한국농화학회지 제37권 제5호(1994)
Agricultural Chemistry and Biotechnology
Vol. 37, No. 5, pp. 385~391(1994)

Contamination of *Fusarium* Mycotoxins in Corn Samples Imported from China

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Abstract : The occurrence of *Fusarium* mycotoxins was surveyed in 68 corn samples imported from China. Four 8-ketotrichothecenes including deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), and nivalenol (NIV) were detected in corn. In addition, the corn samples were contaminated with zearalenone (ZEA), fumonisin B_1 (FB_1), fumonisin B_2, and fumonisin B_3. DON, 15-ADON, 3-ADON, ZEA, and FB_1 were major contaminants in corn, with mean levels of 277, 34, 37, 39, and 123 ng/g, respectively.
(Received July 25, 1994; accepted October 6, 1994).

Introduction

Corn ear rot is caused by species of *Fusarium* and sometimes results in toxic effects on humans and farm animals following consumption of *Fusarium*-infected corn. *F. graminearum* Schwabe (*Gibberella zeae* Petch) and *F. moniliforme* Sheldon (*G. fujikuroi* (Sawada) Wollenw.) are the major causative fungi of corn ear rot.

F. graminearum produces trichothecenes, such as deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), nivalenol (NIV), and 4-acetylnivalenol (4-ANIV, also known as fusarenon-X), as well as an estrogenic mycotoxin, zearalenone (ZEA) (Fig. 1(A, B)). Among these, the mycotoxins often encountered in corn are DON, NIV and ZEA in oriental countries.[8,10-13,16,19] Animals consuming feeds contaminated with ZEA develop reproductive problems,[15] while trichothecene intoxication of humans and animals is characterized by skin irritation, vomitting, diarrhea, and damage to hematopoietic tissues.[7]

On the other hand, *F. moniliforme* is the most prevalent fungus associated with corn in most corn-producing areas of the world. This fungus has been suspected of being involved in human and animal diseases such as human esophageal cancer and equine leukoencephalomalacia (ELEM), and has been proven to be toxic to experimental animals.[14] Bezuidenhout *et al.*[1] characterized the structures of fumonisins (Fig. 2), a new group of mycotoxins, that had been purified from the cultures of *F. moniliforme*. Toxicological investigations resulted in the reproduction of ELEM[14] and porcine pulmonary edema[6] in farm animals after following the administration of pure fumonisin B_1 (FB_1). Poor performance, increased organ weights, diarrhea, multifocal hepatic necrosis, biliary hyperplasia, and rickets have also been observed in broiler chicks and turkey poults fed FB_1.[2,9,22] FB_1 is hepatotoxic and carcinogenic in rats,[21] and caused morphological functional changes in chicken macrophages *in vitro*, which indicates the possibility of an immunosuppressive effect.[17] Short-term carcinogenesis studies in a rat liver bioassay indicated that fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) exhibit toxicological

Key words : Natural occurrence, *Fusarium* mycotoxins, trichothecenes, zearalenone, fumonisins, Chinese corn
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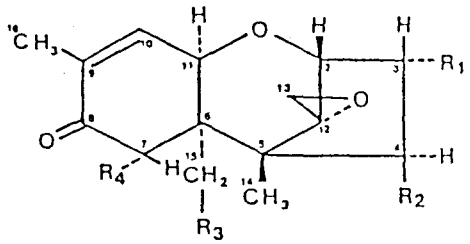
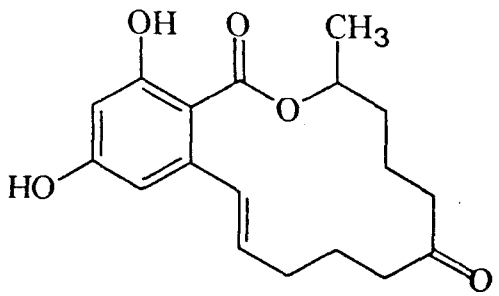


(A)

Compound	R_1	R_2	R_3	R_4
DON	OH	H	OH	OH
3-ADON	OAc	H	OH	OH
15-ADON	OH	H	OAc	OH
3,15-DADON	OAc	H	OAc	OH
NIV	OH	OH	OH	OH
4-ANIV	OH	OAc	OH	OH
4,15-DANIV	OH	OAc	OAc	OH



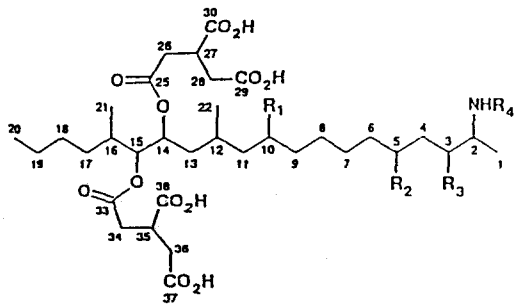
(B)

Fig. 1. Chemical structures of trichothecenes (A) and zearalenone (B).

and cancer-initiating activities similar to those observed for FB_1.[3,4] These observations suggest that fumonisins may pose a threat to human and animal health because fumonisins occur worldwide on corn and corn products.[18,20]

Because corn production is very limited in Korea, it is imported from foreign countries such as China, United States, and Canada. Especially corn used for feedstuffs is mainly imported from China. Although no incident of mycotoxicoses due to consumption of corn from China has been reported, the natural occurrence of *Fusarium* mycotoxins has been highly suspected.

In this report, we attempted to survey the natural occurrence of 8-ketotrichothecenes, ZEA, and fumonisins in corn samples imported from China in order to provide informations on risk assessment of mycotoxins in imported cereals.



Compound	R_1	R_2	R_3	R_4
Fumonisin B_1	OH	OH	OH	H
Fumonisin B_2	H	OH	OH	H
Fumonisin B_3	OH	H	OH	H

Fig. 2. Chemical structures of fumonisins.

Materials and Methods

Corn samples

Corn kernels (68 samples in total) with each 500g were obtained from 4 feedstuff companies, which imported the corn from China in 1993. All the obtained samples were stored at 4℃ until analysis.

Chemicals

Trichothecene mycotoxins, including DON, NIV, 15-ADON, 3-ADON, 4-ANIV, 3,15-diacetyldeoxynivalenol (3,15-DADON), and 4,15-diacetylnivalenol (4,15-DANIV) were kindly supplied by Dr. T. Yoshizawa, Department of Bioresource Science, Kagawa University, Japan. ZEA and FB_1 were purchased from Sigma Chemical Co. (St. Louis, Mo. USA). FB_2 and FB_3 were prepared in our laboratory. The toxins were individually dissolved in methanol at concentration of 1 mg/ml and stored at 4℃. A trimethylsilylating reagent was prepared with an *N*-trimethyl-silylimidazole-*N,O*-bis(trimethylsilyl)acetamide-trimethylchlorosilane at a ratio of 3 : 3 : 2 (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Analysis of trichothecenes and ZEA

Corn samples were extracted by using a previously published procedure.[8] Each ground sample (40 g) was extracted with 160 m*l* of acetonitrile-water (3 : 1, v/v) for 30 min, and the extract was filtered through Whatman no. 1 filter paper. An 80 m*l* aliquot of the filtrate was defatted with the same volume of *n*-hexane and concentrated to dryness. The residue was dissolved in 2 m*l* of methanol and applied onto a Florisil column (2×15 cm) containing 10g of Florisil (60~100 mesh; Fisher Scientific Co., Pittsburgh, USA). The column was washed with 100 m*l* of *n*-hexane and then eluted with 100 m*l* of chloroform-methanol (9 : 1, v/v). The eluate was concentrated to dryness and the residue was redissolved in 2 m*l* of methanol. For trichothecene analysis by gas chromatography-mass spectrometry (GC-MS), 1 m*l* of the sample solution was used, and the rest was used for ZEA analysis by high performance liquid chromatography (HPLC).

A portion of each extract was reacted with the trimethylsilylating reagent and analyzed with a JEOL JMS AX 505 gas chromatograph-mass spectrometer with a selected ion monitoring (SIM) mode. The following equipment and conditions were used for trichothecene analysis: column, DB-5 fused silica column (0.25 mm×30 m, 0.25 μm film; J & W Scientific, Folsom, USA); carrier, helium gas at a flow rate of 2 m*l*/min; column temperature, 120℃ for 5 min and then increased to 270℃ at 5℃/min; injector temperature, 280℃; ion source temperature, 250℃; ionizing voltage, 70 eV; ionizing current, 300 μA; scanning rate, 0.5 scan/s. The fragment ions monitored for quantitation of trichothecenes were *m/z* 422 and 393 for DON, *m/z* 377 and 287 for 3-ADON, *m/z* 392 and 350 for 15-ADON, *m/z* 379 and 289 for NIV, *m/z* 480 and 450 for 4-ANIV, *m/z* 320 for 3,15-DADON, and *m/z* 408 for 4,15-DANIV. Calculation of trichothecene concentration was based on the average area counts of the fragment ions of each standard toxin. Retention time of each toxin was 28.98 min for DON, 30.90 min for 3-ADON, 31.11 min for 4-ANIV, 31.22 min for 15-ADON, 31.66 min for NIV, 33.04 min for 3,15-DADON, and 33.91 min for 4,15-DANIV. The detection limit of the method employed for trichothecene was 1 ng/g. When control corn samples were spiked with 200 ng of each trichothecene per gram of each sample, recoveries were 87% to 95%.

A Shimadzu LC-6A HPLC equipped with a PF-110 spectrofluorometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan) was used for the analysis of ZEA. For HPLC analysis, a Zorbax octadecylsilane (ODS) column (4.6 mm×15 cm; particle size, 5 μm; Dupont Co., Kyoto, Japan) with a mobile phase of 70% aqueous methanol, a flow rate of 1 m*l*/min, an excitation wavelength of 236 nm, and an emission wavelength of 418 nm were used. The detection limit of the method for ZEA was 2 ng/g. When the control corn sample was spiked with 200 ng of ZEA per gram of corn, recovery from corn was 88%.

In order to confirm the presence of 15-ADON, 3-ADON, and NIV in corn, a corn extract which was positive for trichothecenes by GC-MS with SIM was reacted with trimethylsilylating reagent and subjected to GC-MS to obtain the full scan mass spectra.

Analysis of fumonisins

The extraction and detection of fumonisins in corn samples were done by the method of Sydenham *et al.*[18] Briefly, 25g of each corn sample was extracted with 50 m*l* of methanol-water (3 : 1, v/v) for 5 min in a Sorvall Omnimixer. Aliquots (10 m*l*) of each extract were applied to strong anion-exchange(SAX) cartridges (Supelco Inc., Supelco Park, Dellefonte, Pa, USA) at a flow rate of 2 m*l*/min. The fumonisins were eluted from the cartridges with 14 m*l* of 0.5% acetic acid in methanol at a flow rate of 0.8 m*l*/min. The eluate was concentrated to dryness and dissolved in 2 m*l* of methanol. Aliquot (25 μ*l*) of methanol extract was added into 225 μ*l* of *o*-phthaldialdehyde (OPA) reagent and the mixed solution was injected into HPLC within 1 min after adding OPA reagent. For the HPLC analysis of fumonisins, the following equipment and conditions were used: column, Zorbax ODS column (4.6 mm×15 cm); mobile phase, a gradient solvent

system of acetonitrile-water-acetic acid (30 : 90 : 1 and 60 : 39 : 1); flow rate, 1 ml/min; excitation wavelength, 335 nm; emission wavelength, 440 nm. When control corn samples were spiked with 200 ng each fumonisin per gram of corn, recoveries were 90% to 105% in corn.

Results

The natural occurrence of 8-ketotrichothecenes, ZEA, and fumonisins in corn samples is summerized in Table 1. None of 4-ANIV, 3,15-DADON, and 4,15-DANIV were detected in corn by GC-MS with the SIM mode. Fig. 3 shows the chromatograms recorded by SIM of DON, 15-ADON, 3-ADON, and NIV in one of the corn samples. The incidences of toxins in corn samples were 98.5% for DON, 72.1% for 15-ADON, 38.2% for 3-ADON, 13.2% for NIV, 25% for ZEA, 39.7% for FB_1, 5.9% for FB_2, and 2.9% for FB_3. The 68 corn samples were contaminated as follows: 17 samples (25.0%) with DON, 22 samples (32.4%) with DON and 15-ADON, 18 samples (26.5%) with DON, 15-ADON, and 3-ADON, 7 samples (10.3%) with DON, 15-ADON, 3-ADON, and NIV, 2 samples (2.9%) with DON, 15-ADON, and NIV, and one sample with DON and 3-ADON. FB_1, FB_2, and FB_3 were coincidentally found in 2 (2.9%) samples. The mean concentrations of DON and FB_1 in positive samples were 277 ng/g and 123 ng/g, respectively. Other toxins were detected at concentrations of less than 100 ng/g. The maximal levels of toxins detected in corn were 4,565 ng/g for DON, 181 ng/g for 15-ADON, 234 ng/g for 3-ADON, 17 ng/g for NIV, 124 ng/g for ZEA, 1,428 ng/g for FB_1, 88 ng/g for FB_2, and 57 ng/g for FB_3.

Table 1. Natural occurrence of *Fusarium* mycotoxins in 68 corn samples imported from China

Mycotoxins	No.(%) of positive samples	[a]Mean level(range) (ng/g) in positive samples
DON	67(98.5)	277.1(5-4,565)
15-ADON	49(72.1)	34.3(2-181)
3-ADON	26(38.2)	37.0(3-234)
3,15-DADON	0 (0.0)	[b]ND
NIV	9(13.2)	6.1(3-17)
4-ANIV	0 (0.0)	ND
4,15-DANIV	0 (0.0)	ND
ZEA	17(25.0)	39.4(6-124)
FB_1	27(39.7)	122.7(11-1,428)
FB_2	4 (5.9)	66.0(51-88)
FB_3	2 (2.9)	56.5(56-57)

[a]The trichothecenes were quantified by GC-MS with SIM. Fumonisins and ZEA were quantified by HPLC with a fluorescence detector; [b]ND, not detected.

In order to unequivocally verify the presence of 15-ADON, 3-ADON, and NIV, one corn sample which was positive for three trichothecenes in the SIM analysis was chosen and prepared by the procedure described in Materials and Methods. The extract was then subjected to capillary GC-MS. Fig. 4 shows the mass spectra of the trimethylsilyl ethers of 15-ADON, 3-ADON, and NIV. Fig. 4(A) shows the diagnostic ions for 15-ADON at *m/z* 482, 467, 392, and 350. The mass spectra gave the diagnostic ions for 3-ADON at *m/z* 482, 467, 392, 377, and 363 (Fig. 4(B)) and for NIV at *m/z* 600, 585, 482, 392, 379, and 349 (Fig.4(C)). The agreement of retention time coupled with the presence of diagnostic ions verified the presence of the three trichothecenes in the sample extract.

Discussion

The survey on corn samples imported from China revealed a heavy contamination of DON, 15-ADON, 3-ADON, NIV, ZEA, and fumonisins. Among the 8-ketotrichothecenes, the major toxins were DON, 15-ADON, and 3-ADON, although the mean levels of monoacetyl-DON were lower than those of DON. The presence of NIV was also found in corn from oriental countries as a minor contaminant.[8,12,13] The present survey demonstrates that the pattern of the natural occurrence of monoacetyl-DON was different for each country. Kim *et al.*[8] reported that five 8-ketotrichothecenes (DON, 15-DON, NIV, 4-ANIV, and 4,15-DANIV) were contaminated in corn samples from Kangwon province in Korea. Luo *et al.*[12] also reported that DON, 15-ADON, and NIV

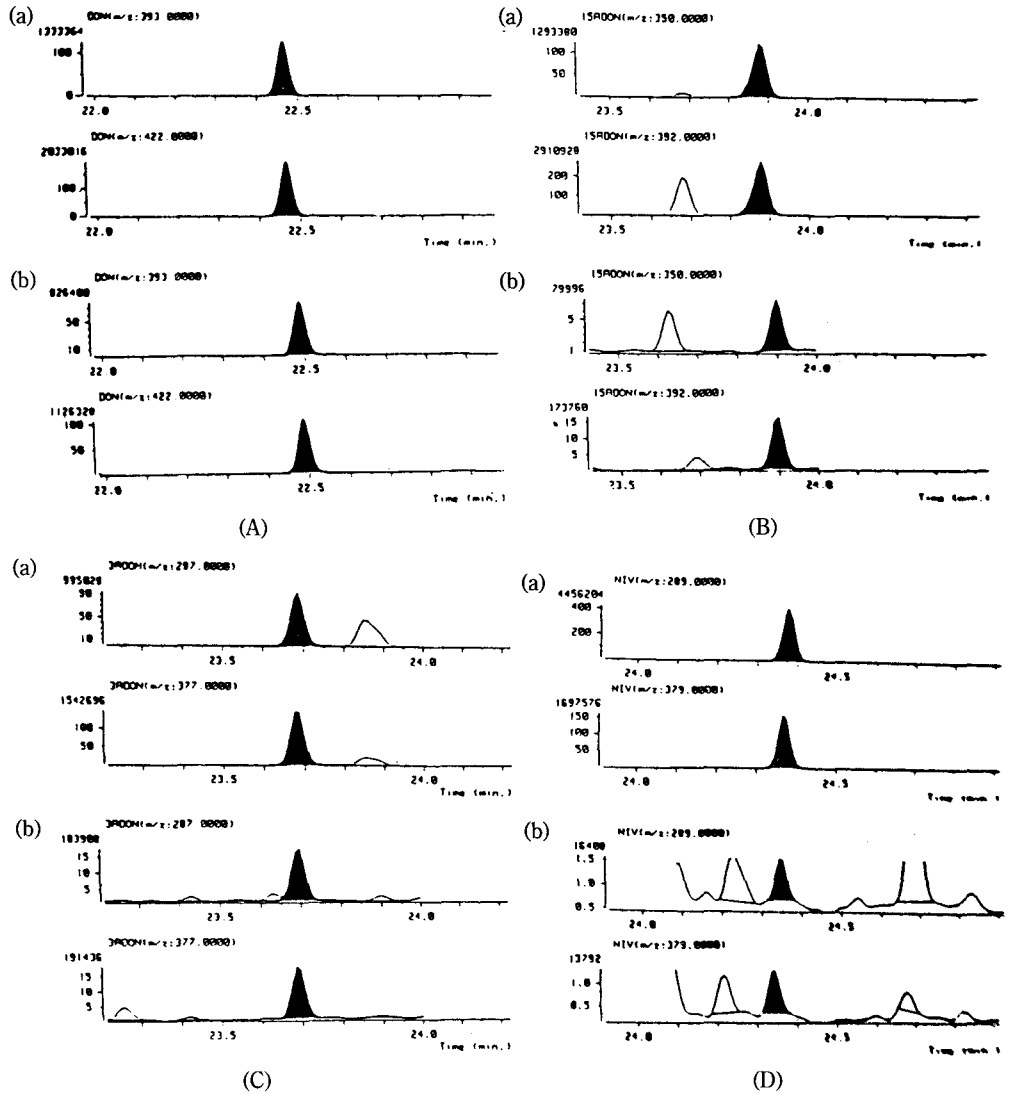


Fig. 3. Chromatograms of trimethylsilyl ethers of DON (A), 15-ADON (B), 3-ADON (C), and NIV (D) in corn samples recorded by SIM. (a) standard toxins; (b) toxins in samples.

were contaminated in corn from high- and low-risk areas for human esophageal cancer in China. In those surveys, only 15-ADON was detected as a monoacetyl-DON. However, both 15-ADON and 3-ADON were detected in this study. Thus, there are regional differences in the natural occurrence of trichothecenes in different countries or even in the same country.[10,19] The acute lethal toxicity of monoacetyl-DON is higher than that of DON; the 50% lethal dose (LD_{50}) of 15-ADON in male mice by oral route was 34 mg/kg[23] and that of 3-ADON in male mice by intraperitoneal route was 49.4 mg/kg.[5] Much attention should be paid to the natural occurrence of monoacetyl-DON as well as DON in Chinese corn.

More remarkable was the co-occurrence of fumonisins and trichothecenes in the Chinese corn samples. Recently, Yoshizawa *et al.*[24] reported fumonisin occurrence in corn from high- and low-risk areas for human esophageal cancer in China. The

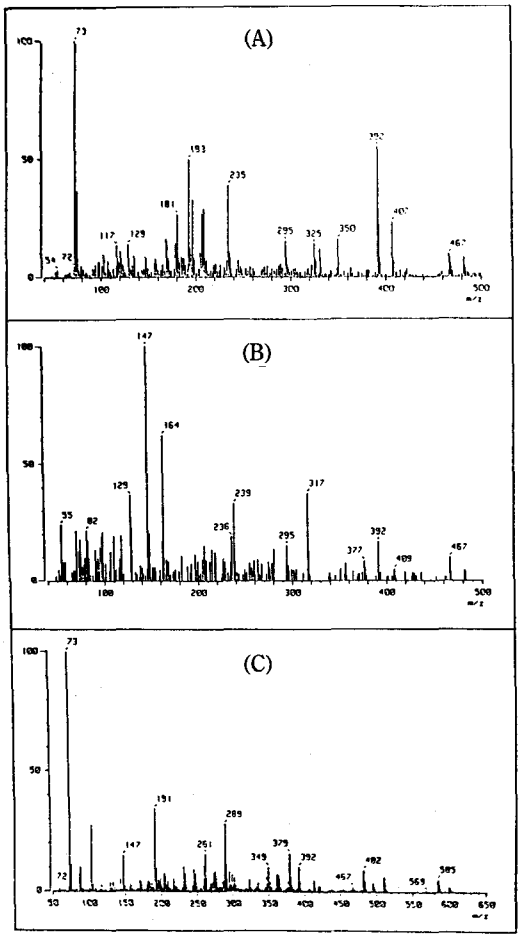


Fig. 4. Full scan mass spectra of trimethylsilyl ethers of 15-ADON (A), 3-ADON (B), and NIV (C) in corn samples.

incidence of fumonisin contamination of high risk-area was about two times higher than that of low risk-area, although the mean levels of fumonisins in positive samples were same in both areas. The incidence of fumonisins of imported corn in this study is similar to that of high risk-area for human esophageal cancer in China.

This is the first report of the natural occurrence of *Fusarium* mycotoxins in corn samples imported from China. Additional surveys on the natural occurrence of the toxins in imported cereals are expected to provide valuable informations on risk assessment of the toxins.

Acknowledgements

This work was supported by a grant from Korea Science and Engineering Foundation (91-05-00-11).

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중국으로부터 수입한 옥수수에서의 *Fusarium* 진균독소오염

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초록 : 중국으로부터 수입한 68개의 옥수수시료에서 *Fusarium*진균독소의 오염을 조사하였다. 수입옥수수에서 deoxynivalenol(DON), 15-acetyldeoxynivalenol(15-ADON), 3-acetyldeoxynivalenol(3-ADON) 및 nivalenol(NIV) 등 4가지의 8-ketotrichothecene이 검출되었다. 또한 zearalenone(ZEA), fumonisin B_1(FB_1), fumonisin B_2(FB_2) 그리고 fumonisin B_3(FB_3)도 검출되었다. DON, 15-ADON, 3-ADON, ZEA 그리고 FB_1이 주요 오염독소였으며, 이들 독소의 평균오염농도는 각각 277, 34, 37, 39, 그리고 123 ng/g이었다.

찾는말 : 자연오염, *Fusarium* 진균독소, trichothecene, zearalenone, fumonisins, 중국산 옥수수