Table 3. Occurrence of AF contamination of pistatio nuts imported into Japan from 2000 to 2003

year	number of samples	number of positive samples	detection ratio (%)	0.2-10 μg/kg	> 10 μg/kg	contravention ratio (%)
2000	877	68	7.75	42	26	2.96
2001	786	40	5.09	28	12	1.53
2002	310	5	1.61	3	2	0.65
2003	369	8	2.17	8	0	0.00

low incidences and low levels of AFB_1 have been found. Therefore, the current risk for liver cancer caused by AFB_1 seems to be very low. However, it is important to establish appropriate regulations and to enforce a monitoring program. At present, many countries regulate AF contamination in food as total AF. In Japan, the time for reconsideration of the regulation as total AF has come.

I would like to thank Mycotoxin Research Association, Nippon Kaiji Kentei Kyoukai and Japan Frozen Food Association for providing information on AF contamination in nuts imported into Japan.

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アフラトキシンの発ガンメカニズムとわが国におけるアフラトキシンの汚染事例

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アフラトキシンは，天然物中で最も発ガン性の強い化合物であるが，そのメカニズムについてはアフラトキシン B_1 を用いて分子細胞学レベルまで研究されている．本報告では 2002 年に発刊した IARC のモノグラフに基づいてアフラトキシン B_1 の発ガンメカニズムを中心に紹介した．また，わが国では現在アフラトキシン B_1 に関しては規制を設けているが，この規制を超える違反例の状況を検疫所のモニタリングシステムの結果から紹介するとともに，最も違反例の多いナッツ類に焦点をあて，その輸入時検査での検出率からわが国のアフラトキシン汚染の現状について考察を行った．その結果，現在の状況ではナッツ類におけるアフラトキシンの検出頻度および濃度も低いことから，アフラトキシン汚染による肝臓がん発症リスクは極めて低水準であることが明らかとなったが，この水準を保つにはより一層のモニタリングの強化と適切な基準値設定が必要であると思われた．

キーワード：アフラトキシン，発ガン性，メカニズム，汚染事例，ピーナッツ，アーモンド，ピスタチオ

Toxicity of mycotoxins related with head blight diseases in wheat and establishment of provisional standard for tolerable level of DON in wheat

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Key words : mycotoxins, head blight deseases, deoxynivalenol, provisional standard

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Introduction

In Japan there are some pathogens responsible for causing fusarium head blight o wheat. These pathogens belong to *Fusarium* species (*Fusarium graminearum F. avenaceum, F. culmorum, F. crookwellense, F. sporotrichioides, F. poae, F. tricinctum, F. acuminatum, F. equiseti, F. semitectum, F. kyushuense, Microdochium nivale (formaerly F.nivale)*). The principal toxins produced by these Fusarium species are trichothecenes, such as deoxynivalenol (DON), 3-acetyledeoxynivarenol (3-ADON), 15-acetyledeoxynivalenol (15-ADON), Diacetoxyscirpenol (DAS), nivalenol (NIV), fusarenon X (FX), T-2 toxin and Zearalenone (ZEN).

Among these mycotoxins, DON, T-2 toxin and HT-2 toxin were evaluated at the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2001. As for DON, the provisional maximum tolerable daily intake (PMTDI) has been established a level of 1 μg/kg of body weight per day, based on the results of a 2-year feeding study in mice. With the determination of PMTDI for DON, the Ministry of Health, Labor and Welfare of Japan has started the study and surveillance on the risk assessment of DON in cereals to set the standard from 2001 and they has recommended a level of 1.1 mg/kg of DON in wheat as the provisional standard in 2002.

This paper aims to introduce the toxicities of mycotoxins related with fusarium head blight and the ground of setting the provisional standard.

Toxicities of trichothecene mycotoxins and zearalenone

Trichothecene mycotoxins, especially T-2, HT-2, DON and NIV possess common biochemical and cellular toxicities. These are 1) the strong inhibitory effect on the protein synthesis by binding to the ribosomes. 2) the inhibitory effect on RNA and DNA synthesis. 3) toxic effects on cell membranes (it seems to be anti-oxidation reaction). Also, their capacity to inhibit protein synthesis is thought to induce apoptosis in thymus, lymphatic and haematopoietic tissue via mitogen activated

protein kinases (MAP kinases)[1,2]. The different trichothecenes differ the inhibitory activity.

For T-2 toxin, HT-2, DON and NIV, general toxicity and immunotoxicity of trichothecenes are considered to be critical effects. The crops contaminated with trichothecens resulted in serious food poinsining with nausea, vomiting and diarrhea. After the World war II, the critical food poisoning occurred by milletin Russia. This disease caused the decrease of circulating white blood cell number. It is called alimentary toxic aleukia (ATA) and a major causal toxin was thought to be T-2 toxin produced by *Fusarium sporotrichioides and F. poae.* The immunotoxicity, which is chronic effect of trichothecenes mycotoxins results in the decrease of host resistance[3]. Selective upregulates serum IgA caused by dietary expose to DON or NIV induces the IgA nephropathy[4]. The effect as the cancer promoter is seems to be responsible for the immunotoxicity[5].

Zearalenone, which is produced by *F. graminaerum and F. culmorum*, interacts with oestrogen receptors. This toxin induces apparent hyperoestrogenism. Even though all of mammalian species are target of zearalenone, female pigs are considered to be most sensitive animal species. Other typical toxicities of zearalenone are not recognizes, but the oral exposure to high dose zearalenone has been reported to cause hepatocellular adeomas in B6C3F1 mice[6] and apoptosis in vitro[7,8].

International regulation of trichothecenes and zearalenone

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and European Commission (EC) had evaluated the risk of trichothecens and zearalenone and established provisional maximum tolerable daily intake (PMTDI) of these toxins (Table 1). JECFA has set PMTDI for deoxynivalenol (DON), T-2 roxin, HT-2 toxin and zearalenone. EC has established a full TDI to DON and temporary TDIs to nivalenol, zearalenon and T-2 toxin and HT-2 toxin.

Setting provisional standard for tolerable level of DON contamination in wheat

Prior to setting PMTDI by JECFA, some investigators in Japan have pointed out a possibility that DON intake over the PMTDI in Japan if no regulation is set for DON contamination levels in domestic wheat in Japan because high levels of DON were found in Japanese domestic wheat frequently. Therefore the Ministry of Health, Labour and Welfare (MHLW) organized the reseach group to survey current situation of DON contamination levels in domestic wheat in 2001-2002. As

Table 1. PMTDI of Trichothecenes Mycotoxins and Zearalenone Established by JECFA or EC

mycotoxin	PMTDI (μg/person kg/day)	
	JECFA	EC
DON	1	1
NIV	-	0.7*
T-2 and HT-2	0.06	0.06*
ZEA	0.5	0.2*

* temporary TDI

Table 2. Tolerable level of DON contamination in wheat and rice satisfying PMTDI established by JECFA (Based on the data obtained in 2002)

Estimated DON concentration	Reduction by processing	DON level in flour (μg/kg)	DON level in unpolished wheat (μg/kg)
only wheat	30 %	557	795
	50 %	557	1110
DON level in rice at 15/39 of that in wheat	30 %	331	473
	50 %	331	662
DON concentration in rice at the same as that of wheat	30 %	201	287
	50 %	201	402

the regulation for DON was urgently needed to prevent adverse health, the Joint Committee of Food Standard and Toxicity Committee under the Food Sanitation Council recommended setting a provisional standard for the tolerable level of DON in wheat. Based on the surveillance data in 2001-2002 and daily average intake of wheat, the research group proposed some simulations about the tolerable level of DON in unpolished wheat (Table 2). On May 21, 2002, the Food Sanitation Council has set the tolerable level of DON in unpolished wheat at 1.1 mg/kg as a provisional standard when the reduction of DON concentration in final products is estimated about 50 %.

The further study on surveillance of DON levels in wheat and rice in 2002-2003 and reduction of DON concentration in processing and cooking

After the setting of provisional standard for unpolished wheat, MHLW organized further study on surveillance of the current situation and reduction of DON concentration in processing and cooking to asses the exposure level of DON in detail. Further surveillance results of DON levels in unpolished wheat in 2002-2003 revealed that the averages of DON concentration in imported wheat (n=178) and domestic wheat (n=199) were 60 ng/g and 160 ng/g, respectively. Taking into consideration of the weighted average of supply flow, the average of DON contamination was calculated as 71 ng/kg. In domestic rice (n=124), the average was 2.6 ng/g. As the DON level in rice was too low, the contribution to the amount of exposure to DON might be neglected.

The reduction study using the natural contaminant wheat showed that milling process reduced DON level by 55.4 %. The cooking study using the natural contaminant flour found that DON level reduced by 71.1 % in cooked noodle but did not in bread. These studies concluded that the retention level of DON from wheat to final product could be conservatively assessed at 44.2 % in bread and at 12.9 % in noodle.

Based on the results of the study performed in 2002-2003, we recalculated level of DON contamination in wheat and flour. When the reduction of DON in final product was took into consideration, the concentration of DON in unpolished was allowed until 1,913 μg/kg for all age and 858 μg/kg for 1-6 years old (Table 3). The research group assessed that the mean intake of DON in Japan were 1.94 μg/day/person for all age and 1.31 μg/day/person for 1-6 years old children. The intakes were ranked 3.7 % of PMTDI for all age and 8.3 % of PMTDI for 1-6 years old children (Table 4).

Table 3. Tolerable level of DON contamination in wheat and flour satisfying PMTDI (Based on the research on 2002- 2003)

The contribution DON contamination in rice	Age (weight) (kg)	Intake of wheat (g)	Intake of rice (g)	not taking into consideration of reduction in noodle and bread (μg/kg)		taking into of consideration of reduction innoodle and bread (μg/kg)	
				in unpolished	in flour	in unpolished	in flour
No	All age (52.6)	94.3		1,251	558	1,913	853
	1-6 years (15.9)	64.1		556	248	850	380
Yes	All age (52.6)	94.3	160.4	1,247	556	1,908	851
	1-6 years (15.9)	64.1	86.0	554	247	846	378

DON level in unpolished wheat (μg/kg)=PMTDI/intake of wheat x reduction in flour x {(the rate for which noodle consumption x the retention in noodle) + (the rate for which bread consumption x the retention in bread) + (the rate for which others product consumption x the retention in others)}
Retention in flour; 44.6 %, The rate for which noodle consumption; 46.5 %, The retention in noodle; 28.9 %, The rate for which bread consumption; 45.6%, The retention in bread; 97.1 %, the rate for which others product consumption; 7.6 %, the retention in others; 100 %

Table 4. The percentage to PMTDI of the intake amount (Based on the research on 2002-2003)

Age	the intake of wheat (g)	body weight (kg)	the intake of DON (μg/day/person)	the percentage to PMTDI (%)
All age	94.3	52.6	1.94	3.7
1-6 years old	64.3	15.9	1.31	8.3

The average of domestic wheat; 0.16 mg/kg, The amout of product; 540,000 ton
The average of imported wheat; 0.06 mg/kg, The amount of product; 4,560,000 ton
The weighted average; 0.071 mg/kg

As the exposure level of DON was very low, MHWL decided to continue the regulation of unpolished wheat regarding DON contamination by using the provisional standard. In order to establish the standard of DON, more data on DON contamination are needed.

Further approach

Recently, many cases of co-contamination occurrence with trichothecenes have been reported in wheat, barley and other cereals[9-14]. Regarding the establishment of DON standard, the effects of combined exposure to several trichothecenes and ZEA should be taken into account in risk assessment.

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赤カビ病に関わるマイコトキシンの毒性と小麦における DON 汚染レベルの暫定基準について

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赤カビ病に起因する主なカビ毒は，デオキシニバレノール，ニバレノール，T-2 トキシンなどのトリコテセン系マイコトキシンとゼアラレノンである．トリコテセン系マイコトトキシンの共通の毒性には，消化管障害，免疫毒性などがあり，ガンへのプロモーター作用を示唆する報告もある．我が国の赤カビ病において最も頻繁に検出されるカビ毒は，デオキシニバレノール，ニバレノールであるが，デオキシニバレノールに関しては 2002 年に暫定基準値が設定された．その根拠を 2 つの厚生科学特別研究（平成 13 年度および平成 14 年度）の結果をもとに示した．

キーワード：マイコトキシン，赤カビ病，デオキシニバレノール，暫定基準値

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The comparison of two clean-up procedures, multifunctional column and immunoaffinity column, for HPLC determination of ochratoxin A in cereals, raisins and green coffee beans

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Abstract

To evaluate a clean-up method of detecting ochratoxin A (OTA) by HPLC, the performances of two different clean-up columns, an immunoaffinity column and a multifuntional column were compared in an inter-laboratory study. As samples, un-contaminated wheat, corn grits, green coffee beans and naturally contaminated raisins were used. The recovery test was performed at two different concentrations of OTA (0.5 and 5.0 μg/kg) except for naturally contaminated raisins. Using the immunoaffinity column, the recovery rates, and relative standard deviations for repeatability ($R.S.D._r$) and reproducibility ($R.S.D._R$) for wheat, corn grits and green coffee beans ranged 59.0–85.8, 4.2–7.8 and 22.9–29.2%, respectively. For naturally contaminated raisins, recovery, $R.S.D._r$ and $R.S.D._R$ were 84.1, 1.8 and 5.1%, respectively. Using the multifunctional column, the recovery rates, $R.S.D._r$ and $R.S.D._R$ for wheat, corn grits and green coffee beans ranged 80.8–185.0, 0.7–6.9 and 15.2–33.9%, respectively. For naturally contaminated raisins, the recovery, $R.S.D._r$ and $R.S.D._R$ were 128.7, 1.1 and 3.7%, respectively. The results suggest that a multifunctional column could be used to detect OTA in wheat and corn grits at a concentration as low as 0.5 μg/kg; however, it was difficult to detect OTA in green coffee beans and raisins at such a low level. Although an immunoaffinity column could be used for all the test samples in this study from a low level to a high level, the recovery rates were lower than with a multifunctional column.

Keywords: Ochratoxin A; Immunoaffinity column; Multifunctional column; HPLC; Clean-up

1. Introduction

Ochratoxin A (OTA) produced by *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Penicillium verrucosum* is a chlorinated isocoumarin compound. Among mycotoxins contaminated in food, drink and feed, OTA is one of the most widespread and hazardous mycotoxins. Risk assessments carried out by Joint FAO/WHO Expert Committee on Food Additives and Commission of the European Union [1,2] have shown that OTA has nephrotoxicity, teratogenic toxicity, immunotoxicity, genotoxicity and carcinogenicity. OTA has also been identified in human breast milk and blood, and has a long half-life in mammalian tissues [3–5]. Ueno et al. [5] reported detecting OTA in blood of Japanese. Therefore, exposure to OTA is common and serious problem in food safety.

Dietary sources of OTA has been found, in many commodities, such as coffee [6,7], beer [8,9], dried fruit [10,11], wine [12,13], cereals [14,15], cocoa [16,17], spices [18,19] and nuts [20].

Many countries have established maximal limits for OTA in food for enforcement. It is essential to develop and validate

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analytical methods for the detection of OTA in individual foodstuff reliably, speedy and simply.

There are numerous methods for the determination of OTA in food-stuffs, such as thin layer chromatography, enzyme-linked immunosorbent assay, high performance liquid chromatography and liquid chromatography–mass spectroscopy. As regards clean-up methods, the demand for immunoaffinity column and multifunctional column has increased for mycotoxin analysis in recent years [21]. Although there are many collaborative studies using immunoaffinity column [22–25], there is little information about multifunctional clean-up column for OTA in individual material [26].

In this paper, the performances of two different clean-up procedures, using an immunoaffinity and a multifunctional column, respectively, were compared in an inter-laboratory study involving six laboratories. Food materials used in this study were wheat, corn grits, raisins and green coffee beans. Except naturally contaminated raisins, all materials were spiked with a low (0.5 μg/kg) or high (5.0 μg/kg) concentration of OTA. The analytical methods were assessed based on parameters of recovery and the precision.

2. Experimental

2.1. Standard and reagents

The stock standard solution was prepared from crystalline OTA (Wako Pure chemical Industries Ltd., Osaka, Japan) at a concentration of ca. 40 μg/mL. The concentration was checked by the method recommended by AOAC International [27], then a working standard solution (2 μg/mL) was prepared in toluene–acetic acid (99 + 1).

The spiking solutions of OTA (1.25 and 0.125 μg/mL) for the recovery test were prepared by dilution with acetonitrile. Water, acetonitrile and methanol of HPLC grade and the other analytical grade reagents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Multisep #229 was purchased from Romer Labs Inc. (Stylemaster Drive Union, MO, USA). The immunoaffinity column (OchraTest WB) was purchased from VICAM (Watertown, MA, USA). Phosphate-buffered saline (PBS) tablets and Tween 20 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Preparation of samples

Naturally contaminated raisins which was homogenized type, used in the Food Analytical Performance Assessment Scheme (FAPAS) Program carried out in 2003, were purchased from Central Science laboratories (York, UK). The assigned concentration of the sample was 13.2 μg/kg according to the FAPAS report. Blank green coffee beans, wheat and corn grits were obtained from commercial sources and previously shown to contain <0.2 μg/kg of OTA using the AOAC official method [25] and the method of Nakajima et al. [28]. All samples except raisins were ground and passed though a mesh of 1 mm. After mixing and homogenizing, they were packed into a bag at 30 g each.

2.3. Fortification procedure

For evaluating recovery, 100 μL of each spiking solution was added to 25 g of "blank" material in a 200-mL flask (final concentration, 0.5 and 5 μg/kg) and kept at room temperature. After 1 h, the spiked sample was extracted using each clean-up method.

2.4. Clean-up with the immunoaffinity column

Twenty-five grams of sample was weighed in a 200-mL glass-stopped Erlenmeyer flask, and extracted with 100 mL of a methanol/aqueous 1% $NaHCO_3$ solution (70:30, v/v). The suspension was shook vigorously for 30 min on a wrist action shaker and passed through a Whatman No. 4 paper filter. Then 8 mL of the filtrate was diluted to 100 mL in PBS containing 0.01% Tween 20 (PBS-Tween) and passed through a Whatman 934 AH glass filter. Fifty milliliters of the diluted extract, equivalent to 1.0 g of sample, was applied to an OchraTest column under gravity. The column was washed with 3 mL of PBS-Tween followed by water, and OTA was eluted with 3 mL of methanol into a silanized vial, at a flow rate of one drop/s. Then the eluate was evaporated to dryness under nitrogen gas at 40 °C.

2.5. Clean-up with multifunctional column

Twenty-five grams of sample was weighed accurately in a 200-mL flask and extracted with 100 mL of acetonitrile–water (84 + 16) by shaking for 30 min on a wrist action shaker. The extract was passed through a Whatman 934AH glass filter, 10 mL of the filtrate was transferred into a new test tube, and 100 μL of acetic acid (analytical grade, 99.7%) was added. After mixing well, the acetic extract was applied to a #229 column. The first 5 mL of eluate was collected and 4.0 mL, equivalent to 1.0 g of sample, was evaporated to dryness under nitrogen gas at 40 °C.

2.6. Conditions for HPLC

The residues obtained with both clean-up procedures were dissolved in 1.0 mL of acetonitrile:water:acetic acid (30:70:1), and the purified sample solution was subjected to an HPLC analysis. When the final sample solution was cloudy, the solution was filtered through a 0.45 um pore size PTFE syringe filter before analysis by HPLC. The conditions for HPLC are listed in Table 1. The analytical ODS column (100–250 mm × 4.6 mm i.d.) was kept at 45 °C and a mobile phase of acetonitorile–water–acetic acid (55:43:2 v/v) was delivered at a rate of 1.0 mL/min.

Table 1
HPLC conditions for detection of OTA

Column: ODS 250 mm × 4.6 mm, i.d. (3–5 μm)
Column temperature: 45 °C
Flow rate: 1.0 mL/min
Wavelength: Ex 333 nm, Em 460 nm
Injected volumn: 100 μL
Mobile phase: CH_3CN–H_2O–CH_3COOH (55:43:2, v/v)

Detection was performed with a fluorescence detector (excitation wavelength 333 nm and emission wavelength 460 nm). A seven-point calibration curve (0.2–20.0 ng/mL) covering the range of interest for the test sample was established. The calibration curve was to be linear.

2.7. Inter-laboratory study

To evaluate and compare the two clean-up procedures, an inter-laboratory study was carried out using 11 materials (naturally contaminated raisins, two spiked samples of unpolished wheat, two spiked samples of green coffee beans, two spiked samples of corn grits and a blank of each material) in six laboratories within Japan.

2.8. Statistics

Statistical evaluation was performed using the results from the six laboratories (except the results for green coffee beans obtained using the multifunctional column) and parameters of precision, that is, the inter-laboratory relative standard deviation for repeatability ($R.S.D._r$) and for reproducibility ($R.S.D._R$), were deduced as recommended by the AOAC [29].

3. Results and discussion

3.1. Analytical results using the immunoaffinity clean-up column

When the sample was cleaned-up with the immunoaffinity procedure, a clear base line for the chromatogram was obtained from the blanks of all samples used in this study (Fig. 1(a, d and g)).

In all samples spiked with 0.5 and 5 μg/kg of OTA, the peak of OTA was sharp and isolated from other peaks. The limit of determination (LOD) for all materials used in this study (wheat, corn, green coffee beans and raisins) was estimated at 0.1 μg/kg, which was calculated as an *S/N* ratio of 3/1.

As shown in Table 2, the mean recovery of OTA from wheat spiked at a level of 0.5 and 5 μg/kg was 79.6 and 81.5%, from corn spiked at a level of 0.5 and 5 μg/kg was 79.0 and 85.8%, and from green coffee beans spiked at a level of 0.5 and 5 μg/kg was 64.6 and 59.0%, respectively. In naturally contaminated raisins, the mean concentration of OTA was 11.1 μg/kg, and the recovery was 84.1%, which was calculated based on the mean average reported by FAPAS. Except for green coffee beans, the recovery was over 78%. In cereals, the relative standard deviation ($R.S.D._r$) obtained within the six laboratories ranged from 4.2 to 6.8% and the relative standard deviation obtained between laboratories ($R.S.D._R$) ranged from 22.9 to 24.9%. In green coffee beans, the $R.S.D._r$ and $R.S.D._R$ were 5.9, 7.8, 24.6 and 29.2%, respectively. In naturally contaminated raisins, the $R.S.D._r$ and $R.S.D._R$ were 1.8 and 5.1%, respectively. Regarding to IUPAC/AOAC/ISO/CEN standards, for an analytical method of detecting mycotoxins to be recognized as an official standard for the purpose of enforcement, the recovery and the $R.S.D._r$ and $R.S.D._R$ values are required to be in the range of 70–110, <20 and <30%, respectively [30]. The results with the immunoaffinity clean-up procedure satisfied these criteria except for green coffee beans. In green coffee beans, although the recovery was below to 70% at both concentrations (0.5 and 5.0 μg/kg), the $R.S.D._r$ and $R.S.D._R$ values were less than 20 and 30%,

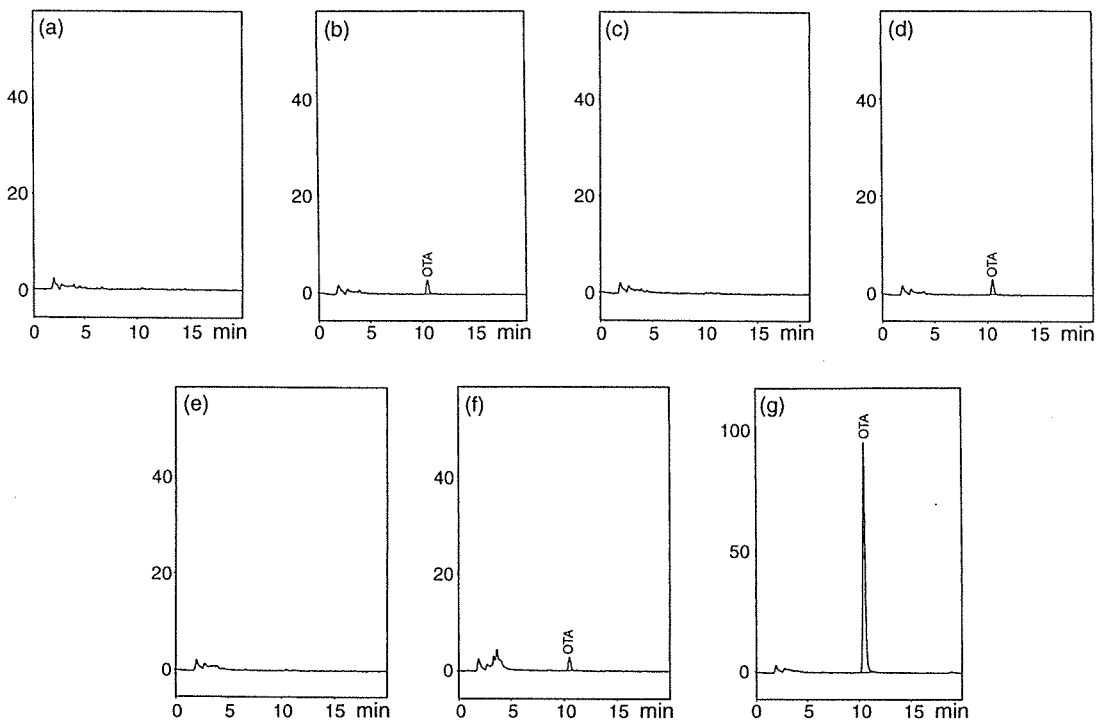

Fig. 1. Chromatograms of OTA detected in food samples using an immunoaffinity clean-up column: (a) blank wheat, (b) wheat spiked with 0.5 μg/kg of OTA, (c) blank corn, (d) corn spiked with 0.5 μg/kg of OTA, (e) blank green coffee beans, (f) green coffee beans spiked with 0.5 μg/kg of OTA, (g) naturally contaminated raisins. For chromatographic conditions see text.

Table 2
Results of an interlaboratory study using an immunoaffinity clean-up column for detection of OTA

Number of laboratory	Spiked wheat				Spiked corn				Spiked green coffee beans				Naturally contaminated raisins	
	0.5 μg/kg		5.0 μg/kg		0.5 μg/kg		5.0 μg/kg		0.5 μg/kg		5.0 μg/kg			
1	78.0	82.0	88.2	88.8	86.0	80.0	95.2	83.2	78.0	84.0	68.8	72.0	13.76	13.73
2	72.0	70.0	78.8	73.0	88.0	78.0	81.6	84.0	58.0	64.0	65.2	56.0	9.78	9.88
3	77.0	74.0	76.3	68.0	71.2	70.2	73.8	75.2	51.2	60.4	49.2	50.6	8.11	8.59
4	86.0	78.0	85.8	90.6	60.0	60.0	88.0	72.0	40.0	46.0	41.6	47.8	11.88	12.11
5	80.0	72.0	71.6	70.2	86.0	80.0	87.4	88.6	58.0	68.0	51.4	54.0	9.50	9.21
6	92.0	94.0	95.0	91.6	96.0	92.0	99.2	101.4	84.0	84.0	75.0	76.0	13.55	13.71
Mean value (μg/kg)													11.1	
Mean recovery (%)	79.6		81.5		79.0		85.8		64.6		59.0		84.1	
Precision (%)														
S_r	3.7		3.4		4.0		5.9		4.9		3.5		0.2	
R.S.D.$_r$	4.8		4.2		5.0		6.8		7.8		5.9		1.8	
S_R	18.2		19.8		19.7		21.3		18.9		14.5		0.6	
R.S.D.$_R$	22.9		24.4		24.9		24.8		29.2		24.6		5.1	

respectively. Recently, Scudamore MacDonald [24] reported a collaborative study of HPLC using immunoaffinity column. They compared two brand immunoaffinity column and obtained that there was no significant difference between them on the recovery, R.S.D.$_R$ and R.S.D.$_r$ in wheat sample spiked 3.7 μg/kg of OTA. The collaborative study using barley, the rate of recovery was 93 ± 10% at a concentration of 5 μg/kg of OTA [23]. From an inter-laboratory study for raisins, the recovery, R.S.D.$_R$ and R.S.D.$_r$ were reported to be 72.2, 4.9 and 14%, respectively [31]. In this study, the recoveries, R.S.D.$_R$ and R.S.D.$_r$ were the same level as these obtained from other collaborative studies in cereals (wheat and corn) and raisins. Vargas and Santos [25] reported the collaborative study using green coffee beans, the recovery rates from samples spiked with 4.48 μg/kg of OTA was 92.8%. The reason why the recovery of OTA in green coffee in this study was lower than that in their study is considered that the caffeine in coffee affects the immuno-reaction because the dilution rate of extract applied to immunoaffinity column used in this study was half of that used in their study.

3.2. Analytical results using a multifunctional clean-up column

The clean-up procedure with the multifunctional column did not give a plane baseline in the chromatogram of any materials used in this study. The presence of numerous peaks disrupted

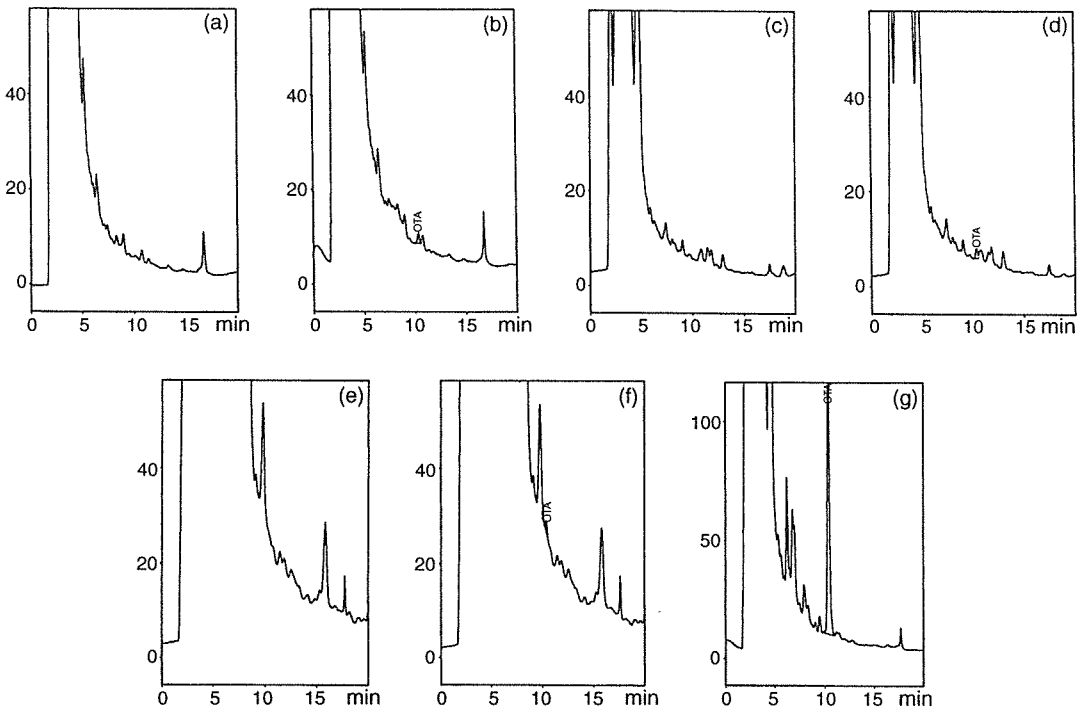

Fig. 2. Chromatograms of OTA detected in food samples using a multifunctional clean-up column: (a) blank wheat, (b) wheat spiked with 0.5 μg/kg of OTA, (c) blank corn, (d) corn spiked with 0.5 μg/kg of OTA, (e) blank green coffee beans, (f) green coffee beans spiked with 0.5 μg/kg of OTA, (g) naturally contaminated raisins. For chromatographic conditions see text.

Table 3
Results of an interlaboratory study using a multifunctional clean-up column for detection of OTA

Number of laboratory	Spiked wheat				Spiked corn				Spiked green coffee beans				Naturally contaminated raisins	
	0.5 μg/kg		5.0 μg/kg		0.5 μg/kg		5.0 μg/kg		0.5 μg/kg		5.0 μg/kg			
1	84.0	84.0	92.4	88.0	86.0	84.0	81.6	82.6	186.0	164.0	101.0	101.2	17.75	17.68
2	96.0	96.0	90.4	91.8	80.0	74.0	82.4	82.8	346.0	348.0	112.0	113.2	16.24	16.66
3	79.8	83.2	90.1	91.6	89.6	88.2	78.5	78.2	129.8	158.2	92.7	99.6	17.23	17.40
4	94.0	98.0	92.2	90.6	66.0	66.0	79.2	79.0	N.I.	N.I.	92.2	89.4	16.31	16.21
5	108.0	106.0	104.0	102.4	96.0	91.0	91.2	93.0	N.I.	N.I.	82.0	92.4	N.I.	N.I.
6	70.0	68.0	92.0	90.8	76.2	72.0	81.8	81.8	74.0	74.0	95.2	91.8	17.14	17.34
Mean value (μg/kg)													17.0	
Mean recovery (%)	88.9		93.0		80.8		82.6		185.0		97.0		128.7	
Precision (%)														
S_r	1.7		1.6		2.7		0.6		12.7		3.8		0.2	
R.S.D.$_r$	1.9		1.7		3.3		0.7		6.9		3.9		1.1	
S_R	13.5		22.4		19.8		18.1		53.2		22.8		0.6	
R.S.D.$_R$	15.2		24.1		24.5		21.9		33.9		23.3		3.7	

N.I.: the peak correspoding to OTA was not identified.

identification of the peak corresponding to OTA (Fig. 2). However, the mean recoveries of all spiked samples except for the green coffee beans spiked with 0.5 μg/kg of OTA were more than 80.0%. In naturally contaminated raisins, one laboratory could not identify OTA because of the many peaks. The mean concentration of OTA in five laboratories was 17.0 μg/kg, which gave a rate of recovery of 128.7% based on the value assigned by the FAPAS report (Table 3).

The recovery, R.S.D.$_r$ and R.S.D.$_R$ values of wheat were 88.9–93.0, 1.7–1.9 and 15.2–24.1%, respectively. For corn, the recovery was 80.8%, the R.S.D.$_r$ was 3.3% and the R.S.D.$_R$ was 24.5% in the sample spiked with 0.5 μg/kg, and the recovery was 82.6%, the R.S.D.$_r$ was 0.7% and R.S.D.$_R$ was 21.9% in the sample spiked with 5.0 μg/kg of OTA. For green coffee beans, in the sample spiked with the low concentration, OTA could not be identified in two laboratories owing to the large number of peaks and the recovery was 185.0%. However, in the sample spiked with high concentration of OTA, all participants recognized OTA in the chromatogram and the recovery, R.S.D.$_r$ and R.S.D.$_R$ were 97.0, 3.9 and 23.3%. The recovery, R.S.D.$_r$ and R.S.D.$_R$ values for naturally contaminated raisins in five laboratories were 125.9, 1.1 and 3.7%, respectively. Based on *S/N* value of 3/1, the limit of determination was estimated at 1.5–2.0 μg/kg for wheat and corn. Since the clean-up using a multifunctional column is a new procedure, there is little information about the application to commodities. Buttinger et al. [26] who developed this column, reported that this procedure could be used to clean-up cereals, red wines, raisins and green coffee. Our results, indicate that it is difficult to clean-up green coffee and raisins using a multifunctional column at a level of 0.5 μg/kg.

4. Conclusions

More than 10 countries have proposed or enacted regulations for OTA. There is much variation in the level of tolerance among countries. The EU has set the tolerable level in raw cereals at 5 μg/kg, while the USA has proposed a level for cereals of 20 μg/kg. Generally, surveillance requires a more sensitive method of detection than enforcement does.

We compared the performances of an immunoaffinity column and a multifunctional column. The immunoaffinity column is complicated but could be used to clean-up cereals, green coffee and raisins at a low concentration of OTA. Although the use of a multifunctional column is simple and rapid, it could not be applied to use for green coffee and raisins at a low concentration of OTA. These results suggest that one has to choose a suitable clean-up procedure depending on the purpose and material.

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Degradation of mycotoxins using microwave-induced argon plasma at atmospheric pressure

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Abstract

Mycotoxins are toxic secondary metabolites of fungi causing health problems in humans, animals and agricultural products. Therefore, the inactivation or degradation of mycotoxins in contaminated foods and feedstuffs is a major global concern. This study was designed to investigate the degradation of three different mycotoxins, aflatoxin B1 (AFB1), deoxynivalenol (DON) and nivalenol (NIV) by using our self-designed microwave-induced argon plasma system at atmospheric pressure. After plasma treatment, the remnants of mycotoxins were analyzed by thin-layer chromatography and high performance liquid chromatography and their cytotoxicity was assessed using mouse macrophage RAW264.7 cells. The mycotoxins, AFB1, DON, and NIV were completely removed after 5 s of plasma treatment. Moreover, the cytotoxicity of mycotoxins was significantly reduced with the progress in the treatment time. These results suggest that this plasma system may have strong potentials to degrade mycotoxins and can be effectively used in the process of foods and feedstuffs.

PACS: 52.50 Sw; 52.70-m
Keywords: Mycotoxin; Aflatoxin B1; Deoxynivalenol; Nivalenol; Microwave-induced argon plasma

1. Introduction

Mycotoxins, such as aflatoxins, zearalenone, deoxynivalenol (DON, vomitoxin) and fumonisins, are secondary metabolites of fungi. They exhibit properties of acute, sub-acute or chronic toxicities in animals and/or human, also being carcinogenic, capable of causing mutations in susceptible organisms and teratogenic, capable of causing deformities in developing embryos. Moreover, they cause loss of viability of the seeds and reduce the quality and acceptability of all type of products, limit the storability and decrease the nutritional quality of the foods. Therefore, they are world-wide serious problem for the public health, agriculture and economics [1–4].

Although the prevention of mycotoxin contamination in the field is the main goal of agricultural and food industries, the contaminations of various commodities by fungal species including *Fusarium*, *Aspergillus*, *Alternaria* and *Penicillium* and mycotoxins are unavoidable under certain environmental conditions [2]. Therefore, decontamination/detoxification procedures are essential in order to recuperate mycotoxin-contaminated commodities. Several strategies are available for the detoxification of mycotoxins. These can be classified as physical, chemical and biological approaches [5]. The physical and chemical methods made different degrees of success. Microbes

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or their enzymes could be applied to mycotoxin detoxification; such biological approaches are now widely studied [6,7]. However, all of these methods have their own disadvantages as they undergo undesirable changes during the process for the detoxification of mycotoxins in the food and feedstuffs [6,7]. These situations led to the development of new techniques that are at least as effective as the established ones, and in some part have superior characteristics, such as short processing times, non-toxicity to its operator and medium preservation.

Recently, much attention has been paid to plasma treatment among the methods of removing or inactivating the dangerous materials. It has been used as a well-established technique in a number of processes, e.g., plasma cleaning, etching, coating and sterilizing [8–11]. This paper describes the efficiency of our self-designed microwave-induced argon plasma system at atmospheric pressure for the degradation of mycotoxins, such as afltoxin B1 (AFB1), DON and nivalenol (NIV), the most common to contaminate foods and feedstuffs.

2. Experimental details

2.1. Materials

All reagents, including three mycotoxins, AFB1, DON (3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one, vomitoxin), NIV (3α,4β,7α,15-tetrahydroxy-12,13-epoxytrichothec-9-en-8-one), were purchased from Sigma (St. Louis, MO). Silicagel plate (60 F254, 20 cm×20 cm) for thin-layer chromatography (TLC) was purchased from Merck and Company (Whitehouse Station, NJ). For cytotoxicity test, cell counting kit-8 containing a highly water soluble tetrazolium salt [WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], reduced to a yellow color formazan dye by mitochondrial dehydrogenase of viable cells, was purchased from Dojindo Laboratories (Kumamoto, Japan).

2.2. Microwave-induced argon plasma system

As previously described [9–11], a microwave-induced argon plasma system to generate plasma at atmospheric pressure was used in this study. Briefly, this system consists of a 2.45 GHz, waveguide-based, 1 kW magnetron power supply commonly used in a microwave oven, an applicator including a tuning section, which is required to reduce the reflected power, and the nozzle section made of quartz. The plasma generated at the end of a nozzle was formed by an interaction between the high electrical field, which is generated by the microwave power, the waveguide aperture and the gas nozzle. Argon was used as a working gas for this plasma system, which was chosen because of its inertness, and the gas flow rate was approximately 100 l/min at 8 kgf/cm^2. The intensity of UV light generated by plasma was measured indirectly by using a radiometer/photometer with a solar blind vacuum photodiode. The range was detected from 75 mW/cm^2 (minimum) to 102 mW/cm^2 (maximum) at a wavelength of 254 nm, which was close to that of our previous studies [9–11].

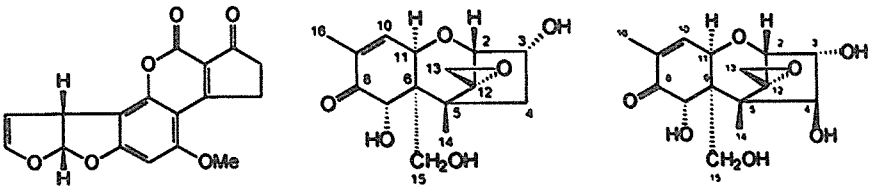
2.3. Mycotoxin degradation by microwave-induced argon plasma

The molecular structures of AFB1, DON, and NIV were shown in Fig. 1 [12,13]. For the degradation test, these mycotoxins were suspended in chloroform. The suspensions were inoculated onto slide glasses and allowed to dry at room temperature for 30 s. The inoculated slide glasses were placed in front of a nozzle and exposed to plasma for 1, 3, 5 and 10 s. After plasma treatment, the slide glasses were transferred into a screw cap glass vial containing 1 ml of chloroform, and thoroughly shaken for 60 s. The extracts were evaporated to dryness and dissolved in two different solutions, 1 ml of acetonitrile for determining the amount of mycotoxins and 1 ml of cell growth media for evaluating the cytotoxic effect of mycotoxins.

2.4. Mycotoxin detection by thin-layer chromatography and high performance liquid chromatography

TLC and high performance liquid chromatography (HPLC) were performed to determine the amounts of mycotoxins following plasma treatment. The extracts from the plasma-treated samples were spotted along with standards on TLC silicagel plates, developed in chloroform:acetone:2-propanol (93:5:2) as mobile phase for AFB1 or in chloroform:methanol (94:6) as mobile phase for DON and NIV. Afterwards, the plates were allowed to air-dry. For AFB1, the developed plate was heated for 10 min at 100–110 °C and then determined by blue fluorescence ultra-violet (UV) light (365 nm). For DON and NIV, the plate was heavily sprayed with 10% aluminum chloride in ethanol, heated for 10 min at 105 °C and then observed under UV light.

Confirmation and quantitative determination of the mytoxins were performed by HPLC according to the previously described



Aflatoxin B1 Deoxynivalenol Nivalenol

Fig. 1. Molecular structures of aflatoxin B1, deoxynivalenol and nivalenol.

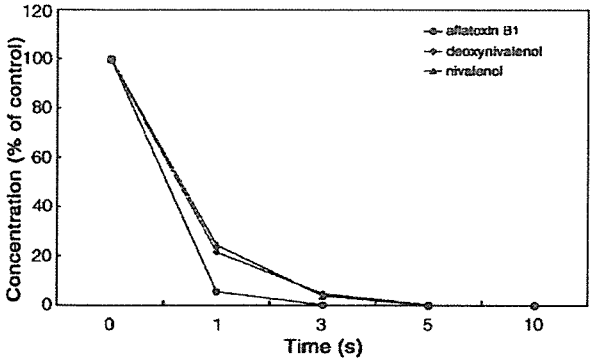


Fig. 2. Degradation effects of microwave-induced argon plasma at atmospheric pressure against aflatoxin B1, deoxynivalenol and nivalenol as assayed by TLC.

method [14]. The HPLC were performed by using a liquid chromatographer (Waters Co., Milford, MA) with a separation module (2695) and a photodiode array detector (2996) to provide confirmation of mycotoxin identity through the comparison of UV spectra with those of standards (Waters Co.). MassLynx, version V3.5 software (Waters Co.) was used to control the chromatograph and to process signal and data. The

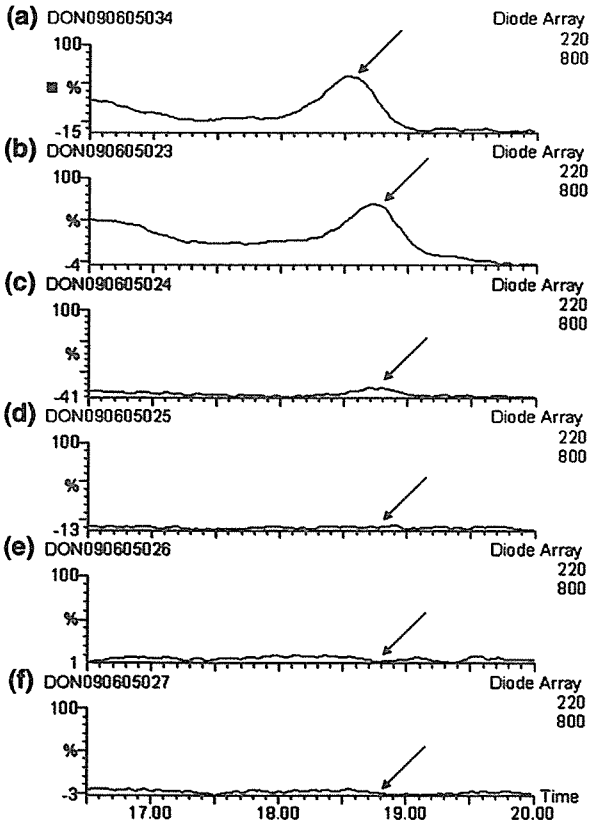


Fig. 3. HPLC chromatograms of deoxynivalenol (DON): standard (a) and DON treated with microwave-induced argon plasma at atmospheric pressure for 0 (b), 1 (c), 3 (d), 5 (e) and 10 (f) s. The arrow represents the UV spectrum of DON.

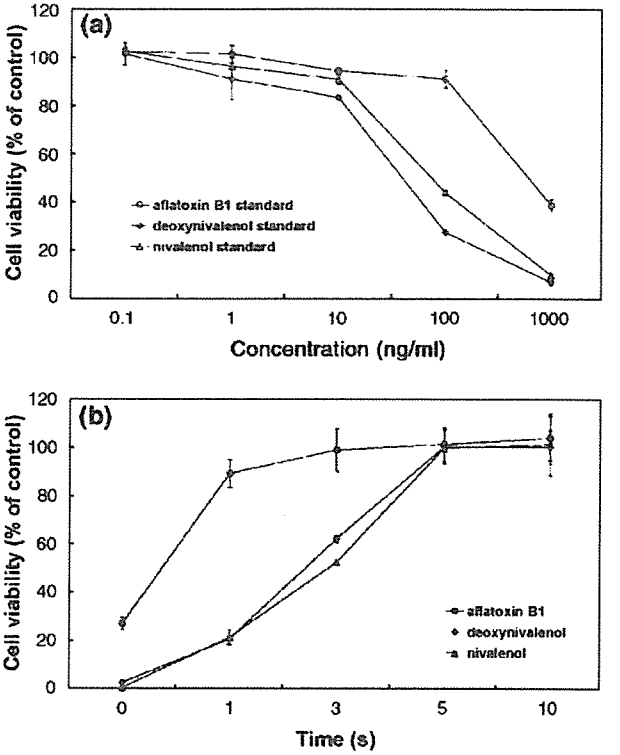


Fig. 4. Cytotoxicity of mycotoxins, such as aflatoxin B1, deoxynivalenol and nivalenol, to cultured mouse macrophage RAW264.7 cells. (A) mycotoxin standards and (B) plasma-treated mycotoxins.

mobile phases were water:acetonitrile:methanol (55:30:15) for AFB1 and water:methanol:acetonitrile (90:5:5) for DON and NIV. The aliquot (10 μl) of the standard and sample solutions prepared was injected into the HPLC. The flow of mobile phases was 1.0 ml/min. AFB1 was detected and quantified by fluorescence detection at an excitation at 365 nm and emission at 430 nm.

2.5. Cytotoxicity of mycotoxins

In order to evaluate the cytotoxicity of mycotoxins following plasma treatment, mouse macrophage cell line, RAW264.7 (TIB-71, American Type Culture Collection, Rockville, MD) was routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and a 1% antibiotic antimycotic solution (including 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were seeded in 96-well plate at 1.0×10^5 cells/well and then incubated for 24 h. After incubation, the extracts of mycotoxins in culture media were added and incubated for further 24 h. According to manufacturer's instruction, the cells were incubated with WST-8 in the last 4 h of the culture period tested at 37 °C in the dark. The absorbance was determined at 450 nm in an ELISA reader (Spectra Max 340, Molecular Device Co., Sunnyvale, CA). Parallel sets of plates received standard mycotoxins as the control.

3. Results and discussion

The most commonly encountered mycotoxins in foods and feedstuffs are aflatoxins, vomitoxin and zearalenone. They can be produced on a wide range of agricultural commodities and under a diverse range of situations. Due to their various toxic effects and their good thermal stability, the presence of mycotoxins in foods and feeds is potentially hazardous to the health of both humans and animals. They have been proven as cause of, or implicated in, mycotoxicoses of either animals or humans.

Various techniques have been devised to remove, destroy or suppress the toxicity of the mycotoxins. These techniques include physical removal of the contaminated portions of the foodstuffs [15], treatment with heat [16–19], chemicals [15,20–23] or radiation [24] in order to convert the toxins into relatively innocuous compounds or the addition of adjuvants [25–27] to suppress or otherwise mask the ill effects of toxins [28]. Although some workers have demonstrated the successful removal of toxins by these methods, these techniques are not always effective, since the mycotoxins can diffuse throughout the materials and are not associated exclusively with damaged, discolored or malformed seeds or materials. In addition, the technologies for removal can be labor- or equipment-intensive and, thus may not always be economically feasible [15–28].

In this study, the degradation effects of microwave-induced argon plasma at atmospheric pressure against mycotoxins, AFB1, DON and NIV, were investigated. TLC analysis revealed that plasma treatment resulted in a significant time-dependent decrease in the concentrations of AFB1 (R_F 0.79), DON (R_F 0.63) and NIV (R_F 0.48) (Fig. 2). The fluorescent spot of each mycotoxin showed that these mycotoxins were completely degraded within 5 s regardless of their types. These results were confirmed by HPLC analysis. Although DON and NIV were degraded relatively slowly compared to AFB1, plasma treatment resulted in a complete degradation of all the mycotoxins. The HPLC chromatogram of DON demonstrated that the UV spectrum of non-treated DON (Fig. 3b) had a peak of mountain shape like that of a standard (Fig. 3a), while the peaks of plasma-treated DON were rapidly decreased with the progress in the treatment time (Fig. 3c, d), and they were not detected after 5 s of treatment (Fig. 3e, f). The HPLC result of AFB1 or NIV showed the pattern similar to that of DON (data not shown).

The microwave-induced plasma system used in this study required much less exposure time for mycotoxin degradation than other methods, such as visible or UV light and gamma ray [15–24]. The UV irradiation and etching by plasma may be responsible for degrading and removing the mycotoxins [3,24]. This plasma system has many advantages, such as increased ionization by reactive species and relatively high intensity of UV light (75–102 mW/cm^2), low average temperature (75–130 °C) and easy operation.

As shown in Fig. 4(a), the standards of mycotoxins, AFB1, DON and NIV resulted in a significant dose-dependent decrease in the viability of RAW264.7 macrophages. These results imply that these mycotoxins are highly cytotoxic to mammalian cells even at micromolar concentrations. In contrast, plasma treatment appreciably reduced the viability loss of macrophages in a time-dependent manner, indicating that the mycotoxin-induced cytotoxicity was completely decreased after 5 s (Fig. 4b). These results suggest that the reduction of cytotoxicity may be involved in the degradation or removal of mycotoxins by plasma treatment. Although further study would not be performed on foods and feedstuffs contaminated with mycotoxins, the microwave-induced plasma system used in this study might be effectively used in the degradation or removal of mycotoxins.

4. Conclusion

This study confirmed the effects of our self-designed microwave-induced argon plasma system at atmospheric pressure on the degradation or removal of mycotoxins, such as AFB, DON and NIV. From the results, it would seem that the plasma system cannot only degrade mycotoxins from contaminated foods and feedstuffs, but also sterilize microorganisms as a source of mycotoxins. Although the physicochemical properties and structural changes of plasma-treated mycotoxins were not defined, this study represents a first step in showing that our plasma system can be applied to specific situations where the removal or inactivation of mycotoxins is required.

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Note

Effect of Cooking Process on the Deoxynivalenol Content and Its Subsequent Cytotoxicity in Wheat Products

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The retention of deoxynivalenol in noodles and bread made from naturally-contaminated flour was examined by a chemical analysis (HPLC) and bioassays. The retention level of deoxynivalenol obtained from both assays was reduced by boiling process, although only the bioassays showed it to have been reduced by baking. This study is the first to estimate the exposure to deoxynivalenol from the consumption of the final products of wheat flour in Japan.

Key words: bread; cytotoxicity; deoxynivalenol; high-performance liquid chromatography; noodle

Deoxynivalenol (DON), a kind of trichothecene mycotoxin, is a frequent contaminant of cereal crops worldwide.[1)] DON and other trichothecene mycotoxins affect animal and human health. The effects include feed refusal, a decrease in body weight, immunomodulation such as the enhancement of IgA production and reduction of host resistance against infection.[2,3)] Thus, an effective treatment for eliminating DON from food products is essential to minimize exposing humans to the toxin. In terms of the treatment methods, heating is known to have little effect in reducing the DON content due to its thermal stability, as has been observed in baked bread and cookies.[4–6)] In contrast, boiling could be expected to be an effective means of reducing the DON level because the toxin is water-soluble.

However, it is doubtful that this reduction in DON level would result in equally reduced harmful effects to health because food processing methods such as heating and boiling can themselves produce new toxic compounds. It has recently been reported that new DON and fumonisin compounds were found in food products.[7,8)] These compounds were mycotoxin-food matrix complexes generated by chemical and biological reactions during food processing. Howard *et al.*[9)] have found new compounds generated from fumonisins in the final corn product and examined their toxicity toward experimental animals. Yumbe-Guevara *et al.*[8)] have found a heat-induced derivative of DON in roasted barley. This derivative showed strong cross-reactivity to an antibody against acetylated DONs, but could not be detected by GC–MS.

As these reports indicate, cooking processes have the possibility of generating new compounds which are structurally different to the native compounds, but whose toxicity is unknown. However, such common analytical methods as HPLC, GC–MS, TLC and ELISA could not detect these compounds. To evaluate the potential health damage from food products containing mycotoxins, it is necessary to conduct assays by chemical and biological methods.

In this study, we measured the residual DON level in food products by chemical and biological methods. We chose noodles and bread as food products made from wheat because these are the major wheat products consumed in Japan.

The flour used for cooking the noodles and bread in this study was milled from naturally contaminated domestic wheat harvested in 2001, in which the DON concentration was 0.71 mg/kg for bread and 0.86 mg/kg

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; GC–MS, gas chromatography and mass spectroscopy; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography

for noodles. DON-free flour for blanks was a commercial product (8.5%, protein content, Nissin Food Products Co., Osaka, Japan) that was a mixture of Australian-standard wheat and Japanese domestic wheat. This flour contained less than 0.1 mg/kg of DON, the detection limit for an HPLC analysis.

Bread was prepared by mixing the following ingredients consisting of DON-contaminated flour (Haruyokoi, 13.0%, protein concentration, 14% moisture basis) or blank flour, water (196 ml), sugar (14.0 g), salt (5.6 g), yeast (2.8 g), non-fat dried milk solids (5.6 g), and shortening (14.0 g). The baking procedure involved mixing the dough to an optimum stage in a mixer, resting for 20 min, kneading and leavening, resting for a further 108 min, repeating this step twice, and then baking at 160 °C for 35 min. The Japanese-style noodle dough commonly used in food such as "udon" was prepared by mixing the DON-contaminated flour (Hokushin, 8.0–9.0% protein concentration) or blank flour, sodium chloride (4% by weight of flour) and water (45% by weight of flour). The dough was then trimmed with a noodle-making machine for home use (UD-10 model, Izumiseiki Seisakusho Co., Nagano, Japan) and stored at −30 °C prior to use. The boiling treatment involved each noodle equivalent to 50 g of the flour being boiled for 10 min in 1 liter of tap water. The noodles were dried at 40 °C for 3 h to remove any remaining water and the boiling water solid, and were then freeze-dried. The lyophilized samples were stored at −30 °C until needed for the DON analysis.

The bread, noodle dough, boiled noodles and boiling water solids were ground for 5 min with a Waring blender in 200 ml of an extraction solution (acetonitrile: water, 85:15). The naturally contaminated and blank flour samples were extracted with the same solution by shaking at 250–300 rpm for 30 min at room temperature. Each extract was cleaned by passing through a multifunctional column (Autoprep® MF-T 1500, Showa Denko., Tokyo, Japan), as described by Trucksess *et al.*[10] before being subjected to the HPLC analysis or bioassays.

HPLC analysis was performed in an Inertsil® ODS-3 column (250 mm × 4.6 mm i.d., 5 μm; GL Sciences., Tokyo, Japan) held at 40 °C with a mobile phase of acetonitrile:methanol:water (5:5:90). The sample injection volume was 20 μl. DON was detected at 220 nm with a UV detector. The bioassays were carried out by using Swiss mouse 3T3 fibroblasts (JCRB9019; Health Science Research Resources Bank, Osaka, Japan). Cytotoxicity was determined with the BrdU and WST-8 bioassays. The BrdU assay assesses DNA synthesis, while the WST-8 assay assesses metabolic activity. The cells were cultured in DMEM containing 10% FCS, 4 mmol L-glutamine and antibiotics, and maintained in a humidified incubator at 37 °C under a 5% CO_2 atmosphere. The cells were seeded in each well of a 96-well plate (Corning, NY, USA) at a density of 5×10^4 cells/well. The culture medium contained a DON standard solution and was replaced with a fresh medium (100 μl/well) after 24 h of culture, before being incubated for another 24 h. To obtain a standard curve, a pure DON solution (Sigma Chemical Co., St. Louis, MO, USA) or the DON-free flour extract spiked with DON was serially 2-fold diluted, and 100-μl aliquots of each dilution were added to the cell culture in triplicate. The samples for the assay were diluted, and 100-μl aliquots of each sample added to the cell culture in triplicate, resulting in a final sample concentration of 250 mg/ml. As a negative control, the DON-free noodle extract (DON-free extract) was diluted to 250 mg/ml. A BrdU ELISA cell proliferation kit (Roche Molecular Biochemicals, Basel, Switzerland) was used for the BrdU assay according to the manufacturer's instructions. A cell counting kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan) were used for the WST-8 assay containing WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), a water-soluble tetrazolium salt which is reduced by the cellular dehydrogenase activity of viable cells to produce a yellow formazan dye. The cell density was 2.0×10^4 cells/well. After 24 h of incubation, the sample and DON standard media changed to a fresh medium with the CCK-8 solution, before incubating for 1 h at 37 °C. The absorbance of each well was measured at 450 nm, with a reference wavelength of 620 nm, by using an automatic microplate reader (Spectra Max 340; Molecular Device, Sunnyvale, CA, USA). The results from the chemical and cytotoxicity assays were subjected to on statistical analysis (one-way analysis of variance (ANOVA)).

Widestrand *et al.*[11] have developed a cell culture technique for screening a low concentration of *Fusarium* mycotoxins, including DON, by using Swiss 3T3 mouse fibroblasts. They found that a DNA synthesis assay (BrdU assay) was more sensitive for detecting the toxic effect of these toxins than the metabolic activity assay based on the ability of mitochontrial dehydrogenase (MTT assay) and the damage assay (LDH assay). They have recently applied the bioassay to detect *Fusarium* trichothecenes in real cereal samplesand showed that the IC_{50} value in the BrdU assay was 666 μg/kg of DON in a wheat extract.[12] In the present study, a metabolic activity assay (WST-8 assay) was found to have the same sensitivity as the BrdU assay for detecting DON (data not shown), so we evaluated the cytotoxicity at each stage of cooking noodles and baking bread by using the BrdU assay and WST-8 assay to assess whether or not the cooking process generated new toxic compounds from DON. The standard curves obtained from pure DON and the DON-free extract spiked with DON showed a dose–dependent response in both bioassays (data not shown). There was no significant difference between these standard curves, indicating that the flour matrix did not affect the cytotoxicity of DON (data not shown). The IC_{50} values from these assays with the DON-free flour extract spiked with DON were 623 μg/

Table 1. DON Retention at Each Processing Stage of Noodles Measured by an HPLC Analysis and Cytotoxicity Assays

Assay method	HPLC		WST-8		BrdU	
	DON conc. (mg/kg)	DON retention (%)	DON conc. (mg/kg)	DON retention (%)	DON conc. (mg/kg)	DON retention (%)
Raw flour	0.86 ± 0.03	100.29 ± 3.65	0.86 ± 0.03	100.29 ± 3.65	0.86 ± 0.08	100.29 ± 8.78
Before boiling	0.85 ± 0.04	98.55 ± 4.08	0.85 ± 0.04	98.55 ± 4.08	0.85 ± 0.06	98.84 ± 6.78
After boiling	0.26 ± 0.04	30.52 ± 4.08	0.30 ± 0.01	34.53 ± 1.29	0.25 ± 0.04	28.88 ± 5.02
Boiling water	0.36 ± 0.03	41.28 ± 3.89	0.56 ± 0.03	64.97 ± 3.99	0.37 ± 0.04	42.89 ± 4.58

Each value is expressed as the mean ± StandardError (n = 4).

Table 2. DON Retention Level in Bread Calculated from the HPLC Analysis and Cytotoxicity Assays

Assay method	HPLC		WST-8		BrdU	
	DON conc. (mg/kg)	DON retention (%)	DON conc. (mg/kg)	DON retention (%)	DON conc. (mg/kg)	DON retention (%)
Raw flour	0.71 ± 0.05	100.00 ± 7.04	0.69 ± 0.07	100.00 ± 4.10	0.78 ± 0.12	100.00 ± 1.53
Bread	0.77 ± 0.06	108.42 ± 8.45	0.58 ± 0.03	84.05 ± 4.34*	0.72 ± 0.08	92.30 ± 1.03*

Each value is expressed as the mean ± StandardError (n = 4).
Asterisks (*) indicate mean values that are significantly different at ($p < 0.05$) from those of the HPLC value.

kg of DON for the BrdU assay and 555 μg/kg of DON for the WST-8 assay. These results show that both bioassays of the flour extract had similar sensitivity to the BrdU assay of the wheat extract reported by Widestrand *et al.*[12] In addition, since the bioassay could detect the cytotoxicity of various trichothecene mycotoxins, it would be applicable to the detection of possible new toxic compounds derived from DON during the cooking process. In contrast, such chemical analyses as GC–MS and HPLC would have been unable to detect such new compounds because their structures were unknown. Therefore, the bioassays provided a unique and effective means of detecting possible new toxic compounds produced during food processing.

Table 1 shows the concentration of DON and the cytotoxicity at each stage of the noodle cooking process assessed by the HPLC analysis and the bioassays. The DON concentration corresponding to the cytotoxicity of a sample was calculated from a standard curve that had been established by using 3T3 cells exposed to the DON-free flour extract spiked with DON. The raw flour was contaminated with 0.86 ± 0.03 mg/kg of DON, and a similar concentration was found in the dough after conducting three assays. After boiling, the DON concentration in the noodles was 0.26 ± 0.04 mg/kg, and the DON retention in this boiled product was calculated to be at 30.52 ± 4.08% based on the HPLC results. In respect of the cytotoxicity, the WST-8 assay showed the DON concentration to be 0.30 ± 0.01 mg/kg and the DON retention ratio to be 34.53 ± 1.29%. The BrdU assay showed a 0.25 ± 0.04 mg/kg DON concentration and a 28.88 ± 5.02% DON retention ratio. There was no significant difference in DON concentration between these three assays. The DON concentration in boiling water, assayed by HPLC was 0.37 ± 0.04 mg/kg, which corresponds to 42.89 ± 4.58% of the raw DON concentration. The DON retention corresponding to the cytotoxicity assessed by the BrdU assay was similar to that by HPLC. However, the DON retention corresponding to the cytotoxicity assessed by the WST-8 assay was higher than that obtained from either the HPLC analysis or the BrdU assay. These results demonstrate that boiling reduced both the DON concentration and its cytotoxicity, and that the residue of DON leached into the boiling water. Although it is possible that a new compound having metabolic cytotoxicity was generated in the boiling water solids this seems not to be a serious problem for health because the boiling water is commonly discarded.

Nowicki *et al.*[13] have also investigated the effects of processing and cooking on the DON level in noodles prepared from naturally contaminated Canadian Western Red Spring wheat flour. Although the protein concentration was unknown, they have shown that the reduction in level of DON by the chemical analytical method was 40% in Japanese-style noodles after boiling. The difference in reduction between their study and ours probably resulted from the kind of flour, protein concentration or other factors regarding the matrix structure.

Table 2 shows the concentration of DON and the cytotoxicity of raw flour and bread assessed by the bioassays and the HPLC analysis. The DON level in raw flour ranged from 0.69–0.78 mg/kg in the three assays. After making bread, the DON level assayed by HPLC was 0.77 ± 0.06 mg/kg and the retention ratio was 108.42 ± 8.45%. However, the DON levels corresponding to the cytotoxicity obtained from the WST-8 assay and the BrdU assay were 0.58 ± 0.03 mg/kg (84.05 ± 4.34%) and 0.72 ± 0.08 mg/kg (92.30 ± 1.03%), respectively. These results suggest that the DON level in bread was not reduced as a chemical compound but that