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Determination of Fumonisins B$_1$ and B$_2$ and Their Major Hydrolysis Products in Corn, Feed, and Meat, Using HPLC[†]

Rohan A. Thakur and J. Scott Smith*

Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas 66506

A high-performance liquid chromatograph coupled to a fluorescence detector (excitation, 229 nm; emission, 442 nm) was used to analyze *o*-phthaldialdehyde-derivatized fumonisin B$_1$ (FB$_1$), fumonisin B$_2$ (FB$_2$), hydrolyzed FB$_1$ (HFB$_1$), hydrolyzed FB$_2$ (HFB$_2$), and partially hydrolyzed FB$_1$ (PHFB$_1$) from corn (1848 ng/g FB$_1$ and 1092 ng/g FB$_2$), feed (756 ng/g FB$_1$ and 252 ng/g FB$_2$), and spiked meat (720 ng/g FB$_1$, 600 ng/g FB$_2$, 960 ng/g HFB$_1$, 840 ng/g HFB$_2$) samples. Thermospray mass spectrometry was used to confirm the base hydrolysis products of FB$_1$ and FB$_2$. Chromatography was achieved on a C$_{18}$, reversed-phase column. The mobile phases were acetonitrile/water/acetic acid mixtures set at A (40:59:1) and B (60:39:1), pumped in a gradient of 100% A to 100% B (9 min), and followed by a stepup to 100% acetonitrile (C) at the end of 17 min. The flow rate was 1 mL/min. The HPLC method has a retention factor (k) between 2 and 10, a selectivity coefficient (α) between 1.13 and 1.65, and a lower detection limit of 20 ng/mL at a signal to noise ratio of 5:1.

Keywords: *Fumonisins; HPLC; mass spectrometry; metabolites; hydrolysis*

INTRODUCTION

Fumonisins constitute a group of nongenotoxic carcinogens produced primarily by the fungus *Fusarium moniliforme*. Chemically, fumonisins are diesters of propane-1,2,3-tricarboxylic acid and either 2-(acetylamino)- or 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxycosane (see Figure 1). In all cases, both the C-14 and C-15 hydroxy groups are involved in ester formation with the terminal carboxyl group of propane-1,2,3-tricarboxylic acid (Bezuidenhout et al., 1988). Fumonisin B$_1$ (FB$_1$) is the most active cancer-promoting homologue within the fumonisins (Gelderblom et al., 1988). The related homologues differ by lacking one of the free hydroxy groups at either the C-5 or C-10 position of the C-20 aminopentol backbone and are denoted FB$_2$, FB$_3$, FB$_4$, FA$_1$, and FA$_2$.

Fumonisins are of particular concern because they have been linked to human esophageal cancer and have been shown to be atherogenic to nonhuman primates (Shepard et al., 1994). The role of fumonisins in the etiopathogenesis of equine leukoencephalomalacia (Wilson et al., 1992; Ross et al., 1993) and pulmonary edema in pigs (Harrison et al., 1990) has been well established. Furthermore, they are nephrotoxic and hepatocarcinogenic in rats (Gelderblom et al., 1991).

The most readily identifiable metabolites of FB$_1$ are its hydrolyzed (HFB$_1$) and partially hydrolyzed (PHFB$_1$) toxicologically active forms (Shepard et al., 1994; Rice and Ross, 1994; Hopmans and Murphy, 1993; Hendrich et al., 1993). HFB$_1$ has been found in nixtamalized corn-based foods and is toxicologically active (Hopmans and Murphy, 1993). The presence of HFB$_2$ in any matrix has not been reported, and its absence may be attributed to the deficiency of existing methodology. The PHFB$_1$ moiety was detected in the feces of ruminant animals dosed with FB$_1$ (Rice and Ross, 1994). Thus,
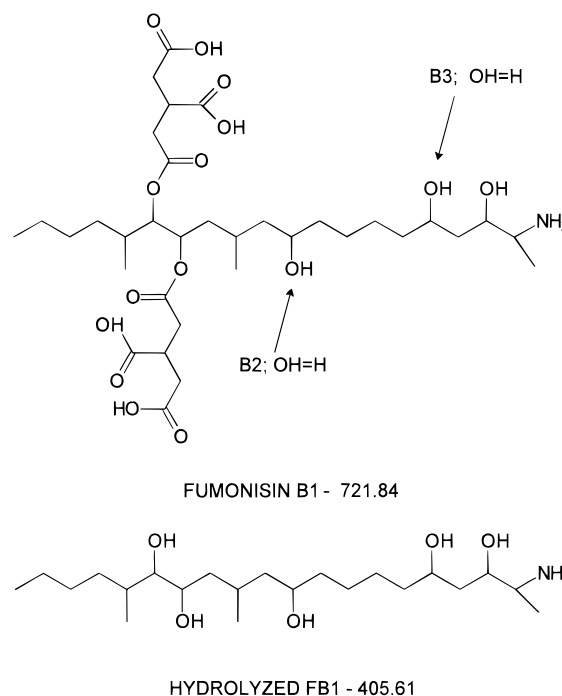

Figure 1. Chemical structures of the fumonisin derivatives.

the toxicity attributed to FB$_1$, its analogue FB$_2$, and their major hydrolysis products HFB$_1$ and HFB$_2$ necessitates the development of a reliable method of analysis for these toxicants from any matrix. The structures of the fumonisin metabolites are presented in Figure 1.

Current methods for fumonisin analysis include thin layer chromatography (Gelderblom et al., 1991), gas chromatography/mass spectrometry, liquid secondary ion mass spectrometry (Plattner et al., 1990), immunosorbent assays (Azcona-Olivera et al., 1992), thermospray mass spectrometry (Thakur and Smith, 1994), and electrospray mass spectrometry (Doerge et al., 1994). However, the water-soluble nature of fumonisins and their metabolites makes them ideally suited for reliable, repetitive analysis using high-performance liquid chromatography (HPLC).

* Author to whom correspondence should be addressed [telephone (913) 532-1219; fax (913) 532-5681; e-mail jsschem@ksu.ksu.edu].

The fumonisin molecule lacks both ultraviolet (UV) absorption and natural fluorescence. Thus, some kind of chemical modification is necessary to make it detectable to common HPLC detectors. A maleyl derivative of FB_1, FB_2, and FB_3 was prepared for HPLC analysis using UV detection (Shepard et al., 1992), but it lacked the desired sensitivity and specificity needed for routine analysis. A fluorescent derivative of FB_1, FB_2, and FB_3 was prepared using fluorescamine (Ross et al., 1991). However the fumonisin—fluorescamine adduct eluted as two peaks because of formation of the acid/alcohol and lactone derivatives, making quantitation unreliable. The stable fluorescent derivative naphthalene-2,3-dicarboxylic acid (NAD) uses highly toxic sodium cyanide as a catalyst for the reaction and, consequently, has received little attention (Scott and Lawrence, 1992).

The use of precolumn derivatization of FB_1, FB_2, and FB_3 with *o*-phthaldialdehyde (OPA) and separation using HPLC followed by fluorescent detection, first developed by Shepard et al. (1990), has been used widely by many investigators. This method was the subject of a collaborative interlaboratory study to determine its reproducibility characteristics (Thiel et al., 1993). It has been successful in determining FB_1, HFB_1, $PHFB_1$, FB_2, and FB_3 in corn, culture medium, blood, plasma, urine, and feces. However, the chromatography needs further improvement for unequivocal quantitative analysis.

Other drawbacks of the OPA derivative are its rapid decay in fluorescence intensity with time and its reactivity with all free primary amines. Thus, the reliability of this method hinges on extensive cleanup and a constant derivative reaction time before sample injection. This paper discusses chromatographic improvements made upon existing HPLC methods using OPA to surmount the problems of matrix interference and optimize the derivative reaction time to enhance the reliability of the results. Naturally contaminated corn and feed and fumonisin-spiked beef were used as matrices from which FB_1, HFB_1, FB_2, and HFB_2 were resolved successfully by the developed method.

MATERIALS AND METHODS

Chemicals. Acetonitrile (HPLC grade), glacial acetic acid (ACS grade), methanol (HPLC grade), potassium hydroxide (ACS grade), ammonium acetate, and hydrochloric acid (HCl, ACS grade) were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Fluoraldehyde (OPA reagent) was obtained from Pierce Chemical Co. (Rockford, IL). Deionized water (HPLC grade) was obtained from a Sybron-Barnstead ion exchange unit. The FB_1 and FB_2 standards were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Stock Solutions. Fumonisin B_1 stock solution was prepared by transferring the contents of the vial (5 mg) to a 10 mL volumetric flask and made to volume with acetonitrile/water (20:80 v/v) to give a stock solution that was nominally 500 μg/mL. The FB_2 stock solution was made up similarly. The stock solutions were used for serial dilutions. Dilutions made with acetonitrile/water (20:80 v/v) were 10, 5, 3, 1, 0.5, and 0.3 μg/mL. Standard curves were plotted using these dilutions and evaluated for linearity by determining the coefficient of determination (r^2).

Base Hydrolysis of the Fumonisins. The HFB_1, HFB_2, and $PHFB_1$ were prepared from 10 μg/mL of stock FB_1 and FB_2 solutions using base hydrolysis and with controlled time and temperature of the reaction. Complete hydrolysis was achieved by mixing 5 mL of the 10 μg/mL of stock FB_1 and FB_2 solutions with 5 mL of 1 N potassium hydroxide (KOH) each and heating the mixtures in a closed vial at 70 °C for 1 h. After cleanup, HFB_1 and HFB_2 were serially diluted to achieve concentrations of 10, 5, 3, 1, 0.5, and 0.3 μg/mL. Partial hydrolysis of FB_1 was done by mixing 1 mL of 10 μg/ mL of stock FB_1 solution with 1 mL of 1 N KOH and letting the solution sit at room temperature for 10 min. The hydrolysis was stopped by acidifying the solutions to pH 4.5 by addition of 2 N HCl. The formation of HFB_1, HFB_2, and $PHFB_1$ was confirmed by direct liquid injection thermospray mass spectrometry (TSP-MS).

Sample Preparation. Samples of corn and feed (a mixture of sorghum, corn, and alfalfa) were obtained from Kansas State University's feed mill facility. To monitor levels of FB_1 and FB_2 in corn and feed, 25 g of sample was blended twice with 35 mL of acetonitrile/water (1:1 v/v) in a Waring blender for 20 min. A total of 20 mL of blended slurry was centrifuged at $20000g_{max}$ in a Safe Guard centrifuge (Clay Adams Inc., New York) for 15 min. The supernatant was used for solid-phase extraction.

Spiking of Beef Samples. A ground beef sample was obtained from the Department of Animal Sciences and Industry's meat-processing facility. Two portions (5 g each) of the ground beef sample were used for analysis. One 5 g portion served as a blank and was used to monitor base levels of fumonisins and/or their hydrolysis products. The other 5 g portion of ground beef was spiked to contain 1 μg/mL each of FB_1, HFB_1, FB_2, and HFB_2. The spiking was achieved by individually pipetting 50 μL of the appropriate stock solutions (100 μg/mL) onto the surface of the ground beef (5 g) sample placed in the blender jar. To ensure dispersion of spiked standards into the beef matrix, the spiked-beef sample was allowed to stand for 5 min before the extraction solvent was added to the blender jar. The spiked-beef sample was then treated as described above.

Isolation of FB_1 and FB_2. Bond-Elut solid-phase anion exchange (SAX) cartridges (3 mL capacity containing 500 mg of sorbent) were obtained from Varian (Harbor City, CA). The SAX cartridge was conditioned by passing 10 mL of methanol (MeOH), followed by 10 mL of MeOH/water (3:1 v/v). The supernatants obtained by centrifuging the corn, feed, and meat samples were then applied to the cartridge. Care was taken that the cartridge did not run dry at the time of the cleanup. The cartridge then was washed with 8 mL of MeOH/water (3:1 v/v), followed by 4 mL of MeOH. These washings were collected and applied to the Amberlite XAD-2 nonpolymeric ion exchange column (see below). FB_1 and FB_2 were eluted from the SAX cartridge by washing with 15 mL of acidified MeOH (5% glacial acetic acid in MeOH). The 15 mL of acidified MeOH eluant was evaporated to dryness under vacuum in a Buchi rotary evaporator (Brinkman Inst., Westbury, NY). The residue was redissolved in 5 mL of MeOH and re-evaporated to dryness under vacuum to ensure complete removal of acetic acid. Finally, the residue was resuspended in 250 μL of acetonitrile/water (40:60 v/v), filtered through a 0.45 μm syringe filter, and stored in a Teflon-lined amber vial at −4 °C.

Isolation of Hydrolysis Products. The Amberlite XAD-2 nonpolymeric ion exchange resin was obtained from Sigma. A 10 cm^3 disposable syringe was plugged with glass wool and filled with 6 g of XAD-2 resin. Another piece of glass wool was inserted to hold the resin bed in place during solvent addition. The resin was washed with 10 mL of MeOH, followed by 10 mL of water. The washings from the SAX procedure that contained the HFB_1 and HFB_2 moieties were titrated to pH <4.5 and then applied to the XAD-2 column. The column then was washed with 10 mL of water, and HFB_1 and HFB_2 were eluted with 15 mL of MeOH. The eluate was evaporated to dryness under vacuum, resuspended in 250 μL of acetonitrile/water (40:60 v/v), and combined with the residues of FB_1 and FB_2. $PHFB_1$ was isolated in the same manner.

HPLC. Analyses were done using a Hewlett-Packard (HP) Series II, 1090A HPLC (Hewlett-Packard, Palo Alto, CA), fitted with a Rheodyne 7125 injector (Rheodyne Inc., Cotati, CA) and having a 20 μL loop. Chromatographic separation was achieved using a double-end-capped, metal-free silica, C_{18} (250 mm × 4.6 mm, 5 μm, Altima) column (Alltech Associates, Deerfield, IL) equilibrated at a temperature of 40 °C. Detection was performed with an HP 1046A programmable fluorescence detector set at an excitation wavelength of 229 nm, an emission

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wavelength of 442 nm, a lamp frequency of 220 Hz, a response time of 4000 ms, and a 418 nm filter.

The mobile phases were acetonitrile/water/acetic acid mixtures, set at A (40:59:1 v/v) and B (60:39:1 v/v), pumped in a gradient of 100% A to 100% B (9 min), and followed by a stepup to 100% acetonitrile at the end of 17 min. The flow rate was 1 mL/min. The fluorescent derivative was prepared by mixing the OPA reagent and the sample in a 2:1 ratio for exactly 1 min.

Derivative Reaction Optimization Time. A 3 μg/mL FB_1 stock solution was mixed with OPA for 0.5, 1, 3, 5, and 10 min. The derivative was injected after the completion of each time period, and the integrated peak area was noted. Triplicate injections were performed at each time interval, and the results obtained were treated statistically.

Thermospray Mass Spectrometry. Mass spectral analyses were performed using an HP 5989A quadrupole mass spectrometer connected to the HPLC via an HP TSP interface. A Model 59970C processor was used for data acquisition and processing. The MS was operated in the filament-on, discharge-off, fragmentor-off mode. The source temperature was set at 225 °C, and the quadrupoles were set at 100 °C. The TSP was set to 98% solvent/mobile phase vaporization, which corresponded to a TSP tip temperature of 178 °C. Linear scanning between m/z 250 and 850 resulted in scan rates of 0.64 scan cycles/s. The MS was tuned prior to analysis using propylene glycol. The mobile phases used were acetonitrile/ 0.1 M ammonium acetate mixtures at a flow rate of 0.8 mL/ min. All analyses were done via direct liquid injections.

RESULTS AND DISCUSSION

Mass Spectral Confirmation of HFB$_1$, HFB$_2$, and PHFB$_1$. Figure 2 shows the filament-on, positive ion (PI), linear scan mass spectra of HFB$_1$ in acetonitrile 0.1 M ammonium acetate (4:1 v/v). The base peak [m/z 406 (M + H$^+$)] is the protonated molecular ion of HFB$_1$. Base hydrolysis cleaves the ester bonds, resulting in the loss of both tricarballylic acid side chains. Thus, the C-14 and C-15 carbons on the hydroxycosane backbone are replaced by hydroxyl (−OH) groups from the alkali, giving HFB$_1$ a molecular mass of 405. The loss of the tricarbalyllic acid side chains makes the HFB$_1$ conducive to positive ion detection, with the probable site of protonation being the primary amine group.

Figure 3 shows the filament-on, PI, linear scan mass spectra of HFB$_2$ in acetonitrile/0.1 M ammonium acetate (4:1 v/v). Fumonisin B$_2$ does not have any functional groups on C-10 (see Figure 1) and, after hydrolysis, yields a backbone that has a molecular weight of 389. The base peak [m/z 390 (M + H$^+$)] is the protonated molecular ion of HFB$_2$. The signals appearing at m/z 424 and m/z 442 are probably [M + NH$_4$ + (H$_2$O)$_n$]$^+$ adducts. The signals observed at m/z 414 (Figure 2) and m/z 398 (Figure 3) were both 9 amu more than the parent ions. Their presence or identity cannot be explained at this time.

Figure 4 shows the filament-on, negative ion (NI), linear scan mass spectra of PHFB$_1$ in acetonitrile/0.1 M ammonium acetate (1:1 v/v). The loss of the single tricarballylic side chain from the parent FB$_1$ results in PHFB$_1$ having a molecular weight of 563. The base peak in the spectrum is at m/z 576. This ion is speculated to be the loss of a neutral, in this case an acid group (−COOH, m/z 45), from the adduct formed between the deprotonated molecular ion (m/z 562) and the acetate ion [(CH$_3$COO)$^-$, m/z 59). Such types of reactions were observed earlier in NI TSP-MS of the parent FB$_1$ (Thakur and Smith,1994).

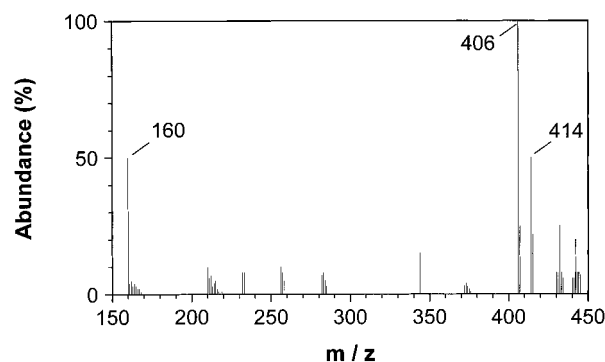**Figure 2.** Thermospray PI, filament-on, direct liquid injection, linear scan mass spectrum of standard HFB$_1$ (10 μg/mL) using acetonitrile/0.1 M ammonium acetate buffer (4:1 v/v).
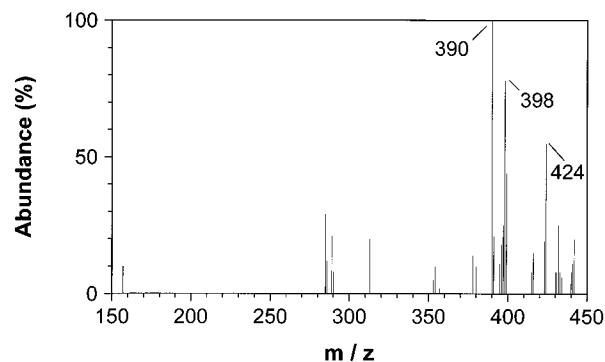
Figure 3. Thermospray PI, filament-on, direct liquid injection, linear scan mass spectrum of standard HFB$_2$ (10 μg/mL) using acetonitrile/0.1 M ammonium acetate buffer (4:1 v/v).
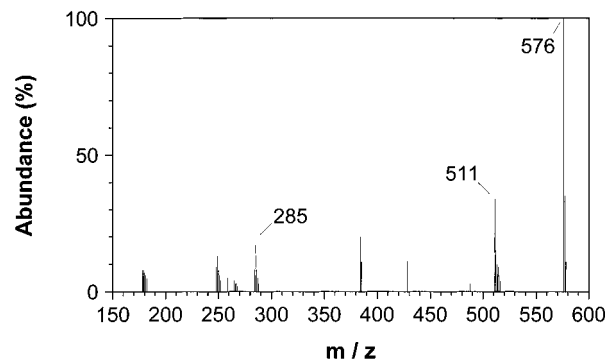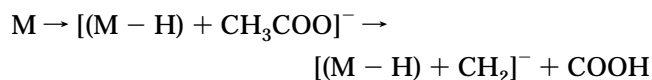
Figure 4. Thermospray NI, filament-on, direct liquid injection, linear scan mass spectrum of standard PHFB$_1$ (10 μg/ mL) using acetonitrile/0.1 M ammonium acetate buffer (1:1 v/v).

$$M \rightarrow [(M - H) + CH_3COO]^- \rightarrow$$
$$[(M - H) + CH_2]^- + COOH$$

Because the hydrolysis was done on standard FB$_1$ (99% purity), the possibility of an impurity is ruled out. However, it is important to note that the presence of the tricarballylic acid side chain renders the PHFB$_1$ molecule sensitive to NI detection, whereas the HFB$_1$ molecule is detected only in the PI detection mode.

Optimization of Reaction Derivatization Time. This study was done to determine the optimum time required to maximize fluorescence intensity at the lowest coefficient of variation. Figure 5 shows the relationship between the fluorescence intensity and various reaction times. The time-dependent instability of the fluorescent adduct formed between the primary amine and OPA results in fluctuation of the fluorescent signal. Although the decay of fluorescence is not linear
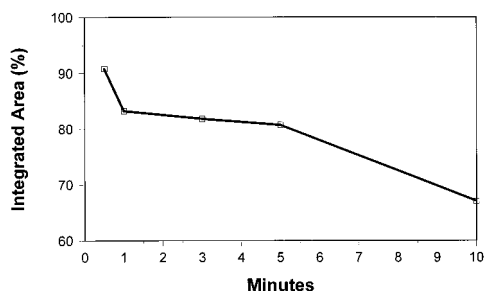
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Figure 5. Time-dependent nonlinear decay of fluorescence intensity of the OPA adduct. Each point represents a mean of triplicate injections for the same time interval. A standard FB_1 (3 μg/mL) was reacted with OPA in a 1:2 ratio in an amber vial before injection.

with respect to time, controlling the time required for derivative formation can reduce the standard error associated with the instability of the fluorescent derivative.

Figure 5 indicates that a reaction time of 1 min before sample injection is best for reproducible results. Longer reaction times (>1 min) showed greater variation and decreased fluorescence intensity. Interestingly, in a similar study performed by Rice et al. (1995) the OPA derivative was found to reach maximum response after 10 min. Whether the rapid decay in fluorescence intensity observed within the first minute in our experiment can be attributed to the premixed Fluoraldehyde reagent cannot be determined. However, a reaction time of <1 min was followed by the participants of an IUPAC collaborative study of a method used to analyze for fumonisins (Thiel et al., 1993), and our data seem to corroborate the shorter reaction time.

Derivative reactions were carried out at room temperature ($\approx$25 °C) and at 5 °C (refrigerator temperature). No statistical difference was found in fluorescence stability of the OPA adduct between the two different temperatures. The temperature-independent

characteristic of the OPA adduct between 4 and 25 °C is typical of primary amines having one hydrogen atom attached to the amino α-carbon (Cronin et al., 1978).

HPLC Analyses. Figure 6A shows the chromatogram of the mixture containing 1 μg/mL FB_1 (11.4 min), FB_2 (15.7 min), HFB_1 (13.1 min), and HFB_2 (19.0 min) standards. In the range 0.3−10 μg/mL, the standard curves were linear. The coefficients of determination (r^2) for FB_1, FB_2 HFB_1, and HFB_2 were 0.9996, 0.9996, 0.9981, and 0.9987, respectively. The lowest detectable limit was 20 ng/mL, at a signal to noise ratio of 5:1. No standard curve was plotted for $PHFB_1$ because it was unlikely to be found in the matrices examined. A 1 μg/mL standard $PHFB_1$ was prepared, derivatized, and injected. $PHFB_1$ had a retention time of 10.3 min. Successful separation of $PHFB_1$ was important, because this metabolite has been detected in the feces of ruminant animals (Rice and Ross, 1994).

Parts B, C, and D of Figure 6 show the chromatograms of the corn, feed, and spiked-beef samples, respectively. All peaks of interest were baseline resolved and devoid of matrix interference. The signals seen between 21 and 23 min are complexes derived from unreacted OPA, which elute when the gradient is stepped up to a 100% organic mobile phase. These signals could be troublesome, especially in isocratic conditions when they may suddenly elute after many runs and appear in the chromatogram as a "mystery peak". We recommend washing the analytical column after analyses with 100% acetonitrile for at least 10 min at a flow rate of 2 mL/min to avoid buildup of such complexes. The highly hydrophobic complexes observed could be attributed to Fluoraldehyde premixed (OPA, Brij-35, sodium borate buffer) and may not present a problem with OPA that is freshly prepared and mixed with the appropriate buffers.

Quantitative analyses were based on standard curves and the method of standards addition (Bader, 1980).
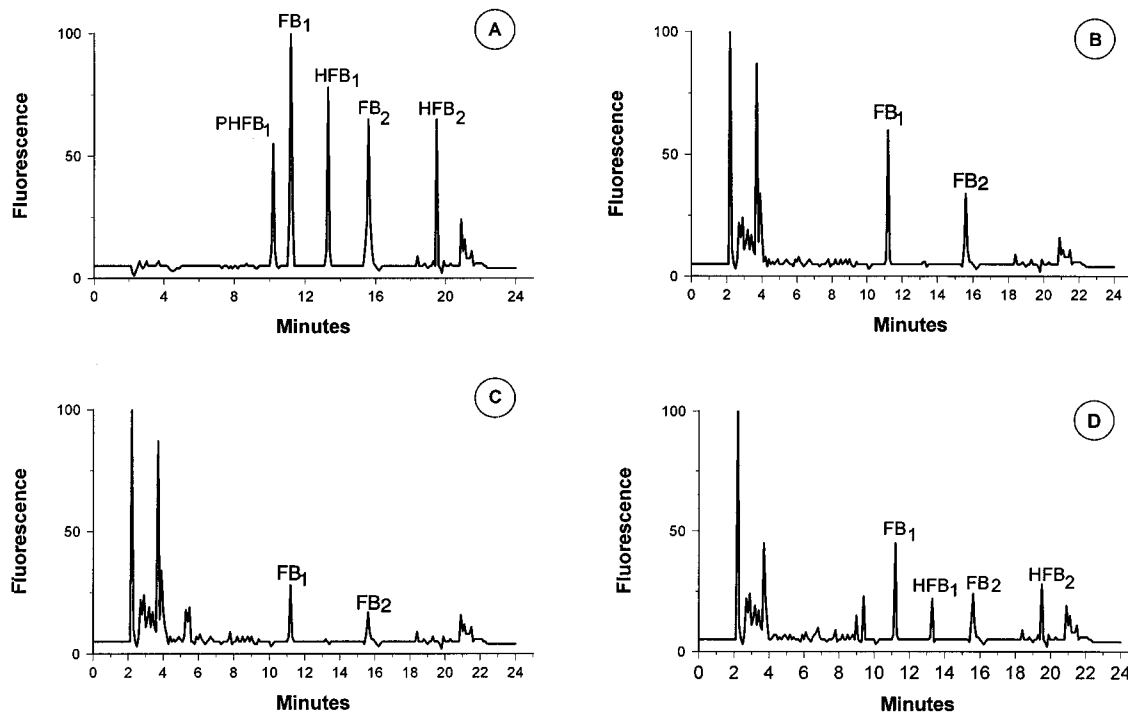

Figure 6. HPLC chromatograms of FB_1, FB_2, HFB_1, HFB_2, $PHFB_1$, and $PHFB_2$ resolved on a C_{18} reversed-phased column using a mobile phase mixture of acetonitrile/H_2O/CH_3COOH, set at A (40:59:1) and B (60:39:1), pumped in a gradient of 100% A to 100% B (9 min) at a flow rate of 1 mL/min. The fluorescence detector was set at excitation and emission wavelengths of 229 and 442 nm, respectively. (A) Standard mixture (1 μ/mL each); (B) corn, (C) feed, and (D) meat extracts spiked with standards.

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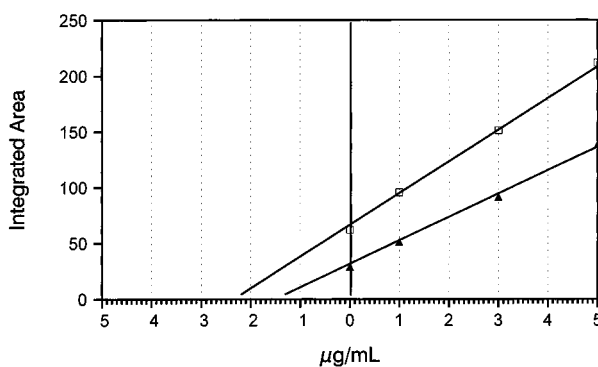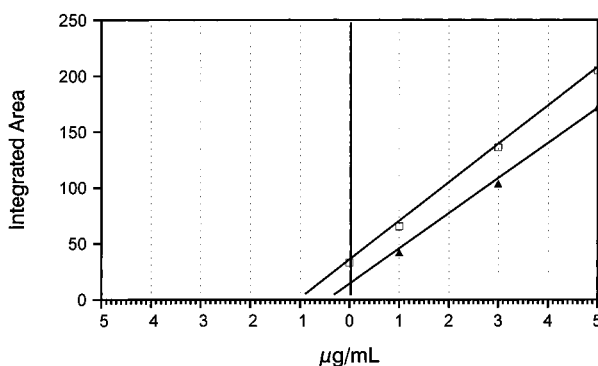*J. Agric. Food Chem.,* Vol. 44, No. 4, 1996 **1051**

Figure 7. Graphical treatment of results from the method of standards addition for the corn sample obtained by HPLC. The point of intersection on the abscissa indicates the concentration at a dilution factor of 0.84. Final concentrations of the sample are about 1.85 μg/mL (2.2 μg/mL $\times$ 0.84) FB$_1$ and 1.09 μg/mL (1.3 μg/mL $\times$ 0.84) FB$_2$. ($\square$) FB$_1$; ($\blacktriangle$) FB$_2$.

Figure 8. Graphical treatment of results from the method of standards addition for the feed sample obtained by HPLC. The point of intersection on the abscissa indicates the concentration at a dilution factor of 0.84. Final concentrations of the sample are about 0.75 μg/mL (0.9 μg/mL $\times$ 0.84) FB$_1$ and 0.25 μg/mL (0.3 μg/mL $\times$ 0.84) FB$_2$. ($\square$) FB$_1$; ($\blacktriangle$) FB$_2$.

This method was used to obtain direct proportionality, and the results were treated graphically. Corn was found to contain 1.84 μg/mL FB$_1$ and 1.09 μg/mL FB$_2$ (Figure 7). The line graphs obtained were treated statistically and had r^2 values of 0.9996 for FB$_1$ and 0.9992 for FB$_2$. Feed was found to contain 0.76 μg/mL FB$_1$ and 0.25 μg/mL FB$_2$. The r^2 values for the line graphs obtained were 0.9992 for FB$_1$ and 0.9990 for FB$_2$ (Figure 8). Both chromatograms were noisy between 2 and 6 min, but no interfering peaks were observed in the region where target analytes eluted.

A background analysis for the presence of FB$_1$, HFB$_1$, FB$_2$, and HFB$_2$ in meat proved to be negative. However, the presence of extraneous peaks between 2 and 9 min emphasizes the importance of longer retention times (t_r) for the target analytes ($t_{r(minimum)}$ = 10.3 min). Results obtained from the method of standards addition were 0.72 μg/mL FB$_1$, 0.60 μg/mL FB$_2$, 0.96 μg/mL HFB$_1$, and 0.84 μg/mL HFB$_2$ (Figure 9). The r^2 values for the line graphs corresponding to FB$_1$, FB$_2$, HFB$_1$, and HFB$_2$ were 0.9997, 0.9906, 0.9812, and 0.9888, respectively. Recoveries from the spiked (1 μg/mL each) meat sample reflected the concentrations determined and were 72% for FB$_1$, 60% for FB$_2$, 96% for HFB$_1$, and 84% for HFB$_2$. Because the emphasis was on chromatography, the recoveries obtained were tolerable for analysis.

Thus, FB$_1$, FB$_2$, HFB$_1$, HFB$_2$, and PHFB$_1$ can be analyzed reliably from natural and biological matrices using the developed HPLC method. Retention factors (k) for FB$_1$ (k = 4.7), FB$_2$ (k = 6.8), HFB$_1$ (k = 5.5), HFB$_2$
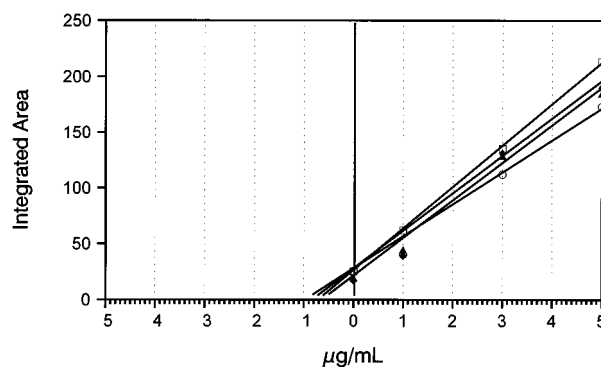

Figure 9. Graphical treatment of results from the method of standards addition for the FB$_1$, FB$_2$, and HFB$_1$ spiked-meat sample obtained by HPLC. The point of intersection on the abscissa indicates the concentration at a dilution factor of 1.2. Final concentrations of the sample are therefore about 0.72 μg/mL (0.6 μg/mL $\times$ 1.2) FB$_1$, 0.6 μg/mL (0.5 μg/mL $\times$ 1.2) FB$_2$, 0.96 μg/mL (0.8 μg/mL $\times$ 1.2) HFB$_1$, and 0.84 μg/mL (0.7 μg/mL $\times$ 1.2) HFB$_2$. ($\square$) FB$_1$; ($\blacktriangle$) FB$_2$; ($\bigcirc$) HFB$_1$; ($\blacklozenge$) HFB$_2$.

(k = 8.8), and PHFB$_1$ (k = 4.1) were within the ideal 2 $\leq k \leq$ 10 region (Dolan, 1994). Despite selective SPE cleanup, interfering peaks were observed between 2 and 6 min for the corn and feed samples and between 2 and 9 min for the beef sample. The $k_{minimum}$ value of 4.1 eliminated the possibility of the target analyte peak mixing with extraneous peaks normally associated with samples within the $\pm$5 min range of the solvent front. This fact is evident from Figure 6B$-$D. The importance of obtaining a $k_{minimum}$ of 4.1 was underscored when biological samples such as feces, urine, and organ tissue (liver, kidneys, pancreas, spleen, gall bladder, tongue) were analyzed using this method (Smith and Thakur, 1995).

The selectivity (α) of the method is between 1.13 and 1.65, with a run time of 23 min and a mobile phase regeneration time of 3 min. Elimination of buffering salts in the mobile phase makes the method amenable to online LC$-$MS analysis and decreases the time required for column equilibration and cleanup, offsetting the slightly longer analysis time ($\leq$26 min). Using acetonitrile results in a maximum system backpressure of 136 bar. The double-end-capped, metal-free, silica column packing combined with a mobile phase pH of 3.5 minimizes silanol interactions and peak tailing. All of these factors make this a reliable HPLC method for the repetitive analyses of fumonisins and their major hydrolysis products.

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