HPLC and FAB Mass Spectrometry Analysis of Fumonisins B_1 and B_2 Produced by *Fusarium moniliforme* on Food Substrates

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Flasks containing moistened, autoclaved corn, unmilled rice, peanuts, soybeans, and laboratory rodent feed were inoculated with *Fusarium moniliforme* NRRL 13616 and incubated in the dark at 25 °C. After 24 days, the cultures were extracted with acetonitrile and water. High-performance liquid chromatography (HPLC) of the extracts, after they had been derivatized with fluorescamine, showed high concentrations of fumonisins B_1 and B_2 (10 242 and 3068 μg/g, respectively) in the corn cultures and moderately high concentrations (206 and 100 μg/g, respectively) in the unmilled rice cultures. HPLC also detected fumonisins B_1 and B_2 (34 and 50 μg/g, respectively) in the rodent feed cultures. Only trace levels (5 μg/g or less) of fumonisin B_1 were detected in the peanut and soybean cultures. Fast atom bombardment mass spectrometry (FAB MS) confirmed the presence of fumonisins B_1 and B_2 in the corn, unmilled rice, and rodent feed cultures.

INTRODUCTION

Fusarium moniliforme Sheldon, which occurs worldwide on grains, is a common contaminant of corn (Marasas et al., 1984). Feeds infested with *F. moniliforme* have been associated with diseases in several animals (Kriek et al., 1981). Corn infested with *F. moniliforme* has been implicated in the high incidence of human esophageal cancer in South Africa (Marasas, 1982) and China (Yang, 1980). Fumonisins B_1 and B_2, which are mycotoxins produced by *F. moniliforme*, have been shown to promote cancer in rats (Gelderblom et al., 1988). Purified fumonisin B_1 also causes leukoencephalomalacia in horses (Marasas et al., 1988; Kellerman et al., 1990), pulmonary edema in swine (Harrison et al., 1990), and liver tumors in rats (Gelderblom et al., 1991).

Isolates of *F. moniliforme* from corn have been shown to produce higher concentrations of fumonisins B_1 and B_2 than similar isolates from other substrates (Thiel et al., 1991; Nelson et al., 1991). The amounts of fumonisins produced by cultures on corn also depend on the strain of *F. moniliforme* (Nelson et al., 1991). Five other species of *Fusarium* also produce fumonisins on corn (Thiel et al., 1991; Nelson et al., 1992).

We grew cultures of a toxigenic strain of *F. moniliforme* on five different substrates (corn, unmilled rice, peanuts, soybeans, and rodent feed) and analyzed them for fumonisins by gradient high-performance liquid chromatography (HPLC) and fast atom bombardment mass spectrometry (FAB MS). Unmilled rice, soybeans, and peanuts have not been reported previously as substrates for fumonisin production, although Abbas et al. (1991) reported fumonisin production on milled long-grain rice. Corn was used as a positive control for determining if proper conditions were used for fumonisin production. Laboratory rodent feed was also tested for fumonisins. Rodent feed cultures were of particular interest since the National Center for Toxicological Research (NCTR) maintains a rodent colony for research purposes and any toxin in the feed could affect research results. Rodent feed used at the NCTR is routinely analyzed for aflatoxins according to the method of Holcomb et al. (1991).

The fumonisins were quantitated by a modified gradient-elution HPLC method with fluorescence detection after derivatization with fluorescamine. It has recently been shown that FAB MS also can be utilized for the analysis of picogram amounts of fumonisin B_1 (Korfmacher et al., 1991). In this paper, we show that FAB MS can be utilized for the determination of both fumonisins B_1 and B_2 in culture extracts.

MATERIALS AND METHODS

Chemicals and Reagents. Organic solvents were of HPLC grade from J. T. Baker, Richardson, TX. Water was double-distilled in glass.

The fluorescamine derivatizing reagent was prepared by adding 40 mg of fluorescamine (Roche Diagnostics, Nutley, NJ) to 10 mL of acetonitrile.

The fumonisin B_1 and B_2 standards (CSIR, Pretoria, South Africa) were used as received. The fumonisins were transferred quantitatively to 100-mL volumetric flasks with acetonitrile/water (50/50), resulting in stock standards with concentrations of 100 μg/mL. Working standards were made up in acetonitrile/water from these stock standards.

The fumonisins are toxic and possibly carcinogenic and should be handled by using safety precautions (AOAC, 1984). These safety guidelines indicate that mycotoxins should be handled with gloves and used only in properly ventilated hoods or gloveboxes.

Culture Preparation. *F. moniliforme* NRRL 13616 was obtained from the U.S. Department of Agriculture, Peoria, IL, and maintained on potato dextrose agar. Coarsely chopped corn was purchased commercially and used without grinding. Unmilled rice, soybeans, and peanuts were obtained from a local farmer and chopped finely with a blender. The laboratory rodent feed (Ralston Purina Co., St. Louis, MO), which consisted of 21% corn with other ingredients, was ground in a Wiley mill equipped with a 2-mm screen.

One hundred grams of each substrate was placed in a 500-mL Erlenmeyer flask with 100 mL of deionized water. The flasks were plugged with cotton, covered with aluminum foil, and

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autoclaved for 15 min. After cooling, they were inoculated with a conidial suspension of *F. moniliforme* that had been grown on potato dextrose agar and suspended in sterile phosphate-buffered saline solution. The cultures were incubated at 25 °C in the dark.

After 24 days, the cultures were freeze-dried and chopped with a blender inside a glovebox for safety. The samples were stored at −20 °C until they were analyzed for fumonisins B_1 and B_2.

Culture Extraction and Cleanup. A 10-g sample of each freeze-dried, blended culture was extracted with 50 mL of acetonitrile/water (50/50) and centrifuged at 2000 rpm for 5 min. A 3-mL-capacity C_{18} Sep-Pak Vac SPE column (Waters Chromatography Division, Millipore Corp., Milford, MA) was preconditioned with 5 mL of methanol followed by 5 mL of 1% aqueous KCl. Two milliliters of the culture supernatant was added to 5 mL of 1% aqueous KCl and passed through the column. The column was washed with 3 mL of 1% aqueous KCl, followed by 2 mL of acetonitrile/1% aqueous KCl (20/80). The fumonisins were eluted from the column with 2 mL of acetonitrile/water (50/50).

Precolumn Derivatization. One hundred microliters of either a fumonisin working standard or the eluate from a C_{18} SPE column was added to a 2-mL screw-cap autosampler vial with 100 μL of 100 mM sodium borate buffer (pH 9.2) and 100 μL of the fluorescamine derivatization reagent. After the vials were vortexed and allowed to stand for 1 min, 0.5 mL of a mixture of acetonitrile/10 mM boric acid (40/60 v/v) was added to each vial. The vials were vortexed again and placed in an autosampler for HPLC analysis.

HPLC Analysis. The gradient HPLC system consisted of two Shimadzu Model LC-600 pumps (Shimadzu Scientific Instruments, Columbia, MD), a Shimadzu Model SIL-9A autoinjector, a Shimadzu Model C-R3A integrator, a Waters Model 470 fluorescence detector (Waters Chromatography Division), and a Microsorb C_8 column (25 cm × 4.6 mm i.d., 5-μm particle size, Rainin Instrument Co., Woburn, MA).

The column was maintained at room temperature with a flow rate of 1.5 mL/min. Detector parameters were as follows: excitation, 390 nm; emission, 475 nm; attenuation, 128; and gain, 100. Fluorescamine derivatives of the fumonisins were analyzed according to a modification of the method by Sydenham et al. (1990). A gradient program for the HPLC was used instead of isocratic elution with the acetonitrile/acetate buffer (Sydenham et al., 1990). Instead, solutions of acetonitrile/1% acetic acid in water were used for both of the mobile-phase solvents; solvent A was 30/70 (v/v), and solvent B was 70/30. Each solvent was filtered and degassed with a 0.45-μm filter before use.

The autosampler of the HPLC was programmed to inject 10-μL samples at 40-min intervals. The analysis time was 35 min, and the equilibration time between samples was 5 min. During the first 3 min, the percent of solvent B was increased from 10% to 20%; in the next 15 min, it was increased to 35%; in the next 1 min, it was increased to 55%; in the next 2 min, it was increased to 70%; and in the next 5 min, it was increased to 100%. Solvent B was then maintained at 100% for 10 min.

Duplicate 10-μL aliquots were injected for each culture so that each reported value represents the mean of two injections.

FAB MS Analysis. The FAB MS system consisted of a Finnigan MAT TSQ 70 triple quadrupole mass spectrometer, equipped with a Finnigan fast atom bombardment (FAB) source and an Ion Tech fast atom gun. Xenon was utilized in the primary atom beam at energies of 8–10 keV, and thioglycerol was used as the matrix. Typically, the matrix was applied to the FAB probe tip followed by 1 μL of the sample. The mass spectrometer was scanned from m/z 200 to 800 at 1 scan/s.

The fast atom bombardment tandem mass spectrometry (FAB MS/MS) analyses were performed under the same conditions as FAB MS, except that the collision gas, argon, was set to about 0.5 mTorr and the collision energy was either 30 or 50 eV. To obtain daughter ion spectra, MS1 (Q1) was set to pass the selected parent ion and MS2 (Q3) was operated under full scanning conditions.

RESULTS AND DISCUSSION

The HPLC gradient elution program was successful in resolving the fluorescamine derivatives of fumonisin B_1 and B_2 standards, which eluted at 22.35 and 24.53 min, respectively (Figure 1A,B). It also resolved the fluorescamine derivatives of fumonisins B_1 and B_2 in the culture extracts. Figure 1C shows an HPLC chromatogram of a fluorescamine derivative of a *F. moniliforme* corn culture extract, showing fumonisins B_1 and B_2. The addition of acetic acid to the mobile phase resulted in sharper peaks with increased intensities for both fumonisins. Although the fluorescamine derivative of fumonisin B_1 has been reported to produce two fluorescent peaks (Ross et al., 1991), the use of acetic acid in the mobile phase greatly reduced the size of the minor peak. The sensitivity increased to approximately 0.5 μg/mL, and the precision for five separate analyses of a 5 μg/g fumonisin B_1 and B_2 standard was determined to be 3.2% and 3.1%, respectively. Average recovery of 98% was obtained for fumonisin B_1 in corn over the range from 10 to 100 μg/g (Table I).

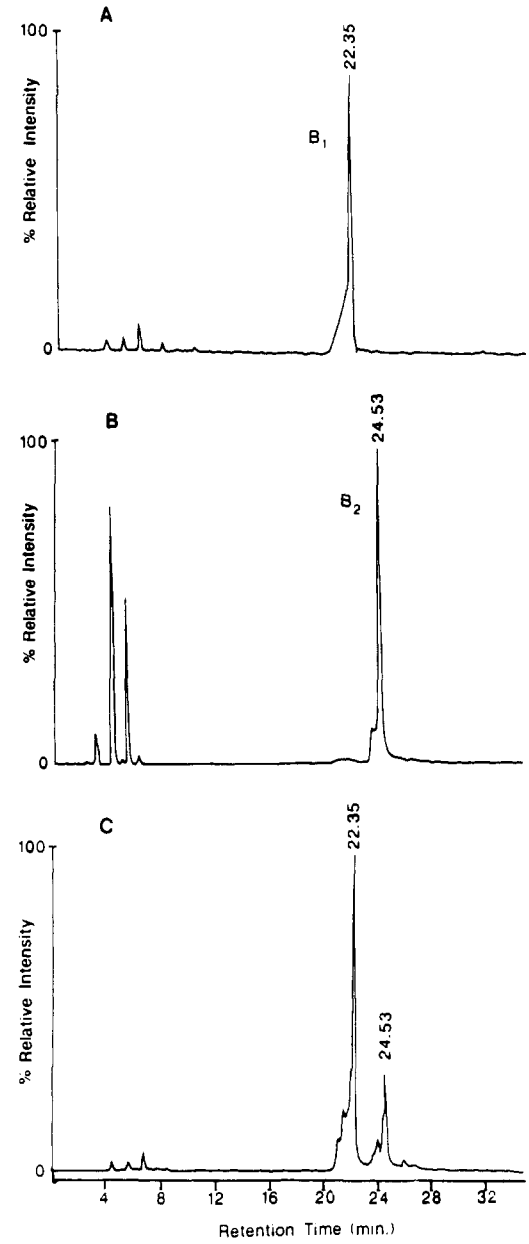

Figure 1. (A) HPLC chromatogram of the fluorescamine derivative of a 10 μg/g fumonisin B_1 standard. (B) HPLC chromatogram of the fluorescamine derivative of a 10 μg/g fumonisin B_2 standard. (C) HPLC chromatogram of the fluorescamine derivative of a *F. moniliforme* corn culture extract, showing fumonisins B_1 and B_2.

Table I. Analysis of Corn Spiked with Fumonisin B_1

μg/g of FB_1 added	recovery,[a] μg/g	$\bar{X}$ + SD, %
10	9.7 ± 0.8	97.0 ± 8.0
100	98.9 ± 2.4	98.9 ± 2.4

[a] Recovery is corrected for background of 3.2 μg/g fumonisin B_1 found in unspiked corn.

Table II. HPLC Detection of Fumonisins B_1 and B_2 Produced by *F. moniliforme* NRRL 13616

substrate	concn of fumonisin,[a] μg/g	
	fumonisin B_1	fumonisin B_2
corn	10242	3068
unmilled rice	206	100
peanuts	5	<1
soybeans	3	<1
rodent feed	34	50

[a] The fumonisin B_1 concentration of the corn before inoculation with *F. moniliforme* was 1.6 μg/g; that of each of the other substrates was <1 μg/g. The fumonisin B_2 concentration for all substrates was <1 μg/g before inoculation.

The concentrations of fumonisins B_1 and B_2 produced in cultures of *F. moniliforme* on corn, unmilled rice, peanuts, soybeans, and rodent feed are shown in Table II. As expected, the corn cultures had the highest amounts of both fumonisins B_1 (10 242 μg/g) and B_2 (3068 μg/g). These results for corn agree with the published values of Sydenham et al. (1990) and indicate that the cultural conditions we used were conducive to fumonisin production.

The cultures of *F. moniliforme* on unmilled rice also contained high amounts of both fumonisins B_1 and B_2 (206 and 100 μg/g, respectively) (Table II). This finding and those of Abbas et al. (1991) on milled long-grain rice suggest that rice-containing foods and feeds should be investigated to determine the natural occurrence of fumonisins in rice products.

The peanut and soybean cultures contained insignificant amounts of 5 μg/g or less of fumonisin B_1 and no fumonisin B_2, either before or after the growth of *F. moniliforme* (Table II).

The laboratory rodent feed cultures contained 34 and 50 μg/g of fumonisins B_1 and B_2, respectively (Table II). The fumonisins may have been produced on the corn component of the rodent feed. Unexpectedly, more fumonisin B_2 than fumonisin B_1 was detected in these samples.

Korfmacher et al. (1991) showed the sensitivity of FAB MS for the detection of fumonisin B_1; in the present study, FAB MS was also found to be a sensitive technique for the detection of fumonisin B_2. In the FAB mass spectrum of the fumonisin B_2 standard (Figure 2), the $[M + H]^+$ ion can be observed as the base peak at m/z 706.

Figure 3 shows the mass spectra obtained for the FAB MS analyses of the unmilled rice and corn culture extracts. The $[M + H]^+$ ions at m/z 706 and 722 for fumonisins B_2 and B_1, respectively, are evident. The additional peaks can be attributed to K adduct ions. The peaks at m/z 744 and 760 correspond to the $[M + K]^+$ ions for fumonisins B_2 and B_1, respectively. The peaks at m/z 782 and 798 correspond to the $[M + 2K - H]^+$ ions for fumonisins B_2 and B_1, respectively. The peak at m/z 836 corresponds to the $[M + 3K - 2H]^+$ ion for fumonisin B_1. The predominance of the K adduct ions can be explained by the sample preparation procedure, in which aqueous KCl was used to activate the C_{18} SPE cleanup column.

Similar results were obtained from the FAB MS analysis of rodent feed cultures, confirming the presence of fumonisins B_1 and B_2 in these cultures.

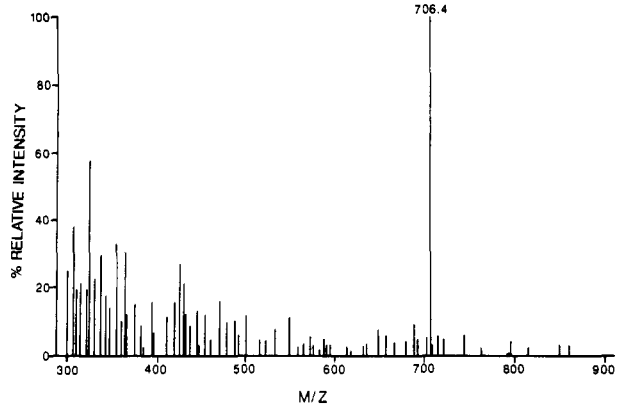

Figure 2. FAB mass spectrum of a fumonisin B_2 standard.

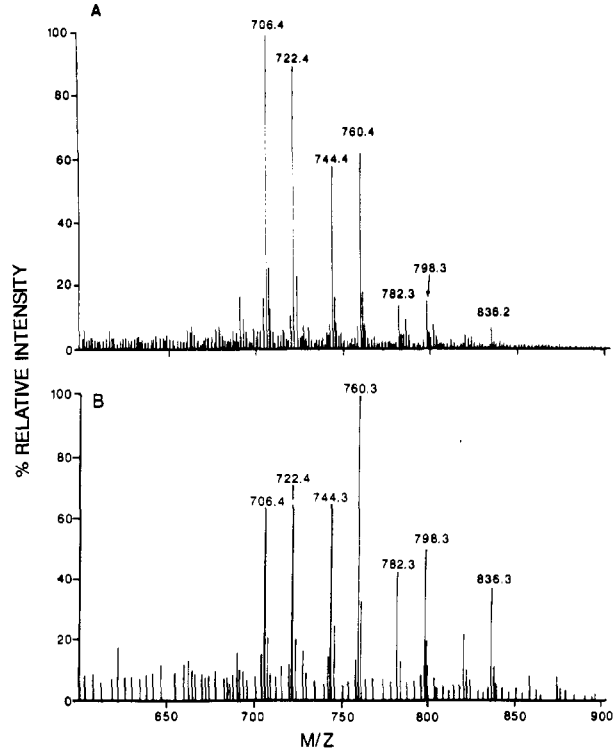

Figure 3. (A) FAB mass spectrum of a *F. moniliforme* rice culture extract, confirming fumonisins B_1 and B_2. (B) FAB mass spectrum of a *F. moniliforme* corn culture extract, confirming fumonisins B_1 and B_2.

Since FAB MS did not confirm the low levels of fumonisin B_1 in the peanut and soybean cultures, FAB tandem MS/MS in the single-reaction monitoring mode (Korfmacher et al., 1991) was utilized. This method confirmed the presence of trace levels of fumonisin B_1 in peanut and soybean cultures.

The results show that fumonisins B_1 and B_2 could be detected in corn, unmilled rice, and rodent feed cultures of *F. moniliforme* with high sensitivity by HPLC and FAB MS.

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