtrate was loaded onto the IAC at one drop per second, and the column was washed with 2.5% sodium chloride plus 0.5% sodium bicarbonate followed by a 10 mmol/liter ammonium acetate aqueous solution. The samples were then analyzed according to the method used for grains in fiscal years 2005 to 2007.

Extraction and analysis of fumonisins. Each sample (except for raw, frozen, and canned corn, corn soups, beer, and asparagus) was thoroughly mixed and ground to a fine powder. Samples were analyzed according to the method used in our previous report *(36)*. Before the extraction procedure, the rice and powdered corn soup were digested by α-amylase and by α-amylase and β-mannosidase, respectively, according to the method described by Akiyama et al. *(1)*. A sample of 20 g was extracted with 100 ml of methanol-water (3:1) by shaking for 15 min (or 60 min for the soybeans); the raw, frozen, and canned corn and asparagus extractions were homogenized for 3 min. For the beer, 20 g of the sample was diluted to 100 ml with methanol-water (3:1). The extract was filtered with Whatman no. 4 filter paper, and 10 ml of filtrate was applied to a Bond Elut LRC SAX ion-exchange cartridge (Varian, Palo Alto, CA). Ten milliliters of filtrate from frozen and canned corn, cornflakes, and buckwheat dried noodles was diluted more than fivefold, and the entire solution was applied to the ion-exchange cartridge. The pH of the millet filtrate was adjusted to 6.0 to 6.5 and applied to the cartridge. The cartridge was conditioned with 8 ml of methanol and then with 8 ml of methanol-water (3:1). The filtrate was applied to the cartridge at a flow rate of one or two drops per second. The cartridge was washed with 8 ml of methanol-water (3:1) and then with 8 ml of methanol, and the fumonisins were eluted with 14 ml of methanol–acetic acid (99:1). The eluate was evaporated at approximately 40°C and dried under a nitrogen stream. The residue was dissolved in 1 ml (or 5 ml for beer) of acetonitrile-water (1:1), mixed well, and filtered with a membrane filter; 5 μl was loaded onto a Zorbax Eclipse XDB-C18 column (inside diameter: 150 by 2.1 mm, 5 μm; Agilent, Palo Alto, CA) at 40°C for liquid chromatography–mass spectrometry (LC-MS) analysis. The mobile phase was a binary gradient of solvent A (0.1% formic acid in water) and solvent B (acetonitrile) programmed as follows: at 0 min, 25% B; at 5 min, 50% B; at 8 min, 50% B; at 10 min, 25% B. The flow rate was set at 0.2 ml/min. Electrospray ionization was used for ionization in the mass spectrometric analysis. The capillary voltage and fragmenter voltage were set to 3 kV and 220 V, respectively. Nitrogen was used as both the nebulizer gas and the drying gas. The mass spectrometer was used in the selected ion monitoring mode and detected the positive ions $[M + H]^+$ of FB_1 (*m/z* 722), FB_2, and FB_3 (*m/z* 706).

For the corn snacks, 10 g of sample was extracted with 60 ml of water-methanol-acetonitrile (2:1:1) by homogenizing for 1 min. The extract was filtered with Whatman no. 4 filter paper. Sixty milliliters of hexane saturated with acetonitrile was added to the filtrate, and the solution was shaken for 5 min. The lower layer of the shaken solution was diluted to 100 ml with acetonitrile, and 10 ml of the solution was additionally diluted to 50 ml with water. Twenty-five milliliters of the diluted solution was applied to a Bond Elut C_{18} cartridge (Varian) at a flow rate of one or two drops per second. The cartridge was washed with 10 ml of water and then with 10 ml of water-acetonitrile (4:1), and the fumonisins were eluted with 10 ml of acetonitrile–trifluoroacetic acid (1,000:1). The eluate was evaporated at approximately 40 C and dried under a nitrogen stream. The residue was dissolved in 1 ml of acetonitrile-water (1:1), mixed well, filtered with a membrane filter, and injected into the LC-MS system. The LC-MS conditions were the same as those used for the earlier samples. The flattened barley was filtered with a membrane filter (core size, 0.45 μm) and directly analyzed by LC-MS.

RESULTS AND DISCUSSION

Table 1 shows the results of the survey of OTA. The mean recoveries for each commodity ranged from 65.6 to 113.5%. Samples were considered contaminated if OTA was detected at the upper concentration of each LOQ. OTA was detected in wheat flour, pasta, oatmeal, rye, buckwheat flour and dried noodles, raisins, wine, beer, green and roasted coffee beans, instant coffee, coffee beverages, chocolate, cocoa, and coriander; it was not detected in the other products. OTA was detected in more than 90% of the samples of instant coffee and cocoa, in more than 70% of the samples of pasta and chocolate, and in more than 50% of the samples of buckwheat dried noodles, raisins, and beer. Codex Alimentarius has established a maximum level for OTA in grain *(6)*, and the European Union has set a maximum level for OTA in a variety of commodities *(8)*. Because there is currently no maximum level for OTA in Japan, it is important to monitor OTA levels to prevent adverse health effects. This survey revealed OTA contamination in several products. Although the incidence of contamination in some commodities (such as instant coffee, cocoa, pasta, chocolate, buckwheat dried noodles, and beer) was relatively high, the contamination levels were under the maximum levels set by the European Union and were similar to the levels found in other surveillance studies *(3, 4, 12, 14, 17, 20, 21, 23, 27, 29, 38, 39, 43)*. Only one sample (12.5 μg/kg) of raisins had a contamination level that was outside of the maximum level.

Rice is a food staple in Japan, and daily consumption is high. OTA was not detected in domestic polished rice in this survey or in a study reported by Tabata et al. *(38)*. However, in a survey conducted across the sea in Korea, which has a climate similar to that in the north of Japan, 13 of 148 polished rice samples were contaminated with OTA at levels of 0.9 to 6.0 μg/kg *(26)*. In addition, OTA has been detected in rice around the world, and it must continue to be monitored.

Large quantities of pasta and buckwheat dried noodles are also consumed in Japan. OTA was found in 70% of the pasta samples at a mean concentration of 0.47 μg/kg; it was found in 55% of the buckwheat dried noodle samples at a mean concentration of 0.31 μg/kg.

Corn and corn products are thought to be a predominant source of OTA exposure. The JECFA has examined a European-based worldwide survey for OTA and reported a weighted mean concentration of OTA in corn of 7.5 μg/kg *(12)*. Miraglia and Brera *(19)* have reported a mean concentration of 0.719 μg/kg in OTA-positive corn samples from the European Union; other researchers have identified

TABLE 1. *Natural ochratoxin A in retail food in fiscal years 2004 to 2007*[a]

Commodity	LOQ (μg/kg)	No. of analyzed samples					No. of contaminated samples	Mean of positives (μg/kg)	Maximum (μg/kg)
		2004	2005	2006	2007	Total			
Wheat flour	0.1	50	50	30	30	160	79	0.26	1.00
Pasta	0.1	NA	20	20	40	80	56	0.47	1.66
Oatmeal	0.1	20	14	10	10	54	7	0.79	2.50
Rye	0.1	10	10	10	10	40	18	0.64	2.59
Buckwheat flour	0.1	10	20	5	NA	35	15	0.50	1.79
Buckwheat dried noodles	0.1	NA	40	25	42	107	59	0.31	1.48
Polished rice	0.1	50	30	10	10	100	0	—	—
Rice cracker	0.1	NA	NA	21	NA	21	0	—	—
Frozen or canned corn	0.1	30	20	NA	NA	50	0	—	—
Popcorn grain	0.1	5	5	5	NA	15	0	—	—
Cornflakes	0.1	20	15	10	NA	45	0	—	—
Corn grits	0.1	5	5	5	5	20	0	—	—
Barley	0.1	NA	NA	NA	11	11	0	—	—
Millet	0.1	NA	NA	NA	11	11	0	—	—
Raisins	0.1	11	10	10	21	52	31	0.93	12.5
Wine	0.05	10	23	20	30	83	15	0.29	1.29
Grape juice	0.05	NA	14	10	10	34	0	—	—
Beer	0.01	20	20	21	20	81	45	0.031	0.445
Green coffee beans	0.1	11	10	NA	NA	21	5	0.40	0.76
Roasted coffee beans	0.1	9	10	10	20	49	18	0.55	2.75
Instant coffee	0.1	NA	10	26	30	66	63	0.72	4.23
Coffee beverage	0.02	NA	NA	10	31	41	11	0.026	0.039
Chocolate	0.1	NA	41	34	40	115	84	0.31	1.75
Cocoa	0.1	NA	NA	21	17	38	37	0.89	3.45
Dried bonito	0.1	NA	NA	22	NA	22	0	—	—
Coriander	0.5	NA	NA	NA	5	5	1	0.93	0.93
Dried figs	0.1	NA	NA	NA	5	5	0	—	—

[a] LOQ, limit of quantification, which was determined from the height of the signal peak of OTA that corresponded to 10 times that of the background noise; NA, not analyzed; —, no numerical data.

contamination in corn outside of the European Union *(30)*. However, OTA was not detected in any of the corn products examined in our study.

The JECFA found a mean OTA concentration of 0.18 μg/kg in cacao products (such as chocolate and cocoa) *(12)*. Table 1 shows that in the 4 years of this survey, OTA concentrations exceeded these mean levels in both cocoa (0.89 μg/kg) and chocolate (0.31 μg/kg). These results indicate that in Japan, cocoa and chocolate are higher contributors of OTA than corn.

OTA is generally found at higher concentrations in green coffee than in roasted coffee (because the roasting process destroys some OTA) *(22, 28)*. However, as seen in Table 1, our survey found different results. This may indicate that the OTA in low-contaminated samples was not reduced by a short roasting time as described by Romani et al. *(28)*, or it may indicate that the roasted coffee beans were originally highly contaminated with OTA. On the other hand, instant coffee was more highly contaminated with OTA than roasted coffee beans (0.72 μg/kg versus 0.55 μg/kg, respectively); this corresponds to the results found by Lombaert et al. *(16)*. OTA contamination of instant coffee is a common problem around the world, and Korea has recently set a maximum level of OTA in several foods, including instant coffee.

Although sample numbers were limited, a high level of OTA contamination was detected in coriander (0.93 μg/kg) when compared with other commodities; the same result has been reported in the European Union *(19)*. However, the consumption of spices such as coriander is extremely low in Japan, and they are therefore not seen to be important contributors of OTA.

Concentrations of OTA in other contaminated samples were similar to or lower than those reported by other researchers. Considering consumption and contamination levels, cereals (pasta and buckwheat dried noodles), cacao products (chocolate and cocoa), coffee products (instant coffee), and raisins were the primary contributors to OTA exposure in Japan.

Figure 1 shows the annual mean concentration of OTA contamination in fifteen commodities, and Figure 2 shows the incidence of contamination in those commodities. The mean concentrations of OTA varied significantly from year to year in oatmeal (0.15 to 1.47 μg/kg), rye (0.32 to 1.05 μg/kg), raisins (0.31 to 1.64 μg/kg), wine (0.11 to 0.34 μg/kg), and roasted coffee beans (0.22 to 1.04 μg/kg). The incidence of contamination varied significantly from year to year in oatmeal (7 to 30%), buckwheat dried noodles (25 to 71%), wine (3 to 60%), and roasted coffee beans (25 to 70%). In general, it is presumed that OTA contamination is greater in red wine than in white wine *(2, 4)*. To confirm this hypothesis, we surveyed almost equal numbers of red and white wines each year. Significant annual variations in

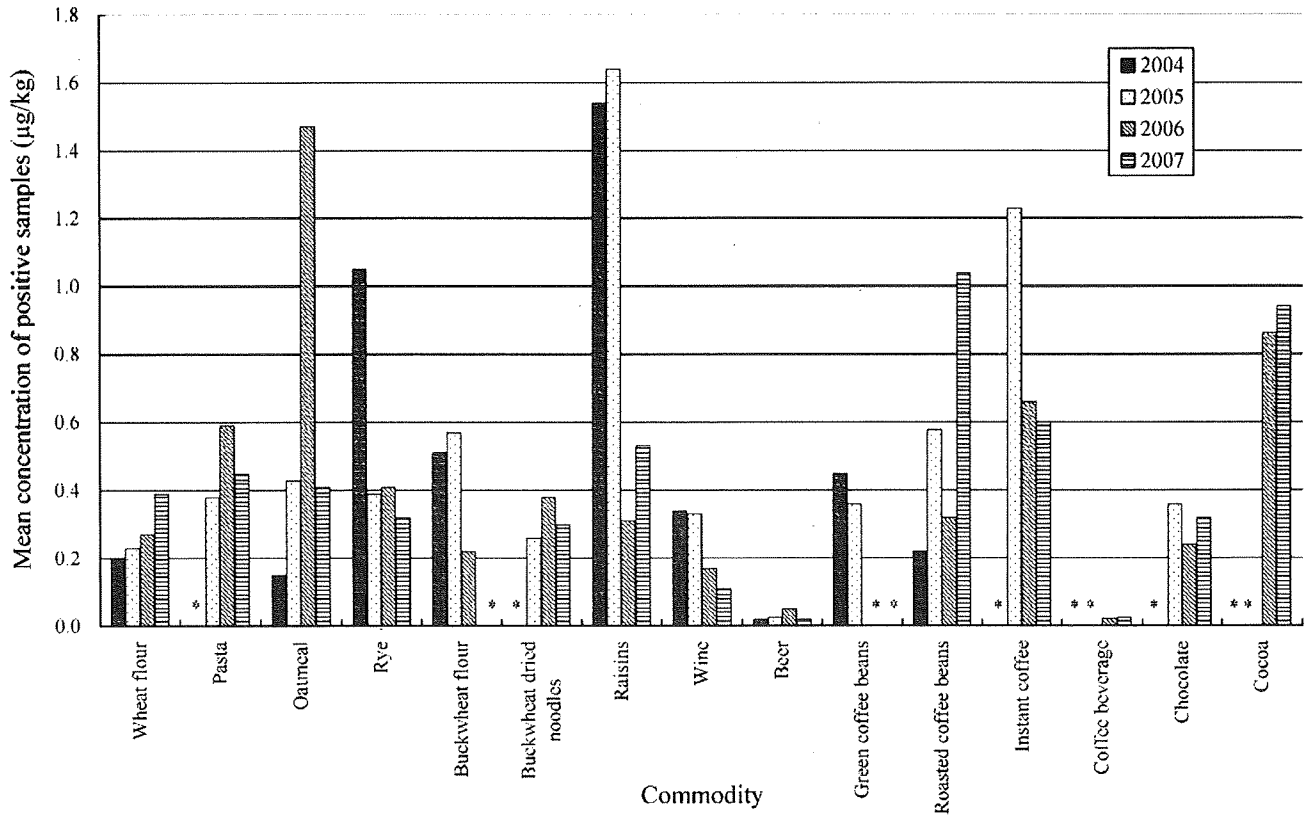

FIGURE 1. *Annual intergradation of the mean concentration of ochratoxin A contamination during fiscal years 2004 to 2007. *, Not analyzed.*

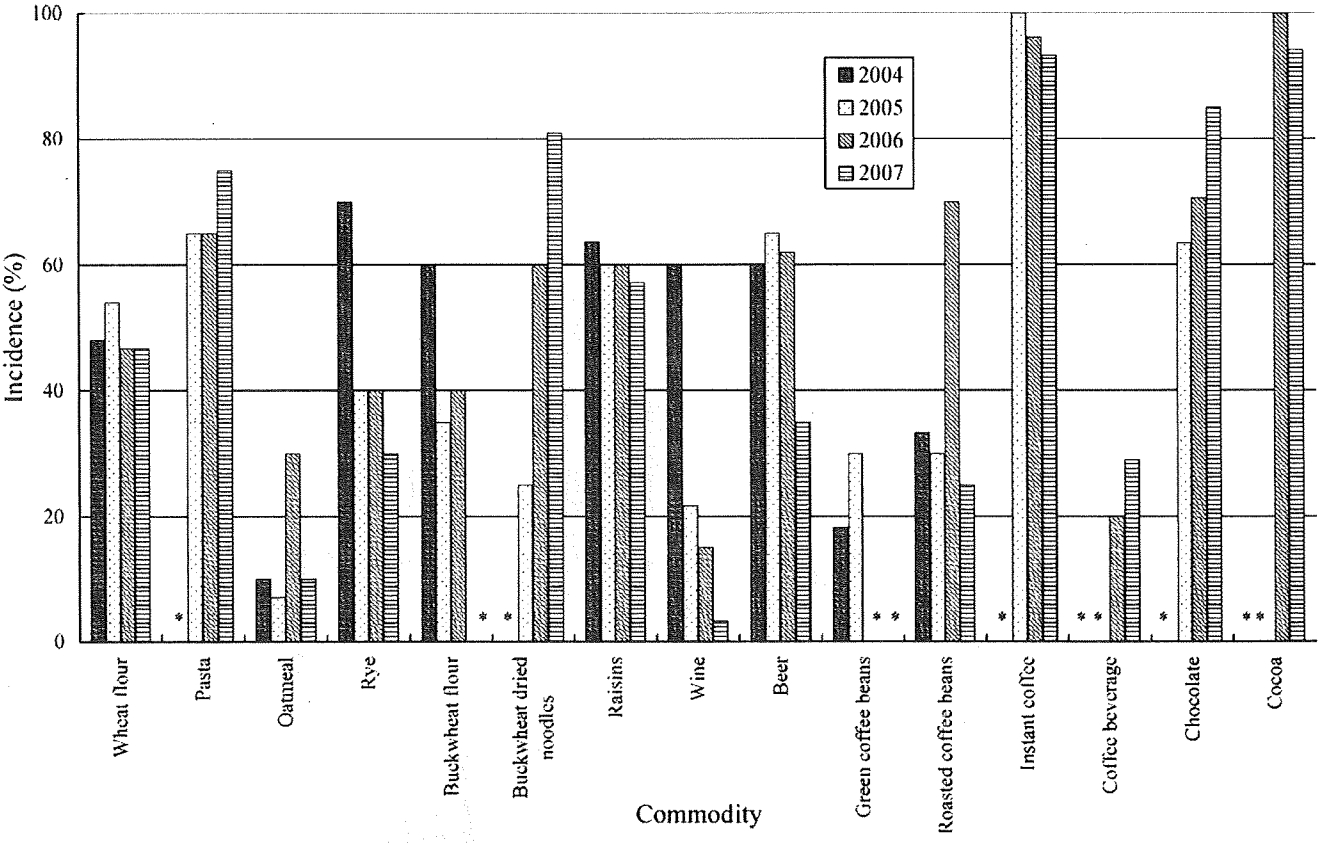

FIGURE 2. *Annual intergradation of the incidence of ochratoxin A contamination during fiscal years 2004 to 2007. *, Not analyzed.*