Preparative LC Isolation and Purification of Fumonisin B_1 from Rice Culture

Filmore I. Meredith,*,† Charles W. Bacon,† Ronald D. Plattner,‡ and William P. Norred†

Toxicology and Mycotoxin Research Unit, R. B. Russell Agricultural Research Center, USDA/ARS, P.O. Box 5677, Athens, Georgia 30613, and National Center for Agricultural Utilization Research, USDA/ARS, 1815 North University Street, Peoria, Illinois 61604

Procedures are presented for the production and rapid isolation of fumonisin B_1 (FB_1) from fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3), and for the purification of the mycotoxin FB_1 from cultures of *Fusarium moniliforme* MRC 826 grown on rice. The toxin was extracted from rice culture with acetonitrile:water (1:1; 5mL/g of culture material), filtered, and reduced in volume on a rotary evaporator to remove the acetonitrile. A three-step liquid chromatographic (LC) method was developed for the isolation of FB_1, FB_2, and FB_3, and the purification of FB_1. Preparative reversed-phase LC was used to isolate and partially purify the FB_1. In the first step, the extract was applied to a Waters Bondapak PrepPak 500 C_{18} reversed-phase cartridge and eluted with a methanol:water gradient. Fractions containing partially purified FB_1 were collected, reduced in volume, and subjected to a second preparative LC procedure. The extract was applied to two Bondapak PrepPak cyano cartridges, and isocratic elution with water:0.5% pyridine was used in the purification step. Recovery of the total FB_1 was 97% from the first isolation step and was 93% from the second purification step. Analytical high-performance liquid chromatography and fast-atom bombardment/mass spectrometry were used in determining the purity of the FB_1. Recovery of the high purity FB_1 (95% or greater) was 77% or 2811 mg/3647 mg of starting material.

Keywords: *Fumonisin; FB_1; FB_2; FB_3; mycotoxin*

INTRODUCTION

Considerable worldwide interest in the mycotoxin fumonisin B_1 (FB_1) isolated from *Fusarium moniliforme* f. sp. Sheldon, has resulted from recent investigations implicating its possible role in the etiology of esophageal cancer, which occurs at abnormally high rates in the Transkei region of South Africa (Marasas et al., 1988a; Rheeder et al., 1992; Gelderblom et al., 1988; Bezuidenhout et al., 1988). Studies have shown that feeding corn screenings and moldy corn contaminated with *F. moniliforme* to animals can reproduce effects seen in outbreaks of certain animal diseases. The FB_1 purified from *F. moniliforme* cultures and culture material containing fumonisin have been shown to be involved with equine leukoencephalomalacia (ELEM), a fatal disease of horses in which the white and gray matter of the brain developed liquefactive lesions (Kellerman et al., 1988; Marasas et al., 1988b; Ross et al., 1991). Fumonisin culture material and FB_1 have been associated in swine with porcine pulmonary edema (PPE) (Harrison et al., 1990; Kriek et al., 1981), and both mycotoxins were shown to be toxic to broiler chicks, ducklings, and turkey poults (Javed et al., 1993; Brydon et al., 1987). Investigations with rats fed purified culture material and naturally contaminated corn produced changes in kidney and liver cells with only small amounts of the toxins (Voss et al., 1989, 1993).

Very little is known about the mode of action and effects of these toxins in biological systems of different animal species. Development of chemical isolation and purification methods are needed to obtain fumonisins in sufficient quantities and high purity for large scale biological studies, such as feeding trials and other toxicology studies. Cawood et al. (1991) developed a preparative method for obtaining FB_1 with Amberlite XAD-2, silica gel, and C_{18} reversed-phase chromatography. Recovery was 80% and purity was >90% for FB_1 prepared by this procedure; however, recovery values were not presented on the concentration of FB_1 recovered at 90% purity or above. A procedure was reported for isolation of high-purity FB_1 from corn culture with Amberlite XAD-2, silica gel, and high-performance liquid chromatography (HPLC) equipped with a preparative C_{18} column (Vesonder et al., 1990). In this paper, we describe a three-step improved preparative liquid chromatography (LC) procedure for the separation of FB_1 from two very similar toxins, fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3; Figure 1) and for the purification of FB_1 with purity of 95% or better and a total recovery of >90%.

MATERIALS AND METHODS

Rice Culture. The inoculum from the fungus *F. moniliforme* MRC 826 was cultured on potato dextrose agar for 7 days. Uncle Ben's Converted Rice was prepared as follows: To each 2.8-L wide-mouthed Fernbach flask, 300 g of rice and 300 mL of distilled water were added. The flasks were allowed to sit overnight (12 h) and then were autoclaved for 30 min. Each flask was inoculated with 5 mL of an aqueous suspension of conidia (10^9/mL), stoppered loosely with a cotton plug, and incubated at 26 °C for 28−35 days in the dark. The flasks were shaken briefly once daily during the initial incubation period until day 10.

The rice culture was extracted in the ratio of 100 g of culture material to 500 mL of acetonitrile:water (1:1, v/v). The rice culture was stirred occasionally during the extraction and, after 4 h, was filtered under reduced pressure through Whatman No. 4 paper. The culture material was resuspended in fresh solvent at the same ratio of culture material to acetonitrile and allowed to stand overnight. The culture material was filtered as described previously, and the extracts

* Author to whom correspondence should be addressed.

† Toxicology and Mycotoxin Research Unit.

‡ National Center for Agricultural Utilization Research.

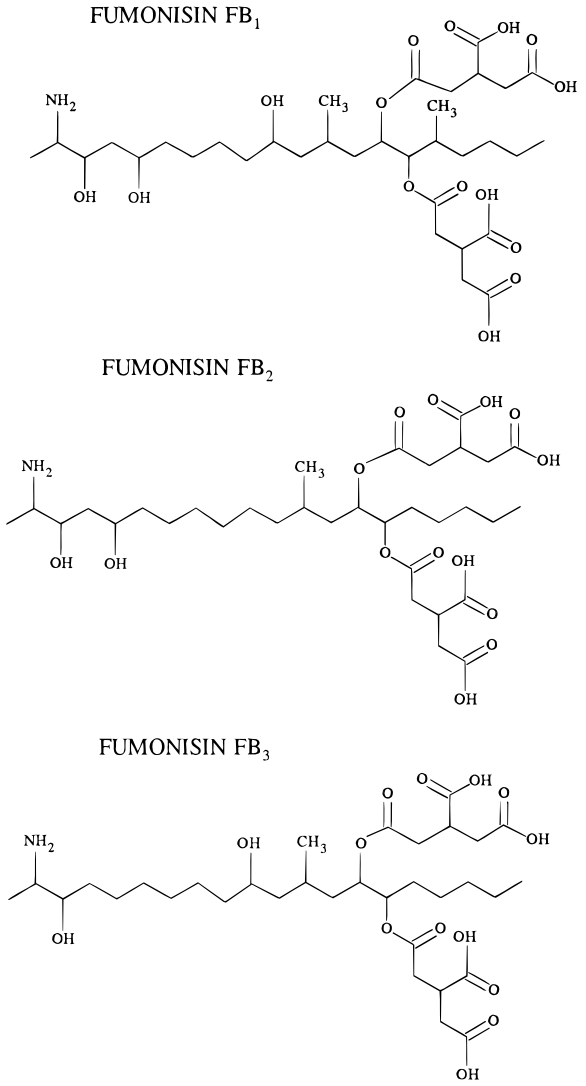

Figure 1. Chemical structures of FB_1, FB_2, and FB_3.

combined. The extract was reduced to a little less than half its volume on a 10-L vacuum rotary evaporator, with the water bath temperature at 38 °C. This procedure made the final acetonitrile concentration ~18−20%. The extract was placed into 4-L brown jugs and stored at 2 °C until the preparative LC separations were conducted. The extract was a deep red color from the colored pigments present in the solution (Steyn et al., 1979).

Analytical HPLC. Fumonisins were separated isocratically on a Hewlett-Packard 1090 HPLC connected to a Rainin C_{18} reversed-phase guard column and C_{18} reversed-phase 10 cm × 4.6 mm column that contained packing material of 3 μm particle size. Eluting solvents were 25% water containing 1% orthophosphoric acid and 75% methanol at a flow rate of 0.8 mL/min. Samples were derivatized with the preinjection program capabilities of the 1090 HPLC. The first step was drawing into a sample loop 10 μL of *o*-phthalaldehyde (OPA) (fluoraldehyde, Pierce Chemical Company), 6 μL of sample, and an additional 10 μL of OPA, mixing the three components, waiting 1 min for the derivatization to take place, and injecting the material onto the column. The sample loop was washed with 50 μL of methanol prior to the preinjection sequence and it was also washed with eluting solvent after the injection. The needle, that removed the sample and different derivatizing agents from the vials, was washed with water before entering each vial to obtain each new chemical or sample. Derivatives were detected with a Hewlett-Packard 1046A fluorescence detector at 304 nm excitation and emission cutoff filter at 440 nm.

Preparative HPLC. A Waters Delta 4000 preparative LC system equipped with a gradient elution controller and a gradient mixer was used in the first isolation and purification

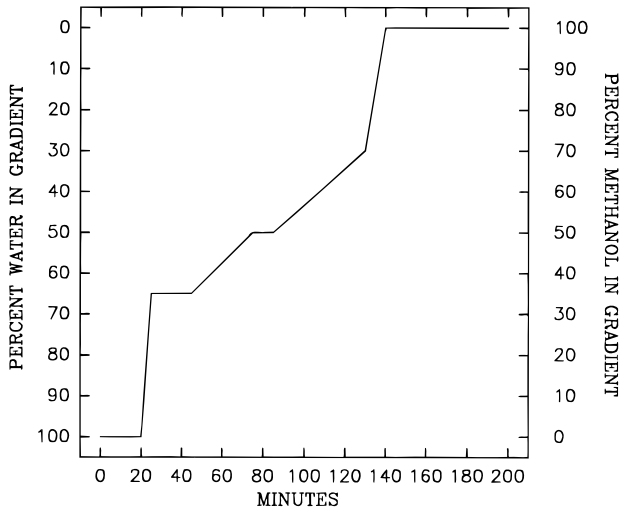

Figure 2. Percent water in the water:methanol stepwise gradient elution used with the reversed-phase preparative C_{18} column of the first isolation procedure.

step. We found that it was important to install a gradient mixer in addition to the "T" that is normally use in mixing the solvents from the pump heads to prevent the formation of bubbles (and erratic flow rates) as methanol was introduced into the elution solvent (water). A Waters Bondapak PrepPak 500/C_{18} reversed-phase cartridge (47 × 300 mm) was conditioned with 5 void volumes (1 void volume = 501 mL) of methanol and 6 void volumes of water before the sample was added.

The sample extract (representing 350 g of rice culture material) was diluted 1:2 with water (3 L of extract diluted with 6 L of water for a total volume of 9 L) and pumped onto the column at a rate of 50 mL/min. The extract, which was a deep red to black color before the dilution, turned a cloudy, light red-brown color upon addition of water. The sample pumped onto the C_{18} column contained 4422 mg of FB_1 as determined by analytical HPLC. After applying the sample to the column, a water:methanol gradient (Figure 2) was begun with a flow rate of 30 mL/min. Samples were collected by volume.

Twenty fractions were collected and analyzed by analytical HPLC to determine which fractions contained FB_1, FB_2, or FB_3. The FB_1 concentration was determined by comparison with a highly purified "standard" prepared as previously described (Plattner et al., 1990). Fractions containing FB_1 were placed on a vacuum rotary evaporator to remove the methanol from the sample. The sample volume was reduced to ~50% of the original volume. Water bath temperature was kept at 38 °C or below to avoid methylation of FB_1. Fractions that contained FB_2 and FB_3 were concentrated under reduced pressure and low heat (30 °C) to remove the methanol, acetonitrile was added to a final concentration of ~20%, and the solutions were stored under refrigeration for isolation at a later date.

The second procedure used for FB_1 purification was an isocratic preparative LC method. A Waters Prep LC/System 500A equipped with two Waters Bondapak PrepPak 47 × 300 mm CN cartridges connected in series was operated at a flow rate of 50 mL/min. The elution solvent was 0.5% pyridine in water. The columns were conditioned with 2 L of methanol, 1 L of acetonitrile and 8 L of water:0.5% pyridine before the sample was introduced.

Fractions from the first chromatography procedure containing FB_1 were combined to give two fractions, A (fractions 5−8) and B (fractions 9−13). Fraction B contained ~3647 mg of FB_1 as determined by analytical HPLC and was chosen for further purification on the CN columns. Fraction A could also be treated similarly to obtain additional pure FB_1. The collection of the fractions was started as soon as the sample was introduced to the column. Fractions isolated from the CN columns were analyzed by analytical HPLC, and those that contained FB_1 were freeze-dried and weights of material recovered were determined. Accurately weighed (microbalance) samples of the isolated FB_1 were analyzed by analytical

Table 1. Characteristics of Fumonisin Fractions Separated by Preparative HPLC with a Reversed-Phase Column and a Water:Methanol Elution Gradient

			concentration, mg		
fraction	time, min	volume, mL	FB_1	FB_2	FB_3
1	0−30	900	np[a]	np	np
2	30−45	450	np	np	np
3	45−60	450	np	np	np
4	60−70	300	np	np	np
5	70−80	300	24	np	np
6	80−90	300	102	np	np
7	90−95	150	150	np	np
8	95−100	150	200	np	np
9	100−105	150	350	np	np
10	105−110	150	600	np	np
11	110−120	150	978	np	np
12	120−125	150	1328	np	np
13	125−130	150	391	np	np
14	130−135	150	106	np	p
15	135−140	150	54	p	p
16	140−150	300	10	p	p
17	150−160	300	np	p	np
18	150−160	300	np	np	np
19	160−170	300	np	np	np
20	170−200	900	np	np	np

[a] np, not present. [b] p, present.

HPLC and compared with a reference standard of FB_1 to determine the purity of the final preparations.

The fractions containing FB_1 were placed onto a preparative 1 × 45 cm column packed with C_{18} from a Waters Bondapak PrepPak 500 C_{18} cartridge. The column was washed with 500 mL of water at 30 mL/min to remove the pyridine from the FB_1. The fumonisin FB_1 was eluted from the column with 70% acetonitrile:water. The acetonitrile was removed from the sample by rotary evaporation at low heat (38 °C) and freeze-dried.

Mass Spectrometry. Samples were analyzed by fast-atom bombardment/mass spectrometry (FAB/MS) on a Finnigan TSQ-700 spectrometer equipped with an Ion Tech fast-atom gun. A sample of the isolated and purified FB_1 was dissolved in water to a concentration of 1 mg/mL. A 1 μL aliquot of the sample was placed into a small drop (~1 μL) of glycerol on the FAB/MS probe, and the FAB spectrum was recorded. Quantitation was done by the method of Plattner and Branham (1994). A second aliquot (25 μL) of the sample was mixed with an equal volume of deuterium-labeled FB_1 (Plattner and Branham, 1994), and the ratio of the signals at m/z 722 (FB_1) and m/z 728 (deuterated FB_1) were recorded. The average ratio of three FAB/MS analyses of the sample was divided by the average ratio of signals at m/z 722 and m/z 728 for three analyses of a mixture of equal amounts of FB_1 and deuterium-labeled FB_1 to determine sample purity (Plattner and Branham, 1994).

RESULTS AND DISCUSSION

Rice was used as a substrate in place of corn as we felt that a cleaner extract with fewer small fine particles, was obtained. This decrease in small fine particles allowed the extract to filter more quickly and resulted in fewer problems in the valves of the preparative pump. Also, fewer pigments were produced, as indicated by less color in the rice extract compared with in the corn extract, thus allowing FB_1 to be purified more easily.

The 20 fractions that were collected from the water:methanol gradient elution on reversed-phase C_{18} are listed in Table 1, with the time that each fraction was collected, volume of each fraction, and indication of which fraction contained FB_1, FB_2, and FB_3. Fractions 5−16 contained varying quantities of FB_1, with fraction 12 containing the greatest amount (1328 mg). Fractions 14−18 contained a mixture of FB_1, FB_2, and/or FB_3. The total quantity of FB_1 isolated in all the fractions for the first isolation step was 4293 mg, for a recovery of 97.1% of the 4422 mg in the original extract. The color of the fractions of interest varied from colorless, slight red, orange-red, to dark red.

Table 2. Characteristics of Fumonisin Fractions Separated by Isocratic Preparative HPLC with Two Cyano Columns and a Water:0.5% Pyridine Elution Gradient

				purity, %	
fraction	time, min	volume, mL	concentration, mg	HPLC	FAB/MS
1	0−10	500	60	50	40.3
2	10−15	250	125	64	—[a]
3	15−20	250	140	78	—
4	20−25	250	180	89	—
5	25−30	250	221	95	93
6	30−35	250	320	95	—
7	35−40	250	440	96	—
8	40−45	250	567	97	—
9	45−50	250	831	97	104
10	50−60	500	432	98	101.6
11	60−80	1000	71	85	—
12	80−100	1000	—	—	—
total			3387		

[a] —, not determined.

Twelve fractions were collected from the CN cartridges (Table 2). The total quantity of FB_1 isolated in fractions 1−11, which contained the compound of interest (FB_1), was 3387 mg. This amout represents a recovery of 92.8% of the concentration of FB_1 from fraction B (combined fraction 9−13; 3647 mg) applied to the CN cartridges. Fractions 1−12 were colorless, but fractions 1−4 contained various impurities, as indicated by the percent area of only 50% for FB_1 that was determined by analytical HPLC of fraction 1; however, the percent area or purity increased with each successive fraction. Purity of FB_1, as determined by FAB/MS, was compared with that of deuterated-labeled FB_1. This procedure has a precision of 95% (or greater; Plattner and Branham, 1994). The purity of fraction 5 expressed as the ratio of FB_1 to deuterated-FB_1, was 93%, as determined by FAB/MS, and 95%, as determined by HPLC. Fraction 10 had a purity of 101.6%, as determined by FAB/MS, and 98%, as determined by HPLC. The percent recovery of the FB_1 that had a purity determined by analytical HPLC of 95% or better was 77% (2811 mg from the CN cartridges and 3647 mg of fraction B) and the overall recovery of the high purity FB_1 was 64% (2811 mg and total FB_1 applied of 4422 mg).

Spectra generated by FAB/MS are a good way of confirming FB_1, because they show abundant protonated molecules and little fragmentation (Plattner and Branham, 1994). The FAB/MS spectrum of fraction 10 is presented in Figure 3 in which the largest signal represents the protonated molecule m/z 722 (FB_1). FB_1 fragmentation ions are described by Powell and Plattner (1995). HPLC analysis using OPA derivatization did not produce any peaks other than that for FB_1. Therefore, no other OPA reacting components are present.

The procedure described allows large-scale isolation of FB_1 in gram quantities with a reasonable purity for toxicological studies. However, FB_2 and FB_3 cannot be purified with the CN preparative columns. To keep FB_2 and FB_3 in solution for application to the columns, at least 10−12% acetonitrile must be present. This amount of organic solvent allows the impurities to be washed through the column with the compound of interest.

SUMMARY

Fusarium moniliforme MRC 826 produces about the same concentration of fumonisins on rice (12 mg of FB_1/g

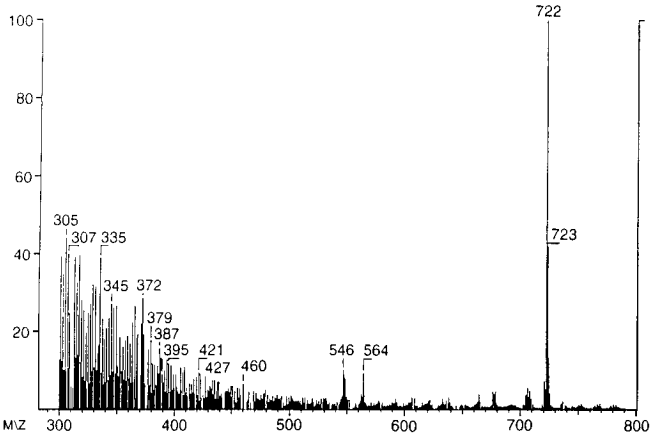

Figure 3. FAB/MS spectrum of FB_1 in fraction 10 isolated from *Fusarium* moniliforme MRC 826 grown on rice culture. The major signal, *m/z* 722, represents FB_1.

of culture material) as on corn. The primary advantages of rice culture material over corn culture material are that rice produces less particulate material therefore, fewer problems occur with the LC pumps; and rice culture produces fewer pigments and other contaminates, which makes purification of FB_1 easier. The Cawood et al. (1991) and Vesonder et al. (1990) procedures for purification of FB_1 require several steps and the use of acid and methanol during the chromatography, which can result in degradation of fumonisin during the isolation. The extract applied to the preparative C_{18} cartridge represented 350 g of rice culture and the total FB_1 applied to the cartridge was 4492 mg. The recovery of FB_1 for the first LC purification step was 97% (4293/4492 mg). The percent recovery of FB_1 from fraction B in the second LC purification step was 92.8% (3387/3647 mg) of which 77% was 95% pure (2811/3647 mg).

Additional studies will be conducted to further improve preparative procedures for the large scale preparation of FB_2 and FB_3 in sufficient quantities for biological studies.

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Registry No. Supplied by the Author: Acetonitrile, 75-050; glycerol, 56-81-5; methanol, 67-56-1; orthophosphoric acid, 56-81-1.

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