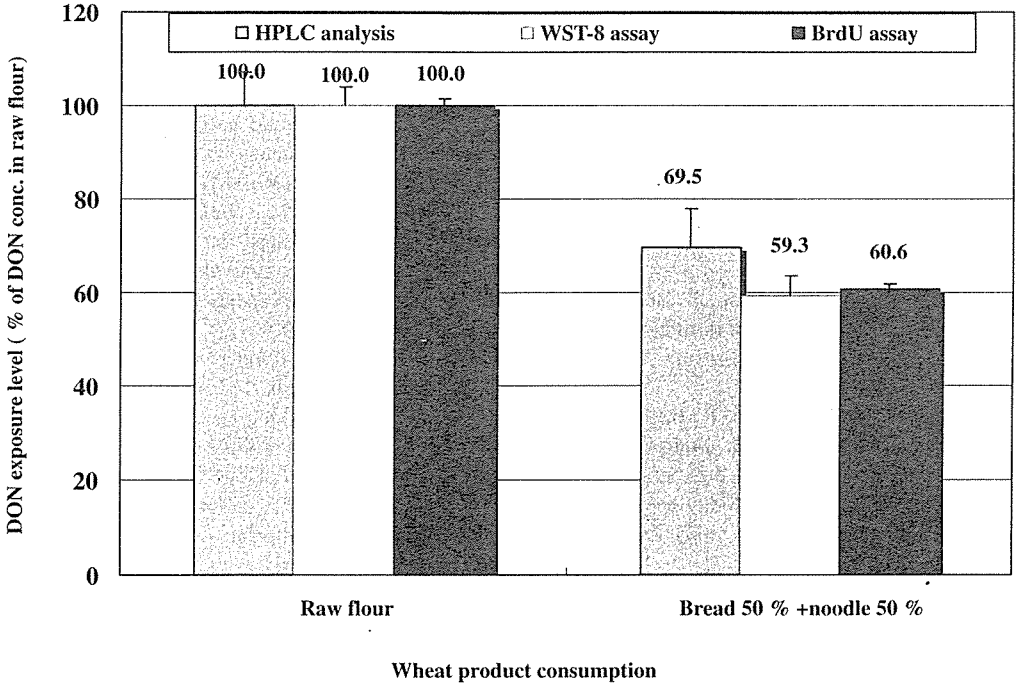

Fig. 1. Estimated DON Exposure Level from Final Wheat Products in Japan.
Bread 50% + noodle 50% indicates the estimated exposure to DON when people ingest bread and noodles equally as wheat products, being calculated by using the following formula: DON exposure level with bread 50% + noodle 50% = (mean of DON retention in bread)/2 + (mean of DON retention in noodles)/2.

rather the biological toxicity was significantly reduced. This fact indicates the possibility that a new complex produced in bread during cooking, such as a DON-binding protein or DON-binding carbohydrate had less cytotoxicity than DON itself. Many studies on the reduction of DON in the baking process have demonstrated that baking could not reduce the level of DON, and the DON level was simply measured by chemical analytical methods.[14–16] The present study is the first report to show the possibility that the baking process reduces the cytotoxicity of DON.

To estimate the risk of exposure to DON in Japan, we calculated the total retention level of DON based on the consumption of wheat products in Japan. A national nutritional survey has shown that 50% of wheat was consumed as bread and other 50% as noodles in Japan.[17] When the DON retention level in each final product was assayed by a chemical or biological analysis, its exposure level from the final wheat products consumed in Japan was calculated as 69.5% by the chemical analysis, 59.3% by the WST-8 assay and 60.6% by the BrdU assay of the DON level in raw flour (Fig. 1).

In conclusion, this study is the first to report the DON retention level at each cooking stage in noodles and bread determined by a chemical analysis and bioassays. The experiments with naturally contaminated wheat flour revealed that boiling significantly reduced not only the DON concentration, but also its cytotoxicity. In contrast, baking maintained the DON concentration at the same level as that of raw flour although reducing its cytotoxicity. The risk of exposure to DON was estimated 69.5% or less of the DON level in raw flour if people eat noodles and bread half and half as the final wheat products, which reflects the current consumption of final wheat products in Japan.

Acknowledgments

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References

1) Scott, P. M., The natural occurrence of trichothecenes, 1. In "Trichothecene Mycotoxicosis: Pathophysiologic Effects," ed. Beasley, V. R., CRC Press, Boca Raton, pp. 1–26 (1989).
2) Rotter, B. A., Prelusky, D. B., and Pestka, J. J., Toxicology of deoxynivalenol (vomitoxin). *J. Toxicol. Environ. Health*, **48**, 1–34 (1996).
3) Sugita-Konishi, Y., Hara-Kudo, Y., Kasuga, F., and Kumagai, S., Effect of trichothecenes on host defense against infectious diseases. *Mycotoxins*, **47**, 19–23 (1998).
4) Boyacioglu, D., Hettierachchy, N. S., and D'Appolonia, B. L., Additives affect deoxynivalenol (vomitoxin) flour during breadmaking. *J. Food Sci.*, **58**, 416–418 (1993).
5) Neira, M. S., Pacin, A. M., Martinez, E. J., Molto, G.,

and Resnik, S. L., The effects of bakery processing on natural deoxynivalenol contamination. *Int. J. Food Microbiol.*, **37**, 21–25 (1997).

6) Young, L. C., Subryan, L. M., Potts, D., McLaren, M. E., and Gobran, F. H., Reduction in levels of deoxynivalenol in contaminated wheat by chemical and physical treatment. *J. Agric. Food Chem.*, **34**, 659–664 (1986).
7) Bullerman, L. B., and Ryu, D., Stability of fumonisins in food processing. *Adv. Exp. Med. Biol.*, **504**, 195–204 (2002).
8) Yumbe-Guevara, B. É., and Yoshizawa, T., Reduction of deoxynivalenol and nivalenol content during roasting of naturally contaminated barley. *Mycotoxins*, **53**, 87–93 (2003).
9) Howard, P. C., Couch, L. H., Patton, R. E., Eppley, R. M., Doerge, D. R., Churchwell, M. I., Marques, M. M., and Okerberg, C. V., Comparison of the cytotoxicity of several fumonisin derivatives in a 28-day feeding study with female B6C3F(1) mice. *Toxicol. Appl. Pharmacol.*, **185**, 153–165 (2002).
10) Trucksess, M. W., Reasy, D. E., Pender, M. K., Ligmond, C. A., Wood, G. E., and Page, S. W., Determination and survey of deoxynivalenol in white flour, whole wheat, flour and bran. *J. AOAC Int.*, **79**, 883–887 (1996).
11) Widestrand, J., Lundh, T., Pettersson, H., and Lindberg, J., Cytotoxicity of four trichothecenes evaluated by three colorimetric bioassays. *Mycopathologia*, **147**, 149–155 (1999).
12) Widesstrand, J., Lundh, T., Pettersson, H., and Lindberg, J. E., A rapid and sensitive cytotoxicity screening assay for trichothecenes in cereal samples. *Food Chem. Toxicol.*, **41**, 1307–1313 (2003).
13) Nowecki, T. M., Gaba, D. G., Dexter, J. E., Matsuo, R. R., and Clear, R. M., Retention of the fusarium mycotoxin deoxynivalenol in wheat during processing and cooking of spaghetti and noodles. *J. Cereal Sci.*, **8**, 189–202 (1988).
14) Scott, P. M., Kanhere, S. R., Lau, P. Y., Dexter, J. E., and Greenhaigh, R., Effects of experimental flour milling and bread making on retention of DON. *Cereal Chem.*, **60**, 421–424 (1983).
15) Young, J. C., Fulcher, R. G., Hayhoe, J. H., Scott, P. M., and Dexter, J. E., Effects of milling and baking on DON content of Canadian wheats. *J. Agric. Food Chem.*, **32**, 659–664 (1984).
16) Hazel, C. M., and Patel, S., Influence of processing on trichothecene levels. *Toxicol. Lett.*, **153**, 51–59 (2004).
17) "The National Nutrition Survey in Japan, 2002," Ministry of Health, Labour and Welfare, Daiichi-shuppan, Tokyo, p. 92 (2002).

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Occurrence of Aflatoxins, Ochratoxin A, and Fumonisins in Retail Foods in Japan

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ABSTRACT

We conducted a survey of aflatoxin B_1, B_2, G_1, and G_2, ochratoxin A, and fumonisin B_1, B_2, and B_3 contamination in various foods on the retail market in Japan in 2004 and 2005. The mycotoxins were analyzed by high-performance liquid chromatography, liquid chromatography–mass spectrometry, or high-performance thin-layer chromatography. Aflatoxins were detected in 10 of 21 peanut butter samples; the highest concentration of aflatoxin B_1 was 2.59 μg/kg. Aflatoxin contamination was not found in corn products, corn, peanuts, buckwheat flour, dried buckwheat noodles, rice, or sesame oil. Ochratoxin A was detected in oatmeal, wheat flour, rye, buckwheat flour, green coffee beans, roasted coffee beans, raisins, beer, and wine but not in rice or corn products. Ochratoxin A concentrations in contaminated samples were below 0.8 μg/kg. Fumonisins were detected in popcorn, frozen corn, corn flakes, and corn grits. The highest concentrations of fumonisins B_1, B_2, and B_3 in these samples were 354.0, 94.0, and 64.0 μg /kg, respectively.

Mycotoxin contamination in agricultural products is a significant threat to human health and results in enormous economic losses from exclusion of contaminated products from food and feed chains. Among numerous mycotoxins, aflatoxins (AFs), ochratoxin A (OTA), and fumonisins are of high priority for control because of their frequent and worldwide distribution in agricultural products.

AFs are potent hepatotoxic and hepatocarcinogenic compounds produced by *Aspergillus flavus* and *Aspergillus parasiticus.* In Asia and Africa, human hepatic cancers and acute fatal diseases, including hepatic lesions, have been associated with consumption of foods heavily contaminated with these toxins *(4, 12).* Commodities frequently contaminated include peanuts, tree nuts, spices, corn, rice, cottonseed, dry fruits, and copra *(6).* OTA is produced by *Penicillium verrucosum* and various species of *Aspergillus* and is regarded as the causal agent of endemic nephropathy and urinary tract tumor in the Balkans *(7).* A variety of commodities are contaminated with OTA; relatively high levels of contamination have been found in corn, rye, and coffee *(7).* Fumonisins are produced by *Fusarium verticillioides, Fusarium proliferatum,* and other *Fusarium* species. Fumonisin contamination in corn has been observed in many areas of the world *(8),* and an association between human esophageal cancer and high concentrations of fumonisin in corn has been reported in China and Africa *(3, 19, 24).*

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Although contamination of food with these mycotoxins has been studied extensively in Europe and North America, relatively little information has come from Asia. In Japan, a survey of AF contamination has been conducted for a variety of foods *(1, 10, 20, 21),* but data on OTA and fumonisin contamination in local and imported foods are very limited *(13, 18).* In 2004 and 2005, we conducted a survey of AF, OTA, and fumonisin contamination in various foods collected from retail markets in Japan to evaluate the risk to the health of Japanese consumers.

MATERIALS AND METHODS

Sampling. Rice and wheat samples were harvested in the 2004 fiscal year except for 2 samples of rice (2003 fiscal year) were supplied from the Ministry of Agriculture, Forestry and Fisheries. All other samples were purchased in a random manner from local supermarkets and small retail shops in all parts of Japan from the summer of 2004 to winter of 2005. Samples were stored at 4°C until analysis.

Reagents. AFB_1, AFB_2, AFG_1, AFG_2, and OTA standards were purchased from Sigma-Aldrich Co. (St. Louis, Mo.). Fumonisin B_1 (FB_1) and FB_2 were purchased from CALBIOCHEM (San Diego, Calif.), and FB_3 was from PROMEC Medical Research Council (Tygerberg, South Africa). Methanol and acetonitrile were high-performance liquid chromatography (HPLC) grade. Water was purified in a Milli-Q system (Millipore Co., Bedford, Mass.). All other reagents were of the highest analytical grade available.

Standard solutions. Original standard solutions of AFB_1, AFB_2, AFG_1, and AFG_2 were prepared in toluene-acetonitrile (9:1), and a standard solution of OTA was prepared in toluene–acetic acid (99:1). The concentration was determined according to the AOAC International method *(23)*. An AF mixed stock solution (4 μg/ml for each toxin) and a OTA stock solution (1 μg/ml) was prepared by dilution with acetonitrile and toluene–acetic acid (99:1), respectively. A fumonisin mixed stock solution (20 μg/ml for each toxin) was prepared by dilution with acetonitrile-water (1:1).

Extraction and analysis of AFs. AFs in collected samples were analyzed at five independent laboratories. Twenty-five grams of finely ground sample (except for peanut butter and sesame seed oil) was extracted with 100 ml of methanol-water (8:2) and 5 g of sodium chloride by shaking for 30 min. The extract was filtered through no. 4 filter paper (Whatman, Clifton, N.J.). Ten milliliters of filtered solution was diluted to 50 ml with phosphate-buffered saline (PBS, pH 7.4) and filtered through a Whatman 934AH glass microfiber filter. Twenty milliliters of filtered solution was loaded onto an AflaTest P column (Vicam, Watertown, Mass.) at a flow rate of 1 drop per s. After washing with PBS and then purified water, the column was dried by pushing air into the column with a syringe. AFs were eluted with acetonitrile into a silanized amber vial (4.0 ml, Supelco, Bellefonte, Pa.). The eluate was evaporated to dryness under a gentle stream of nitrogen gas 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 derivatization, the residue was dissolved in 1.0 ml of injection solvent and injected into the HPLC system, which consisted of an LC-10AD pump, a SIL-10A autoinjector (100-μl loop), a CTO-10AC column oven, an RF-10AXL fluorescence detector (excitation, 360 nm; emission, 450 nm), a DGU-3A degasser, a CBM-10A communication bus module, and a class LC-10 chromatography data system (Simadzu, Kyoto, Japan). The analytical column (4.6 mm by 250 mm by 5 μm; Inertsil ODS-3V, GL Sciences, Inc., Tokyo, Japan) was kept at 45°C with a mobile phase of acetonitrile-methanol-water (1:3:6) at a flow rate of 1.0 ml/min for TFA derivatization or with acetonitrile-methanol-water (2:3:5) at a flow rate of 0.7 ml/min for photochemical reactor derivatization. The photochemical reactor for enhanced detection consisted of a short-wave UV lamp and a knitted Teflon tube (0.25 mm by 20 m, Aura Industry Inc., New York) and was set between the analytical column and the detector. The calibration curve was prepared by plotting the peak height against the concentration of AF standards. Quantification of each AF in sample solution was performed by measuring the peak height at each retention time and comparing these curves with a relevant calibration curve.

For peanut butter and sesame seed oil samples, the method of Kamimura et al. *(9)* was used for analysis. A 10- or 20-g sample was extracted with 50 or 100 ml of chloroform. The extract was loaded on florisil columns, from which AFs were 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. OTA in collected samples was analyzed at five independent laboratories with a standardized analytical method. Each sample (except for raisins, beer, and wine) was thoroughly mixed and ground to a fine powder. Raisins were slurred with water (five parts raisins to four parts water, wt/wt) to form a homogenous paste. Beer was degassed in an ultrasonic bath for 30 min. Extraction solvents used in this survey were 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. Twenty-five grams of sample (45 g of raisins paste) was extracted in 100 ml (80 ml for raisins paste) of extraction solvent by shaking for 30 min. The extract was filtered through Whatman no. 4 paper. Ten milliliters of filtered solution was diluted to 50 ml (100 ml for coffee) with PBS or with PBS plus 0.01% Tween 20 (PBS-Tween) for coffee and raisin samples and filtered through a Whatman 934AH glass microfiber filter. Twenty milliliters of filtrate (40 ml for coffee) was loaded onto an OchraTest column (Vicam) at a flow rate of 1 drop per s. The column was washed with PBS (PBS-Tween for coffee and raisin samples) and then with purified water. For beer and wine, 10 g of sample solution was diluted with 10 ml of 1% polyethylene glycol 8000 plus 5% sodium bicarbonate and filtered through a Whatman 934AH glass microfiber filter. Ten milliliters of filtrate was loaded onto the OchraTest column at a flow rate of 1 drop per s, and the column was washed with 2.5% sodium chloride plus 0.5% sodium bicarbonate followed by purified water. OTA was eluted into the Silanized amber vial with three washes (1 ml each time) of methanol–acetic acid (99:1). The eluate was evaporated to dryness under a gentle stream of nitrogen gas 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 (4.6 by 250 mm by 5 μm; Inertsil ODS-3V, GL Sciences) 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. Standard OTA solutions for HPLC or 100 μl of test sample solution were injected into the HPLC system. The calibration curve was prepared by plotting the peak height against the concentration of OTA standards. Quantification of OTA was performed with the same manner used for the AF analysis.

Extraction and analysis of fumonisins. A 20-g sample was weighed and extracted with 100 ml methanol-water (3:1) by shaking for 15 min. The extract was filtered with Whatman no. 4 filter paper and applied to Bond Elut LRC SAX cartridge (Varian, Palo Alto, Calif.). The cartridge was conditioned with methanol and then with methanol-water (3:1). The filtrate was applied to the cartridge at a flow rate of 1 or 2 drops per s. The cartridge was washed with methanol-water (3:1) and then methanol, and fumonisins were eluted with methanol–acetic acid (99:1). The eluate was evaporated at ca. 40°C and dried under a nitrogen stream. The residue was dissolved in 1 ml of acetonitrile-water (1:1), mixed well, and filtered with a membrane filter, and 5 μl was loaded onto a ZORBAX Eclipse XDB-C18 column (inside diameter, 150 by 2.1 mm; Agilent, Palo Alto, Calif.) at 40°C for liquid chromatography–mass spectrometry 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. The nebulizer gas and drying gas were both nitrogen. The mass spectrometer was used in the selected ion monitoring mode, detecting the positive ions $[M + H]^+$ of FB_1 (*m/z* 722), FB_2, and FB_3 (*m/z* 706).

Confirmation of identity. The identities of AFB_1 and AFG_1 in the sample were confirmed by the method based on the quenching of their fluorescence in reversed-phase HPLC. AFB_1 and AFG_1 were detected as AFB_{2a} and AFG_{2a}, respectively, by precolumn derivatization with TFA or in-line postcolumn derivati-

TABLE 1. *Recovery of total aflatoxins from each spiked individual matrix*

Commodity	Spiked sample		Recovery (%)[a]			
	Concn (μg/kg)	*n*	AFB_1	AFB_2	AFG_1	AFG_2
Raw corn	0.1	3	79.8 ± 0.7	80.3 ± 1.4	74.5 ± 1.0	74.6 ± 1.1
	5.0	3	83.7 ± 1.1	82.1 ± 1.2	76.0 ± 0.9	75.6 ± 1.3
Canned corn	0.1	2	65.0	72.0	64.5	70.5
	5.0	2	66.9	68.7	69.5	70.2
Rice	0.1	2	82.0	83.9	84.4	89.9
	5.0	2	83.7	87.8	82.3	87.1
Peanuts with shells	0.1	6	65.0 ± 10.8	85.0 ± 12.3	66.0 ± 16.2	85.0 ± 20.0
	5.0	6	80.0 ± 5.9	105.0 ± 5.9	102.5 ± 2.9	99.3 ± 1.5
Peanuts without shells	0.2	3	98.3 ± 4.8	113.7 ± 3.5	88.3 ± 3.3	91.8 ± 8.3
	5.0	3	94.4 ± 0.4	99.1 ± 1.8	98.5 ± 1.1	97.7 ± 0.3
Peanut flour	0.1	3	89.0 ± 0.8	90.2 ± 1.0	80.1 ± 1.5	82.2 ± 1.1
	5.0	3	89.3 ± 2.9	90.1 ± 2.1	77.8 ± 10.2	79.0 ± 7.7
Buckwheat flour	0.1	3	78.1 ± 1.3	78.6 ± 1.0	73.7 ± 4.1	82.7 ± 1.1
	5.0	3	68.7 ± 0.8	75.6 ± 0.3	74.0 ± 1.6	80.3 ± 0.8
Buckwheat dried noodles	0.1	3	72.8 ± 3.4	84.2 ± 0.6	77.6 ± 1.4	86.3 ± 0.5
	5.0	3	72.7 ± 0.4	79.8 ± 1.6	72.9 ± 2.1	79.4 ± 1.6
Popcorn grain	0.1	3	85.0 ± 4.0	77.3 ± 3.0	85.1 ± 3.0	82.6 ± 2.0
	5.0	2	67.6	72.8	84.8	84.7
Cornflakes	0.1	3	95.8 ± 2.0	82.3 ± 6.1	87.5 ± 5.0	85.1 ± 4.1
	5.0	2	78.8	75.4	77.9	79.3
Corn grits	0.1	3	88.1 ± 2.8	86.3 ± 3.0	88.7 ± 2.0	90.9 ± 2.0
	5.0	2	76.5	76.5	98.8	97.7
Peanut butter	0.2	3	98.3 ± 11.4	79.0 ± 6.7[b]	84.2 ± 13.8[c]	101.2 ± 6.2
	5.0	3	93.8 ± 3.1	93.5 ± 2.5[d]	92.1 ± 3.1[e]	92.3 ± 2.8
Sesame oil	0.2	2	90.6	102.8	76.7	98.0
	5.0	2	103.7	107.2	75.5	84.0

[a] For $n \geq 3$, values are expressed as mean ± residual standard deviation. For $n = 2$, values are expressed as mean only.
[b] Spiked dose was 0.1 μg/kg.
[c] Spiked dose was 0.3 μg/kg.
[d] Spiked dose was 2.5 μg/kg.
[e] Spiked dose was 7.5 μg/kg.

zation of the photochemical reactor used in this survey. The disappearance of the AFB_1 or AFG_1 peaks by non-TFA derivatization confirmed the original presence of AFB_1 or AFG_1 in the sample. The decrease of peak height for AFB_1 or AFG_1 with the photochemical reactor UV lamp off confirmed the original presence of AFB_1 or AFG_1. For peanut butter and sesame oil, the positive sample detected by HPLC analysis was spotted onto a silica gel plate (Merck KGaA, Darmstadt, Germany) for high-performance thin-layer chromatography.

The presence of OTA was confirmed by OTA–methyl ester formation as follows. The remaining 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 and then evaporated to dryness. The residue was dissolved in injection solvent and injected into the HPLC system. The identity of OTA in the sample was confirmed by the presence of an OTA–methyl ester peak at delayed retention time and the disappearance of the OTA peak.

RESULTS AND DISCUSSION

The recoveries of each standard mycotoxin spiked to the individual matrix are shown in Tables 1 through 3. The recoveries of AFs from the matrix were 65.0 to 103.7% for AFB_1, 68.7 to 113.7% for AFB_2, 64.5 to 102.5% for AFG_1, and 70.2 to 101.2% for AFG_2 (Table 1). The OTA recoveries were 60.4 to 107.9% (Table 2). The recoveries of fumonisins were 67.7 to 136.0% for FB_1, 70.5 to 133.0% for FB_2, and 70.3 to 136.0% for FB_3. The limit of quantification was calculated by the signal-to-noise ratio as more than 10:1.

AF contamination was detected in 10 of 21 peanut butter samples (Table 4). Six and 2 of the 10 positive samples were imported and domestic products, respectively, but the origin of the other two products was unknown. AFB_1, AFB_2, AFG_1, and AFG_2 were detected in four samples, AFB_1 and AFB_2 were detected in three samples, and only AFB_1 was detected in the other three samples. AF was not detected in corn, cornflakes, corn flour, corn grit, popcorn grains, canned or frozen corn, peanuts with shell, peanuts without shell, buckwheat flour, dried buckwheat noodle, rice, or sesame oil (Table 4). These results indicate that peanut butter sold in Japan is frequently contaminated with AFs.

OTA was detected in oatmeal, wheat flour, rye, buckwheat flour, green coffee beans, roasted coffee beans, raisins, beer, and wine but not in rice, cornflakes, corn grits, popcorn grain, or canned or frozen corn (Table 5). Frequent contamination was noted in raisins, beer, rye, and wine. All

TABLE 2. *Recovery of ochratoxin A from each spiked individual matrix*

Commodity	Spiked sample Concn (μg/kg)	*n*	OTA recovery (%)[a]
Canned corn	0.1	5	100.0 ± 0.1
	0.5	7	100.0 ± 0.4
Rice	0.1	2	88.2
	5.0	2	82.8
Rye	0.1	2	86.1
	5.0	2	87.3
Wheat flour	0.1	2	104.6
	5.0	2	87.8
Oatmeal	0.1	3	90.0 ± 11.1
	5.0	3	89.2 ± 2.6
Buckwheat flour	0.2	3	101.3 ± 3.1
	5.0	3	91.2 ± 1.2
Popcorn grain	0.1	6	99.6 ± 9.6
	5.0	6	100.0 ± 0.3
Cornflakes	0.1	6	83.3 ± 8.6
	5.0	6	99.9 ± 0.4
Corn grits	0.1	6	100.0 ± 0.1
	5.0	6	100.1 ± 0.3
Raisins	0.1	3	96.7 ± 15.8
	5.0	3	89.5 ± 8.7
Wine	0.05	3	94.7 ± 4.9
	5.0	3	107.9 ± 2.3
Beer	0.01	3	98.4 ± 2.8
	0.5	3	97.5 ± 0.5
Green coffee beans	0.1	3	102.7 ± 19.4
	5.0	3	80.6 ± 3.4
Roasted coffee beans	0.1	3	78.3 ± 11.5
	5.0	3	60.4 ± 5.0

[a] For $n \geq 3$, values are expressed as mean ± residual standard deviation. For $n = 2$, values are expressed as mean only.

TABLE 3. *Recovery of fumonisins B_1, B_2, and B_3 from each spiked individual matrix*

Commodity	Spiked sample concn (μg/kg)	Recovery (%)[a] FB_1	FB_2	FB_3
Raw corn	10	136.0	133.0	136.0
	1,000	82.1	81.1	85.7
Canned corn	10	103.6	98.9	94.5
	1,000	88.0	85.6	86.6
Buckwheat dried noodle	10	124.5	123.6	120.3
	1,000	82.8	84.1	96.4
Popcorn grain	10	117.0	103.0	101.0
	1,000	67.7	70.5	70.3
Cornflakes	10	112.9	79.1	71.9
	1,000	92.6	75.7	78.6
Corn soups	10	84.6	116.0	101.3
	1,000	122.3	90.3	96.0
Corn grits	1,000	70.7	71.4	73.3
Flattened barley	10	81.5	89.5	90.4
	1,000	77.9	72.7	73.9

[a] Values are expressed as mean.

contaminated rye and raisin samples were from imported products, whereas contaminated beer, buckwheat flour, and wine samples were from both imported and domestic products. Concentrations of OTA in wine of more than 1 μg/kg have frequently been observed *(7, 11)*, but the concentration in this study was less than 0.8 μg/kg.

Reports of OTA contamination in buckwheat are very limited. In a survey conducted in Germany in 1995 through 1998, only 3 of 14 buckwheat samples were contaminated at more than 0.01 μg/kg, with a maximum of 12.1 μg/kg *(7)*. In our study, buckwheat flour was very frequently contaminated with OTA, although its concentration was lower than that reported in the German study, indicating the importance of broadening the survey of OTA contamination of domestic and imported buckwheat in Japan over succes-

TABLE 4. *Natural aflatoxins in retail foods, 2004 and 2005*

Commodity	No. of samples analyzed	No. of contaminated samples	Concn (μg/kg) in contaminated samples Mean (range, no. of samples) AFB_1	AFB_2	AFG_1	AFG_2	LOQ[a]
Raw corn	10	0	—	—	—	—	0.1
Frozen or canned corn	50	0	—	—	—	—	0.1
Rice	53	0	—	—	—	—	0.1
Peanuts with shells	30	0	—	—	—	—	0.1
Peanuts without shells	30	0	—	—	—	—	0.1
Peanut flour	10	0	—	—	—	—	0.1
Buckwheat flour	12	0	—	—	—	—	0.1
Buckwheat dried noodle	39	0	—	—	—	—	0.1
Popcorn grain	10	0	—	—	—	—	0.1
Cornflakes	20	0	—	—	—	—	0.1
Corn grits	10	0	—	—	—	—	0.1
Peanut butter	21	10	1.07 (0.17–2.59, 10)	0.27 (0.16–0.52, 7)	0.40 (0.17–0.81, 4)	0.21 (0.12–0.46, 4)	0.1
Sesame oil	10	0	—	—	—	—	0.1

[a] LOQ, limit of quantification.

TABLE 5. *Natural ochratoxin A in retail food, 2004, and 2005*

Commodity	No. of samples analyzed	No. of contaminated samples	Concn (μg/kg) in contaminated samples	LOQ (μg/kg)[a]
Frozen or canned corn	30	0	—	0.1
Rice	50	0	—	0.1
Rye	10	7	1.05 (0.28–2.59)	0.1
Wheat flour	50	24	0.20 (0.10–0.48)	0.1
Oatmeal	20	2	0.15 (0.13, 0.18)	0.1
Buckwheat flour	10	6	0.51 (0.16–1.79)	0.1
Popcorn grain	5	0	—	0.1
Cornflakes	20	0	—	0.1
Corn grits	5	0	—	0.1
Raisins	11	7	1.54 (0.18–12.5)	0.1
Wine	10	6	0.34 (0.07–0.72)	0.05
Beer	20	12	0.02 (0.01–0.05)	0.01
Raw coffee	11	2	0.45 (0.14–0.76)	0.1
Roasted coffee	9	3	0.22 (0.11–0.33)	0.1

[a] LOQ, limit of quantification.

sive years. Concentrations of OTA in other positive samples were similar to or lower than those reported by many European and American researchers *(2, 6, 11, 14, 16, 22)*.

Fumonisins were detected in popcorn, frozen corn, cornflakes, and corn grits but not in the other products (Table 6). Popcorn and corn grits were the foods most frequently contaminated food by fumonisins. The highest concentration among the corn products was found in popcorn, with maximums of 354.0 μg/kg for FB_1, 94.0 μg/kg for FB_2, and 64.0 μg/kg for FB_3. However, concentrations in these samples were relatively low compared with those reported in other countries *(8)*. All contaminated popcorn, frozen corn, and corn grit samples were from imported products, but contaminated cornflake samples were from both imported and domestic products. In a recent study, no contamination was found in domestic beer *(18)*.

The origin of the materials used in the processed domestic products was unknown, but raw materials such as rice and raw corn were confirmed to be domestic products. AFs have been detected in imported rice in Japan *(10)* but have not been detected in domestic rice until recently *(20)*, and the presence of these toxins was confirmed in this study. AFs were found in peanut butter, with maximums of 2.59, 0.52, 0.81, and 0.46 μg/kg for AFB_1, AFB_2, AFG_1, and AFG_2, respectively. Although AF concentrations found in peanut butter retailed in 1988 through 1992 in Japan *(20)* were lower than those noted in this study, it was concluded that some peanut butter products distributed in Japan may continuously be contaminated with low concentrations of AFs. In a survey in the Republic of Cyprus, 21 of 74 peanut butter samples were positive for AF, with a maximum AFB_1 concentration of 73 μg/kg *(5)*. Siame et al. *(17)* reported that in Botswana the average AF concentration in peanut butter was 23 μg/kg. In Sudan, heavy contamination of peanut butter with AFs has been regarded as a risk factor for hepatic cell cancer *(15)*. Although risks to human health from such low concentrations of AFs in peanut butter as noted in this study may be very low, measures to reduce further AF contamination are needed.

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TABLE 6. *Natural fumonisins in retail foods, 2004 and 2005*

Commodity	No. of samples analyzed	No. of contaminated samples	Concn (μg/kg) in samples: Mean (range, no. of samples) FB_1	FB_2	FB_3	LOQ[a]
Raw corn	18	0	—	—	—	10
Canned or frozen corn	51	2	26.4 (16.8–36.0, 2)	14.8 (14.8, 1)	—	10
Buckwheat dried noodle	30	0	—	—	—	2.0
Popcorn grain	15	15	57.2 (5.0–354.0, 15)	16.9 (2.0–94.0, 14)	12.8 (2.0–64.0, 11)	2.0
Cornflake	30	9	27 (13–59, 9)	—	—	10
Corn soups	29	0	—	—	—	10
Corn grits	10	10	51.1 (17.8–73.8, 10)	21.1 (17.9–29.1, 10)	13.1 (8.9–18.0, 10)	2.0
Flattened barley	20	0	—	—	—	10

[a] LOQ, limit of quantification.

REFERENCES

1. Adachi, Y., M. Hara, N. H. Kumazawa, K. Hirano, I. Ueno, and K. Egawa. 1991. Detection of aflatoxin B1 in imported food products into Japan by enzyme-linked immunosorbent assay and high performance liquid chromatography. *J. Vet. Med. Sci.* 53:49–52.
2. Biffi, R., M. Munari, L. Dioguardi, C. Ballabio, A. Cattaneo, C. L. Galli, and P. Restani. 2004. Ochratoxin A in conventional and organic cereal derivatives: a survey of the Italian market 2001–02. *Food Addit. Contam.* 21:586–591.
3. Chu, F. S., and G. Y. Li. 1994. Simultaneous occurrence of fumonisin B1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. *Appl. Environ. Microbiol.* 60:847–852.
4. International Agency for Research on Cancer. 1993. Aflatoxins. *IARC Monogr.* 56:245–395.
5. Ioannou-Kakouri, E., M. Aletrari, E. Christou, A. Hadjioannou-Ralli, A. Koliou, and D. Akkelidou. 1999. Surveillance and control of aflatoxin B1, B2, G1, G2, and M1 in foodstuffs in the Republic of Cyprus: 1992–1996. *J. AOAC Int.* 82:883–892.
6. Joint FAO/WHO Expert Committee on Food Additives. 1998. Safety evaluation of certain food additives and contaminants in food: aflatoxin, p. 359–469. *In* Proceedings of the 49th Meeting of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization, Geneva.
7. Joint FAO/WHO Expert Committee on Food Additives. 2001. Safety evaluation of certain food additives and contaminants in food: ochratoxin A, p. 281–415. *In* Proceedings of the 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization, Geneva.
8. Joint FAO/WHO Expert Committee on Food Additives. 2001. Safety evaluation of certain food additives and contaminants in food: fumonisins, p. 103–279. *In* Proceedings of the 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization, Geneva.
9. Kamimura, H., M. Nishijima, K. Yasuda, H. Ushiyama, S. Tabata, and S. Matsumoto. 1995. Simple, rapid cleanup method for analysis of aflatoxins and comparison with various methods. *J. AOAC Int.* 68:458–461.
10. Lipigorngoson, S., A. Norhayati, and T. Yoshizawa. 2003. Limited survey for aflatoxin contamination of polished rice imported into Japan. *Mycotoxins* 53:95–101.
11. Lombaert, G. A., P. Pellaers, G. Neumann, D. Kitchen, V. Huzel, R. Trelka, S. Kotello, and P. M. Scott. 2004. Ochratoxin A in dried vine fruits on the Canadian retail market. *Food Addit. Contam.* 21:578–585.
12. Muture, B. N., and G. Ogana. 2005. Aflatoxin levels in maize and maize products during the 2004 food poisoning outbreak in Eastern Province of Kenya. *East Afr. Med. J.* 82:275–279.
13. Nakajima, M., H. Tsubouchi, and M. Miyabe. 1999. A survey of ochratoxin A and aflatoxins in domestic and imported beers in Japan by immunoaffinity and liquid chromatography. *J. AOAC Int.* 82:897–902.
14. Ng, W., M. Mankotia, P. Pantazopoulos, R. J. Neil., and P. M. Scott. 2004. Ochratoxin A in wine and grape juice sold in Canada. *Food Addit. Contam.* 21:971–981.
15. Omer, R. E., L. Verhoef, P. Van't Veer, M. O. Idris, A. M. Y. Kadaru, E. Kampman, A. Bunschoten, and F. J. Kok. 2001. Peanut butter intake GSTM1 genotype and hepatocellular carcinoma: a case-control study in Sudan. *Cancer Causes Control* 12:23–32.
16. Rosa, C. A. R., C. E. Magnoli, M. E. Fraga, A. M. Dalcero, and D. M. N. Santana. 2004. Occurrence of ochratoxin A in wine and grape juice marketed in Rio de Janeiro, Brazil *Food Addit. Contam.* 21: 358–364.
17. Siame, B. A., S. F. Mpuchane, B. A. Gashe, J. Allotey, and G. Teffera. 1998. Occurrence of aflatoxins, fumonisin B1, and zearalenone in foods and feeds in Botswana. *J. Food Prot.* 61:1670–1673.
18. Suga, K., N. Mochizuki, and H. Yamashita. 2004. Analysis of fumonisin B1 and B2 in beer and raw materials of beer by liquid chromatography/tandem mass spectrometry. *Shokuhin Eiseigaku Zasshi* 45:255–258.
19. Sydenham, E. W., P. G. Thiel, W. H. O. Marasas, G. S. Shephard, D. J. Van Schalkwyk, and K. R. Koch. 1990. Natural occurrence of some *Fusarium* mycotoxins in corn from low and high esophageal cancer prevalence areas of the Transkei, Southern Africa. *J. Agric. Food Chem.* 38:1900–1903.
20. Tabata, S., H. Kamimura, A. Ide, H. Hashimoto, M. Iida, Y. Tamura, and T. Nishima. 1993. Aflatoxin contamination in foods and foodstuff in Tokyo: 1986–1990. *J. AOAC Int.* 76:32–35.
21. Taguchi, S., S. Fukushima, T. Sumimoto, S. Yoshida, and T. Nishimune. 1995. Aflatoxins in foods collected in Osaka, Japan, from 1988 to 1992. *J. AOAC Int.* 78:325–327.
22. Tangni, E. K., S. Ponchaut, M. Maudoux, R. Rozenberg, and Y. Larondelle. 2002. Ochratoxin A in domestic and imported beer in Belgium: occurrence and exposure assessment. *Food Addit. Contam.* 19:1169–1179.
23. Truckness, N. W. 2000. Natural toxins, p. 6. *In* AOAC official methods of analysis, 17th ed. AOAC International, Gaithersburg, Md.
24. Yoshizawa, T., A. Yamashita, and Y. Luo. 1994. Fumonisin occurrence in corn from high- and low-risk areas for human esophageal cancer in China. *Appl. Environ. Microbiol.* 60:1626–1629.

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RCM

Development of a liquid chromatography/time-of-flight mass spectrometric method for the simultaneous determination of trichothecenes, zearalenone and aflatoxins in foodstuffs

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A liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS) method based on time-of-flight MS (TOFMS) with a real-time reference mass correction technique was developed for the simultaneous determination of *Fusarium* mycotoxins (nivalenol, deoxynivalenol, fusarenon X, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, HT-2 toxin, T-2 toxin, diacetoxyscirpenol, zearalenone) and *Aspergillus* mycotoxins (aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2) in corn, wheat, cornflakes and biscuits. Samples were cleaned up with a MultiSep #226 column. Detection of the mycotoxins was carried out in exact mass chromatograms with a mass window of 0.03 Th. Calibration curves were linear from 2 to 200 ng · mL^{-1} for trichothecenes and zearalenone, and 0.2 to 20 ng · mL^{-1} for aflatoxins, by 20 μL injection. The limits of detection ranged from 0.1 to 6.1 ng · g^{-1} in foodstuffs analyzed in this study. The LC/TOFMS method was found to be suitable for the screening of multiple mycotoxins in foodstuffs rapidly and with high sensitivity, and its performance was demonstrated for the confirmation for target mycotoxins. Copyright © 2006 John Wiley & Sons, Ltd.

Many crops are susceptible to fungal attack not only in the field, but also during storage. These fungi may produce a toxic secondary metabolite, called a mycotoxin. Major mycotoxins found in cereals are aflatoxins (AFs), ochratoxins, trichothecenes (TRs), fumonisins (FMs) and zearalenone (ZEN). These mycotoxins frequently contaminate food commodities simultaneously. Since *Fusarium* species can produce a variable range of two or more toxins, such as TRs, FMs and ZEN, the co-occurrence of several TRs and ZEN is a problem in food safety.[1–4] The co-occurrence of *Fusarium* toxins and *Aspergillus* mycotoxins such as AFs has also been reported in food commodities.[5,6]

Although these mycotoxins cause health problems in humans as well as in animals at a sub-acute or acute dose, the chronic effects at low levels of exposure are of great concern, such as those affecting immunotoxicity and resulting in the decrease of host resistance to infectious diseases and cancer.[7–9]

TRs are produced by *Fusarium graminearum*, *F. culmorum*, *F. sporotrichioides*, *F. equiseti*, and *F. poae* and can be characterized into four types (A–D). Among the four types, type-A and type-B are known to be major *Fusarium* mycotoxins in contaminated foodstuffs and feed.[1,2,4,10,11] The type-A group contains the highly toxic T-2 toxin (T-2), HT-2 toxin (HT-2) and diacetoxyscirpenol (DAS) and type-B contains nivalenol (NIV), deoxynivalenol (DON), fusarenon X (FUS-X), 3-acetyldeoxynivalenol (3ADON) and 15-acetyldeoxynivalenol (15ADON).

ZEN is also produced by *Fusarium* species such as *F. graminearum* and has been known to have estrogenic effects.[12] Having the capacity for binding to an estrogen receptor, ZEN and its metabolites lead to alterations in the reproductive tract and a variety of symptoms, such as decreased fertility, or increased embryolethal resorptions in pig and other animals may be observed. With respect to carcinogenesis, it has been reported that long-term chronic toxicity induced hepatocellular adenomas and pituitary adenomas in mice,[13] although the International Agency for Research in Cancer (IARC) concluded that there was limited evidence in experimental animals.[14]

AFs are produced by mainly *Aspergillus flavus* and *A. parasiticus*, and there are many analogs. Among these analogs, the four aflatoxins, aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2), are commonly detected in both food and feed. These AFs are potent carcinogens and were classified as human liver

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carcinogens by the IARC on the basis of epidemiological evidence in 1993.[14,15]

In order to assess exposure to these mycotoxins, it is essential to develop a rapid and reliable method for the simultaneous determination of co-occurring multiple mycotoxins. However, there have been few reports of methods for the simultaneous determination of more than one family of mycotoxins. Driffield *et al.*[16] have reported a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method using electrospray ionization (ESI) for the detection of NIV, DON, ZEN, ochratoxinA and AFB_1 in pig livers. Berger *et al.*[17] established an LC/ion trap method for the quantitative detection of nine type-A and type-B TRs. Recently, a liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry (LC/APCI-MS/MS) method for the determination of seven TRs and ZEN simultaneously was published.[18] The LC/MS and LC/MS/MS methods have been increasingly applied to multiple mycotoxin analysis.

However, there are some problems in the simultaneous analysis of multiple mycotoxins using LC/MS and LC/MS/MS. When many compounds with different molecular weights are detected simultaneously using a quadrupole instrument, the demand for scanning over a wide mass range will result in decreased sensitivity. Also, when selected ion monitoring (SIM) with LC/MS or multiple reaction monitoring (MRM) modes with LC/MS/MS are used to increase sensitivity, there may be a poorer confirmation of identity because full mass spectra have not been acquired.

Time-of-flight mass spectrometry (TOFMS) has the capability, in contrast to quadrupole or ion trap instruments, of detecting a wide mass range without losing significant sensitivity. Furthermore, TOF instruments provide enhanced full mass range spectra sensitivity and accuracy due to their higher mass resolution.[19] This also makes it possible to distinguish among isobaric ions and increases confidence in the identification of analytes by giving an estimate of the elemental composition of each ion.[20–22] The information obtained from TOF analysis has the additional advantage that quantitation can be performed on any ion observed in the acquired mass range.[23,24] These properties of LC/TOFMS analysis should be of great advantage for the screening of foodstuffs containing multiple mycotoxins with different molecular weights.

In this study we established a rapid and reliable method utilizing LC/TOFMS analysis for the screening of the nine *Fusarium* mycotoxins including ZEN and *Aspergillus* mycotoxins in corn, wheat, cornflakes and biscuits simultaneously. In addition, the value of the higher mass resolution of LC/TOFMS in routine analysis was demonstrated by the confirmation of target mycotoxins by accurate mass measurements.

EXPERIMENTAL

Chemicals

The mycotoxins, NIV, DON, FUS-X, 3ADON, 15ADON, DAS, HT-2, T-2, ZEN, AFB_1, AFB_2, AFG_1 and AFG_2, were purchased from Sigma-Aldrich Japan (Tokyo, Japan). LC/MS-grade methanol and reagent-grade ammonium acetate were purchased from Wako Chemicals (Osaka, Japan). Pure water was purified with a Milli-Q system (Millipore, Tokyo, Japan). Purine and hexakis(1*H*,1*H*,3*H*-tetrafluoropentoxy)-phosphazene mixture solutions as lock mass compounds were obtained from Agilent Technologies (Santa Clara, CA, USA). The MultiSep® cartridge column #226 (Romer Labs, Inc., Union, MO, USA) was purchased from Showa Denko Ltd. (Tokyo, Japan).

Liquid chromatography

An Agilent 1100 series LC system (Agilent Technologies, Waldbronn, Germany), including a vacuum solvent degassing unit, a binary high-pressure gradient pump, an automatic sample injector, a column thermostat, and a photodiode array, was used. LC separation was performed on a 150 × 2.1 mm i.d. column packed with 3.5 μm Zorbax Eclipse XDB C18 (Agilent Technologies, Santa Clara, CA, USA) at 40°C. The LC mobile phase was aqueous 10 mM ammonium acetate (A) and methanol (B). The initial gradient condition was 90% A and 10% B changing linearly to 100% B in 40 min. After analysis, the column was equilibrated for 15 min. The flow rate was 200 $\mu L \cdot min^{-1}$ and the injection volume was 20 μL.

Mass spectrometry

Mass spectrometry was performed using an Agilent 1100 series MSD TOF instrument equipped with an orthogonal spray APCI source (Agilent Technologies, Santa Clara, CA, USA). The nebulizer gas as well as drying gas (350°C) was nitrogen generated from pressurized air by a Balston model 75–72 nitrogen generator (Balston, Haverhill, MA, USA). The capillary voltage for ion transmission, fragmentor voltage for in-source-fragmentation, and vaporizer temperature were all optimized using the analytical column with mycotoxin standard mixtures at 100 $ng \cdot mL^{-1}$. The nebulizer gas, the drying gas, the capillary voltage, the fragmentor voltage and the vaporizer temperature were set at 50 psi, 6 $L \cdot min^{-1}$, 4000 V, 100 V and 350°C, respectively. The skimer 1 and entrance lens voltages in the ion source of the MSD TOF were automatically optimized by a calibration standard using the calibrant delivery system at 0.1 $mL \cdot min^{-1}$, and were set to 27 and 62 V, respectively. The MSD TOF was operated in the positive ion mode. Profiling mode mass spectra were acquired over the scan range *m/z* 100–1000 with a spectral acquisition rate of 0.89 s per spectrum and the mass resolution was from 3800 FWHM at *m/z* 121 to 10000 FWHM at *m/z* 922 in the lock mass solution. The calibration of the spectral range was performed using an APCI calibrant solution (Agilent Technologies, Santa Clara, CA, USA) and a fifth-order non-linear calibration curve was usually adopted. To perform the real-time lock mass correction, a lock mass solution including purine ($C_5H_4N_4$ at *m/z* 121.050873, 10 $\mu mol \cdot L^{-1}$) and hexakis(1*H*,1*H*,3*H*-tetrafluoropentoxy)-phosphazene ($C_{18}H_{18}O_6N_3P_3F_{24}$ at *m/z* 922.009798, 2 $\mu mol \cdot L^{-1}$) was infused using a syringe pump (Harvard Apparatus Inc., Holliston, MA, USA) at a flow rate of 3 $\mu L \cdot min^{-1}$. Quantitative analysis was performed using the exact mass chromatograms of the base peak ions with a 0.03 Th window. As the mass chromatograms were extracted from data that had been corrected by lock mass solution, the

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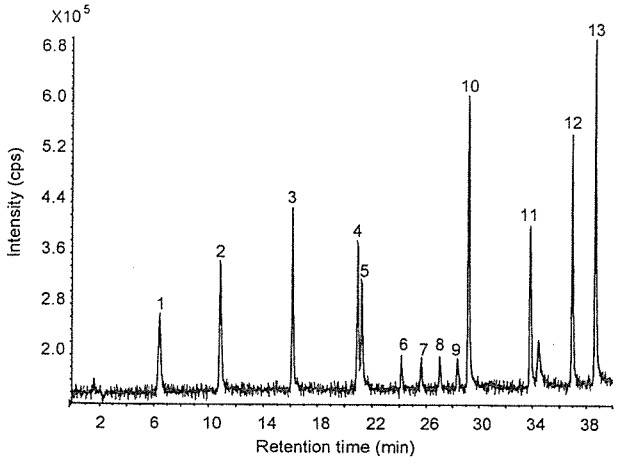

Figure 1. Total ion chromatogram obtained by LC/TOFMS of mycotoxin standard mixture solution containing 1 $\mu g \cdot mL^{-1}$ of TRs and ZEN, and 0.1 $\mu g \cdot mL^{-1}$ of AFs. 1, NIV; 2, DON; 3, FUS-X; 4, 3ADON; 5, 15ADON; 6, AFG_2; 7, AFG_1; 8, AFB_2; 9, AFB_1; 10, DAS; 11, HT-2; 12, T-2; 13, ZEN.

fluctuations in *m/z* value (usually below ±0.01 Th) during the long-term period did not exceed this mass width.

Sample preparation

The four AF mixture standard solution at 1 $\mu g \cdot mL^{-1}$ and eight TRs and ZEN mixture standard solution at 10 $\mu g \cdot mL^{-1}$ for stock and fortification experiments were dissolved in acetonitrile. Then they were stored at 4°C in the dark until use. For preparation of a mixed working standard solution, an appropriate amount of individual stock standard was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 1 mL of mobile phase.

The samples (corn, wheat, cornflasks, biscuits) were obtained from a local retail store in Japan. For fortification experiments, 10 μL of each stock standard solution were spiked into 10 g of blank samples (=1 $ng \cdot g^{-1}$ for AFs, and 10 $ng \cdot g^{-1}$ for TRs and ZEN) before extraction. Three replicates of one level were prepared.

The extraction and cleanup for mycotoxins from samples were carried out as follows. A total of 10 g of each sample was weighed in a 50 mL centrifuge tube. After adding 40 mL acetonitrile/water (85:15), the tube was shaken for 30 min on an automatic shaker. 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. The first 3 mL of eluate was discarded, but the next 2 mL were collected and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 0.5 mL mobile phase.

RESULTS AND DISCUSSION

The development of the LC/TOFMS method

The separation of all mycotoxins (eight TRs, four AFs and ZEN) was compared by using several reversed-phase columns. Figure 1 shows the total ion chromatogram of the mycotoxins obtained with the Zorbax Eclipse XDB C18 (150 × 2.1 mm, 3.5 μm) column, demonstrating that this column and the condition used gave a good separation of all the mycotoxins. Thus, this column was used throughout this study.

Ionization of the mycotoxins was examined with ESI and APCI in both positive and negative ion modes. The concentration of each mycotoxin was 1 $\mu g \cdot mL^{-1}$ for the nine *Fusarium* mycotoxins and 0.1 $\mu g \cdot mL^{-1}$ for the four *Aspergillus* mycotoxins. It was found that APCI in positive ion mode provided the optimum response for all the *Fusarium* mycotoxins, and ESI in positive ion mode provided slightly better signal intensity for the four AF compounds than APCI. Thus, APCI in positive ion mode was employed to analyze the 13 mycotoxins studied simultaneously. The combination of ammonium acetate and methanol was employed as the mobile phase, because this gave a better response in APCI for all the mycotoxins.

Mass measurement accuracy of mycotoxins

Achieving an accurate mass calibration was carried out by using a solution of several compounds whose masses are

Table 1. Calculated accurate masses and mass errors of 13 mycotoxins

Mycotoxin	Formula	Observed base peak ion	Calculated accurate mass	RMS mass error (ppm)[a]	
				100 $ng \cdot mL^{-1}$	10 $ng \cdot mL^{-1}$
NIV	$C_{15}H_{20}O_7$	$[M+NH_4-H_2O]^+$	312.14416	1.33	0.25
DON	$C_{15}H_{20}O_6$	$[M+H]^+$	297.13326	2.12	1.32
FUS-X	$C_{17}H_{22}O_8$	$[M+H]^+$	355.13874	0.71	−1.81
3ADON	$C_{17}H_{22}O_7$	$[M+H]^+$	339.14382	1.09	−0.38
15ADON	$C_{17}H_{22}O_7$	$[M+NH_4]^+$	356.17037	−1.91	−2.18
AFG_2	$C_{17}H_{14}O_7$	$[M+H]^+$	331.08122	−1.29	−2.32
AFG_1	$C_{17}H_{12}O_7$	$[M+H]^+$	329.06557	1.58	0.97
AFB_2	$C_{17}H_{14}O_6$	$[M+H]^+$	315.08631	−0.04	−0.69
AFB_1	$C_{17}H_{12}O_6$	$[M+H]^+$	313.07066	−1.16	0.44
DAS	$C_{19}H_{26}O_7$	$[M+NH_4]^+$	384.20167	−0.72	−0.98
HT-2	$C_{22}H_{32}O_8$	$[M+NH_4]^+$	442.24354	0.12	−2.12
T-2	$C_{24}H_{34}O_9$	$[M+NH_4]$	484.25410	−0.43	−2.49
ZEN	$C_{18}H_{22}O_5$	$[M+H]^+$	319.15400	0.62	0.01

[a] RMS mass error was calculated on the basis of five replicates.

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known with great accuracy to produce accurate mass measurements. However, even the most minute changes in energy applied to the ions can cause a noticeable mass shift. Thus, real-time lock mass correction was used to cancel out these factors. With this technique, two compounds of known mass are introduced into the ion source at the same time as the samples and the instrument software constantly corrects the measured mass of the target unknown compounds using the known masses as references.

In this study, a lock mass acetonitrile solution including $10\,\mu mol \cdot L^{-1}$ purine and $2\,\mu mol \cdot L^{-1}$ hexakis(1*H*,1*H*,3*H*-tetrafluoropentoxy)phosphazene was infused using a syringe pump at a flow rate of $3\,\mu L \cdot min^{-1}$.

Accuracy was evaluated on the measured mass in terms of RMS mass error. For this purpose, standard solutions of mycotoxins at 10 and $100\,ng \cdot mL^{-1}$ were analyzed five times. Table 1 shows the formulae, the theoretical accurate mass of the base peak ions and the RMS mass error of each mycotoxin.

As shown in Table 1, the accurate mass measured by LC/TOFMS of NIV was attributed to the $[M + NH_4–H_2O]^+$ ion with a trueness of 1.33 ppm at $100\,ng \cdot mL^{-1}$. The use of high-resolution TOFMS allowed identification of this ion whereas this would not be possible with a low-resolution mass spectrometer because the difference in the calculated masses of $[M + NH_4–H_2O]^+$ and $[M]^+$ is only 0.02 Th. The

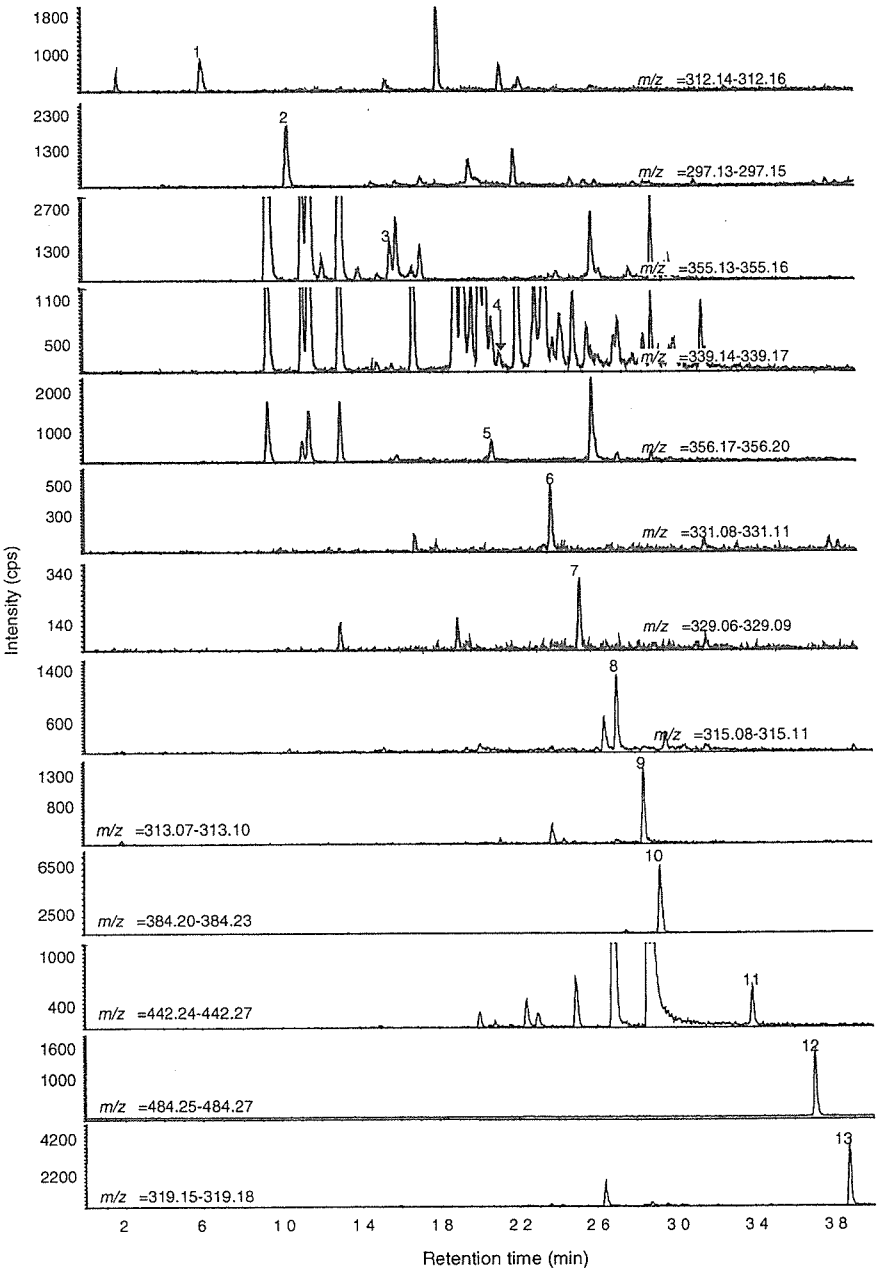

Figure 2. Exact mass chromatograms obtained by LC/TOFMS of mycotoxin in corn extract containing $10\,ng \cdot g^{-1}$ of TRs and ZEN, and $1\,ng \cdot mL^{-1}$ of AFs. 1, NIV; 2, DON; 3, FUS-X; 4, 3ADON; 5, 15ADON; 6, AFG_2; 7, AFG_1; 8, AFB_2; 9, AFB_1; 10, DAS; 11, HT-2; 12, T-2; 13, ZEN.

Rapid Commun. Mass Spectrom. 2006; 20: 1422–1428
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Table 2. RMS mass errors of 13 mycotoxins in foodstuffs

	RMS mass error (ppm)[a]			
Mycotoxin	Wheat	Biscuits	Corn	Cornflakes
NIV	2.32	1.03	2.34	2.49
DON	1.46	3.32	−1.50	−2.92
FUS-X	1.00	−3.20	−0.68	0.15
3ADON	2.27	−2.44	−1.56	3.21
15ADON	−0.22	−1.06	−2.46	−1.34
AFG_2	−3.71	−3.10	−0.39	4.32
AFG_1	3.21	0.97	−1.15	0.36
AFB_2	0.58	−0.36	−0.68	−3.51
AFB_1	−3.21	−2.12	−0.52	−2.43
DAS	−0.21	−0.98	−0.46	−1.76
HT-2	−3.20	−2.81	−3.26	−4.39
T-2	−2.49	−1.46	−1.87	−0.63
ZEN	−0.01	0.01	−0.31	1.23

[a] RMS mass error was calculated on the basis of three replicates.

RMS mass errors of all mycotoxins in this study were in the range of −2.49 to 2.12 ppm. These accurate masses greatly increased the confidence in the correct assignment of the compounds of interest.

Quantification of mycotoxins

LC/TOFMS has been applied for confirmatory analysis mainly because of the well-known limitations, such as narrow dynamic linear range, obtained with this type of mass spectrometer. However, improvements in this instrumentation that have involved new digital sampling techniques have overcome this drawback. Furthermore, the increased resolving power can increase selectivity by separating isobaric interferences from target ions of interest. In this work, an exact mass chromatogram for each mycotoxin was created with a 0.03 Th mass window to obtain maximum selectivity of LC/TOFMS for 13 mycotoxins.

Figure 2 shows the exact mass chromatograms of the mycotoxins in the corn extract spiked with 10 $ng \cdot g^{-1}$ of TRs and ZEN, and 1 $ng \cdot g^{-1}$ of AFs, because the matrix compounds in corn were the most complex among the foodstuffs used in this study.

Table 3. Linearity of LC/TOFMS analysis for 13 mycotoxin standards

	Calibraion equation	
Mycotoxin	$y = ax$*	r^2*
NIV	$y = 693x$	1.0000
DON	$y = 1890x$	0.9999
FUS-X	$y = 1630x$	0.9999
3ADON	$y = 2870x$	0.9996
15ADON	$y = 769x$	0.9997
AFG_2	$y = 4180x$	0.9991
AFG_1	$y = 2930x$	0.9995
AFB_2	$y = 15230x$	0.9999
AFB_1	$y = 9040x$	0.9997
DAS	$y = 14320x$	0.9998
HT-2	$y = 1008x$	0.9998
T-2	$y = 4530x$	0.9998
ZEN	$y = 14320x$	0.9999

* r^2 is the correlation coefficient, x is the injected concentration in $ng \cdot mL^{-1}$ and y is the peak signal intensity.

To determine which column is suitable for cleanup in the multiple mycotoxin analysis, MultiSep #227 and MultiSep #226 columns were compared. Using MultiSep #227 the TRs were purified, but AFs and ZEN were not (data not shown). On the other hand, using MultiSep #226 there were some peaks in the chromatograms of corn samples at the exact mass chromatograms of FUS-X and 3ADON, but they did not affect the quantitative analysis of these mycotoxins. Our results are in agreement with those of Berthiller *et al.*, who selected MycoSep #226 for the purification of TRs and ZEN from maize.[18]

The selectivity of each mycotoxin was assessed in each foodstuff. Because interference from matrix compounds might affect the mass accuracy and signal intensity of the analyte, this matrix effect was evaluated for each mycotoxin in all the matrices used in this study (corn, wheat, cornflakes and biscuits) by comparing the exact mass chromatogram and accurate mass of base peak ions obtained from standard solutions in pure water and in the matrix extract. As shown in Table 2, the mass error of each mycotoxin in all the foodstuffs used in this study was within acceptable limits for present purposes (within ±5 ppm), and also reduction and enhancement of the TOFMS responses were not observed for the foodstuffs. These results suggest that there is an absence of matrix effect in terms of signal intensity and mass accuracy in our experimental conditions.

Calibration curves were created by using a mycotoxin standard solution since there is no matrix effect on signal intensity. To test the linearity of the calibration curves, various concentrations of each mycotoxin ranging from 2 to 200 $ng \cdot mL^{-1}$ for TRs and ZEN, and 0.2 to 20 $ng \cdot mL^{-1}$ for AFs, were used. As shown in Table 3, calibration curves for all the mycotoxins were linear over the working range. Squared correlation coefficients (r^2) were higher than 0.999 for the seven point calibration curves.

Method validation

To evaluate recoveries, the proposed method was applied to the analysis of spiked samples of mycotoxin-free foodstuffs. Each foodstuff was spiked at a final concentration of 10 $ng \cdot g^{-1}$ for TRs and ZEN, and 1 $ng \cdot g^{-1}$ for AFs. Mean recovery of each mycotoxin in each foodstuff ranged from 71 to 133%, as shown in Table 4. At the same concentration, within-laboratory repeatability was performed (n = 5) indicating that relative standard deviations (RSDs) ranged from 2.1 to 7.1% (Table 4).

The limits of detection (LODs) of each mycotoxin in foodstuffs were determined by the signal corresponding to three times the background noise on each mass chromatogram. The LODs of TRs, AFs and ZEN were in the range of 0.5–6.1, 0.1–0.3 and 0.2–0.8 $ng \cdot g^{-1}$, respectively (Table 5).

Application of the LC/TOFMS method to foodstuff samples

The LC/TOFMS method was applied in the quantitation and confirmation of these mycotoxins in the foodstuffs under investigation. As shown in Fig. 3, DON was identified in

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Table 4. Recovery of 13 mycotoxins in foodstuffs

	Recovery[a] ± RSD (%)[b]			
Mycotoxin	Wheat	Biscuits	Corn	Cornflakes
NIV	76 ± 4.3	82 ± 4.1	81 ± 3.9	88 ± 6.2
DON	132 ± 3.9	110 ± 2.9	111 ± 4.4	91 ± 5.1
FUS-X	118 ± 5.2	121 ± 6.5	96 ± 3.7	116 ± 5.8
3ADON	117 ± 4.9	128 ± 7.1	115 ± 4.6	128 ± 5.2
15ADON	122 ± 3.9	113 ± 4.3	105 ± 2.9	129 ± 4.5
AFG_2	73 ± 2.1	122 ± 3.7	132 ± 3.5	80 ± 3.9
AFG_1	99 ± 3.2	84 ± 2.7	91 ± 2.7	94 ± 2.1
AFB_2	83 ± 4.9	114 ± 4.1	98 ± 3.7	89 ± 4.2
AFB_1	84 ± 2.6	111 ± 4.4	105 ± 3.5	112 ± 3.6
DAS	111 ± 2.9	126 ± 3.1	133 ± 3.6	108 ± 3.9
HT-2	128 ± 3.8	115 ± 2.9	108 ± 2.3	91 ± 2.7
T-2	108 ± 2.7	115 ± 3.1	115 ± 3.6	105 ± 3.3
ZEN	71 ± 4.1	89 ± 3.7	109 ± 4.7	81 ± 4.5

[a] Recovery was calculated on the basis of three replicates.
[b] RSD was calculated on the basis of five replicates at 10 ng · g^{-1} for TRs and ZEN, and 1 ng · g^{-1} for AFs within 1 day.

Table 5. Limits of detection (LOD) for standard solution and spiked foodstuffs

	LOD (ng · g^{-1})				
Mycotoxin	Standard	Wheat	Biscuits	Corn	Cornflakes
NIV	2.3	3.8	5.9	2.4	6.1
DON	0.6	1.0	1.4	0.5	1.3
FUS-X	0.5	1.4	0.9	2.9	3.3
3ADON	1.2	3.6	1.2	4.9	4.1
15ADON	1.5	1.7	1.4	2.1	2.7
AFG_2	0.1	0.1	0.1	0.1	0.2
AFG_1	0.1	0.1	0.1	0.1	0.2
AFB_2	0.1	0.1	0.1	0.1	0.2
AFB_1	0.1	0.1	0.1	0.1	0.3
DAS	0.1	0.3	0.3	0.3	0.3
HT-2	0.5	0.6	0.5	0.9	0.6
T-2	0.2	0.2	0.2	0.2	0.2
ZEN	0.2	0.7	0.6	0.8	0.7

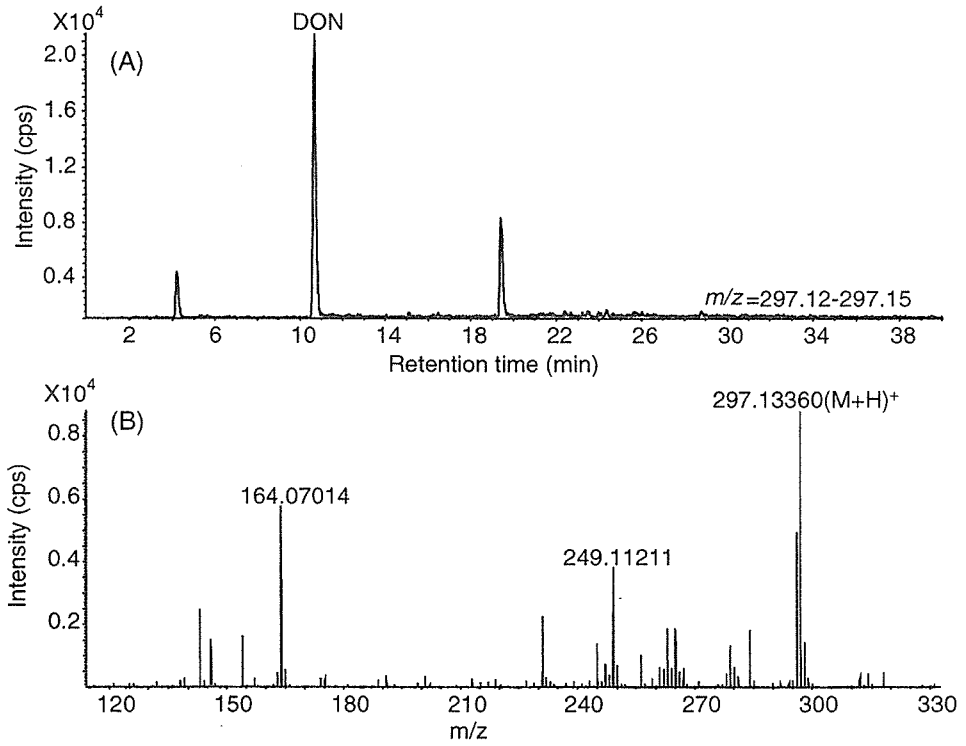

Figure 3. Exact mass chromatogram (A) and full mass spectrum (B) of wheat sample contaminated with 112 ng · g^{-1} of DON.

wheat samples. The calculated amount was 112 ng · g^{-1}, and the mass error of the protonated molecule (*m/z* 297.13360) was only 1.12 ppm. This accurate mass enabled the unequivocal confirmation of DON in any foodstuff.

CONCLUSIONS

The LC/TOFMS method developed in this study was demonstrated to be a powerful rapid screening method for 13 mycotoxins in complex foodstuff matrices with higher resolution. It was found that the condition using high-resolution TOFMS with a 0.03 Th window is suitable to obtain highly selective and simultaneous detection of multiple mycotoxins in real food samples in a rapid and reliable way. Further research is in progress to clarify which foods and feeds are suitable for the determination of these mycotoxins by LC/TOFMS, and also to validate the application of the method in routine analysis.

REFERENCES

1. Krska R, Baumgartner S, Josephs R. *Fresenius. J. Anal. Chem.* 2001; **371**: 285.
2. D'Mello JPF, Placinta CM, Macdonald AMC. *Anim. Feed Sci. Technol.* 1999; **80**: 183.

Rapid Commun. Mass Spectrom. 2006; **20**: 1422–1428
DOI: 10.1002/rcm

3. Müller H-M, Reimann J, Schumacher U, Schwadorf K. *Food Add. Contam.* 1998; **15**: 801.
4. Tanaka T, Hasegawa A, Yamamoto S, Lee U, Sugiura Y, Ueno Y. *J. Agric. Food Chem.* 1988; **36**: 979.
5. Lovelace CE, Nyathi CB. *J. Sci. Food Agri.* 1977; **28**: 288.
6. Wang DS, Liang YX, Nguyen TC, Le DD, Tanaka T, Ueno Y. *Nat. Toxins* 1995; **3**: 445.
7. Joint FAO/WHO Expert Committee on Food Additives 47th Session Ed., 'Safety evaluation of certain mycotoxins in food', Geneva, 2001; 419–556 (ISBN 92-4-166047).
8. Sugita-Konishi Y, Hara-Kudo Y, Kasuga F, Kumagai S. *Mycotoxins* 1998; **47**: 19.
9. Hsia CC, Wu ZY, Li YS, Zhang F, Sun ZT. *Oncol. Rep.* 2004; **12**: 449.
10. Biselli S, Hummert C. *Food Add. Contam.* 2005; **22**: 752.
11. Schollenberger M, Müller H-M, Rüfle M, Suchy S, Planck S, Drochner W. *Int. J. Food Microbiol.* 2005; **97**: 317.
12. Joint FAO/WHO Expert Committee on Food Additives 53th Session Ed., 'Safety evaluation of certain food additives and contaminants', Geneva, 2000; 393–481.
13. National Toxicity Program (1982), NTP carcinogenesis bioassay of zearalenone in F344/N rats and F6C3F1 mice (Technical Report Series No. 235), Department of Health and Human Resources: Research Triangle Park, NC.
14. *Some Naturally Occurring Substances: Food Items and Constituents. Heterocyclic Amines and Mycotoxins*, IARC monographs on the evaluation of carcinogenic risk of chemicals in humans, IARC: Lyon, 1993.
15. Castegnaro M, Wild CP. *Nat. Toxins* 1995; **3**: 327.
16. Driffield M, Hird SJ, MacDonald SJ. *Asp. Appl. Biol.* 2003; **68**: 205.
17. Berger U, Oehme M, Kuhn F. *J. Agric. Food Chem.* 1999; **47**: 4240.
18. Berthiller F, Schuhmacher R, Buttinger G, Krska R. *J. Chromatogr. A* 2005; **1062**: 209.
19. Morris HR, Paxton T, Dell A, Langhorne J, Berg M, Bordoli RS, Hoyes J, Bateman RH. *Rapid Commun. Mass Spectrom.* 1996; **10**: 889.
20. Wu J, McAllister H. *J. Mass Spectrom.* 2003; **38**: 1043.
21. Calbiani F, Careri M, Elviri L, Mangia A, Zagnoni I. *J Chromatogr. A* 2004; **1058**: 127.
22. Tolonen A, Uusitalo J. *Rapid Commun. Mass Spectrom.* 2004; **18**: 3113.
23. Clauwaert KM, Van Bocxlaer JF, Major HJ, Claereboudt JA, Lambert WE, Van den Eeckhout EM, Van Peteghem CH, De Leenheer AP. *Rapid Commun. Mass Spectrom.* 1999; **13**: 1540.
24. Hall WL, Jeanes YM, Pugh J, Lodge JK. *Rapid Commun. Mass Spectrom.* 2003; **17**: 2797.

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