Isolation of the Fumonisin Mycotoxins: A Quantitative Approach

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A method for the preparative-scale isolation of the fumonisin B (FB) mycotoxins, from corn cultures of *Fusarium moniliforme*, is described and quantitatively evaluated. Eighty percent of FB_1 and 60% of FB_2 were recovered after extraction with CH_3OH/H_2O (3:1). The fumonisins, including the newly discovered FB_3 and FB_4, were purified using Amberlite XAD-2, silica gel, and reverse-phase C_{18} chromatography. The Amberlite XAD-2 purification step proved to be the most effective cleanup procedure, while subsequent chromatography on silica gel and RP C_{18} effectively separate the individual fumonisins to a purity of over 90%. The relatively low final yield (40%) of FB_1 and FB_2 may be ascribed to (1) the strong affinity of FB_1 for silica gel, (2) the low initial recovery (60%) of FB_2, and (3) the formation of monomethyl and dimethyl esters of FB_1 and FB_2, as well as their interference in the purification of the individual fumonisins. The *N*-acetyl derivatives of FB_1 and FB_2 were also purified and shown to be metabolites of *F. moniliforme*.

INTRODUCTION

Recent investigations on the mycotoxins produced by *Fusarium moniliforme* Sheldon indicate that the fumonisin mycotoxins could be responsible for the major toxicological and carcinogenic effects caused by corn cultures of the fungus in experimental animals (Gelderblom et al., 1988a,b, 1991; Kellerman et al., 1990; Marasas et al., 1988; Voss et al., 1989). At present little is known about the biological effects of these toxins in different animal species. The need to develop efficient and cost-effective methods for purifying sufficient quantities for biological evaluation is therefore of particular importance since the fumonisin mycotoxins occur under natural conditions (Sydenham et al., 1990a,b; Shephard et al., 1990) and could enter the human and animal food chains.

The first paper on the isolation and purification of fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) dealt mainly with the detection of cancer-promoting compounds from cultures of a carcinogenic strain of *F. moniliforme*; the purification techniques were described only qualitatively (Gelderblom et al., 1988a). Extraction of the fumonisin mycotoxins was achieved with CH_3OH/H_2O (3:1) followed by a solvent-partitioning step using $CHCl_3$. The subsequent purification of the aqueous phase was effected on Amberlite XAD-2, silica gel, and reverse-phase (C_{18}) chromatographic columns yielding FB_1 and FB_2, both with a purity of approximately 90%.

This paper describes methods for the quantitative purification of FB_1, FB_2, and the newly discovered fumonisin B_3 (FB_3) from corn cultures of *F. moniliforme* strain MRC 826. In addition, several other structurally related compounds, including fumonisin A_1 (FA_1) and fumonisin A_2 (FA_2), which were previously observed during the purification of FB_1 and FB_2 (Gelderblom et al., 1988a; Bezuidenhout et al., 1988), and fumonisin B_4 (FB_4) were purified and their chemical structures determined.

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MATERIALS AND METHODS

Chemicals. Amberlite XAD-2 and silica gel 60 (Kieselgel 60, 0.063–0.200 mm) were purchased from Merck SA. The reverse-phase (RP) packing material (C_{18}; Bondesil; 40 μm) was obtained from Analytichem International, Harbour City, CA, or prepared from silica gel 60 (0.063–0.200 mm) according to the method of Kingston and Gerhart (1976). All of the organic solvents were of analytical grade and glass-distilled prior to use during the final column purification steps on silica gel and RP C_{18}.

Fungal Cultures. Corn cultures of *F. moniliforme* strain 826 were prepared as described previously (Alberts et al., 1990) and incubated in the dark for 6 weeks at 25 °C. Cultures were oven-dried at 50 °C, ground, and stored at 4 °C until required.

Fumonisin Standards. FB_1, FB_2, and FB_3 (Figure 1) were purified according to the method described in the present paper. For use as analytical standards the fumonisins were further subjected to two successive column separations on silica gel (second column) and reverse-phase (C_{18}) columns as described below. The purity of both standards was verified by nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC) (Alberts et al., 1990), and silica gel thin-layer chromatography (TLC), and they are considered for the purpose of this study as 100% pure.

Detection and Quantification of the Fumonisins. All of the sample extracts and column fractions were analyzed by silica gel TLC for the presence of the different fumonisins using $CHCl_3/CH_3OH/H_2O/CH_3COOH$ (55:36:8:1) and/or $CHCl_3/CH_3OH/CH_3COOH$ (6:3:1) as developing solvents. The TLC plates were developed by spraying with a *p*-anisaldehyde [0.5 g of *p*-anisaldehyde in $CH_3OH/CH_3COOH/H_2SO_4$ (8:10:5)] and/or a ninhydrin (0.2% in ethanol) solution and heated at 120 °C until color development. On the basis of the TLC analyses, column fractions were combined in such a manner that the most effective separation between the individual fumonisins was accomplished. Subsamples of the combined column fractions and sample extracts were kept throughout the purification process for the quantification of FB_1, FB_2, and FB_3.

Quantification of the fumonisins in the sample extracts and combined column fractions was achieved by HPLC analyses of the maleyl derivatives as described previously (Alberts et al, 1990). The mobile phases used consisted of (1) CH_3OH/0.1 M KH_2PO_4 (7:3; 10 mM $MgCl_2$; pH 3.5) for FB_1 analyses and (2) CH_3OH/0.1 M KH_2PO_4 (18:7; 10 mM $MgCl_2$; pH 3.5) for FB_2 and FB_3 analyses.

Extraction and Solvent Partitioning. Culture material (1 kg) was extracted twice with ethyl acetate (1 L) by blending (Waring blender) and filtering (Whatman No. 4). The residue

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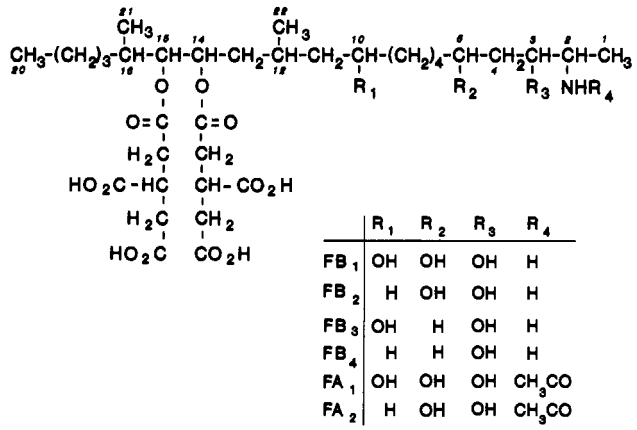
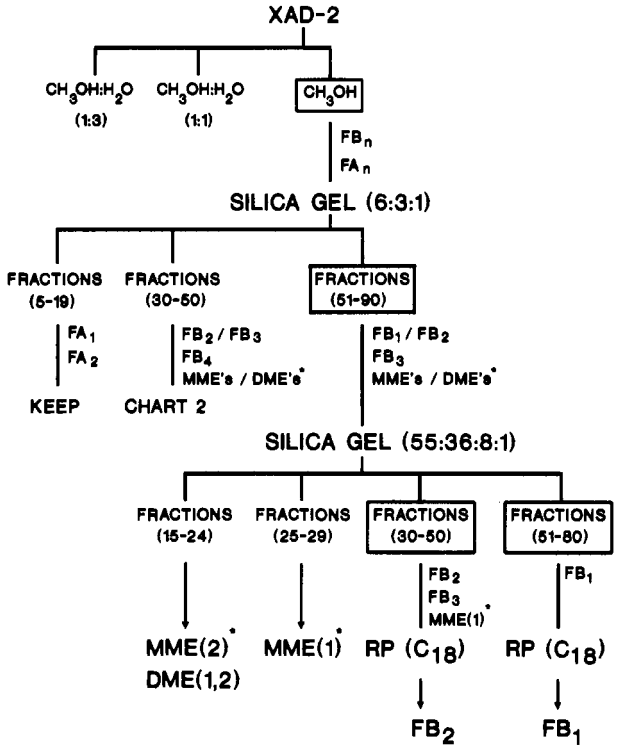


	R_1	R_2	R_3	R_4
FB_1	OH	OH	OH	H
FB_2	H	OH	OH	H
FB_3	OH	H	OH	H
FB_4	H	H	OH	H
FA_1	OH	OH	OH	CH_3CO
FA_2	H	OH	OH	CH_3CO

Figure 1. Chemical structures of the different fumonisins produced by *F. moniliforme*.

Chart I. Schematic Representation of the Procedures Used for the Isolation of Fumonisins B_1 (FB_1) and B_2 (FB_2)



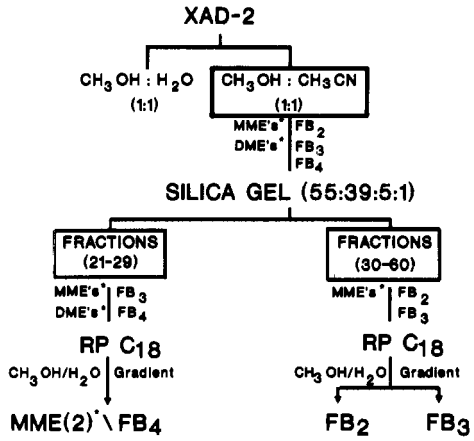
* MMEs/DMEs, monomethyl esters/dimethyl esters; MME-(1), monomethyl ester of FB_1; MME(2), monomethyl ester of FB_2; DME(1,2), dimethyl ester of FB_1 and FB_2, respectively.

was extracted once with 1 L and twice with 1.5 L of CH_3OH/H_2O (3:1) as described above. The combined extracts were evaporated to dryness under vacuum at 50 °C; the residual material was dried at 60 °C and stored at 4 °C for FB_1 and FB_2 analyses. The dried culture extract was dissolved in CH_3OH/H_2O (1:3, 200 mL) at 50 °C and partitioned with $CHCl_3$ (3 × 100 mL) as described previously (Gelderblom et al., 1988a). The aqueous phase was evaporated to dryness under vacuum at 50 °C. A schematic diagram of the column chromatographic purification procedures for the different fumonisins is outlined in Charts I and II.

Purification of FB_1. *Amberlite XAD-2.* The above extraction residue (125 ± 32 g, Table I) was dissolved in CH_3OH/H_2O (1:3; 100 mL) and applied to an Amberlite XAD-2 column (7 × 87 cm; sample:resin 1:15) previously equilibrated with CH_3OH/H_2O (1:3). After the column was washed successively with CH_3OH/H_2O (1:3; 1 L) and CH_3OH/H_2O (1:1; 1.5 L), the fumonisin mycotoxins were eluted with CH_3OH (1 L).

Silica Gel 60. First Column [$CHCl_3/CH_3OH/CH_3COOH$ (6:3:1) as Eluant]. The residue obtained from the methanol fraction

Chart II. Schematic Representation of the Procedures Used for the Isolation of Fumonisins B_2 (FB_2), B_3 (FB_3), and B_4 (FB_4)



* MMEs, monomethyl esters, DMEs, dimethyl esters; MME-(2), monomethyl ester of FB_2.

Table I. Quantification of the Extraction of FB_1 and FB_2 from Fungal Culture Material

sample	wt, g	FB_1 concn,[a] g	FB_2 concn,[a] g
corn culture	1000	2.24 ● 0.40	1.12 ± 0.33
ethyl acetate	81.1 ± 3.4	nd[b]	nd[b]
CH_3OH/H_2O (3:1)	125.2 ± 17.6	1.82 ± 0.5 (81.3%)[c]	0.67 ± 0.2 (59.8%)[c]

[a] Values represent means ± standard deviation of two different samples each done in triplicate. [b] nd, not detected. [c] The percentage of FB_1 and FB_2 recovered in the CH_3OH/H_2O (3:1) fraction is given in parentheses.

(10 ± 0.6 g) was first fractionated on a silica gel column (5.5 × 85 cm; 1100 g) using $CHCl_3/CH_3OH/CH_3COOH$ (6:3:1) as the mobile phase. The sample was dissolved in the eluant (70 mL) and applied to the column which contained anhydrous Na_2SO_4 (ca. 50 g) on top of the silica gel. After elution of 1.3 L of mobile phase at a flow rate of 3.5 mL/min, fractions (50 mL) were collected and analyzed by TLC. Fractions containing FA_1 and FA_2 (5–19), FB_2 and FB_3 (30–50), and FB_1, FB_2, and FB_3 (51–90) were combined separately (Chart I) and the solvents evaporated at 50 °C under reduced pressure. The subsequent purification of the FB_2/FB_3 mixture (fractions 30–50) and the other structurally related compounds is described separately.

Second Column [$CHCl_3/CH_3OH/H_2O/CH_3COOH$ (55:36:8:1) as Eluant]. The main FB_1 fraction (51–90; 4.8 ± 0.22 g) was purified by silica gel column chromatography as described above, but without the Na_2SO_4, using $CHCl_3/CH_3OH/H_2O/CH_3COOH$ (55:36:8:1) as eluant. The sample, dissolved in 20 mL of the eluant, was applied to the column and eluted at 2 mL/min. After elution of 1.2 L of mobile phase, the flow rate was changed to 3.5 mL/min while fractions (50 mL) were collected and analyzed by TLC. Fractions 50–80 (2.14 ● 0.32 g) contained only FB_1. The fractions containing a mixture of FB_2, FB_3, and other related compounds, such as monomethyl esters (MMEs) and dimethyl esters (DMEs), were combined separately (Chart I).

Reverse Phase (C_{18}). The final purification of FB_1 was achieved on a RP C_{18} column (1.5 × 50 cm; 200 g) equipped with end fittings and using CH_3OH/H_2O (1:1) as eluant. The eluant flow was maintained at 1.5 mL/min with the aid of a peristaltic pump. The pH of the sample, dissolved in CH_3OH/H_2O (1:1; 20 mL), was adjusted to 3.5 with 1 N HCl prior to application to the column. Fractionation was effected by running a 800-mL linear gradient from CH_3OH/H_2O (1:1) to CH_3OH/H_2O (4:1) at a flow rate of 1.5 mL/min. Fractions (15 mL) were collected after 400 mL of the gradient had eluted from the column. The purity of FB_1 (1.33 ± 0.44 g), which eluted as a single peak from the column (fractions 8–15), was determined by HPLC (Alberts et al., 1990).

Purification of FB_2. *Silica Gel.* During the first chromatographic separation on silica gel the FB_3-containing fractions (30–50) and the FB_1-containing fractions (51–90) each contained

similar amounts of FB_2. The purification of FB_2 from the FB_2/FB_3 sample will be described separately. The FB_2 that coeluted with FB_1 and some FB_3 was fractionated on a silica gel column as described above (second column). As indicated in Chart I, FB_2 eluted first from the column (fractions 30–50) followed by FB_1, while a small amount of FB_3 coeluted with FB_2.

Reverse Phase (C_{18}). The C_{18} material, prepared from silica gel as described by Kingston and Gerhart (1976), was equilibrated with CH_3OH/H_2O (1:1) and packed in a column (3 × 27 cm) supplied with end fittings. The sample, at a pH of 3.5, was fractionated using a 800-mL linear gradient from CH_3OH/H_2O (1:1) to CH_3OH while the flow was maintained at 1.5 mL/min. Fractions (15 mL) were collected after 300 mL had eluted from the column. FB_2, with a purity of 90%, was obtained in fractions 17–30. FB_3, which coeluted with FB_2 during the course of the second silica gel column fractionation, was obtained in fractions 5–14 with a purity of only 20%.

Purification of FB_3. *Amberlite XAD-2.* The material containing FB_2 and FB_3 (fractions 30–50, Chart I), obtained from the first silica gel column purification, was further fractionated on Amberlite XAD-2 (Chart II). The Amberlite XAD-2 column (4.5 × 30 cm) was equilibrated with CH_3OH/H_2O (1:1), and the sample (3.24 ± 0.2 g), dissolved in the same solvent, was applied after the pH had been adjusted to 3.5 with 0.1 N HCl. After the column was washed with 400 mL of CH_3OH/H_2O (1:1), the fumonisins were eluted with CH_3OH/CH_3CN (1:1; 250 mL). Fractions containing both FB_2 and FB_3 were combined and the solvents evaporated in vacuo at 50 °C.

Silica Gel 60. A silica gel column (5 × 45 cm; 300 g) was equilibrated with $CHCl_3/CH_3OH/H_2O/CH_3COOH$ (55:39:5:1). The material obtained from the Amberlite XAD-2 column (2.16 ± 0.20 g) was applied to the column, and after elution of 450 mL (1.0 mL/min), fractions (30 mL) were collected. Fractions 30–60, containing a mixture of FB_2 and FB_3, were pooled and the solvents evaporated at 50 °C.

Reverse Phase (C_{18}). Final separation of FB_2 and FB_3 was achieved on the RP C_{18} column described above. The column was first regenerated by successive elution with CH_3OH (250 mL) containing 0.1 M $MgCl_2$ and H_2O (250 mL) and subsequently equilibrated with CH_3OH/H_2O (1:1). After application of the sample (1.99 ± 0.10 g, pH 3.5), separation was achieved by gradient elution as described earlier using a 800-mL gradient from CH_3OH/H_2O (1:1) to CH_3OH. FB_3 (0.38 ± 0.02 g), which eluted first, chromatographed as a single spot on TLC and was subjected to further analyses (HPLC and NMR) to confirm its purity. FB_2 (0.37 ± 0.01 g) with a purity of 92% was obtained from subsequent fractions.

Purification of Structurally Related Compounds. In the course of purifying FB_1, FB_2, and FB_3 several other compounds that reacted similarly to the fumonisins with both *p*-anisaldehyde and ninhydrin were observed by silica gel TLC. On the basis of their reaction with ninhydrin these compounds were divided into ninhydrin-positive and ninhydrin-negative compounds.

Ninhydrin Positive Compounds. TLC analysis of the CH_3OH fraction from the Amberlite XAD-2 column and the fractions obtained during its subsequent purification on silica gel (first column) indicated the presence of several spots from compounds with higher R_f values than FB_1 and FB_2 (Table IV). These spots were eventually shown to consist of a mixture of the monomethyl and dimethyl esters of both FB_1 and FB_2 and another fumonisin given the trivial name of FB_4 (Figure 1). The same fractionation steps used during the purification of FB_2 and FB_3 were applied for the isolation of FB_4 (Chart I). Subsequent fractionation of FB_4, after the RP C_{18} purification step, was performed on a silica gel column using $CHCl_3/CH_3OH/H_2O/CH_3COOH$ (55:36:8:1) as eluant. The FB_4 was finally fractionated through a Sep-Pak C_{18} cartridge as described below and subjected to structural analyses.

After the second silica gel column, a pure preparation of a mixture of monomethyl esters (MMEs) of FB_1 containing some inorganic compounds was obtained (Chart I). The fraction containing the mixture of DMEs of FB_1 and FB_2 and the MME of FB_2 was further fractionated on a silica gel column (2 × 60 cm) using $CHCl_3/CH_3OH/H_2O/CH_3COOH$ (55:36:8:1) as the eluant. This chromatographic system achieved good separation between the DME of FB_1 and the MME and DME of FB_2. Final purification of the MMEs and DMEs of FB_1 and FB_2 for structure elucidation by NMR was achieved on a C_{18} Sep-Pak cartridge (Waters, Millipore Corp., Milford, MA). The cartridge was washed with CH_3OH and equilibrated with CH_3OH/H_2O (1:1). After application of the sample (pH 3.5), 20 mL of the eluant was passed through the cartridge. The compounds were eluted using CH_3OH. The solvent was evaporated and the pure compounds subjected to structural analyses.

Ninhydrin Negative Compounds. Fractions obtained during chromatography on the first silica gel column revealed two spots on TLC with R_f values of 0.38 and 0.46, respectively, upon spraying with *p*-anisaldehyde (Table IV). These compounds, called FA_1 and FA_2 (Bezuidenhout et al., 1988) (Figure 1), were further purified on an Amberlite XAD-2 column (4.5 × 30 cm) using the same elution procedures as described for the purification of FB_3. The material, still containing both FA_1 and FA_2, was subsequently fractionated on a silica gel column (2 × 65 cm) using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (65:25:6:4) as eluant. TLC analyses of the fractions indicated that FA_1 (fractions 30–40) and FA_2 (fractions 20–27) were cleanly separated and each combined fraction exhibited a single spot on TLC. The final purification of each compound was achieved on a RP C_{18} column (3 × 30 cm) equilibrated with CH_3OH/H_2O (1:1). The compounds were eluted in CH_3OH/H_2O (3:1) after the column had been washed with the equilibration solvent (250 mL).

The ratio of sample to RP packing material was approximately 1:100, whereas for silica gel it was 1:250. The pH of all the samples was adjusted to 3.5 prior to fractionation on Amberlite XAD-2 and RP C_{18} columns. For samples of 50 mg or less final purification was accomplished using Sep-Pak C_{18} cartridges.

RESULTS AND DISCUSSION

In the present study the fundamental extraction and purification steps reported previously (Gelderblom et al., 1988a) were used, with minor modifications, for the isolation of FB_1, FB_2, FB_3, and FB_4 and other structurally related compounds. Chemical structures of the newly discovered fumonisins B_3 and B_4 are given in Figure 1.

Extraction of the fumonisins was carried out using CH_3OH/H_2O (3:1) after the culture material had been extracted with ethyl acetate to remove lipid-soluble material. The latter step was very effective in that, although approximately 8% of material was recovered from the ethyl acetate extract, no fumonisin mycotoxins were extracted (Table I). Subsequent extraction with aqueous CH_3OH resulted in an 81.3% recovery of FB_1 from the culture material (Table I). The recovery of the analytical technique for the quantitation of FB_1 in fungal cultures, using the same extraction solvent, is on the order of 85% (Alberts et al., 1990). In contrast, a far lower yield of FB_2 (59.8%) was obtained which could partly be ascribed to the extraction solvent used, as it was selected for the isolation and quantification of FB_1 (Alberts et al., 1990). From this study it would appear that aqueous methanol is not a good extraction solvent for FB_2, and further investigation is necessary.

A high-percentage recovery of both FB_1 (98%) and FB_2 (97.5%) was obtained from the aqueous phase after the $CHCl_3$ partitioning step, while a low (1.1-fold) purification was achieved. The aim of the solvent-partitioning step is, as in the case with the ethyl acetate extraction, to remove lipid-soluble material that could interfere during the Amberlite XAD-2 column purification step.

Chromatographic separation on the Amberlite XAD-2 column was performed by a stepwise increase in the CH_3OH content of the eluting solvent (Chart I). Fractionation on this column is based on group separation. The bulk (>94%) of the fumonisins was recovered in the final methanol eluate (Tables II and III), while a high degree of purification was achieved (12.1-fold for FB_1, 8.7-fold

Table II. Quantification of FB_1 and FB_2 during Successive Column Purification Steps

purification step	sample wt,[a] g	fumonisin wt,[a] g	recovery,[b] %	fumonisin concn, %	purification[c] degree	purification[c] fold
solvent partitioning	125.0 ± 32.6	FB_1 2.64 ± 0.28	98	2.1		1.1
		FB_2 1.23 ± 0.17	97.5	1.0		
XAD (CH_3OH)	(i) 10.0 ± 0.6	FB_1 2.55 ± 0.18	98	25.5	12.1	12.5
	(ii) 13.3 ± 0.1	FB_2 1.16 ± 0.16	94	8.7	8.7	9.6
silica gel (6:3:1)	(i) 4.75 ± 0.22	FB_1 1.90 ± 0.15	74.5	40.0	1.6	26
	(ii) 5.60 ± 0.22	FB_2 0.68 ± 0.03	58	12.1	1.4	22
silica gel (55:36:8:1)	(i) 2.14 ± 0.32	FB_1 1.30 ± 0.35	68	60.7	1.5	58
	(ii) 1.30 ± 0.07	FB_2 0.49 ± 0.14	72	37.7	3.1	96
RP (C_{18})	(i) 1.33 ± 0.44	FB_1 1.20 ± 0.29	91	90.2	1.5	94
	(ii) 0.50 ± 0.04	FB_2 0.46 ± 0.01	94	92.0	2.4	250

[a] All values represent means ± standard deviation calculated from performing each purification in triplicate. The quantitation of the recoveries of FB_1 and FB_2 was done in two separate triplicate series. [b] Percentage fumonisin recovered during each purification step. [c] Degree of purification represents the increase in fumonisin concentration achieved by each purification step, while the fold represents the extent of purification of each fumonisin after the solvent-partitioning step.

Table III. Quantification of FB_2 and FB_3 during Successive Column Purification Steps

purification step	sample wt,[a] g	fumonisin wt,[a] g	recovery,[b] %	fumonisin concn, %	purification[c] degree	purification[c] fold
solvent partitioning	125 ± 32.6	FB_2 1.23 ± 0.17		1.0		
		FB_3 0.62 ± 0.08		0.5		
XAD (CH_3OH)	13.3 ± 0.1	FB_2 1.16 ± 0.16	(a) 94	8.7	8.7	9.6
		FB_3 0.61 ± 0.08	(b) 98	4.6	9.2	9.6
silica gel (6:3:1)	3.24 ± 0.17	FB_2 0.49 ± 0.06	(a) 42	15.1	1.7	39
		FB_3 0.52 ± 0.08	(b) 85	16	3.5	39
XAD (CH_3OH/CH_3CN)	2.16 ± 0.20	FB_2 0.46 ± 0.09	(a) 94	21.3	1.4	58
		FB_3 0.51 ± 0.08	(b) 98	23.6	1.5	58
silica gel (55:39:5:1)	1.99 ± 0.10	FB_2 0.35 ± 0.02	(a) 76	17.6	0.8	63
		FB_3 0.36 ± 0.03	(b) 71	18.1	0.8	63
RP (C_{18})	(a) 0.37 ± 0.02	FB_2 0.33 ± 0.03	(a) 94	89.2	5.1	(a) 338
	(b) 0.38 ± 0.02	FB_3 0.36 ± 0.02	(b) 99	94.7	5.2	(b) 328

[a] All values represent means ± standard deviation calculated from performing each purification step in triplicate. [b] Percentage fumonisin recovered during each purification step. [c] Degree of purification represents the increase in fumonisin concentration achieved by each purification step, while the fold represents the extent of purification of each fumonisin after the solvent-partitioning step. Quantitation of FB_2 and FB_3 eluted from the XAD-2 column was performed on two different CH_3OH eluates (a and b).

for FB_2, Table II; 9.2-fold for FB_3, Table III). This chromatographic procedure is therefore by far the most effective purification step for removing residual material other than the fumonisins, although no separation between the individual fumonisins is obtained. In addition, polar components that would interfere with the subsequent purifications on silica gel were effectively removed from the aqueous extract.

The most effective separation between the different fumonisins was obtained on silica gel by using two different mobile phases. Both column separations resulted in approximately 70% recovery of FB_1 (Table II). During the first silica gel column separation, using $CHCl_3/CH_3OH/CH_3OOH$ (6:3:1) as the eluant, most of the pigmented material (Steyn et al., 1979) present in culture extracts of *F. moniliforme* MRC 826 was separated from FB_1, FB_2, and FB_3. When this purification step was omitted, some of the pigments were present in the final FB_1 preparation. This purification step did not completely separate FB_1 from FB_2 and FB_3, but as the aim of this study was directed at the purification of FB_1, the fractions were combined separately, thus yielding two main fractions. One fraction (fractions 51–90) contained the bulk of the FB_1 as well as about 50% of the FB_2 and far less FB_3, while the other fraction (fractions 30–50) consisted mainly of FB_3 and some FB_2 (Chart I). The recovery of FB_3 from the first silica gel column was 85% (Table III). The FB_2 was recovered quantitatively from the first silica gel column with 58% being obtained from the fractions containing FB_1 (Table II) and 42% from those containing FB_3 (Table III). The lower recovery for FB_1 (74.5%, Table II) can be explained by the long elution time and its tendency to streak from silica gel.

The advantage of the second silica gel column purification step in the isolation of FB_1 was that FB_1 was completely separated from FB_2 as shown by TLC. However, the material recovered in the combined FB_1 fractions contained much methanol-insoluble material, presumably salts, which only dissolved in aqueous methanol at a pH below 3.5. The subsequent RP C_{18} column step was therefore aimed mainly at removing the inorganic material while the gradient elution of FB_1 also removed some yellow pigments that coeluted with FB_1 from the second silica gel column. A high recovery (91%) and relatively low purification (1.5-fold) of FB_1 was achieved in this chromatographic purification step (Table II).

Although the present method was developed mainly to purify FB_1, it became clear that, with minor modifications in the sequence of the columns and eluants used, FB_2 and FB_3 could also be purified without any major difficulties. Approximately 50% of the FB_2 was purified to over 90% purity by applying the same column purification procedures as for FB_1 (Chart I; Table II). The introduction of another Amberlite XAD-2 column in the purification of the main FB_2/FB_3 sample (containing the other half of the FB_2) was to effect the removal of CH_3OH-insoluble material which coeluted with this fraction from the first silica gel column. As discussed earlier, this material elutes mainly with FB_1 from the second silica gel column. The use of CH_3CN in place of CH_3OH during the second Amberlite XAD-2 chromatographic step was necessary because of the lower polarity of FB_2 and FB_3 in comparison to that of FB_1 (Table IV). A very high recovery of FB_2 (94%) and FB_3 (98%) from the Amberlite XAD-2 column was achieved (Table III). FB_2 and FB_3 coeluted from both the second Amberlite XAD-2 column and the subsequent

Table IV. Chemical and Chromatographic Characteristics of the Fumonisins and Structurally Related Derivatives

compound	R_f value[a]	FB_1	FB_2
FB_1	0.23 (0.15)[b]		
FB_2	0.30 (0.20)		
FB_3	0.30 (0.26)		
FB_4	0.37 (0.31)		
MMEs(1)[c]	0.35 (0.27)	monomethyl ester	
MMEs(2)[c]	0.42 (0.34))		monomethyl ester
DMEs(1)[c]	0.48 (0.42)	dimethyl ester	
DMEs(2)[c]	0.54 (0.46)		dimethyl ester
FA_1	0.38 (0.71)	*N*-acetyl derivative	
FA_2	0.46 (0.76)		*N*-acetyl derivative

[a] Silica gel TLC using $CHCl_3$:CH_3OH:H_2O:CH_3COOH (55:36:8:1) as developing solvent. [b] Values in parentheses represent R_f values with $CHCl_3$:CH_3OH:CH_3COOH (6:3:1) as developing solvent. [c] MMEs, monomethyl esters; DMEs, dimethyl esters.

silica gel column but were finally separated on RP C_{18}. The latter step is the only chromatographic procedure which effectively separates FB_2 from FB_3. As indicated in Chart I, FB_4 is also purified during these chromatographic procedures.

Fumonisins B_1, B_2, and B_3 are the major naturally produced fumonisins in corn cultures of *F. moniliforme* strain MRC 826. FB_4, FA_1, and FA_2 are produced only in minor quantities. A major disadvantage of the main separation between the different fumonisins on silica gel is the fact that several structurally related compounds (MMEs and DMEs) are formed from FB_1, FB_2, and probably FB_3 as a result of the presence of CH_3OH and CH_3COOH in the mobile phase (Table IV; Chart I). The carboxylic acid group involved in the formation of each ester is not known. The formation of the MMEs complicates the purification of FB_2 and FB_3 especially during their fractionation on silica gel as they eluted in the same region, as indicated by the R_f values obtained by TLC (Table IV). The same problem is encountered on the C_{18} columns where good separation was obtained between FB_1, FB_2, and FB_3 but not between FB_3 and the MMEs of FB_1 and FB_2, respectively. The purification of FB_4, which appears to be produced only in minor quantities by the fungus, is hampered by the same difficulties. No problems were encountered in the purification of the dimethyl ester derivatives of both FB_1 and FB_2 as they are eluted much earlier from silica gel (higher R_f values).

FA_1 and FA_2 were obtained after the first silica gel column separation, and NMR spectral analyses indicated that these compounds are the *N*-acetyl derivatives of FB_1 and FB_2, respectively. These compounds have previously been reported as metabolites produced by *F. moniliforme* in culture (Bezuidenhout et al., 1988). This finding is confirmed in the present study as there is no indication that either FB_1 or FB_2 is converted into these compounds in the presence of CH_3COOH even when incubated at fairly high temperatures (60 °C).

Although the method described in this paper is not optimal with respect to the quantities of FB_1, FB_2, and FB_3 that are purified, it does provide ample amounts of these mycotoxins for biological evaluation in experimental animals. The high concentration of FB_1 present in culture material of this specific fungal isolate facilitates the purification of FB_1 over that of FB_2 and FB_3 as indicated by the much greater ease (lower purification fold; Table II) with which pure FB_1 can be obtained. At present the fumonisin production profiles of other isolates of *F. moniliforme* are under investigation in an attempt to select those isolates which will allow optimal purification of each individual fumonisin. Other solvent systems for the purification of the fumonisins on silica gel are also under investigation to prevent the formation of the structurally related compounds, thereby increasing the final yield of the pure fumonisins.

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Registry No. FA_1, 117415-48-2; FA_2, 117415-47-1; FB_1, 116355-83-0; FB_1 monomethyl ester, 136458-38-3; FB_1 dimethyl ester, 136458-39-4; FB_2, 116355-84-1; FB_2 monomethyl ester, 136520-22-4; FB_2 dimethyl ester, 136458-40-7; FB_3, 136379-59-4; FB_4, 136379-60-7.