Analysis of Fumonisin B_1 in Rodent Feed by Gradient Elution HPLC Using Precolumn Derivatization with FMOC and Fluorescence Detection

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A new, sensitive, and stable method has been developed for the analysis of fumonisin B_1 in rodent feed. Fumonisin B_1, which is the major fumonisin metabolite produced by the fungus *Fusarium moniliforme*, has been implicated in human and animal diseases. Fumonisin B_1 was extracted from spiked rodent feed with acetonitrile/water (50/50) and then cleaned up with tandem C_{18} Sep-Pak Vac and strong anion-exchange (SAX) columns. Fumonisin B_1 was eluted off the SAX column with 1% acetic acid in methanol and quantitated via gradient elution HPLC using precolumn derivatization with FMOC and fluorescence detection. The minimum detectable amount in the rodent feed was 0.2 ppm. Recovery values in spiked rodent feed averaged 83% over the range 2–20 ppm.

INTRODUCTION

Fumonisins are metabolites of the fungus *Fusarium moniliforme*, which is a common fungal contaminant on corn. Corn infected with *F. moniliforme* has been indicated in the high incidence of human esophageal cancer in Transkei, South Africa (Marasas, 1982) and in China (Yang, 1980). Gelderblom et al. (1988) isolated and identified fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) from *F. moniliforme* cultures and showed that these metabolites possess cancer-promoting activity. Marasas et al. (1988) found that FB_1 is the toxic metabolite that causes leukoencephalomalacia (LEM) in horses. Harrison et al. (1990) reported that FB_1 induces pulmonary edema in swine, and Gelderblom et al. (1991) determined that FB_1 causes hepatocarcinogenic and hepatotoxic effects in rats.

The National Center for Toxicological Research (NCTR) maintains a colony of rodents for research, and these rodents are fed a corn-based diet. A sensitive and stable method for analyzing fumonisin B_1 was needed for assurance that fumonisin B_1 was not present in the diet to affect research studies.

Analytical procedures for quantitative analysis of fumonisin B_1 in various media include mainly three HPLC methods. The first is that of Sydenham et al. (1990), who used HPLC with UV detection after derivatization with maleic anhydride to analyze for fumonisin B_1. They also used a second method, HPLC with fluorescence detection, after derivatization with fluorescamine. Ultraviolet detection of the maleate derivative was not sensitive, and the fluorescent derivative with fluorescamine had a disadvantage of producing two peaks for fumonisin B_1 in the HPLC chromatogram. Shephard et al. (1990) published a very sensitive third method, using HPLC and fluorescence detection after derivatization with *o*-phthaldialdehyde (OPA). Sydenham et al. (1992) improved on the HPLC method using the OPA derivative and were the first to use the OPA derivative to analyze for fumonisin B_3. This method appeared to work with feed samples; however, the fluorescent derivative formed was unstable. Just recently, Scott and Lawrence (1992) published a very sensitive method using HPLC and fluorescence detection after derivatization with 4-fluoro-7-nitrobenzofurazan (NBD-F); however, this derivative does not appear to be very stable either.

Since a sensitive and stable method for analysis of fumonisin B_1 was not available, we developed a new method using gradient elution HPLC with fluorescence detection after derivatization with (9-fluorenylmethyl) chloroformate (FMOC).

EXPERIMENTAL PROCEDURES

Apparatus. The gradient HPLC system consisted of two Model LC-600 Shimadzu pumps and a Shimadzu Model SIL-9A autoinjector (Shimadzu Scientific Instruments, Columbia, MD). A guard column with a 2-mm frit was placed between the autoinjector and the 25 cm × 4.6 mm 5-μm Microsorb C_8 column (Rainin Instrument Co., Woburn, MA). A Waters Model 470 fluorescence detector (Waters Chromatography Division, Milford, MA) was used. The integrator used was a Shimadzu Model C-R3A.

Reagents. Citrate buffer was prepared by placing citric acid (15 mM) and tetramethylammonium chloride (10 mM) in deionized water and adjusting the pH to 4.7 with 6 M NaOH.

The mobile phases were (A) acetonitrile/citrate buffer (30/70) and (B) acetonitrile/citrate buffer (70/30). Each mobile phase was degassed by passing through a 0.45-μm filter. The derivatizing reagent was prepared by placing 0.12 g of FMOC in 40 mL of acetonitrile.

The boric acid buffer used in forming the FMOC derivative was prepared by making up a 1 M solution of boric acid in deionized water and adjusting the pH to 7.5 with 6 M NaOH.

The 1% aqueous KCl was prepared by adding 5 g of Fisher ACS grade KCl to 500 mL of deionized water.

The fumonisin B_1 standard (CSIR, Pretoria, South Africa) was used as received in 10-mg vials. A stock standard of fumonisin B_1 was prepared by quantitatively transferring the fumonisin B_1 from the vial to a 100-mL volumetric flask using acetonitrile/water (50/50) to give a final concentration of 100 μg/mL or 100 ppm. Working standards were made up from this stock standard in acetonitrile/water (50/50).

Safety Note. The fumonisin mycotoxins are suspected carcinogens and should be handled with caution.

The C_{18} Sep-Pak Vac column (Waters No. 20805) and the strong anion-exchange (SAX) column (Analytichem Bond Elut, Jones Chromatography, Lakewood, CO, No. 1210-2044) were used for cleanup of feed sample extracts.

The rodent feed (NIH-31) was obtained from Ralston Purina Co., St. Louis, MO.

Extraction of Rodent Feed. A 50-g sample of rodent feed was extracted with 200 mL of acetonitrile/water (50/50) in a 500-

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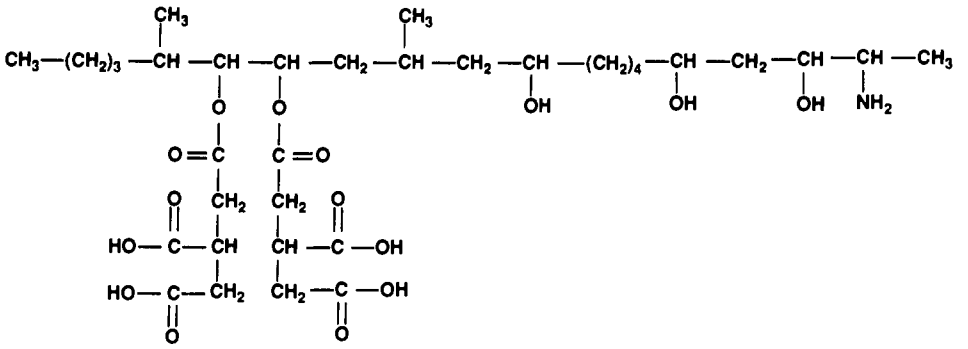

Figure 1. Structure of fumonisin B_1.

mL blender for 2 min at medium speed. A 20-mL portion of this extract was centrifuged at 2000 rpm for 5 min, after which time 2 mL of the extract was added to 5 mL of 1% aqueous KCl and passed through a 3-mL C_{18} Sep-Pak Vac column, which had been preconditioned with 5 mL of methanol followed by 5 mL of 1% aqueous KCl (Rottinghaus et al., 1992). The column was then washed with 3 mL of 1% aqueous KCl, followed by 2 mL of acetonitrile/1% aqueous KCl (20/80). The fumonisin B_1 was eluted off the C_{18} column with 2 mL of methanol/water (70/30). The 2 mL of C_{18} eluate was then diluted to 4 mL with methanol/water (70/30) and passed through a SAX column at a flow rate of 2 mL/min that had been preconditioned with 8 mL of methanol/water (70/30). The column was then washed with 8 mL of methanol/water (70/30), followed by 3 mL of methanol. The fumonisin B_1 was then eluted off the SAX column with 14 mL of 1% acetic acid in methanol. The SAX eluate was evaporated to dryness under N_2 and then brought up to 0.5 mL in acetonitrile/water (50/50) and held for precolumn derivatization. The fumonisin B_1 standard was taken through the C_{18} and SAX columns the same as the sample extracts and held for precolumn derivatization.

Precolumn Derivatization and Gradient HPLC. For the precolumn derivatization, a 400-μL fraction of each of the cleaned up sample extracts and standards was added to separate 2-mL screw-cap autosampler vials. Then 100 μL of the boric acid buffer (pH 7.5) and 100 μL of the FMOC derivatizing reagent were added to each vial. Each reaction mixture was allowed to react for 30 s and then extracted two times with 1 mL of pentane; the pentane layers were discarded. The vials containing the derivatized samples and standards were then placed in the autosampler for HPLC analysis.

The autosampler was programmed for 10-μL injections with 40 min allowed for each sample, including a 5-min equilibrating time between samples.

The gradient program was ramped from 10% to 40% B in 9 min, then increased to 70% B over the next 8 min, followed by a rapid increase to 100% B in 1 min, with a final hold of 17 min. The total analysis time was 35 min. The LC column was kept at room temperature with a flow rate of 1.5 mL/min. The fluorescence detector had the excitation and emission wavelengths set at 263 and 313 nm, respectively, with the attenuation set at 128 and the gain set at 10.

Recovery Experiments. Triplicate 50-g feed samples were spiked with 2, 10, and 20 ppm of FB_1 by adding appropriate volumes of spiking standard to respective 50-g feed samples to give the desired concentrations. The feed samples were then taken through the analytical procedure.

RESULTS AND DISCUSSION

A highly sensitive fluorescent derivative can be obtained with fumonisin B_1 by the reaction of a fluorescent reagent with the primary amine of the fumonisin B_1 (Figure 1) molecule. Shephard et al. (1990) showed that a highly fluorescent derivative could be obtained by reacting the fumonisins with OPA reagent in the presence of a mercaptan. The resulting method turned out to be sensitive; however, the derivative is not stable. Our objective was to find another fluorescent reagent to react with the primary amine of fumonisin B_1 to give a highly sensitive and stable derivative that would react at low temperatures in a relatively short time. The FMOC reagent is used routinely as a fluorescent derivatizing reagent for primary amines in the analysis of amino acids (Bartok et al., 1992; Haynes et al., 1991). The FMOC reagent and primary amine compounds normally react in less than 1 min at room temperature to give a highly fluorescent derivative (Betner and Foldi, 1988, 1986).

Table I. Stability of FMOC Derivative of Fumonisin B_1 (1 ppm Standard)

time, h	$\bar{X}$ ± SD peak height, mm	peak height of fresh FB_1 standard
0	72 ● 1.0	
5	85 ± 1.0	81
25	91 ± 1.5	89
28	93 ± 2.0	92
49	91 ± 2.6	96
52	92 ± 1.5	97
72	96 ● 2.6	96

Table II. Reproducibility of FMOC Derivative of Fumonisin B_1 (1 ppm Standard)

replicate	area	peak height
1	148 450	11 685
2	156 402	11 964
3	163 653	12 658
4	154 442	11 810
5	147 938	11 336
$\bar{X}$	154 177	11 890
SD	4.2	4.1

Initially, the FMOC derivatives of fumonisin B_1 completely saturated the fluorescence detector of the HPLC. Decreasing the injection volume from 50 to 10 μL and increasing the integrator attenuation brought the HPLC chromatograms back to scale; however, the FMOC derivative was not resolved from the injection peak. Changing the column flow rate and mobile phase still did not resolve the FMOC derivative under isocratic conditions on the HPLC. Finally, the FMOC derivative was resolved by gradient elution HPLC with (30/70) acetonitrile/citrate buffer and (70/30) acetonitrile/citrate buffer (Figure 2A).

The FMOC derivative was stable for at least 72 h (Table I). The reproducibility determined from replicate analyses of a 1 ppm of FB_1 standard was approximately 4% (Table II). Sensitivity of the FMOC derivative of FB_1 standards was around 20 ppb with good linearity from 0.05 to 10 ppm.

Recovery values from spiked rodent feed averaged 83% over a range from 2 to 20 ppm of FB_1 (Table III). However, the detection limit increased to approximately 0.2 ppm because of background compounds from the media. These recoveries are for the rodent feed only and are not absolute recoveries since the FB_1 standards were passed through the tandem C_{18} and SAX columns in the same way as the feed extracts.

Table III. Analysis of Rodent Feed Spiked with Fumonisin B_1

$\mu g/g$ of FB_1 added	recovery, $X \pm SD$	
	$\mu g/g$	%
0	<MDL[a]	<MDL
2	1.76 ± 0.14	88 ± 6.9
10	8.40 ± 0.96	84 ± 9.6
20	15.30 ± 1.50	76 ± 7.5

[a] MDL for method as applied to rodent feed is 0.2 $\mu g/g$.

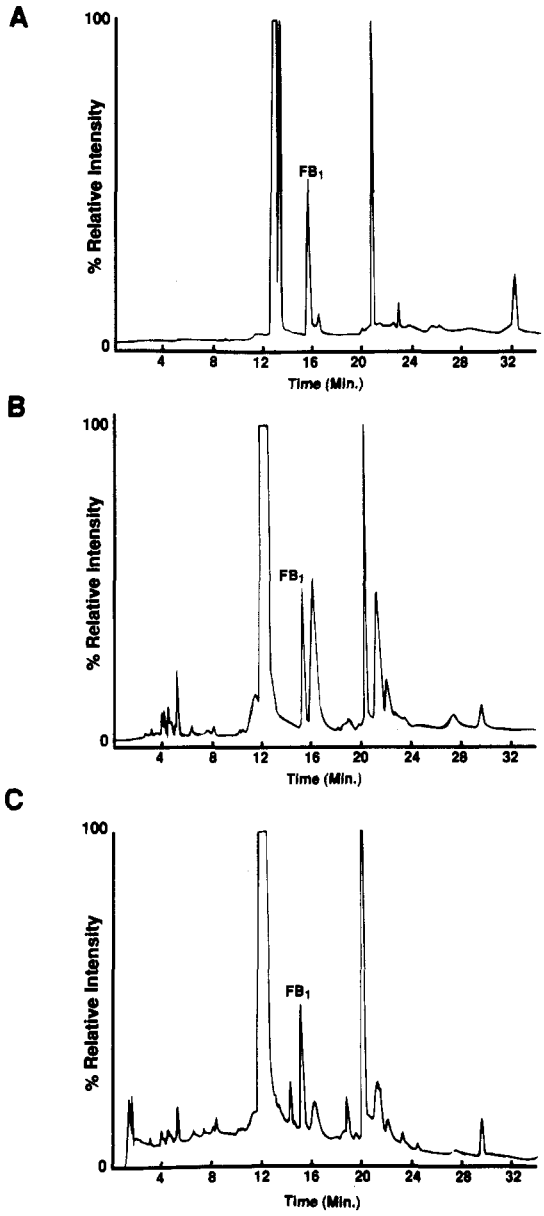

Figure 2. (A) HPLC chromatogram of the FMOC derivative of a 1 ppm of fumonisin B_1 standard. (B) HPLC chromatogram of a 5 ppm of fumonisin B_1 standard after C_{18} and SAX cleanup columns. (C) HPLC chromatogram of extract from rodent feed spiked with 10 ppm of fumonisin B_1.

The HPLC chromatograms of the tandem SPE (C_{18} and SAX) cleanup of the feed extracts show a background peak at the same retention time as FB_1. This background peak is below the detection limit of the method. Figure 2B shows the HPLC chromatogram of a FB_1 standard containing 5 ppm. Figure 2C shows the HPLC chromatogram of a cleaned-up extract from a feed sample that had been spiked with 10 ppm of FB_1. Since 0.5 g-equiv of the feed extract was cleaned up, the 10 ppm spiked sample may be compared with a 5 ppm of FB_1 standard. Figure 3B shows an HPLC chromatogram at reduced integrator attenuation of a cleaned-up extract (0.5 g-equiv) of rodent feed as received from the supplier, indicating a trace peak that could be FB_1 at a concentration of less than 0.2 ppm. Figure 3A shows an HPLC chromatogram of a 1 ppm standard of FB_1 and FB_2 indicating that this method can be utilized for analysis of both FB_1 and FB_2.

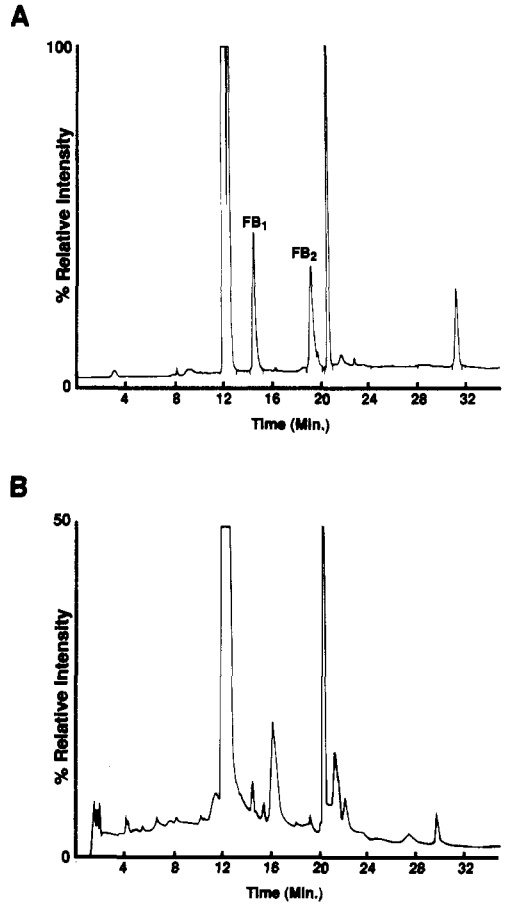

Figure 3. (A) HPLC chromatogram of the FMOC derivative of a 1 ppm of fumonisin B_1 and fumonisin B_2 standard. (B) HPLC chromatogram of extract from unspiked rodent feed.

Table IV. Fumonisin B_1 in Corn Meal Samples Using FMOC Method

sample	fumonisin B_1, ppm	sample	fumonisin B_1, ppm
A	0.68	J	1.54
C	0.76	N	2.80
D	0.76	O	1.04
E	0.58	Q	1.08
F	0.38		
I	2.54	av	1.22

The key to lowering the detection limit was cleanup of interfering materials in the feed extracts. For our studies, the feed extracts were cleaned up with both C_{18} and SAX columns before derivatization and analysis on HPLC. This was necessary to prevent high background peaks that were obtained when only C_{18} cleanup was used. The resulting detection limit of 0.2 ppm is adequate for feeds; however, in the analysis of foods a detection limit of 0.02 ppm is needed.

This method was also used to analyze 10 brands of commercially available corn meal. The fumonisin B_1 values varied from 0.38 to 2.80 ppm, with an average value of 1.22 ppm (Table IV). These values are in agreement with reported fumonisin B_1 results for commercial corn meal samples obtained by HPLC after derivatization with

OPA (Stack and Eppley, 1992; P. F. Ross, NVSL, USDA, Ames, IA, personal communication, 1992; Sydenham et al., 1991).

Our procedure provides a new, sensitive, and reproducible procedure for the analysis of fumonisin B_1 in corn-based rodent feeds. The FMOC derivative is stable, which makes this method amenable to autoinjection of samples.

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