Detection of Two Naturally Occurring Structural Isomers of Partially Hydrolyzed Fumonisin B_1 in Corn by On-Line Capillary Liquid Chromatography−Fast Atom Bombardment Mass Spectrometry

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Fifteen corn kernel and corn screening samples were analyzed for the presence of fumonisin toxins. Samples were extracted with acetonitrile/water (1:1) and cleaned up with C_{18} solid phase extraction (SPE) cartridges. Analysis of fumonisins was carried out by on-line capillary liquid chromatography−fast atom bombardment mass spectrometry (capillary LC/FAB/MS). Two isomers of partially hydrolyzed fumonisin B_1 ($PHFB_1$) were detected in five samples, and *N*-acetyl fumonisin B_2 (FA_2) was detected in one sample. Hydrolyzed fumonisin B_1 (HFB_1) was also detected in one sample, but further confirmation is needed.

Keywords: *Mycotoxin; fumonisin; capillary LC/FAB/MS*

INTRODUCTION

Fumonisins (Figure 1) are a family of mycotoxins produced mainly by *Fusarium moniliforme* Sheldon (Bezuidenhout et al., 1988; Gelderblom et al., 1988, 1992; Thiel et al., 1991; Platter et al., 1992; Branham and Plattner, 1993), a common fungal contaminant of corn worldwide (Marasas et al., 1984). Consumption of *F. moniliforme* contaminated corn has been proven to be the causal factor of equine leukoencephalomalacia (ELEM) in horses (Kriek et al., 1981) and also to be correlated with the high incidence of human esophageal cancer in South Africa (Marasas et al., 1984) and China (Yang, 1980). Fumonisin B_1 (FB_1), the most abundant naturally occurring analogue among several discovered fumonisins, causes ELEM in horses (Kellerman et al., 1990; Wilson et al., 1992) and pulmonary edema in swine (Harrison et al., 1990; Colvin and Harrison, 1992).

Chemically, FB_1 is a diester of propane-1,2,3-tricarboxylic acid (TCA) and 2-amino-12,16-dimethyl-3,5,10,-14,15-pentahydroxylcosane. The C-14 and C-15 hydroxyl groups are involved in ester formation with the terminal carboxy group of propane-1,2,3-tricarboxylic acid (Bezuidenhout et al., 1988). Other B-series fumonisins are fumonisin B_2 (FB_2), B_3 (FB_3), and B_4 (FB_4) (Bezuidenhout et al., 1988; Gelderblom, 1992; Plattner et al., 1992), which are the C-10 and C-5 dehydroxy and the C-5,10 didehydroxy analogues of FB_1, respectively. The A-series fumonisins include the *N*-acetates of FB_1 (FA_1) and FB_2 (FA_2). Fumonisin C_1 (FC_1), which lacks the amino-end terminal methyl group of FB_1, has been reported (Branham and Plattner, 1993).

Since the first report of the natural occurrence of FB_1 in corn in 1990 (Sydenham et al., 1990), contamination of corn and corn-based feed or food samples by FB_1 and FB_2 as well as FB_3 has been reported by many laboratories (Plattner et al., 1990; Sydenham et al., 1991; Bane et al., 1992; Pittet et al., 1992; Thiel et al., 1992; Hopmans et al., 1993; Rice et al., 1994), while the natural occurrence of FB_4, A-fumonisins, and C-fumonisins has not been reported. Hydrolyzed FB_1 (HFB_1) was detected in tortilla chips, masa, and canned yellow corn (Hopmans et al., 1993). It was suggested that the occurrence of HFB_1 in these food samples resulted from hydrolysis of both TCA groups from FB_1 during the food preparation process, which involved a step with high pH and heat. Two structural isomers of partially hydrolyzed FB_1 ($PHFB_1$), formed by the hydrolysis of one of the TCA groups, were detected in the feces of vervet monkeys dosed with FB_1 (Shephard et al., 1994) and corn cultural material of *F. moniliforme* treated with Ca(OH) (Sydenham et al., 1995). $PHFB_1$ and HFB_1 were also found in fecal samples collected from cattle, sheep, and rats fed with diets mixed with *F. moniliforme* cultural material (Rice et al., 1994).

Liquid chromatography (LC) with fluorescence detection has been commonly used for the analysis of fumonisins in contaminated food or feed products. Derivatization of fumonisins, however, is required for the LC analysis (Thiel et al., 1991; Stack and Eppley, 1992;

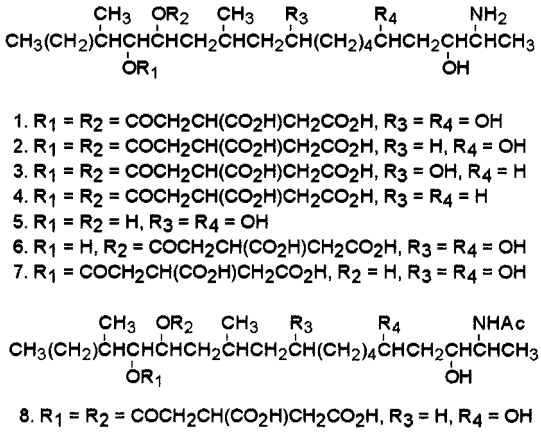

Figure 1. Chemical structures of (1) fumonisin B_1 (FB_1), (2) fumonisin B_2 (FB_2), (3) fumonisin B_3 (FB_3), (4) fumonisin B_4 (FB_4), (5) hydrolyzed FB_1 (HFB_1), (6 and 7) partially hydrolyzed FB_1 ($PHFB_1$), and (8) *N*-acetyl FB_2 (FA_2).

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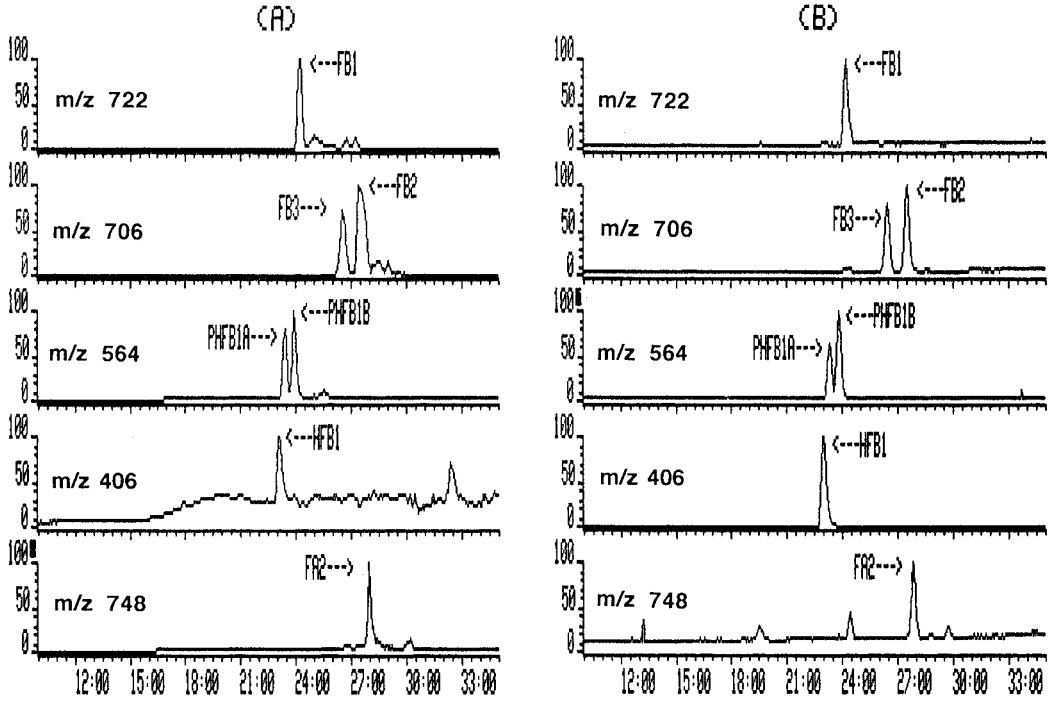

Figure 2. Capillary LC/FAB/MS mass chromatograms (*m/z* 722, 706, 564, 406, and 748) of (A) extract of sample FS870 and (B) fumonisin standard mixture containing FB_1, FB_2, FB_3, $PHFB_1$, HFB_1, and FA_2.

Sydenham et al., 1992; Hopmans et al., 1993; Rice and Ross, 1994). Analysis by gas chromatography/mass spectrometry (GC/MS) also requires hydrolysis followed by derivatization of fumonisins (Plattner et al., 1990). Nonderivatized fumonisins have been analyzed by mass spectrometry using various ionization techniques including fast atom bombardment (FAB) (Korfmancher et al., 1991; Chen et al., 1992; Mirocha et al., 1992; Holcomb et al., 1993), ion spray (IS) (Chen et al., 1992), thermospray (TS) (Korfmancher et al., 1991), liquid secondary ion (LSI) (Bezuidenhout et al., 1988; Plattner et al., 1990), and electrospray (ES) (Korfmacher et al., 1991; Caldas et al., 1995; Sydenham et al., 1995). Liquid chromatography coupled with ion spray mass spectrometry (LC/IS/MS) (Chen et al., 1992) and electrospray mass spectrometry (LC/ES/MS) (Doerge et al., 1994; Plattner, 1995; Musser, 1996) have been proven to be useful methods to analyze fumonisins in fungal cultural materials and contaminated food samples.

This paper reports the natural occurrence of the two isomers of $PHFB_1$ and of FA_2 in corn samples. Evidence for the natural occurrence of HPB_1 in corn is also presented. This paper also describes a microcapillary resolution and FAB detection of the fumonisins.

MATERIALS AND METHODS

Analytical Standards. FB_1 was provided by Dr. Robert M. Eppley of the Food and Drug Administration (Washington, DC). FB_2 and FB_3 were purchased from the Division of Food Sciences and Technology, Council for Scientific & Industrial Research, Pretoria, South Africa. FA_1 and FA_2 were provided by Dr. Hamed K. Abbas (USDA, ARS, Stoneville, MS).

Sample Preparation. Fifteen corn kernel or corn screening (broken corn kernels) samples collected in 1990 were ground to fine powder with a Stein Laboratories Mill (Fred Stein Laboratories, Atchison, KS). Twenty-five grams of each ground sample was extracted with 50 mL of acetonitrile/water (1:1) on a shaker for 1 h. A 2 mL aliquot of the filtered extract was diluted with 6 mL of distilled water and acidified to ca. pH 4 with 0.1 N HCl. The diluted and acidified extract was applied to a Waters C_{18} Sep-Pak Classic solid phase extraction (SPE) cartridge (Waters, Milford, MA), previously washed with 5 mL of acetonitrile followed by 5 mL of distilled water. The loaded cartridge was then washed with 2 mL of acetonitrile/water (3:17). Fumonisins were eluted with 2 mL of acetonitrile/water (7:3). Twenty-five grams of ground clean corn was spiked with 100 μL of FB_1 standard solution (1 mg/mL) as a control. The same extraction and cleanup procedure was carried out for the control extract preparation.

Preparation of $PHFB_1$ and HFB_1 from FB_1. Five hundred milligrams of FB_1 was dissolved in 1 mL of 0.5 M NaOH and kept at room temperature for 15 min. The solution was then acidified to pH 4 with 1 N HCl and passed through Amberlite XAD-2 resin (Aldrich, Milwaukee, WI) packed in a disposable glass pipet and washed with acetone followed by distilled water. A mixture of FB_1, $PHFB_1$, and HFB_1 was eluted with 2 mL of MeOH.

Analysis of Fumonisins. The fumonisin mixture prepared from the hydrolysis of FB_1 was mixed with standards of FB_2, FB_3, FA_1, and FA_2 to make a standard mixture. The standard mixture and the cleaned up corn extracts were analyzed by on-line capillary liquid chromatography−fast atom bombardment mass spectrometry (capillary LC/FAB/MS) on a VG 7070EQ mass spectrometer (VG Analytical, Wythenshawe, Manchester, U.K.) equipped with a VG dynamic FAB ion source and an Ion Tech Xenon saddle field gas gun (Ion Tech, Teddington, Widdlesex, U.K.) operated at 8−9 kv. The mass spectrometer was interfaced with two Shimadzu LC-600 liquid chromatograph pumps (Shimadzu, Kyoto, Japan) through a 20 cm length, 320 μm i.d. reverse phase capillary LC column. The column was packed with 3 μm Phase Sep ODS2 C_{18} (Phase Separations, Norwalk, CT). A 50 μm i.d. fused silica capillary was used as delivery tubing between the column and the mass spectrometer. Samples were injected through a Valco CI4W internal sample injector (Valco Instruments, Houston, TX) with 0.5 μL sample volume. The total flow rate (mixer flow rate) was 160 μL/min, but a 124 mm length, 1.2 mm i.d. stainless steel tubing packed with 5 μm Phase Sep ODS2 C_{18} was used as a preinjector splitter to obtain a capillary column flow rate of 4 μL/min. The Shimadzu LC-600 is capable of delivering 1 μL/min; however, the higher total flow rate was necessary to achieve a repeatable gradient mixing in a relatively short time. The capillary column flow rate was measured at the end of the 50 μm i.d. delivery capillary by measuring the time of filling a 20 μL micropipet with a mobile

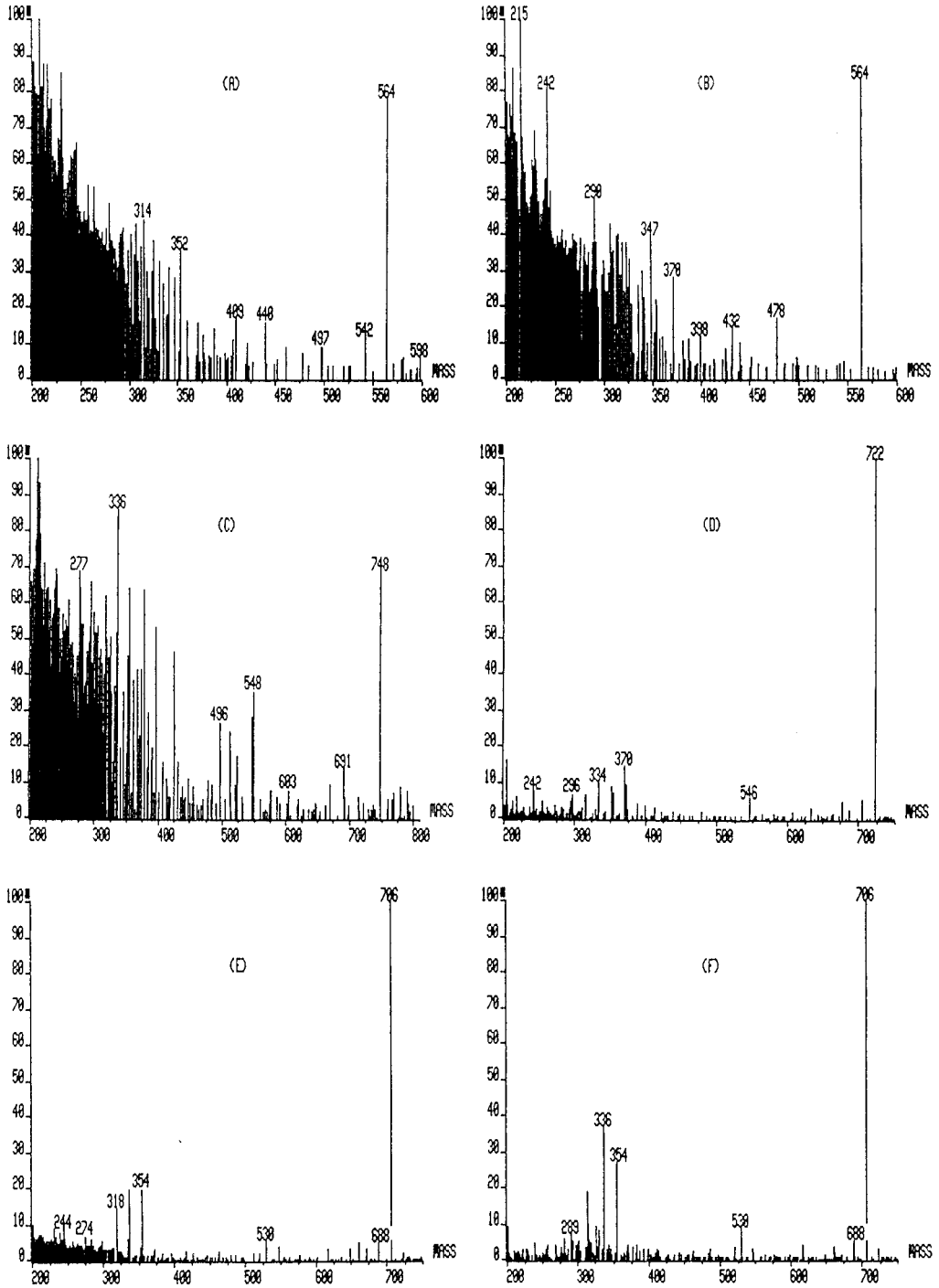

Figure 3. Background-subtracted FAB mass spectra of (A) $PHFB_1$a with $[MH]^+$ at *m/z* 564, (B) $PHFB_1$b with $[MH]^+$ at *m/z* 564, (C) FA_2 with $[MH]^+$ at *m/z* 748, (D) FB_1 with $[MH]^+$ at *m/z* 722, (E) FB_2 with $[MH]^+$ at *m/z* 706, and (F) FB_3 with $[MH]^+$ at *m/z* 706 in the extract of sample FS870.

phase. The desired capillary column flow rate was obtained by adjusting the total flow. The solvent system consisted of (A) water/glycerol/trifluoroacetic acid (TFA) (98:2:0.1) and (B) water/acetonitrile/glycerol/TFA (20:78:2:0.1). Two water/acetonitrile gradient programs were used for fumonisin analysis. All the samples and the standard mixture were run in the gradient system A: isocratic solvent A for 1 min followed by ramping the solvent B concentration from 0% to 80% in 14 min and maintaining the solvent B concentration at 80% for another 5 min. To achieve better separation for all the fumonisins, the extract of one corn screening sample FS870 and the standard mixture were also run in a longer gradient program B: isocratic solvent A for 1 min followed by ramping the solvent B concentration from 0% to 80% in 29 min and maintaining the concentration for another 5 min. All the samples and the standard mixture were analyzed by capillary LC/FAB/MS in single ion monitoring (SIM) mode with sampling time set at 80 ms and interchannel time at 20 ms. FAB mass spectra of sample FS870 and fumonisin standards were obtained in full scan mode. The cleaned up sample extracts were directly analyzed in SIM mode without further sample concentration. For full scan FAB analysis, a 500 μL aliquot of the extract of sample FS870 was evaporated to dryness in N_2 and redissolved in 200 μL of acetonitrile/water (7:3) to make a concentrated sample. FAB spectra of FB_1, FB_2, FB_3, $PHFB_1$, and FA_2 were also obtained in full scan mode through capillary LC/FAB/MS.

RESULTS AND DISCUSSION

The protonated molecular ions ($[MH]^+$) for FB_1, FB_2, FB_3, $PHFB_1$, HFB_1, and FA_2 are *m/z* 722, 706, 706, 564,

406, and 748, respectively. Figure 2A shows the mass chromatogram of those masses in the capillary LC/FAB/MS analysis of the corn screening sample FS870 by the gradient program B, suggesting the existence of FB_1, FB_2, FB_3, two isomers of $PHFB_1$, HFB_1, and FA_2. The peak retention time at each mass (24:10 for FB_1 at *m/z* 722, 26:25 for FB_3 at *m/z* 706, 27:29 for FB_2 at *m/z* 706, 23:16 and 23:47 for isomers of $PHFB_1$ at *m/z* 564, 22:58 for HFB1 at *m/z* 406, and 27:51 for FA_2 at *m/z* 748) agreed with the corresponding fumonisin standard peak in the standard mixture analysis (Figure 2B). The mass chromatogram of *m/z* 564 in Figure 2A shows two peaks indicating the presence of two isomers of $PHFB_1$ in the corn screening sample. The two isomers of $PHFB_1$ with retention times of 23:16 and 23:47 were designated $PHFB_1$a and $PHFB_1$b, respectively. In this gradient program, the two $PHFB_1$ isomers eluted before FB_1 with a near base line separation from FB_1. In the short gradient program A, however, the retention time of FB_1 fell between the retention times of $PHFB_1$a and $PHFB_1$b, and the peaks were partially resolved from the FB_1 peak.

The identification of $PHFB_1$ and FA_2 as well as FB_1, FB_2, and FB_3 in the sample FS870 was confirmed by their full FAB spectra (Figure 3). Panels A and B show spectra of $PHFB_1$a and $PHFB_1$b, respectively. Both spectra give $[MH]^+$ at *m/z* 564. Panel C shows the FAB spectrum of FA_2 with the $[MH]^+$ at *m/z* 748. No fragment ions from the $[MH]^+$ were observed in the spectra due to the relatively small amounts of the compounds and high background signals in the low mass area. The FAB spectrum of FB_1 shown in the panel D has a strong $[MH]^+$ at *m/z* 722 and minor fragment ions at *m/z* 546 and 370, corresponding to loss of one and two TCA groups from $[MH]^+$, respectively. Fragment ions resulting from sequential losses of H_2O from the ion at *m/z* 370 were also observed in the spectrum. All those fragment ions in the spectrum of FB_1 have been observed in the collisionally activated dissociation (CAD) daughter ion spectrum of *m/z* 722 from a FAB/MS/MS analysis of FB_1 by Korfmacher et al. (1991). Similar fragmentation patterns were seen in the spectra of FB_2 (panel E) and FB_3 (panel F). Both spectra show a strong $[MH]^+$ at *m/z* 706 and a minor fragment ion $[MH - TCA]^+$ at *m/z* 530 as well as $[MH - 2TCA]^+$ at *m/z* 354. Water molecule loss from $[MH]^+$ and sequential H_2O losses from the fragment ion at *m/z* 354 were also seen in the spectra. HFB_1 was not detected in the full scan analysis, apparently due to the very small quantity of the compound in the sample.

Korfmacher et al. (1991) detected an impurity component in a purified extract of corn culture material of *F. moniliforme* containing FB_1 and proposed the structure of $PHFB_1$ for the compound based on the CAD daughter ion spectrum of the ion at *m/z* 564 from a FAB/MS/MS analysis. This compound was suggested to be a new fumonisin-like compound in the corn culture material or an artifact caused by the sample purification procedure. In our case, the existence of $PHFB_1$ as an artifact is unlikely since a simple and mild procedure was used in the sample preparation. In order to eliminate the possibility of an artifact, a clean corn sample spiked with FB_1 (4 ppm) was extracted and cleaned up with the same procedure as fumonisin-contaminated corn samples. Capillary LC/FAB/MS analysis of the extract of this FB_1-spiked sample did not detect any $PHFB_1$ or HFB_1.

Table 1. Comparison of Peak Area of $PHFB_1$ (Combination of Two Isomers) and FB_1 in Corn Samples Analyzed by Capillary LC/FAB/MS

sample	$PHFB_1$	FB_1	$PHFB_1$:FB_1
FS886	862	919	1:1.1
FS875	155	565	1:3.6
FS870	2155	4010	1:1.9
FS869	524	5320	1:10.2
FS867	998	7012	1:1.7

A total of 15 samples including corn kernels and corn screening were analyzed for FB_1, FB_2, FB_3, and $PHFB_1$ by capillary LC/FAB/MS in SIM mode. All the samples contained FB_1. FB_2 was detected in nine samples and FB_3 in seven samples. Four corn screening samples and one corn kernel sample contained two isomers of $PHFB_1$. Approximately equal amounts of $PHFB_1$a and $PHFB_1$b were detected in each of the five samples. Quantitation of $PHFB_1$ was not done since a quantitative standard was not available. Peak areas of FB_1 and $PHFB_1$ were compared to obtain an estimate for the relative levels of $PHFB_1$ in the samples (Table 1). Ratios of $PHFB_1$ (combination of two isomers) to FB_1 based on peak area ranged from 1:1.1 to 1:10.2. Samples in which $PHFB_1$ was not detected contained less than 0.1 μg/g FB_1.

We concluded that the two structural isomers of partially hydrolyzed FB_1 can be found in naturally contaminated corn samples. The existence of these two compounds in corn could be significant on the basis of their quantities relative to that of FB_1. The toxicology of these two compounds has not been determined. FA_2, the *N*-acetyl derivative of FB_2, was also reported as a natural contaminant of corn screening for the first time. FA_2 but not FA_1 was found in the sample and this agrees with the fact that the sample contained more FB_2 than FB_1, even though FB_1 usually is the most abundant fumonisin found in both fungal cultures and field samples. The possible existence of a very small amount of HFB_1 in the corn screening samples is also suggested. Further confirmation for this compound is needed, however.

This study also indicates that on-line reverse phase capillary LC/FAB/MS is a suitable method to analyze fumonisins in corn or corn-based samples. Samples can be directly analyzed after a simple extraction and cleanup procedure without derivatization. Sample concentration is not needed when analyzed in SIM mode because of its high sensitivity.

ABBREVIATIONS USED

CAD, collisionally activated dissociation; ES, electrospray; ELEM, leukoencephalomalacia; FA_1, *N*-acetyl fumonisin B_1; FA_2, *N*-acetyl fumonisin B_2; FAB, fast atom bombardment; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3; FB_4, fumonisin B_4; FC_1, fumonisin C_1; GC/MS, gas chromatography/mass spectrometry; HFB_1, hydrolyzed fumonisin B_1; IS, ion spray; LSI, liquid secondary ion; $PHFB_1$, partially hydrolyzed fumonisin B_1; SIM, single ion monitoring; SPE, solid phase extraction; TCA, tricarboxylic acid; TFA, trifluoroacetic acid; TS, thermospray.

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