Rapid Purification of Fumonisins and Their Hydrolysis Products with Solid-Phase Extraction Columns

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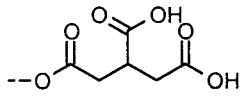
Fumonisins B_3 and B_4 (FB_3 and FB_4) were recovered from the 50:50 acetonitrile/water extract of corn cultures of a strain of *Fusarium moniliforme* that does not make FB_1 or FB_2 by stirring the extract with IRA-68, a weak anion-exchange resin. The fumonisins were desorbed with 5% acetic acid in the same solvent. After dilution with water, the desorbed fumonisins were separated into FB_3 (FB_3 and FA_3) and FB_4 (FB_4, FC_4, and FA_4) fractions with a tC_{18} solid-phase extraction (SPE) cartridge. The FB_3 fraction was then separated into FB_3 and FA_3 by using an NH_2 SPE cartridge and eluting with 5% acetic acid and increasing amounts of acetonitrile in water. Finally, FB_1 and FA_3 were hydrolyzed with calcium hydroxide. After recovery from the reaction mixture using a tC_{18} cartridge, the hydrolyzed and partially hydrolyzed analogues were separated and the unreacted fumonisins recovered by using an NH_2 cartridge, initially in the normal-phase mode with increasing amounts of water in acetonitrile and then in the reversed-phase mode after the addition of 5% acetic acid to the solvent and eluting in the reverse order.

Keywords: *Fumonisins; Fusarium moniliforme; corn; hydrolysis; isolation*

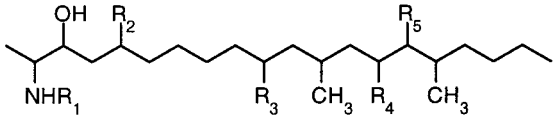
INTRODUCTION

Fumonisins have been widely studied since their initial discovery as toxic metabolites of *Fusarium moniliforme* isolated from corn (Bezuidenhout et al., 1988). Fumonisins are commonly found in grains used for animal feed and can occur in processed corn products consumed by humans (Nelson et al., 1993; Bullerman and Tsai, 1994). Fumonisin B_1 (FB_1) is the predominant fumonisin found in naturally contaminated corn and usually constitutes ~70% of the total amount of fumonisins present. Purified FB_1 causes a range of toxic responses in animals including equine leukoencephalomalacia, porcine pulmonary edema, and hepatosis and nephrotoxicity in rodents (Nelson et al., 1993; Norred and Voss, 1994). FB_1 has also been associated with high incidences of human esophageal cancer in South Africa (Rheeder et al., 1992) and China (Chu and Li, 1994). Because the other fumonisins are not as readily available in purified form, much less is known about their toxicity. The implications of the fumonisins in human and animal health have been reviewed recently (Norred and Voss, 1994; Marasas, 1995).

Recently there has been a growing interest in the hydrolysis products of the fumonisins. Hydrolyzed fumonisins ($HFB_{1, 2, 3, \text{and } 4}$) lack both tricarballylic acid (TCA) groups that are esterified to the C-14 and C-15 hydroxy groups in the intact molecules (Figure 1). In partially hydrolyzed fumonisins ($PHFB_{1, 2, 3, \text{and } 4}$), only one of the TCA groups has been removed. These molecules exist as an equilibrium mixture of the C-14 and C-15 esters. Exposure to hydrolyzed and partially hydrolyzed fumonisins can occur because they exist in the diet or are formed by the metabolism of consumed fumonisins. Hopmans and Murphy (1993) found HFB_1 in tortilla chips, masa, and canned yellow corn after a C_{18} solid-phase extraction (SPE) cartridge cleanup procedure. Scott and Lawrence (1996) used a combined strong-anion exchange (SAX) and a C_{18} SPE cartridge to purify HFB_1 found in 9 of 31 samples of alkali-processed corn foods. Maragos et al. (1997) used an affinity column to clean up 23 corn-based samples and detected HFB_1 in tortilla mix, masa, and distiller's dried grains. The two isomers of $PHFB_1$ have been detected



Tricarballylic Acid (TCA)



	R_1	R_2	R_3	R_4	R_5
FB_1	H	OH	OH	TCA	TCA
HFB_1	H	OH	OH	OH	OH
$PHFB_{1a}$	H	OH	OH	OH	TCA
$PHFB_{1b}$	H	OH	OH	TCA	OH
FB_2	H	OH	H	TCA	TCA
FB_3	H	H	OH	TCA	TCA
FA_3	$COCH_3$	H	OH	TCA	TCA
HFA_3	$COCH_3$	H	OH	OH	OH
$PHFA_{3a}$	$COCH_3$	H	OH	OH	TCA
$PHFA_{3b}$	$COCH_3$	H	OH	TCA	OH
FB_4	H	H	H	TCA	TCA

Figure 1. Chemical structures of fumonisins.

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in 5 of 15 corn kernel and corn screening samples from naturally contaminated corn (Xie et al., 1997). HFB_1 and $PHFB_1$ have also been found in the feces of vervet monkeys fed FB_1 (Shephard et al., 1994, 1995). HFB_1 was found in the feces of cattle, sheep, and rats fed diets containing cultured material, but only cattle and sheep feces contained $PHFB_1$ (Rice and Ross, 1994). The excretion of HFB_1 and the total amount of HFB_1 after hydrolysis in the urine and feces of rats has been monitored after feeding of diets containing FB_1, HFB_1, and an FB_1−fructose adduct (Hopmans et al., 1997).

The amounts of hydrolyzed and partially hydrolyzed fumonisins found in naturally contaminated corn are much less than the amounts of intact fumonisins. Common processing methods used to manufacture corn-based products can convert the intact fumonisins into the hydrolyzed forms and cause increased human exposure. Most of the hydrolyzed and partially hydrolyzed fumonisins in corn-based products probably arise from chemical processing with minor contributions from thermal processing. Nixtamalization is the process of treating corn with $Ca(OH)_2$ and heat. It is traditionally used in the production of masa. Hendrich et al. (1993) studied the effect of nixtamalized *Fusarium proliferatum*-fermented corn-based diets on rats. Nixtamalization removed most of the FB_1 from the diet. HFB_1 was the major toxic fumonisin found after nixtamalization, but the amount of HFB_1 formed was less than the amount of FB_1 lost. Although most of the FB_1 was removed, the toxicity of the nixtamalized diet was similar to that of the starting *F. proliferatum*-fermented corn-based diet. Sydenham et al. (1995a) saw similar reductions in the amount of FB_1 in the solid residue when they studied the alkaline hydrolysis with $Ca(OH)_2$ as a possible method of decontamination. The majority of FB_1 in ground corn was hydrolyzed and recovered as HFB_1 in the aqueous fraction. $PHFB_1$ was also isolated and purified from *F. monoliforme* culture material after treatment with $Ca(OH)_2$ (Sydenham et al., 1995b). The stability of FB_1 (Jackson et al., 1996a) and FB_2 (Jackson et al., 1996b) in aqueous systems during thermal processing has also been studied. They found that fumonisins are fairly heat-stable, but at temperatures >150 °C (baking or frying) there can be a significant reduction in the amount of the fumonisins and an increase in the level of their hydrolysis products. However, FB_1 was heat stable under most of the conditions used to bake corn muffins or fry corn chips, and hydrolysis products were not produced (Jackson et al., 1997).

Previously, we reported on a method of purification for fumonisins that utilized only SPE cartridges (Poling and Plattner, 1996). Culture material from a unique strain of *Gibberella fujikoroi* mating population A (anamorph *F. moniliforme*) isolated during a survey of this mating population (Plattner et al., 1996) was used as the starting material. This strain (A-0819, also called KSU819) accumulated high levels of FB_3 and FB_4, but virtually no FB_1 or FB_2. Extract from corn fermented with this strain was purified by the sequential use of NH_2 and tC_{18} cartridges to give FB_3 and FB_4 fractions containing >90% fumonisins. The FB_3 fraction consisted of a mixture of 80% FB_3, 16% FA_3, 1−2% FC_3, and a few percent of the various methyl esters of FB_3 formed by exposure to MeOH. The separation of the fumonisins from their N-acetylated analogues using NH_2 SPE cartridges will be discussed in the present paper. A batch process using a weak anion-exchange resin will also be described that can be used to replace the NH_2 cartridge. This method allows the rapid concentration of fumonisins from large volumes of corn extract with a similar degree of purification. Finally, the complete separation of hydrolyzed and partially hydrolyzed fumonisins using an NH_2 cartridge, first in the normal-phase and then in the reversed-phase modes, will be presented. These compounds can occur in a variety of processed corn products. This method provides an easy procedure to prepare hydrolyzed and partially hydrolyzed fumonisins for use as analytical standards.

EXPERIMENTAL PROCEDURES

Safety Warning. Fumonisins are cancer promoters, interfere with lipid metabolism, and are toxic to several animal species. Fungal cultures and extracts should be handled with appropriate care.

Fungal Strains and Culture Conditions. Strains used in this study were from the culture collection at the Fusarium Research Center at Pennsylvania State University or from the collection of Dr. John Leslie (Kansas State University). The isolates of *F. moniliforme* were grown on cracked corn using published procedures (Nelson et al., 1991).

Extraction. Corn cultures were extracted with 5 mL of CH_3CN/H_2O (50:50) for each gram of corn as previously described (Poling and Plattner, 1996). Extracts from several flasks of corn fermented with A-0819 (KSU819) were combined and contained ~1.1 mg/mL FB_3.

HPLC Analysis of *o*-Phthaldialdehyde (OPA) Derivatives. Gradient elution from 60 to 80% methanol in 0.1 M NaH_2PO_4 adjusted to pH 3.35 and fluorescence detection as previously described (Poling and Plattner, 1996) were used for the OPA derivatives. All samples except those from the alkaline hydrolysis were diluted with CH_3CN/H_2O (50:50) to the appropriate concentrations. When following the hydrolysis of FB_1 and FB_3, samples were diluted 1:10 with 0.5% HOAc in CH_3CN/H_2O (50:50) to dissolve the $Ca(OH)_2$ before formation of the OPA derivative. The fumonisins eluted at the following times: $PHFB_1$, 5.7 and 6.1 min (unresolved from HFB_1); HFB_1, 6.1 min; FB_1, 6.9 min; $PHFB_3$, 10.9 and 11.3 min; HFB_3, 11.7 min; FB_3, 12.1 min.

HPLC Analysis of Underivatized Fumonisins Using an Evaporative Light Scattering Detector (ELSD). A SpectraSYSTEM P4000 pump connected to an AS3000 autosampler (Thermo-Separation Products, Fremont, CA) was used to generate a linear gradient with a flow rate of 0.5 mL/min. Analyses were performed using a 33 × 4.6 mm, 3 μm, Pecosphere 3.3 cm CR18 column (0258-0195, Perkin-Elmer, Norwalk, CA). The eluting solvent was held at 30:70:0.1 CH_3CN/H_2O/trifluoroacetic acid for 1 min, and then a 15 min linear gradient was run to 45:55:0.1 CH_3CN/H_2O/trifluoroacetic acid, which was maintained an additional 5 min. The column was connected to a Varex Mark III ELSD detector (Alltech Associates, Deerfield, IL). The tube temperature was 110 °C and the gas flow maintained at 2.85 SLPM. The fumonisins eluted at the following times: HFB_1, 3.3 min; $PHFB_1$, 4.0 and 4.8 min; FB_1, 6.0 min; HFB_3, 7.7 min; $PHFB_3$, 8.2 and 9.4 min; FB_3, 10.6 min; HFA_3, 10.0 min; $PHFA_3$, 10.5 and 11.7 min; FA_3, 12.5 min.

LC/MS Analysis of Underivatized Fumonisins. A SpectraSYSTEM P4000 pump was coupled to a Finnigan-MAT TSQ 700 or LCQ mass spectrometer via an electrospray interface (ESI) (Finnigan-MAT, San Jose, CA). An Inertsil ODS-3 15 cm × 3.0 mm i.d. column (0396-150 x 030, MetaChem Technologies, Torrance, CA) was used for the ESI measurements. FB_1 and FB_3 and their hydrolysis products were analyzed using the TSQ 700 and the following conditions. A ternary solvent system was used: solvent A was MeOH/1 mM ammonium formate (5:95), solvent B was H_2O/HOAc (100:1), and solvent C was MeOH. A 10 min linear gradient from 39:5:56 to 20:5:75 A/B/C was used, and the final composition was held for 19 min. The flow rate was 0.4 mL/min. FA_3 and its

hydrolysis products were analyzed using the LCQ. Solvents B and C are the same as above, and solvent D was H_2O. A 10 min linear gradient from 50:35:15 to 0:35:65 D/B/C was used, and the final composition was held for 20 min. The flow rate was 0.3 mL/min. For both systems, the entire HPLC eluent was introduced into the detector. Mass spectra were obtained by scanning from *m/z* 350 to 850 in 1.5 s. The ESI spray voltage was 4.5 kV and the capillary temperature 220 °C.

Separation of FB_3 and FB_4 Fractions. Amberlite IRA-68 resin (1-0331, Supelco, Supelco Park, Bellefonte, PA) was washed twice with H_2O and then twice with CH_3CN/H_2O (50:50). Typically, 15 g of IRA-68 resin was mixed with 50 mL of solvent each wash. The mixture was allowed to stand for 5−10 min and the excess solvent decanted. The washed IRA-68 resin was then added to 500 mL of extract from corn fermented with strain A-0819. The mixture was stirred magnetically for 2 h, then filtered, and rinsed with 100 mL of CH_3CN/H_2O (50:50). The IRA-68 resin was then resuspended in 75 mL of 5% HOAc in CH_3CN/H_2O (50:50) and stirred for 18−21 h. The mixture was filtered and the resin stirred for an additional 2 h with 30 mL of the same solvent and refiltered. The procedure was repeated with another 500 mL of extract. The filtrates were combined and diluted to CH_3CN/H_2O (10:90). The combined filtrate was split, and each half was loaded onto a tC_{18} cartridge [Sep-Pak Vac 35 cm^3 (10 g) tC_{18} cartridge, WAT043350, Waters Corp., Milford, MA] conditioned with 100 mL of MeOH, followed by 100 mL of CH_3CN/H_2O (10:90), and eluted sequentially with 100 mL aliquots of 10:90, 15:85, 25:75, 35:65, a second 35:65, and 45:55 CH_3CN/H_2O. The 35:65 aliquots contained the FB_3 fraction, and the 45:55 aliquots contained the FB_4 fraction. The FB_3 fraction was diluted to CH_3CN/H_2O (10:90) and loaded onto a conditioned 10 g tC_{18} cartridge. The cartridge was washed with 100 mL of CH_3CN/H_2O (10:90), and the fumonisins were eluted with 60 mL of MeOH. The MeOH was removed with a rotary evaporator. The residue was dissolved in 20 mL of CH_3CN/H_2O (50:50) and freeze-dried. The FB_3 fraction weighed 1.13 g.

Separation of FB_3 and FA_3. Three hundred milligrams of the freeze-dried FB_3 fraction was dissolved in 50 mL of CH_3CN/H_2O (50:50) and loaded onto a 10 g NH_2 cartridge [Sep-Pak Vac 35 cm^3 (10 g) NH_2 cartridge, WAT54740] that was conditioned with 100 mL aliquots of MeOH and then CH_3CN/H_2O (50:50). The cartridge was then washed sequentially with 100 mL aliquots of 50:50 and 10:90 CH_3CN/H_2O. The fumonisins were eluted with 100 mL aliquots of 5% HOAc in 10:90, 20:80, 30:70, and 40:60 CH_3CN/H_2O. The 5% HOAc 10/90 and 30:70 CH_3CN/H_2O fractions contained FB_3 and FA_3, respectively. The FB_3 fraction was diluted with 100 mL of H_2O and applied to a 10 g tC_{18} cartridge conditioned as above. The cartridge was eluted once with 100 mL of 10:90 CH_3CN/H_2O and twice with 50 mL of 50:50 CH_3CN/H_2O. The void volume was removed with each aliquot, but the cartridge was not allowed to dry out. The first 50 mL aliquot of 50% aqueous CH_3CN was freeze-dried to yield 233 mg of FB_3. The FA_3 fraction was treated similarly except it was diluted with 200 mL of H_2O prior to SPE and eluted with three 20 mL aliquots of 50:50 CH_3CN/H_2O. The FA_3 was in the first two 20 mL aliquots, which were combined and hydrolyzed without further treatment.

Partial Hydrolysis of FB_1. A solution of 108 mg of FB_1 dissolved in 5 mL of 50:50 CH_3CN/H_2O was added to a stirred suspension of 1.5 g of calcium hydroxide in 200 mL of H_2O. After 4.5 h at room temperature, 4 mL of HOAc and 20 mL of CH_3CN were added and the reaction mixture was loaded onto a 10 g tC_{18} cartridge conditioned as above. The tC_{18} cartridge was washed with 100 mL of 10:90 CH_3CN/H_2O, the void volume removed, and elution was performed with 100 mL of 50:50 CH_3CN/H_2O, which was collected in 20 mL fractions. The first 20 mL eluant containing FB_1 and its hydrolysis products was diluted with 30 mL of CH_3CN and applied to a 2 g NH_2 cartridge [Sep-Pak Vac 12 cm^3 (2 g) NH_2 cartridge, WAT054650] that had been conditioned with 20 mL aliquots of MeOH and 80:20 CH_3CN/H_2O. This cartridge was eluted sequentially with 20 mL aliquots of 80:20, 70:30, 10:90, and 1% and 5% HOAc in 10:90 CH_3CN/H_2O. The 10:90 CH_3CN/H_2O fraction was freeze-dried to give 15.1 mg of $PHFB_1$. The 80:20 CH_3CN/H_2O containing HFB_1 was diluted to 10:90 CH_3CN/H_2O and loaded onto a 2 g tC_{18} cartridge [Sep-Pak Vac 12 cm^3 (2 g) tC_{18} cartridge, WAT043380] conditioned with 20 mL aliquots as above. This cartridge was washed with 20 mL of 10:90 CH_3CN/H_2O and eluted with 20 mL of 50:50 CH_3CN/H_2O. The eluate afforded 20 mg of HFB_1 (analyzed by HPLC-ELSD). Another 5 mg of HFB_1 remained in the 10:90 CH_3CN/H_2O load plus wash. Unhydrolyzed FB_1 was recovered from the 5% HOAc in the 10:90 CH_3CN/H_2O eluant of the NH_2 cartridge by diluting with an equal volume of H_2O and loading onto a 500 mg tC_{18} cartridge [Sep-Pak Vac 3 cm^3 (500 mg) tC_{18} cartridge, WAT036815] conditioned with 5 mL aliquots of MeOH and 5:95 CH_3CN/H_2O. This cartridge was washed with 5 mL of 5:95 CH_3CN/H_2O and eluted with 5 mL of 50:50 CH_3CN/H_2O. This fraction contained 30 mg of FB_1 (analyzed by HPLC-ELSD).

Partial Hydrolysis of FA_3. The FA_3, separated from FB_3 above, in 40 mL of 50:50 CH_3CN/H_2O was added to a suspension of 1.5 g of calcium hydroxide in 160 mL of H_2O and stirred for 4 h at room temperature. Four milliliters of HOAc was added, and the reaction mixture was loaded onto a conditioned 10 g tC_{18} cartridge. The cartridge was eluted and the void volume was pulled through each time, once with 100 mL of 10:90 and twice with 50 mL of 50:50 CH_3CN/H_2O. The hydrolysis products were in the first 50 mL of 50:50 CH_3CN/H_2O, which was diluted with 75 mL of CH_3CN and loaded onto a 2 g NH_2 cartridge conditioned as above. This cartridge was eluted sequentially with 20 mL aliquots of 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, and 10:90 CH_3CN/H_2O, followed by 5% HOAc in 10:90, 20:80, 30:70, and 40:60 CH_3CN/H_2O. HFA_3 eluted in the 80:20 CH_3CN/H_2O fraction. $PHFA_3$ was recovered from the combined 60:40 and 50:50 CH_3CN/H_2O eluants. FA_3 eluted in the 40:60, 30:70, and 10:90 CH_3CN/H_2O fractions, which were combined, as well as in the 5% HOAc in 20:80, 30:70, and 40:60 CH_3CN/H_2O fractions, which were also combined. Each of the four final fractions was diluted to 10:90 CH_3CN/H_2O and processed separately on 2 g tC_{18} cartridges conditioned with 20 mL aliquots of MeOH and 10:90 CH_3CN/H_2O. After loading, the cartridges were washed with 20 mL of 10:90 CH_3CN/H_2O and eluted with two 10 mL aliquots of 50:50 CH_3CN/H_2O. The desired products were in the first 10 mL of elution in each case. After freeze-drying, the following yields were obtained: HFA_3, 2.0 mg; $PHFA_3$, 5.3 mg; and FA_3, 9.2 mg (combined weight).

RESULTS AND DISCUSSION

The purification scheme used for FB_3 and its analogues (Figure 2) is similar to those for the other fumonisins. The FB_3 fraction from the combined IRA-68/tC_{18} cartridge procedure contained 77% FB_3 and 14% FA_3. This is comparable to the FB_3 fraction (80% FB_3 and 16% FA_3) obtained from the same extract using the combined NH_2/tC_{18} cartridge procedure (Poling and Plattner, 1996). Amberlite IRA-68 is a weak anion exchanger like the NH_2 cartridges and absorbs only fumonisins with both tricarballylic acid groups present. Our procedure can easily be scaled up to handle large volumes of extract. The fumonisins are absorbed rapidly when the IRA-68 resin is stirred with the extract (<2 h). The fumonisins are also efficiently absorbed by passing the extract over a bed of IRA-68 resin in a column, but the first desorption is slower (>17 h) and the resin is used best in a batch procedure. Typically, 95% of the fumonisins were recovered when the IRA-68 resin was stirred with extracts of cultured corn. In some very highly pigmented extracts, the pH became too alkaline (pH 9) to allow the complete absorption of the fumonisins. It was necessary to adjust the pH of the mixture of the resin and extract down to pH ~5.5 with 1 N HCl to complete the absorption of the fumonisins.

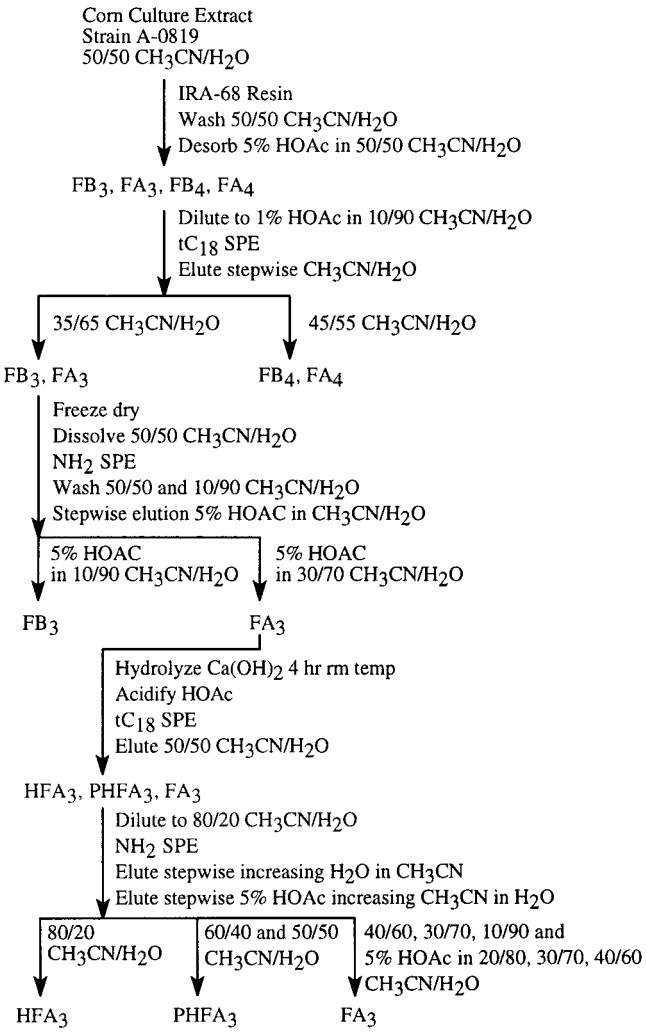

Figure 2. Flow diagram for the purification of FB_3 and its analogues.

The fumonisins can be desorbed from the resin with 5% HOAc in 50:50 CH_3CN/H_2O or 1 N NaCl. The HOAc desorbs less pigment from the resin and needs only to be diluted before application to the tC_{18} cartridge.

Miller et al. (1994) compared four resins, XAD-2, XAD-16, SAX, and DEAE-Sephadex A-25, for the initial cleanup of fumonisins from liquid cultures. The best recovery and cleanup were obtained with the weak anion-exchange resin, DEAE-Sephadex A-25. They also used this resin for the initial cleanup of corn culture extract after it had been freeze-dried and redissolved in water. IRA-68 resin is more economical and can be used directly with extracts from corn cultures. The fractions obtained, using the combined IRA-68/tC_{18} procedure, consist mainly of a single fumonisin and its N-acetylated analogue, which can then be separated easily.

The limiting factor in the combined NH_2/tC_{18} procedure was the amount of fumonisins that could be loaded onto the NH_2 cartridge (Poling and Plattner, 1996). The use of the IRA-68 resin removes this limitation. The tC_{18} cartridges have a very large capacity for fumonisins. A single 10 g tC_{18} cartridge was able to separate a 558 mg FB_3 fraction from a 100 mg FB_4 fraction (FB_4, FA_4, and FC_4). Another illustration of the large capacity of this SPE was when the FB_3 fractions from two separations were combined and loaded onto a 10 g tC_{18} cartridge. Fractions were collected during loading and analyzed to see if breakthrough occurred. The cartridge retained all of the fumonisins and, after elution and freeze-drying, yielded a 1.15 g FB_3 fraction.

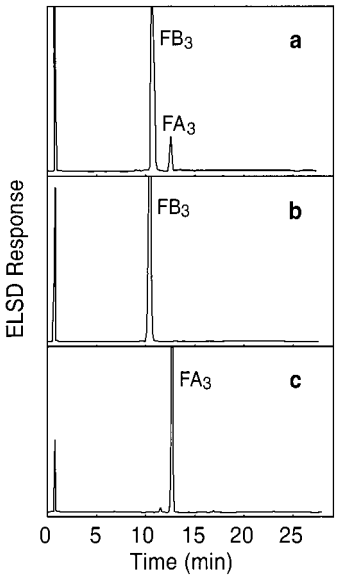

Figure 3. FB_3 fraction (a) from combined IRA-68/tC_{18} SPE; separation of FB_3 (b) from FA_3 (c) by NH_2 SPE.

Fumonisins can be separated from their N-acetylated analogues using NH_2 cartridges, as demonstrated by the complete separation of FB_3 and FA_3, the major components of the FB_3 fraction from the IRA-68/tC_{18} procedure (Figure 3). All of the FB_3 eluted in the 5% HOAc 10:90 CH_3CN/H_2O fraction. Most of the FA_3 eluted in the 5% HOAc 30:70 CH_3CN/H_2O with only small amounts in the earlier and later fractions. The small peak eluting before FA_3 appears to be an O-acetylated analogue of FB_3. The protonated molecular ion and base peak (*m/z* 748) are the same as those of FA_3, but the response is higher than would be expected for a similar amount of the amide and the sodiated molecular ion (*m/z* 770) is only 5% of the base peak compared to 80% for FA_3.

The combined NH_2/tC_{18} cartridge procedure used 5% HOAc in MeOH to elute all of the fumonisins from the NH_2 cartridge (Poling and Plattner, 1996). Using 5% HOAc in 50:50 CH_3CN/H_2O to elute all of the fumonisins from the NH_2 cartridge requires only 40% of the solvent used in the earlier procedure. The separation achieved with the tC_{18} cartridge is not affected by the new eluting solvent. The new method also avoids the formation of minor amounts of fumonisin methyl esters caused by exposure to MeOH.

Sydenham et al. (1996) performed a similar separation of the fumonisins from the N-acetylated analogues using SAX media while trying to account for the higher amounts of fumonisin measured by an immunoassay as compared to HPLC. After performing the standard elution of fumonisins with 1% HOAc in MeOH, they eluted with 5% HOAc in MeOH. The 5% HOAc fraction had little FB_{1-3}, but did respond to the immunoassay. TLC showed two bands that corresponded to FA_1 and FA_2, which was confirmed by GC of the hydrolysis products. The NH_2 cartridge procedure should be useful in similar situations because elution can be achieved with lower concentrations of organic solvent, which can interfere with immunoassays.

We have used the same procedure to remove the last of the FB_1 from FA_1 purified by conventional chromatography, as well as to separate FB_4 and FA_4. Thus,

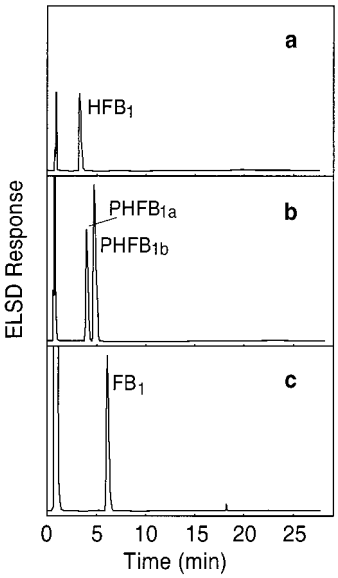

Figure 4. HFB_1 (a) and $PHFB_1$ (b) purified by NH_2 SPE following hydrolysis of FB_1 (c).

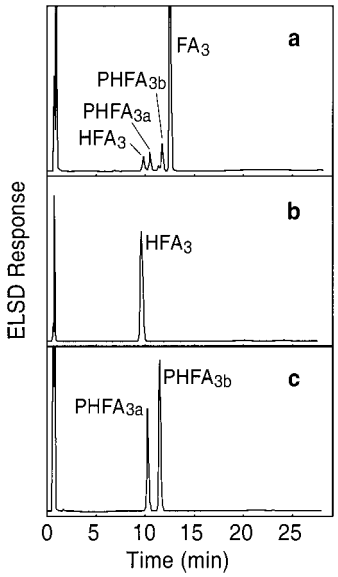

Figure 5. Reaction mixture from the hydrolysis of FA_3 after recovery by tC_{18} SPE (a); HFA_3 (b) and $PHFA_3$ (c) after further separation by NH_2 SPE.

starting with corn cultures of normal strains or unique strains that produce either FB_2 or FB_3 (Plattner et al., 1996) and using the simple cleanup procedures utilizing only IRA-68 resin and tC_{18} and NH_2 cartridges, all of the fumonisins (FB_{1-4}) and their N-acetylated analogues (FA_{1-4}) can be isolated in relatively pure form and with good yields.

A simple purification procedure was then developed to provide a convenient source of hydrolyzed and partially hydrolyzed fumonisins as standards for the identification and quantitation of these compounds in processed foods. The fumonisins were hydrolyzed with calcium hydroxide at room temperature (Sydenham et al., 1995b). The hydrolyses of FB_1 and FB_3 were periodically monitored by HPLC of the OPA derivatives. The maximum amount of partially hydrolyzed fumonisins was produced between 4 and 4.5 h of hydrolysis at room temperature. Sydenham et al. (1995b) followed the hydrolysis of FB_1 in culture material of *F. moniliforme* and observed maximum production of $PHFB_1$ between 4 and 5 h. Less hydrolysis was observed when FA_3 was hydrolyzed for 4 h under the same conditions.

The hydrolyzed, partially hydrolyzed, and unreacted fumonisins were separated using an NH_2 cartridge. Figure 4 shows the ELSD chromatograms of the HFB_1 and $PHFB_1$ fractions from this procedure. Shephard et al. (1994) showed that the ratio of the C-14 to the C-15 esters for $PHFB_1$ was 55:45 at equilibrium. The larger of the $PHFB_1$ peaks was assigned as the C-14 ester ($PHFB_{1b}$) on the basis of this observation. The fractions were also analyzed by LC/MS. Electrospray MS showed a single strong protonated molecular ion at *m/z* 564 for both $PHFB_1$ peaks and at *m/z* 406 for HFB_1. On the basis of the total areas of the *m/z* 406 and 564 peaks, the HFB_1 fraction contains ~1% $PHFB_1$ and the $PHFB_1$ fraction contains ~1% HFB_1. Figure 5 shows the ELSD chromatograms of the hydrolysis products of FA_3 recovered on a tC_{18} cartridge and of the HFA_3 and $PHFA_3$ fractions after NH_2 SPE purification. The larger $PHFA_3$ peak was assumed to be the C-14 ester as in the case of $PHFB_1$, but this needs to be confirmed. LC/MS analysis showed that FA_3, $PHFA_3$, and HFA_3 all have protonated molecular ions at *m/z* 748, 590, and 432, respectively, as the base peaks. The relative intensity of the sodiated molecular ion, which is characteristic of the N-acetylated fumonisins, is 80% for FA_3, 50% for $PHFA_3$, and only 10% for HFA_3. This and the reduced ionization efficiencies of the amides (Poling and Plattner, 1996) make quantitation by electrospray MS more difficult for these compounds. The HFA_3 fraction does not contain any $PHFA_3$. The $PHFA_3$ fraction has ~1% HFA_3 and a small amount of $PHFB_3$. This $PHFB_3$ probably is formed by the hydrolysis of what appears to be an O-acetylated form of FB_3, mentioned above and not from FA_3. The first experiments on hydrolysis and separation using SPE cartridges were performed using the FB_3 fraction (FB_3 and FA_3) from the IRA-68/tC_{18} procedure. A pure $PHFB_3$ fraction was obtained, but part of the $PHFB_3$ was in a mixed fraction of $PHFB_3$ and $PHFA_3$, and the hydrolyzed fraction contained both HFB_3 and HFA_3. The separation of the fumonisins from their N-acetylated analogues by NH_2 SPE should be performed before hydrolysis.

The capacity of the NH_2 cartridges for N-acetylated fumonisins is much less than the capacity for the unacetylated fumonisins. After hydrolysis of FB_1, 30 mg of unhydrolyzed FB_1 was recovered in a single fraction from a 2 g NH_2 cartridge. When hydrolyzed FA_3 was separated on a 2 g NH_2 cartridge, 9.2 mg of FA_3 was recovered but was spread over six fractions. Complete separation of HFA_3 and $PHFA_3$ was achieved, but the recovered FA_3 contained some $PHFA_3$.

By using a combination of the IRA-68 resin and NH_2 and tC_{18} SPE cartridges, the various fumonisins and their N-acetylated analogues can be separated. After a simple hydrolysis, the hydrolyzed and partially hydrolyzed can also be separated and purified. This should provide a convenient source of these fumonisins as standards for their identification and quantitation in processed corn products.

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