Fumonisin Composition in Cultures of *Fusarium moniliforme*, *Fusarium proliferatum*, and *Fusarium nygami*

Steven M. Musser*

Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, D.C. 20204

Ronald D. Plattner

Bioactive Constituents Research, USDA, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, Illinois 61604

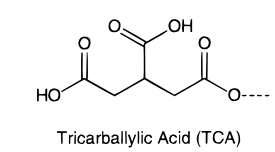
Total fumonisin composition of 22 cultures of *Fusaria* was determined by liquid chromatography−mass spectrometry. All cultures contained primarily FB_{1-3} and FA_{1-2} with little or no variation in the relative percentage of each. In addition to the five principal fumonisins, seven other fumonisins were identified in all cultures; however their concentrations never exceeded 10% relative to FB_1. Of the 22 cultures surveyed, 5 produced low levels (<2 mol %) of the P series of fumonisins and 5 cultures produced high levels of the P series. In cultures producing high levels of FP_1, levels ranged from 20 to 35 mol % of the FB_1 produced by the culture. High-level production of the P series of fumonisins in culture was found to require anaerobic conditions. In cultures grown aerobically, the level of FP_1 never reached more than 2−4% of the FB_1; however cultures grown anaerobically produced levels of 20−35% of FB_1.

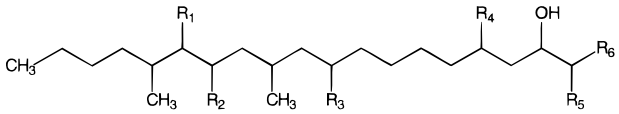
Keywords: *Fumonisins; mass spectrometry; analysis*

INTRODUCTION

Fumonisins are a structurally related group of mycotoxins which are commonly found in corn and corn food products (Chu and Li, 1994; Hopmans et al., 1993). Fumonisins are produced by several species of *Fusarium*; however *F. moniliforme*, *F. proliferatum*, and *F. nygami* are the principal fumonisin-producing strains (Nelson et al., 1991, 1992, 1994). This group of mycotoxins is characterized by a 19−20 carbon aminopolyhydroxyalkyl chain which is diesterified with propane-1,2,3-tricarboxylic acid (tricarballylic acid). The first fumonisins identified were FB_{1-2} (Bezuidenhout et al., 1988); subsequently nine additional structurally related analogs have been described (Figure 1). Among these are amides of fumonisins B_{1-2} (Cawood et al., 1991), 15-keto functionalized FB_1 (Musser et al., 1995), partially hydrolyzed FB_1 (Sydenham et al., 1995), and a newly described series that contains a 3-hydroxypyridinium functionality in place of the amine in the B series (Musser and Plattner, 1996). The structures of fully hydrolyzed FB_1 and FB_2, produced by hydrolysis with base, have been determined (Thakur and Smith, 1996), however they are not known to exist naturally. In general, FB_{1-3} are the most abundant fumonisins found in cultures and naturally contaminated corn; however little analytical data is available comparing the relative abundance of other fumonisins found in corn cultures.

The toxicity of the B series of fumonisins has been extensively studied, and a variety of species-specific toxicities have been reported. Fumonisins have been shown to induce leukoencephalomalacia (ELEM) in horses (Kellerman et al., 1990; Ross et al., 1990) and pulmonary edema in swine (Harrison et al., 1990) and to be hepatotoxic and carcinogenic to rats (Gelderblom et al., 1991; Voss et al., 1995). In addition to the known animal toxicities, the International Agency for Research on Cancer has noted that fumonisins may be carcinogenic to humans (Vainio et al., 1993). While the mechanism of fumonisin toxicity is unknown, recent studies demonstrated that fumonisin B_1 is a potent





	R_1	R_2	R_3	R_4	R_5	R_6	M.W.
FA_1	TCA	TCA	OH	OH	$NHCOCH_3$	CH_3	763
FA_2	TCA	TCA	H	OH	$NHCOCH_3$	CH_3	747
FA_3	TCA	TCA	OH	H	$NHCOCH_3$	CH_3	747
FAK_1	=O	TCA	OH	OH	$NHCOCH_3$	CH_3	603
FB_1	TCA	TCA	OH	OH	NH_2	CH_3	721
FB_2	TCA	TCA	H	OH	NH_2	CH_3	705
FB_3	TCA	TCA	OH	H	NH_2	CH_3	705
FC_1	TCA	TCA	OH	OH	NH_2	H	707
FP_1	TCA	TCA	OH	OH	3HP	CH_3	800
FP_2	TCA	TCA	H	OH	3HP	CH_3	784
FP_3	TCA	TCA	OH	H	3HP	CH_3	784
PH_{1a}	TCA	OH	OH	OH	NH_2	CH_3	563
PH_{1b}	OH	TCA	OH	OH	NH_2	CH_3	563

Figure 1. Chemical structures and molecular weights (MW) of the fumonisins.

* Address correspondence to this author at the Instrumentation and Biophysics Branch, HFS-717, Food and Drug Administration, 200 C Street, SW, Washington, DC 20204 [telephone (202) 205-4371; fax (202) 205-4428; e-mail smm@fdacf.dhhs.gov].

S0021-8561(96)00663-2

inhibitor of sphinganine *N*-acyltransferase, causing the accumulation of sphinganine and sphingosine, ultimately resulting in a disruption of membrane function (Wang et al., 1991; Riley et al., 1994; Ramasamy et al., 1995).

The ubiquitous nature of fumonisins in corn products and the toxicity associated with fumonisin ingestion has resulted in a number of surveys to determine levels of the B series in corn and corn products throughout the world (Shephard et al., 1996; Doko et al., 1995; Murphy et al., 1993). While these surveys find fumonisins in virtually all corn-based foods and feeds, the levels are generally low (<1 ppm). The incidence of reported animal toxicoses linked to fumonisins is low or occurs sporadically (Ross et al., 1992), and although elevated levels of fumonisins are generally seen in samples associated with these toxicities, clear definition of levels that will cause toxicity are not apparent. Typically only FB_1 or a combination of FB_1 and FB_2 are measured in naturally contaminated products, even though a complex array of fumonisins are produced.

Unfortunately most of the analytical methods for fumonisins exclude the detection of one or more of the known fumonisins. For example, the most commonly used analytical method for fumonisins, HPLC, requires derivatization of the amine in the B series (Shephard et al., 1990; Scott et al., 1992; Sydenham et al., 1992); thus this method would not detect amides or any of the P series of fumonisins. To solve this problem, liquid chromatography−mass spectrometry (LC−MS) with electrospray ionization can be used for fumonisin analyses. The technique is capable of detecting and quantitating fumonisins regardless of their chemical structure and without any sample pretreatment (Plattner, 1995; Musser, 1996). Using this method we were able to directly analyze culture extracts from a series of fumonisin-producing strains of *Fusaria* and determine the amounts and relative proportions of fumonisins produced by the individual cultures. This study was undertaken to determine the complete profile of fumonisins produced in solid corn cultures and to identify any fumonisins or growth conditions which might be consistent with the sporadic outbreaks of animal toxicity associated with naturally contaminated corn.

MATERIALS AND METHODS

1. Analytical Standards and Fungal Cultures. Analytical standards of fumonisins were prepared as previously described (Musser et al., 1995, 1996; Musser and Plattner, 1996). *Fusarium* species were obtained as lyophilized cultures from the Fusarium Research Center, the Pennsylvania State University. Cultures were inoculated and grown on coarsely cracked corn in 300-mL Erlenmeyer flasks with Morton closures and incubated in the dark at 25 °C for 4 weeks as reported previously (Nelson et al., 1992). After 4 weeks, 10-g portions (2) of each culture were transferred to sealed 50-mL polypropylene centrifuge tubes and stored for an additional 2 weeks in the dark at room temperature to maximize production of the P series fumonisins. Cultures were then frozen at −50 °C until extraction and analysis. In other experiments, cultures were grown aerobically for 0, 7, 14, or 21 days, after which time they were sealed in polyethylene bags and grown anaerobically in the dark at 25 °C for the remainder of the 28-day growth period.

2. Sample Extraction and Preparation. Frozen culture material (10 g) was extracted with 20 mL of a MeCN:H_2O (75:25) solution. The solutions containing culture material were shaken several times and allowed to reach room temperature. The solid culture material was crushed with a glass rod, producing a slurry. Approximately 1 mL of this solution was removed and filtered through a 0.02-μm membrane filter (Whatman International Ltd., Maidstone, England). Extraction of the solid material remaining after filtration showed, by LC−MS analysis, the same pattern and relative abundance of individual fumonisins as the original extract, but at levels 5−10% of the original extract. No additional cleanup of the filtered extracts was performed prior to analysis by LC−MS.

3. Liquid Chromatography. A Hewlett-Packard (Palo Alto, CA) Model 1050 LC pump was used to provide linear gradients and a constant flow rate of 200 μL/min. All chromatography was performed on a YMC Inc. J-sphere ODS-L80 column (2 × 250 mm) packed with 4 μm particles. Chromatographic elution was accomplished with a gradient system beginning with 20% aqueous MeCN for 5 min and then a linear gradient to 50% aqueous MeCN at 45 min; a buffer concentration of 40 mM formic acid (pH 2.5) was maintained throughout the gradient. Under these conditions, FB_1 elutes at 25 min, FP_1 elutes at 29.5 min, FB_3 elutes at 31.5 min, and FB_2 elutes at 34.5 min.

4. Mass Spectrometry. A Finnigan (San Jose, Ca) Model TSQ-7000 triple-quadrupole mass spectrometer with the standard Finnigan electrospray ion source was used for MS detection. The entire LC column effluent was directed into the ion source. Nitrogen was used as a nebulizing gas, and the electrospray inlet temperature was 230 °C. The instrument was scanned over the range 300−900 amu at 1 s/scan. Quantitation of FB_1 was done by the external standard method described previously (Plattner et al., 1995). MS−MS experiments were performed with xenon as the collision gas and a collision energy of 21 V.

RESULTS AND DISCUSSION

Extracts of cracked corn cultures from eight strains of *Fusarium moniliforme*, eight strains of *Fusarium proliferatum*, and six strains of *Fusarium nygami* were analyzed by LC−MS. Cultures were selected for analysis on the basis of previous reports which showed them to be among the highest producers of FB_1 (Nelson, 1991, 1992). The mole percent amounts of fumonisins relative to FB_1 that are present in each culture extract are shown in Table 1. Response factors (mole percent) for the A and P series of fumonisins were calculated on the basis of response of standards with FB_1 and found to be 2 and 1, respectively. Response factors for the B series of fumonisins were found to be 1, as previously reported (Musser, 1996). By quantitating the amount of FB_1 present in each culture, it was possible, by using response factors, to calculate the mole percent of each of the other fumonisins present. In most cases FB_{1-3} and FA_{1-2} were found to be the most abundant fumonisins. While most of the cultures produced several hundred ppm of fumonisins, five cultures produced less than 100 ppm of FB_1. These low-yield cultures produced very different fumonisin patterns than did the cultures that produced high levels of fumonisins. In the low-yield cultures all of the A and B series are of approximately equal or greater proportion than FB_1, while in high-yield cultures FB_1 is the most abundant fumonisin.

Although the A and B series of fumonisins are the most abundant fumonisins produced in culture, there are several other fumonisins such as FC_1 and FAK_1 which are consistently produced at low levels (<10%) relative to B_1. In the course of identifying those fumonisins produced at low levels, several new fumonisins were identified in the culture extracts. Compounds belonging to the fumonisin class could easily be identified on the basis of their MS−MS spectra (Figure 2). The information from MS−MS experiments shows two important things. First, if the unknown compound is a fumonisin, the molecular ion undergoes collision-

Table 1. Fumonisin Composition of Individual Cultures[a]

	(ppm FB_1)	FB_2	FB_3	FB_4	FB_5	FA_1	FA_2	FA_3	FAK_1	FBK_1	FC_1	FP_1	FP_2	FP_3	PH_{1ab}
F. monil.	M2232 (3346)	48	15	8	1	6	2	<1	<1	3	3	nd	nd	nd	3
	M2285 (3430)	52	24	11	1	10	8	1	1	2	2	22	10	4	2
	M2552 (10)	87	95	106	nd	75	85	55	nd	4	nd	34	28	29	nd
	M2672 (414)	53	21	11	<1	10	6	1	1	3	3	29	17	8	2
	M3038 (832)	59	28	14	2	28	14	2	1	2	2	nd	nd	nd	2
	M5042 (710)	44	11	8	1	41	20	2	<1	<1	2	nd	nd	nd	2
	M5114 (2775)	33	23	6	3	8	3	<1	<1	1	2	19	4	3	5
	M5697 (321)	46	21	9	<1	18	14	2	1	3	2	nd	nd	nd	2
F. prolif.	M1342 (250)	25	7	2	1	21	7	2	4	3	nd	nd	nd	nd	3
	M1597 (575)	31	6	3	1	14	6	1	1	1	nd	nd	nd	nd	1
	M3420 (1017)	59	17	13	3	12	5	2	<1	3	3	nd	nd	nd	2
	M3438 (198)	41	5	3	1	21	16	1	4	2	nd	1	<1	nd	2
	M3504 (117)	62	8	5	1	11	8	<1	2	4	nd	2	<1	nd	3
	M5612 (67)	107	12	20	nd	73	59	5	5	1	nd	1	1	nd	2
	M5689 (531)	36	8	4	1	12	4	1	3	4	<1	<1	<1	nd	3
	M5977 (906)	23	21	6	2	14	6	2	2	5	2	36	8	6	3
F. nygami	M1375 (8)	59	60	56	nd	11	18	8	nd	1	nd	nd	nd	nd	nd
	M1438 (248)	74	1	5	1	6	4	nd	2	4	2	nd	nd	nd	4
	M2019 (562)	36	<1	2	1	22	2	nd	4	1	3	<1	nd	nd	<1
	M3300 (1)	136	143	244	67	87	150	nd	nd	nd	nd	nd	nd	nd	nd
	M3310 (82)	307	3	25	<1	5	13	nd	nd	4	nd	nd	nd	nd	nd
	M3325 (340)	642	3	58	<1	7	14	nd	nd	4	<1	nd	nd	nd	4

[a] Values represent mole percent relative to FB_1.

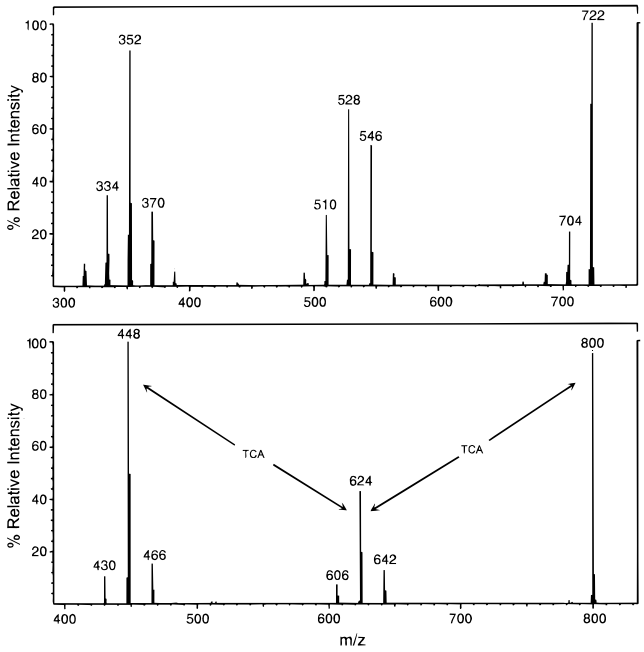


Figure 2. MS−MS spectra of fumonisins B_1 (top) and P_1 (bottom).

induced dissociation resulting in the loss of one TCA if the compound is a monoesterified fumonisin (FAK_1 and PH_1) or consecutive loss of two TCA functionalities ($MH^+ - 175$ and $MH^+ - 335$) if the compound is diesterified (FB_{1-3}, FA_{1-3}, and FP_{1-3}). Second, modifications to the alkyl chain such as fewer hydroxy functions can be differentiated from modifications to the TCA functionalities such as methyl esters. Modifications to the alkyl backbone show losses of TCA, while modifications to the TCA functions show losses of TCA plus the modification. Using this technique we were able to confirm the presence of FB_4 (MW= 693, $t_R = 40.1$ min), a trihydroxyfumonisin which has been reported to occur in culture but whose structure has not yet been determined (Nelson et al., 1993). In addition to FB_4, two new fumonisins were identified in the culture extracts. The first is a B series compound designated FB_5 (MW = 737, $t_R = 22.3$ min) which has a hexahydroxyalkyl backbone and the second, designated FBK_1

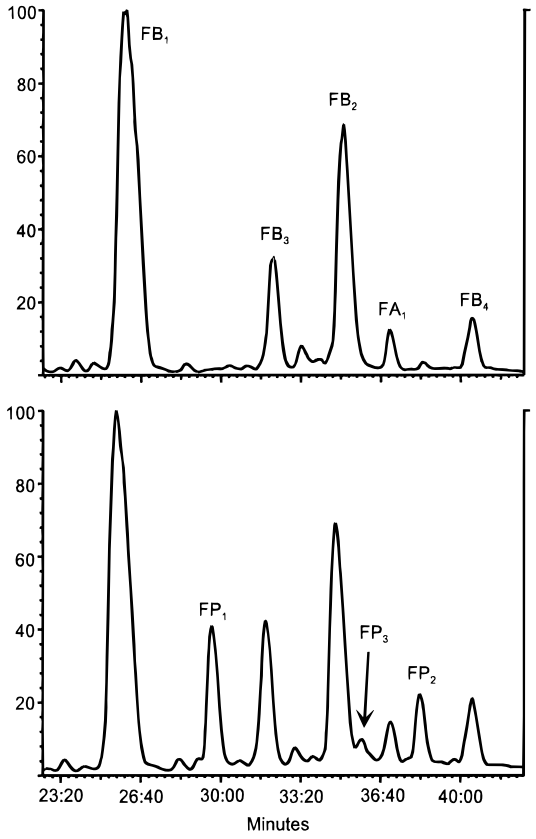


Figure 3. Total ion chromatograms for a culture extract (M2232) which does not produce the P series of fumonisins and a culture extract (M2285) which does produce the P series.

(MW = 562, $t_R = 25.2$ min), is a deacetylated form of FAK_1.

Of the fumonisins identified in our initial survey, the P series was the only group which showed any significant variation in production among the cultures surveyed (Figure 3). The P series was either produced at relatively high levels (20−36 mol % of FB_1) in five cultures (M2285, M2672, M5114, M2285, and M5977) or not produced at all (<1 mol %). Initially it was suspected that the P series compounds were either B series precursors or catabolites, and so cultures of a P series producing culture, M2285, were grown over a

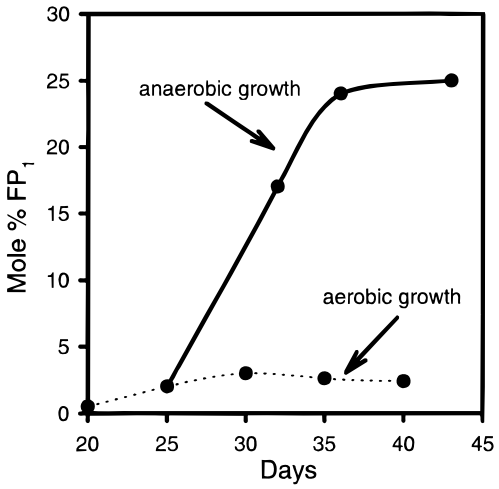


Figure 4. Production of FP_1 relative to FB_1 in cultures grown aerobically for 40 days or cultures grown aerobically for 25 days and anaerobically for 18 days.

Table 2. Fumonisin B_1, B_2, B_3, and P_1 Composition of Cultures Grown for Variable Times under Aerobic and Anaerobic Conditions

culture (aerobic + anaerobic growth time in weeks)	FB_1 (ppm)	FB_2 (ppm)	FB_3 (ppm)	FP_1 (ppm)
M2285 (4 + 0)	4266	403	1773	<50
(3 + 1)	3683	301	1377	1083
(2 + 2)	2529	436	1157	766
(1 + 3)	1506	88	449	517
(0 + 4)	nd	nd	nd	nd
M 3125 (4 + 0)	7397	885	2697	nd
(3 + 1)	5954	557	1928	992
(2 + 2)	5110	497	1607	1272
(1 + 3)	2575	168	603	757
(0 + 4)	nd	nd	nd	nd

period of 40 days, with samples taken for analysis every 5 days starting at day 20. Results of these analyses showed that levels of FP_1 peaked at 30 days but never reached levels greater than 4 mol % relative to FB_1, in sharp contrast to the initial survey results. Because the survey cultures were stored in sealed polypropylene vials for 2 weeks prior to freezing, we suspected that the P series of fumonisins were produced by the fungus under oxygen-limited conditions. To test this hypothesis, cultures were harvested at 25 days and placed in polypropylene bags with samples taken periodically for LC−MS analysis. Results of these analyses (Figure 4) showed that levels of FP_1 began rising rapidly after 7 days of anaerobic growth and peaked at 26 mol % relative to FB_1 between 11 and 18 days. During the period of anaerobic growth, levels of FB_1 as well as the other known fumonisins remained unchanged.

In another more extensive experiment to further test growth conditions, cultures of M2285, a P series producer in our survey and M3125, a strain not in our original survey, were placed in ziplock bags, sealed at the time of inoculation or after 1, 2, or 3 weeks of growth, harvested, and analyzed at 4 and 5 weeks. The results of analysis for FB_1, FB_2, FB_3, and FP_1 in the cultures at 4 weeks are shown in Table 2. The levels of the fumonisins had not changed significantly after 5 weeks of growth (data not shown). When the cultures were grown under anaerobic conditions in sealed bags from inoculation through 4 weeks, little growth occurred and fumonisins were not detected. The levels of fumonisins FB_1, FB_2, and FB_3 increased with time under normal growth conditions but did not continue to rise under anaerobic conditions. Detectable levels of FP_1 were only observed in those cultures which had been oxygen starved. In cultures which had been oxygen starved, FP_1 increased proportionately with the amount of FB_1 produced. Additionally, it did not appear that FP_1 was being produced from the catabolism of FB_1 or any of the other fumonisins found in the study. The metabolic pathway which the fungus uses to produce the P series of fumonisins is not known; however it is different from the pathway used to produce the other known fumonisins and could be important if the P series is found to be toxic.

Interestingly, of the five cultures that produce the P series, three of them, M2285, M2672, and 5114, have been associated with outbreaks of ELEM or human toxicity (Nelson et al., 1991). Of the two other P series producing cultures, one (M2285) was obtained from poultry feed and the other (M5977) was obtained from pearl millet. Since toxicological data for the P series of fumonisins does not exist, connections between their occurrence and mammalian disease cannot be made. However, the anaerobic growth requirements and sporadic production of the P series by different strains of *Fusaria* indicates that the toxicity of these compounds should be evaluated.

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Received for review September 6, 1996. Revised manuscript received December 13, 1996. Accepted December 18, 1996.[⊗] .

JF960663T

[⊗] Abstract published in *Advance ACS Abstracts*, February 15, 1997.