On-Line Immunoaffinity Capture, Coupled with HPLC and Electrospray Ionization Mass Spectrometry, for Automated Determination of Fumonisins

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An automated on-line method for the quantitative detection of fumonisins B_1, B_2, and B_3 and hydrolyzed fumonisin B_1 in corn-based feed was developed using a combination of immunoaffinity capture (IAC)/cleanup, coupled with reversed phase liquid chromatography and electrospray ionization mass spectrometry. Blocking the C2 amine of fumonisin B_1 with 9-fluorenylmethylchloroformate allowed coupling to keyhole limpet hemocyanin through the tricarballylic end, leaving the amino terminus exposed on the immunogenic conjugate after removal of the blocking group. Antiserum had specificity for the C1−C10 fumonisin domain and was used for ELISA and to prepare immunoaffinity columns. The quantitation limit (s/n = 10) for fumonisin B_1 standard was 250 pg using the protonated molecule signal (*m/z* 722). Similar sensitivity was observed for fumonisins B_2 and B_3 (*m/z* 706) and hydrolyzed fumonisin B_1 (*m/z* 406). The on-line IAC/LC/ESI-MS method provided a linear response from the detection limit to 5 ng for fumonisin B_1 and has the capability to analyze low-level contamination of rodent feed samples for fumonisins. The dynamic range can be adjusted as necessary by varying the volume of the sample injected for IAC capture.

Keywords: *Analytical immunology; fumonisins; immunoaffinity; mass spectrometry; immunoassay; site-directed coupling; ELISA; mixtures*

INTRODUCTION

The fumonisins are a group of structurally related toxic metabolites produced by the fungus *Fusarium moniliforme*, which commonly grows on yellow corn. Fumonisins B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3) are considered important contaminants of foods containing corn, and, of these, FB_1 is the most abundant (Figure 1). The ingestion of FB_1-contaminated food causes a variety of toxicities in animals and has been associated with esophageal cancer in humans [reviewed in Ross et al. (1992), Miller (1995), and Shephard et al. (1996)]. The fumonisins are relatively stable and persist through food processing. Furthermore, removal of the propane-1,2,3-tricarboxylic acid side chains produces the corresponding hydrolyzed fumonisins, including hydrolyzed FB_1 (hFB_1), which retains biological activity (Badria et al., 1995; Maragos et al., 1996). The potential toxicity of these sphingosine analogue mycotoxins has resulted in recommendations to restrict animal exposure (Shephard et al., 1996), classification of *Fusarium moniliforme*-derived mycotoxins as possible carcinogens (Vainio et al., 1993), feeding studies to test for potential carcinogenicity in rodents (Gelderblom et al., 1993; Howard et al., 1996), and regulatory interest in methods for the detection and confirmation of fumonisins in foods.

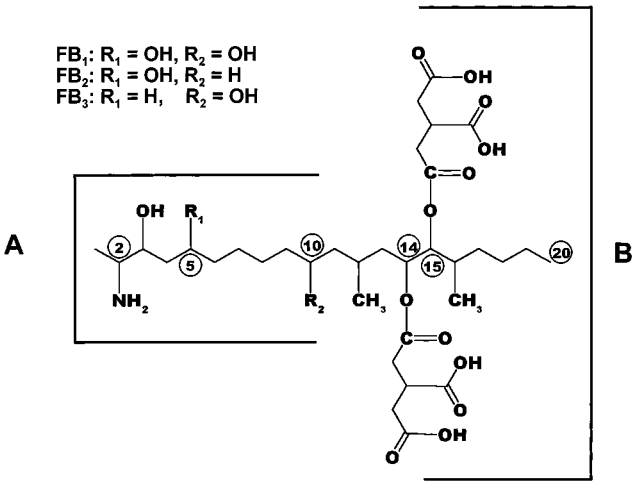

Figure 1. Structures of fumonisins B_1, B_2, and B_3. Two antigenic domains are depicted. Domain A corresponds to C1−C10, and domain B corresponds to C14−C20.

Although the detection of fumonisins is confounded by the lack of a chromophore and the presence of interfering substances in food matrices, analytical detection methods and immunochemical methods for the detection of fumonisins are evolving rapidly. Sensitive and accurate high-pressure liquid chromatography (HPLC) and thin-layer chromatography methods have been developed [reviewed in Scott (1993)], and a standard method for quantifying levels of FB_1, FB_2, and FB_3 in corn was recently accepted by the Association of Official Analytical Chemists (Sydenham et al., 1996a). The procedure uses aqueous methanolic extraction of

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ground corn and solid phase extraction, followed by precolumn *o*-phthalic dialdehyde derivatization and LC determination using fluorescence detection. Several methods based on precolumn fluorogenic labeling with fluorenylmethylchloroformate (Holcomb et al., 1993), *o*-phthalic dialdehyde (Hopmans and Murphy, 1993), and naphthalene-2,3-dicarboxaldehyde (Ware et al., 1994) for the quantitation of fumonisins in rodent feed have been reported. However, these procedures have minimum detection limits for FB_1 in rodent feed of 50−200 ng/g, in part due to the presence of materials in feed that cause fluorescent interference. In addition to the sensitivity limitation, these methods are not optimal for evaluating large numbers of samples because extraction with organic solvents and precolumn derivatization are labor intensive.

Although the exact mechanism of fumonisin B_1-induced toxicity is not known, accumulated evidence indicates that the toxicity is associated with the inhibition of ceramide synthetase (sphinganine *N*-acyltransferase), one of the key enzymes involved in sphingolipid synthesis (Riley et al., 1994; Merrill et al., 1996). This inhibition results in the accumulation of sphinganine in the cells, and increases in the sphinganine/sphingosine ratio have been demonstrated in vitro and in vivo in cells and animals exposed to FB_1. Preliminary rodent toxicity studies in our laboratory demonstrated that low parts per million levels of FB_1 in rodent feed caused detectable alterations in sphingolipid biosynthesis as measured by the ratio of sphingoid bases (Howard et al., 1996). Furthermore, this ratio is directly linked to the apoptotic, hepatotoxic, and nephrotoxic endpoints induced by FB_1 (Tolleson et al., 1996; Bucci and Howard, 1996). On the basis of this experience, limits of 100 ppb FB_1 and 200 ppb total FB_1 congeners ($FB_1 + FB_2 + FB_3$) were established for the untreated control feed for ongoing studies at this institution. Available fluorogenic labeling/LC methods, even in conjunction with immunoaffinity column (IAC) cleanup, were not compatible with these maximum acceptable levels for fumonisins in rodent feed.

A variety of immunochemical methods for determining fumonisins in foods have been reported since the initial report of the development of fumonisin-specific antibodies (Azcona-Olivera et al., 1992). Immunochemical assays for fumonisin include competitive enzyme-linked immunosorbent assays (ELISA) (Fukuda et al., 1994; Pestka et al., 1994; Elissalde et al., 1995; Chu et al., 1995; Usleber et al., 1994; Yeung et al., 1996), antibody-capture ELISAs and indirect competitive ELISAs (Maragos et al., 1996; Sydenham et al., 1996b; Yu et al., 1996), a fiber optic immunosensor assay (Thompson et al., 1996), an immunohistochemical method (Tejadasimon et al., 1995), and a dipstick enzyme-linked immunofluorescent assay (Schneider et al., 1995). These immunoassays report sensitivity from 500 to 0.05 ng/mL, but in general and regardless of format, immunoassays lack the specificity to determine individual fumonisins in mixtures and tend to overestimate levels of fumonisins, presumably due to the presence of common cross-reacting epitopes on compounds other than fumonisins (Pestka et al., 1994; Shelby et al., 1994; Tejadasimon et al., 1995; Sutikno et al., 1996; Sydenham et al., 1996b). Immunoassays nonetheless share the advantages of reduced sample preparation, low cost, speed, sensitivity, and potential for screening samples in the field. Disposable single-use IAC are available commercially (FumoniTest, Vicam, Somerville, MA) and can be used for sample preparation/enrichment followed by derivatization, enabling fluorescent detection of LC separated analytes. This IAC/LC methodology has been reported to have limits of detection of ∼4 ng/g and limits of determination of ∼25 ng/g FB_1 in corn samples (Trucksess et al., 1995).

We recently reported the validation of a method that uses commercially available, single-use IAC for off-line cleanup of corn-based feed samples in conjunction with electrospray ionization mass spectrometry (ESI-MS) for sensitive and unambiguous determination of fumonisins (Churchwell et al., 1997). Here we report site-directed coupling of FB_1 to prepare immunogenic and antigenic conjugates, the development of antisera specific for the C1−C10 domain of fumonisins, and a sensitive *on-line* tandem IAC/LC/ESI-MS assay, based on a reusable immunoaffinity column, that is capable of unambiguous determination of FB_1, FB_2, FB_3, and hFB_1 in a single automated assay.

EXPERIMENTAL PROCEDURES

Safety Note. Fumonisins are potential carcinogens and should be handled with extreme care.

Reagents. The purified fumonisin FB_1 was provided by Robert M. Eppley (CFSAN, FDA, Washington, DC), and purified FB_2 and FB_3 were provided by Ron Plattner (USDA, Peroria, IL). 9-Fluorenylmethylchloroformate (Fmoc-Cl) was purchased from NovaBiochem (La Jolla, CA). Dimethyl sulfoxide (DMSO) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) were purchased from Pierce (Rockford, IL). Rabbit serum albumin (RSA), citric acid, Freund's complete adjuvant, Freund's incomplete adjuvant, and tricarballylic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (La Jolla, CA). Fetal bovine serum was obtained from Gibco (Grand Island, NY). The affinity-purified burro anti-rabbit horseradish peroxidase-conjugated antisera used as the second antibody in ELISA was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Piperidine was purchased from Aldrich (Milwaukee, WI).

Chemical Synthesis. *Fmoc-FB_1* was synthesized by adding a 10-fold molar excess of Fmoc-Cl (35 mM) in 100% acetonitrile to fumonisin (typically 1−10 mg) dissolved in 50% 1 M boric acid, pH 7.5, and 50% acetonitrile (v/v) and stirring for 5 min at room temperature. The Fmoc hydrolysis products were removed from the reaction mixture (total volume 2−3 mL) by extracting the crude mixture with three 1-mL volumes of toluene. The aqueous layer was warmed and evaporated to dryness under argon and then redissolved in water. The Fmoc−fumonisin solution was extracted again, and residual toluene was removed by warming under argon. The purity of Fmoc−fumonisin was determined by HPLC with fluorescence detection and ESI-MS analysis to be >98%, and the structure was confirmed by mass spectra ($M + H^+ = m/z$ 945, $M - H^- = m/z$ 943, Figure 2).

Aminopentol (hFB_1) was prepared by refluxing 10 mg of FB_1 in 50 mL of 1 N NaOH overnight. The mixture was neutralized with HCl and extracted with methylene chloride, the organic layer containing the aminopentol was evaporated to dryness, and the product was dissolved in 2 mL of methanol. A molar excess of Fmoc-Cl (35 mM) in acetonitrile was added to form Fmoc−aminopentol. Fumonisins and hFB_1 used as inhibitors and standards for competitive ELISA and on-line IAC/LC/MS assays were quantified by HPLC after derivatization with Fmoc with fluorescence detection (excitation = 265 nm, emission = 314 nm) and comparison to a standard curve. The HPLC system consisted of a GP40 gradient pump (Dionex, Sunnyvale, CA), a Waters RadialPak cartridge 4μ-C_{18} column (Waters, Milford, MA), a Dionex AD20 UV detector, and a Groton fluorescence detector (Groton, MA). An isocratic

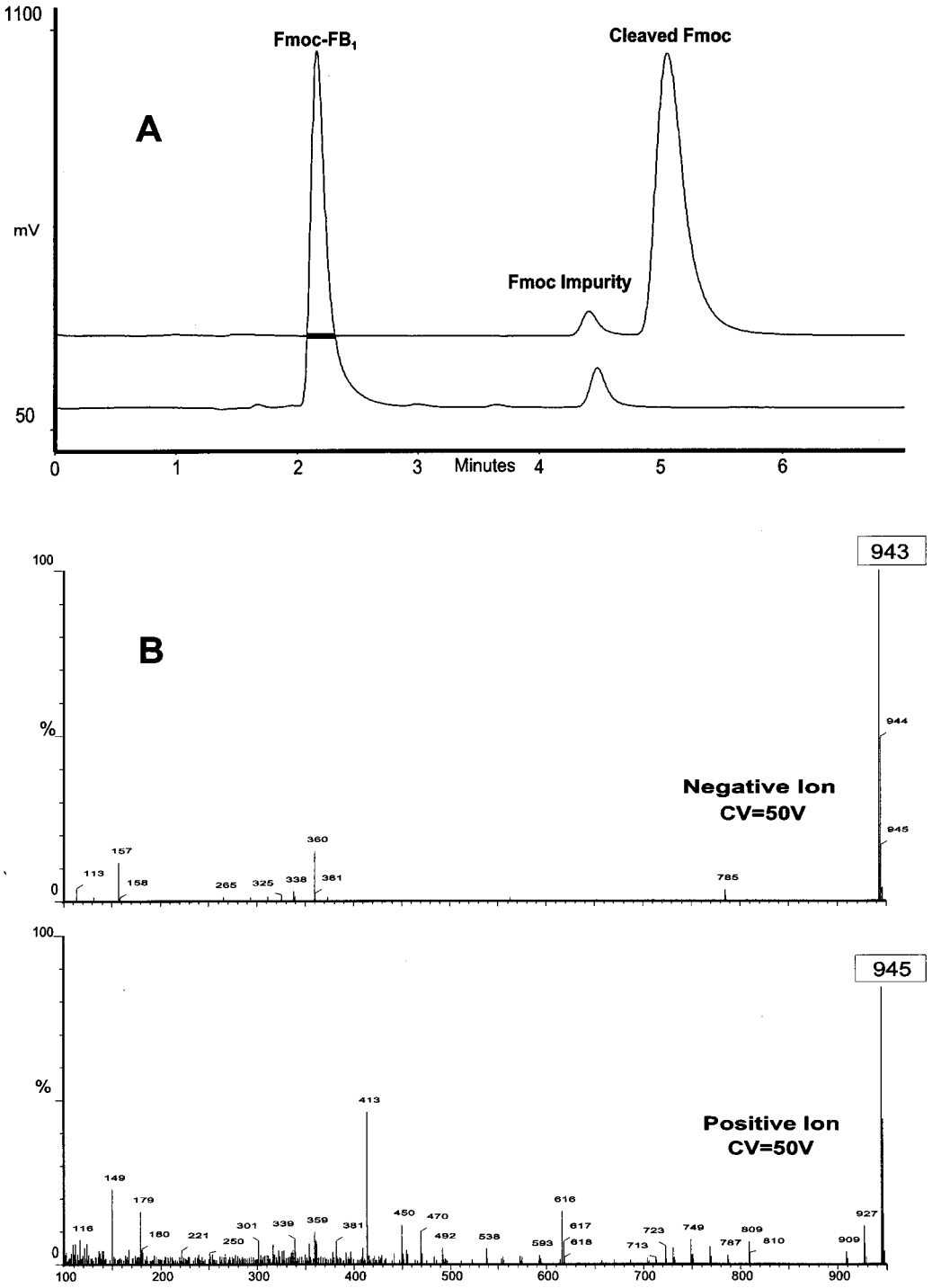

Figure 2. (A) HPLC of FB_1 Fmoc. Fmoc−fumonisin derivatives were quantitated by HPLC with fluorescence detection (excitation = 265 nm, emission = 314 nm). HPLC and comparison to a standard curve prepared from FB_1 Fmoc were used to monitor the products of reactions of Fmoc with fumonisins, deprotection, and quantitation of standards used as inhibitors in competitive ELISA. Liquid chromatography separations were achieved with a Waters RadialPak cartridge 4μ-C_{18} column and isocratic elution with a mobile phase of 50% citric acid buffer, pH 4.7, and 50% acetonitrile at a flow rate of 2 mL/min. (B) MS of FB_1−Fmoc. The identity of FB_1−Fmoc used for synthesis of protein conjugates was confirmed by mass spectrometry ($M + H^+ = m/z$ 945, $M - H^- = m/z$ 943).

gradient of 50% 15 mM citric acid containing 10 mM trimethylammonium acetate, pH 4.7, and 50% acetonitrile at a flow rate of 2.0 mL/min was used to resolve the fumonisins. The Fmoc−aminopentol derivative was purified and quantified by HPLC, and identity was confirmed by ESI-MS analysis (m/z 852).

Synthesis of Protein Conjugates. Since FB_1 has two types of EDC reactive sites (that is, the four carboxyl groups on the two tricarballylic acids at C14 and C15 and the primary amine at C2), the C2 amine was blocked with Fmoc prior to EDC coupling. EDC was used to couple the tricarballylic carboxyl groups of the B domain of Fmoc-FB_1 to R−NH_2 (R = immunogen, assay antigen, or column matrix). To prepare KLH conjugates modified with FB_1 coupled through the tricarballylic acid group (FB_1-c-KLH) for use as immunogen, purified Fmoc-FB_1 (334 nmol) was dissolved in 368 μL of DMSO and 3 mg of EDC was added. The reaction was stirred at room temperature for 1 h, and this mixture was added dropwise to 33.4 mg of KLH in K_2HPO_4/KH_2PO_4 buffer (25 mM, pH 8) with stirring and then allowed to react overnight

at room temperature. The reaction mixture was dialyzed against two 4-L volumes of K_2HPO_4/KH_2PO_4 buffer (25 mM, pH 7.4) overnight to remove any unreacted Fmoc-FB_1 and EDC. To remove the Fmoc protecting group, piperidine (20% v/v) was added and allowed to react for 30 min at room temperature. The reaction mixture was neutralized with HCl and analyzed by HPLC to verify complete release of Fmoc, and the substitution level was determined by the amount of Fmoc released. Using the HPLC conditions described above, the piperidine−Fmoc product had a retention time of 5.5 min (see Figure 2A). The immunogen mixture was dialyzed overnight against two 4-L volumes of K_2HPO_4/KH_2PO_4 buffer (25 mM, pH 7.4) to remove cleaved Fmoc and piperidine. FB_1-c-RSA conjugates used as coating antigen in ELISA were prepared in a similar manner except 1.5 μmol of Fmoc−FB_1 was dissolved in 1.64 mL of DMSO and reacted with 8 mg of EDC prior to reaction with 10 mg of RSA. Protein concentrations were determined using the Coomassie blue dye-binding assay using bovine serum albumin as standard (Bradford, 1976). The substitution levels for KLH and RSA were 20.5 and 2.3 mol of FB_1/mol of protein, respectively.

Immunization. Polyclonal antibody was produced in male albino rabbits immunized with FB_1−KLH conjugates. For the primary immunization the immunogen (1.0 mg of protein/mL in isotonic buffered saline, 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4; IBS) was emulsified with an equal volume of Freund's complete adjuvant, and 2.0 mL of this emulsion (1 mg of immunogen) was divided among 20−30 intradermal injection sites on the back and two intramuscular injection sites. A series of two booster immunizations, at 4 week-intervals followed the primary immunization. Booster immunizations were similar to primary immunization except immunogen was emulsified with Freund's incomplete adjuvant. Blood was drawn from the ear (10−15 mL) 10−14 days after each booster immunization.

ELISAs. The titer and specificity of sera were determined by ELISA using FB_1-c-RSA as solid phase assay antigen. Microtiter plates (96-well; Nunc-Maxisorb, PGC Scientific, Frederick, MD) were coated overnight with FB_1-modified RSA (60 μL containing 100 ng of RSA-c-FB_1 per assay well) by drying the antigen solution at 37 °C. Coated wells were blocked with 3% (w/v) nonfat dry milk in IBS containing 5% fetal bovine serum, washed with deionized water, and then incubated for 90 min at 37 °C with sera diluted in IBS containing 5% fetal bovine serum (dilution buffer). After washing to remove unbound antibody, detection of anti-fumonisin antibodies was accomplished using horseradish peroxidase conjugated burro anti-rabbit IgG (second antibody), followed by washing to remove unbound second antibody and conversion of the substrate 3,3′,5,5′-tetramethylbenzidine dihydrochloride to a chromophore. Substrate conversion was monitored with a kinetic microplate reader (Thermomax, Molecular Devices, Sunnyvale, CA) at 650 nm or at 450 nm after acidification. To optimize for competitive ELISA assays, a checkerboard dilution scheme (serial dilutions of solid phase coating antigen versus serial dilutions of antisera) was used to select a combination of coating antigen concentration and primary antibody dilution that produced an A_{450} of ≈1.0 with adsorbed assay antigen in clear excess and limiting antibody concentration. The relative potency of structurally related fumonisin analogues to inhibit binding of antisera to FB_1-modified RSA assay antigen was determined by competitive ELISA. Inhibitory potency was defined as the inhibitor concentration required to reduce binding of the primary antibody by 50% (IC_{50}).

Affinity Purification of Anti-FB_1 Antibodies. Site-directed coupling was used to modify an amine-containing cross-linked polystyrene/divinylbenzene column matrix (Poros NH, PerSeptive Biosystems, Framingham, MA) such that FB_1 was bound via carboxyl groups on the propane-1,2,3-tricarboxylic acid side chains. This matrix was used to affinity isolate antibodies with major specificity for the C1−C10 domain of FB_1. Briefly, 20 mg of EDC was added to 2.5 μmol of Fmoc−FB_1 dissolved in 2 mL of K_2HPO_4/KH_2PO_4 buffer (25 mM, pH 8). This mixture was added to 2 mL of a 50% slurry of the column matrix in K_2HPO_4/KH_2PO_4 buffer (25 mM, pH 8), and the reaction slurry was placed on a rocking platform at room temperature overnight. The column matrix was washed to remove unbound reactants, and the binding of FB_1 to column matrix was verified by monitoring the release of the protecting Fmoc group by cleavage with 20% piperidine. The FB_1-modified column matrix was washed to remove cleaved Fmoc and piperidine and slurry-packed in a 0.8 mL bed volume poly ether ether ketone (PEEK) column (Perspective Biosystems). One milliliter of neat rabbit anti-FB_1 antisera with specificity for the C1−C10 domain of FB_1 was loaded on the column at 1 mL/min. The column was washed at 2 mL/min with 10 column volumes of IBS. Nonspecifically bound proteins were eluted with IBS containing 0.5 M NaCl. Specific antibodies were eluted with glycine hydrochloride, pH 2.6, and antibody-containing fractions were immediately adjusted to pH 7.4 with Tris base.

Antibody Immunoaffinity Column. Affinity-purified anti-FB_1 from 1 mL of neat serum was immobilized on 500 μL of Poros protein A affinity matrix (IgG binding capacity ≈ 30 mg of IgG/mL of matrix) in 100 mM triethanolamine solution, pH 8.0 (conjugation buffer). The reaction was conducted in a 15 mL polypropylene centrifuge tube, and washes and buffer exchanges were accomplished by centrifugation at 2000*g* and removal of the supernatant. After the matrix was washed two times with conjugation buffer, the immobilized antibody was covalently coupled to the protein A matrix by reaction with 15 mM dimethyl pimelimidate hydrochloride (DMP) in 5 mL of conjugation buffer for 30 min at room temperature with gentle rocking. Excess DMP was quenched by washing with 5 mL of 100 mM monoethanolamine, pH 8.5, followed by two washes with IBS. The antibody affinity matrix was slurry-packed in 2.1 × 30 mm PEEK columns (Perspective Biosystems), and the packed IAC were stored in IBS containing 0.05% (w/v) sodium azide.

Sample Preparation. Rodent feed (NIH-31) was obtained from Ralston Purina Co. (St. Louis, MO). Approximately 2 kg of feed pellets was thoroughly mixed and ground in a V-blender for 30 min. A 10 g portion was extracted with 40 mL of acetonitrile/water (50:50) in a 250 mL blender cup for 2 min. A 20 mL aliquot of this extract was centrifuged at 5000*g* for 5 min, and then 2 mL of the extract was dried, adjusted to 5 mL with IBS, and filtered through a 0.4 μm Spin-X 100 (PGC Scientific, Frederick, MD). Samples (0.1 g of feed equivalent/mL) were stored at 4 °C.

Instrumentation for On-Line Analysis and Mass Spectrometry. The system for on-line analysis of fumonisins by IAC/LC/ESI-MS was composed of an automated HPLC system, PeakNet application's software, an AS 3500 autosampler (Dionex), two pumps [a GP40 gradient pump (Dionex) for the aqueous IAC mobile phase and an ISCO Model 260 syringe pump (ISCO, Lincoln, NE) for the reversed phase mobile phase], two Rheodyne 9750 TPMV six-port switching valves (Rheodyne, Cotati, CA), and three columns [a 2.1 × 30 mm PEEK affinity column packed with Poros protein A matrix (Perspective Biosystems) modified with affinity-purified anti-FB_1, a 2.1 × 30 mm PEEK trapping column packed with Poros R-10 (Perspective Biosystems), and an analytical column [Ultracarb 3 ODS (20) column, 2.0 × 150 mm, C_{18}, 3 μm, Phenomenex, Torrance, CA]] (Figure 3; Table 1). A Platform II single quadrupole mass spectrometer (Micromass, Altrincham, U.K.) was equipped with an atmospheric pressure ionization source, and an electrospray interface was used. The total LC effluent (0.2 mL/min) from the C_{18} analytical column was delivered into the ion source (150 °C) through the electrospray capillary (3.3 kV), using nitrogen as the nebulizing and drying gas. Positive ions were acquired in selected-ion monitoring mode (dwell time, 0.3 s; span, 0.02 μ; and interchannel delay time, 0.03 s) while the sampling cone-skimmer voltage was varied to produce in-source CID simultaneous with the acquisition of the corresponding selected ion. The protonated molecules for FB_1 (*m*/*z* 722), FB_2 and FB_3 (both *m*/*z* 706), and hFB_1 (*m*/*z* 406) were acquired at 40 V, and fragment ions for FB_1 (*m*/*z* 352 and 334) were acquired at 70 V.

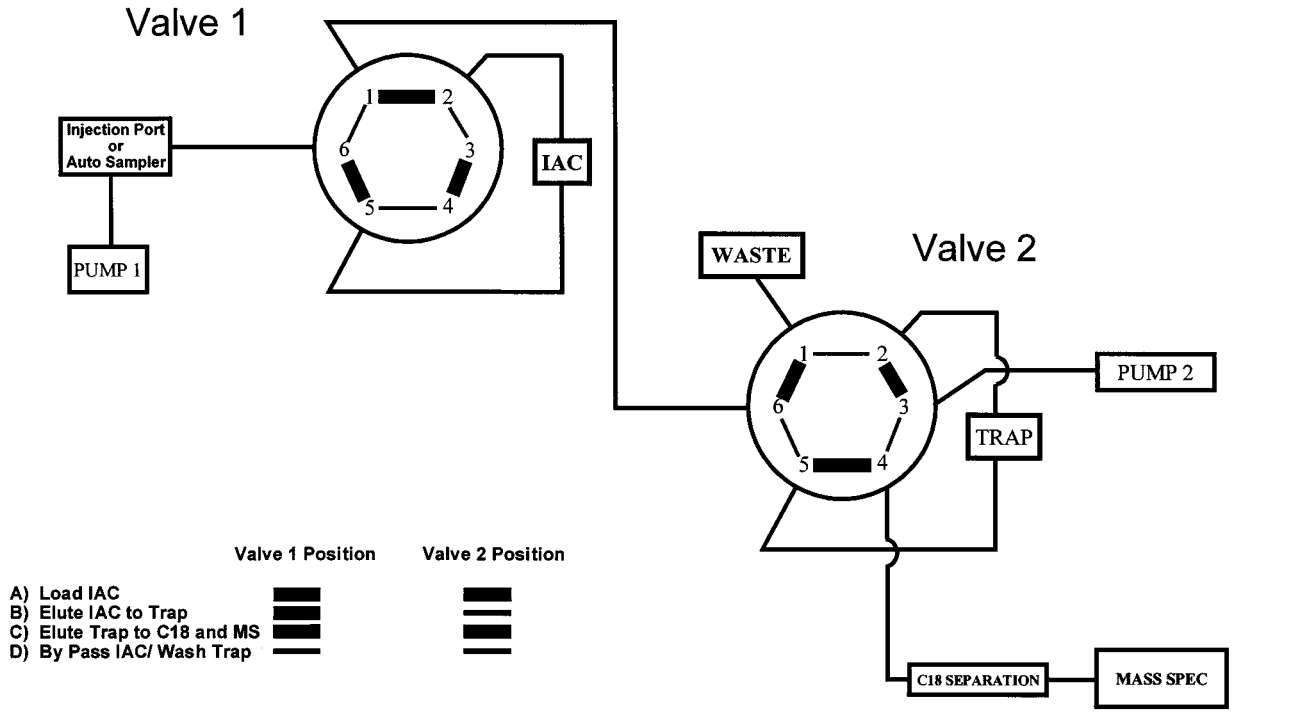

Figure 3. On-line IAC/LC/ESI-MS configuration. Diagram of on-line method for the determination of FB_1, FB_2, FB_3, and hFB_1 in standards or corn-based feed. Analytes in aqueous buffer are captured on a 100 μL immunoaffinity column with specificity for the C1−C10 domain of FB_1, washed to remove confounding materials in the matrix, and eluted to a reversed phase trap column. The trap column is eluted with an organic/volatile buffer, and the relevant analytes are resolved on a reversed phase analytic column with detection by ESI-MS and selected ion monitoring.

Table 1. Procedure for Determination of Fumonisin Congeners in Feed Samples by IAC/LC/ESI-MS

step	valve 1[a]	valve 2[b]	events[c]
1	IAC in-line	on	(P-1) equilibrate IA column with IBS by pumping 100% C through the IA column to waste for 5 min at 1.5 mL/min *(P-2) equilibrate trap column and C_{18} analytic column by pumping 55% 0.1% formic acid/45% acetonitrile through trap and C_{18} analytic column*
2	IAC in-line	on	(P-1) load IA column by injecting 100 μL of feed extract (10 mg of feed equivalent) while pumping 100% C for 1 min at 0.2 mL/min *(P-2) same as above*
3	IAC in-line	on	(P-1) flush IA column with 100% C for 3 min at 1.5 mL/min to remove unretained materials from IA column *(P-2) same as above*
4	IAC in-line	off	(P-1) elute affinity bound fumonisin congeners from IA column to the trap column with 100% A (5 min), 70% A/30% D for 1.5 min at 1 mL/min with trap column effluent routed to waste *(P-2) equilibrate C_{18} analytic column pumping 55% 0.1% formic acid/45% acetonitrile through C_{18} analytic column*
5	IAC in-line	on	(P-1) wash IA column with 20% A, 80% D for 1 min followed by 100% C (3.5 min) at 1.5 mL/min to waste *(P-2) elute trap column by back-flushing to the C_{18} analytic column where HPLC separation of fumonisin congeners by reversing flow through trap column and pumping 55% 0.1% formic acid/45% acetonitrile through the trap column and C_{18} analytic column at 0.2 mL/min with ESI selected ion MS detection*
6	bypass IAC	on	(P-1) wash line from pump 1 to waste with 100% A for 1 min at 1.5 mL/min *(P-2) same as above*
7	bypass IAC	off	(P-1) wash trap column with 20% A/80% D for 2 min at 1.5 mL/min *(P-2) same as above except trap column is removed from flow*
8	bypass IAC	off	(P-1) equilibrate trap with 100% A for 2 min at 1.5 mL/min *(P-2) same as above*

[a] Valve 1 is a Rheodyne 9750 TPMV six-port switching valve between the autoinjector/injection port and port 6 of valve 2 configured to place the IAC inline or bypass the IAC. [b] Valve 2 is a Rheodyne 9750 TPMV six-port switching valve configured to route mobile phase from pump 1 to waste ("on") or through the trap column to waste ("off") *and* route mobile phase from pump 2 through the C_{18} separation column without going through the trap column ("off") or route mobile phase from pump 2 through the trap column and the C_{18} separation column ("on"). [c] Mobile phases: for pump 1 (P-1), A = 2% (v/v) formic acid, C = isotonic buffered saline (IBS), D = methanol; for pump 2 (P-2), 55% 0.1% formic acid, 45% acetonitrile.

RESULTS AND DISCUSSION

The integrity of long-term studies to determine FB_1 toxicity or carcinogenicity in rodents is dependent on control feed of low, defined fumonisin content. The objective of this work was to develop an antibody-based method capable of determining individual fumonisins at low levels in control feed intended for use in rodent bioassays.

The initial goal was to prepare antisera with the capability to distinguish among FB_1, FB_2, and FB_3.

Several considerations contributed to the approach. With regard to structural similarities among the fumonisins and the substituent groups that potentially contribute to the antigenicity of the fumonisins, the C14 and C15 tricarballylic groups and the C1−C20 backbone are common to FB_1, FB_2, and FB_3; the C5 hydroxyl is common to FB_1 and FB_2; and the C10 hydroxyl is common to FB_1 and FB_3 (Figure 1). Since there are no other primary structural differences, the effort to prepare antisera specific for FB_1 can be restated as the effort to prepare antisera with specificity for the hydroxyls at C5 and C10 or an associated unique conformational epitope.

Molecules as small as FB_1 (molecular mass of 721) may serve as haptens, but they are generally not immunogenic unless coupled to an immunogenic carrier protein. With a single exception (Yeung et al., 1996), all efforts to develop fumonisin-specific antibodies have been based on immunogenic FB_1-carrier protein conjugates conjugated via the reactive amine group on C2 of FB_1 (Figure 1). Previous work by Azcona-Olivera et al. (1992) and Fukuda et al. (1994) demonstrated that coupling to the immunogenic carrier protein at the C2 amine of FB_1 with a short, rigid, gluteraldehyde-generated link resulted in antibodies that cross-reacted with all three fumonisins and did not recognize the FB_1 backbone generated by hydrolysis to remove the two tricarballylic groups. Elissalde et al. (1995) also coupled via the C2 amine of FB_1 but used a flexible 16 atom linkage to prepare immunogen and produced a panel of 15 monoclonal antibodies that had approximately similar reactivities with FB_1 and FB_2 and reacted with FB_3 with ≈2−4-fold less potency. Notably, these antibodies also reacted with hFB_1, which had an inhibitory potency in competitive ELISA 15−180-fold less than that of FB_1. These observations indicate the dominance of the C14 and 15 tricarballylic groups as antigenic determinants in antisera raised against C2-coupled immunogenic conjugates and the increased recognition of the C5 hydroxyl when a long flexible linker is used at C2 to prepare the immunogen.

The two approaches described here differ from previous methods to prepare anti-FB_1 antisera (Azcona-Olivera et al., 1992; Fukuda et al., 1994; Elissalde et al., 1995; Chu et al., 1995; Yu et al., 1996) in that the coupling strategies were intended to orient the relevant (contributing to antigenic individuality) C5 and C10 aspect of FB_1 and the C2 amine in the distal "domain A" out position relative to the immunogenic carrier. This is important because the hFB_1 produced from FB_1 by some food-processing procedures (Badria et al., 1995) has been reported to have biologic activity. Furthermore, the C2 amine of FB_1 has been reported to be essential for biological activity (Abbas et al., 1994; Gelderblom et al., 1993).

FB_1 was derivatized at the C2 amine with Fmoc to provide a fluorescent label for quantitation and to provide a starting material for conjugate synthesis. HPLC was used to purify and quantitate Fmoc derivatives of FB_1 (Figure 2A), and mass spectral analysis was used to verify the identity of Fmoc−FB_1 used for conjugate synthesis (Figure 2B). A site-directed coupling procedure using EDC was used to couple the tricarballylic carboxyl groups of the B domain of FB_1 to KLH for use as immunogenic conjugate (FB_1-c-KLH), to RSA for use as solid phase ELISA assay antigen (FB_1-c-RSA), and to an HPLC matrix for affinity purification

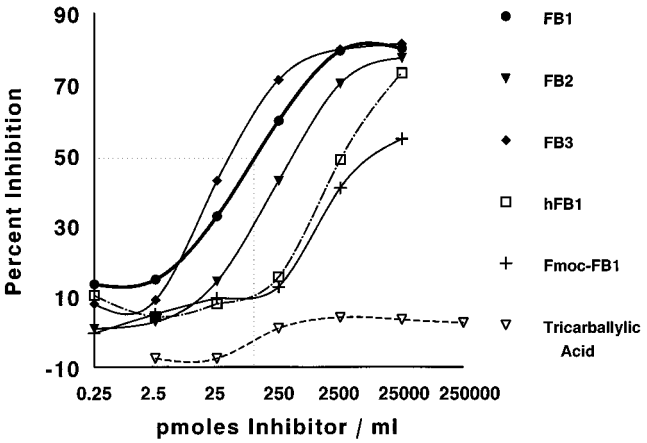

Figure 4. Competitive ELISAs utilized a coating antigen of RSA modified with FB_1 coupled through the tricarballylic acid group (FB_1-c-RSA; 100 ng/well), a 1:20000 dilution of rabbit anti-FB_1 (primary antibody), and structurally related inhibitors in duplicate at final concentrations of 0−25 nmol/well. Bound primary antibody was detected using a 1:6000 dilution of HRP-conjugated burro anti-rabbit Ig. The concentrations of inhibitor required to inhibit 50% of the binding of antibody to FB_1-c-RSA in competitive ELISA (IC_{50}) were FB_3, 40 pmol/mL; FB_1, 120 pmol/mL; FB_2, 399 pmol/mL; hFB_1, 2.6 nmol/mL; and Fmoc-FB_1, 6.0 nmol/mL. Inhibition with tricarballylic acid was not detected at concentrations of up to 250 nmol/mL.

of antibodies. Since FB_1 has two types of EDC reactive sites, the tricarballylic acid carboxyl groups and the primary amine at C2, Fmoc was used to protect the C2 amine and ensure selective coupling at the carboxyl groups. After EDC coupling and dialysis to remove unbound reagents, mild base treatment with piperidine was used to remove Fmoc. The release of Fmoc was measured by HPLC and provided the means to verify covalent binding and to determine substitution levels. This approach resulted in immunogen and antigens in which FB_1 was coupled through one or more tricarballylic acid carboxyl groups with the antigenic determinants of the C1−C10 portion of FB_1 (domain A) presented distal to the carrier.

Antiserum obtained after the third immunization with FB_1-c-KLH had a titer >1:100000 against FB_1-c-RSA in direct ELISA. This antiserum was used at a final dilution of 1:20000 in the competitive ELISA used to determine the relative potency of structurally related analytes to inhibit the binding of anti-FB_1 antibodies to FB_1-c-RSA coated in 96-well plates. The IC_{50} values were as follows: FB_3, 40 pmol/mL; FB_1, 120 pmol/mL; FB_2, 399 pmol/mL; hFB_1 2.6 nmol/mL; and Fmoc-FB_1 6.0 nmol/mL. Inhibition with tricarballylic acid was not detected at up to 250 nmol/mL (Figure 4). The recognition of FB_1, FB_2, FB_3, and hFB_1 and the lack of recognition of tricarballylic acid suggested the antigenic determinants recognized by this antisera are on the C1−C10 portion of fumonisins (domain A). The importance of antigenic determinants in the vicinity of the C2 amine was confirmed by the 50-fold decrease in binding when the C2 amine of FB_1 was blocked with Fmoc (FB_1 versus Fmoc−FB_1). The reason for the greater affinity of the antiserum for FB_3 than FB_1 is not known, but the observation emphasizes the difficulty of distinguishing immunochemically the presence or absence of hydroxyl groups at C5 and C10. The observation is also similar to the increased affinity of some clones of mouse monoclonal antibodies for FB_2 as compared to FB_1 in

mice immunized with FB_1−protein conjugates (Elissalde et al., 1995; Fukuda et al., 1994).

About the same time our laboratory was using Fmoc to block FB_1 and produce immunogen (Newkirk et al., 1996), Yeung et al. (1996) used an essentially analogous approach in which they protected the C2 amine group with 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile, coupled to human serum albumin via carboxylic acid(s) of FB_1 using EDC and then deprotected the amine prior to immunization. The ELISA based on this antisera is among the most sensitive ELISAs for FB_1 reported (Yu et al., 1996; Yeung et al., 1996) and is 130-fold more sensitive in detection of FB_1 (IC_{50} = 0.66 ng/mL) than the ELISA reported here. The apparently higher affinity of these antibodies is probably the result of an increased period for affinity maturation prior to harvesting antibody. Yeung et al. used antiserum obtained after six or seven immunizations made at 1-month intervals, whereas the antisera described here were obtained after the third immunization. Interestingly, both the antisera produced against FB_1 conjugated through carboxyl groups of the C14 and C15 tricarballylic acids (Yeung et al., 1996) and the anti-FB_1-c-KLH described here recognized hFB_1 with ≈20-fold less efficiency than FB_1.

A promising alternative approach for the development of antibodies with specificity for the A-domain of FB_1 involved immunization with a defined C1−C14 fragment. The fragment was prepared using Fmoc to protect the C2 amine of FB_1 and base hydrolysis to remove the C14 and C15 tricarballylic acids, followed by oxidative cleavage between the resultant vicinal hydroxyls at C14 and C15 using sodium metaperiodate. The resultant reactive aldehydes at C14 and/or C15 were reacted with primary amines on KLH to form a Schiff base that was reduced with sodium cyanoborohydride to form a stable covalent linkage, and the Fmoc protecting group was removed with piperidine. It was anticipated that immunization with this KLH conjugate would result in useful anti-domain A antisera. The resultant anti-C1−C14 fragment antisera did not react with RSA in ELISA and had high titer and specificity for RSA modified with C1−C14 fragment using the same coupling process. However, this anti-fragment antiserum did not cross-react with FB_1-c-RSA protein, and the reactivity of anti-fragment antisera with fragment-modified RSA could not be inhibited with FB_1. In subsequent experiments it was determined that the anti-C1−C14 fragment antiserum did not react in solution with FB_1, FB_2, FB_3, or hFB_1 (data not presented). No further developmental work with this antiserum was pursued.

Recently, Maragos et al. (1996) prepared monoclonal antibodies using hFB_1 coupled through the C2 amine to carrier protein as immunogen. The ELISA based on these antibodies had high specificity for hydrolyzed fumonisins but did not recognize intact fumonisins.

Collectively, the ELISA results reported herein and the antibody development work by other investigators described above suggest (1) it is probably not likely that antisera can be prepared that will react with FB_1 and not cross-react with FB_2 and FB_3 and (2) the mosaic of antigenic determinants recognized by both monoclonal and polyclonal antibodies raised against FB_1 immunogenic conjugates coupled through either the C2 amine (Azcona-Olivera et al., 1992; Fukuda et al., 1994; Usleber et al., 1994; Elissalde et al., 1995; Yu et al., 1996) or the tricarballylic carboxyl groups (Newkirk et al., 1996; Yeung et al., 1996) probably include conformational antigenic determinants contributed by the C14 and C15 tricarballylic acids. This is consistent with recent theoretical calculations describing the three-dimensional structure of fumonisins (Beier et al., 1995). This structural model shows a cage-like configuration for FB_1 in which the two esterified trimethylpropane-1,2,3-tricarboxylic acid side chains at C14 and C15 fold back toward the C2 primary amine. In this model, the hydroxyl groups and the acid groups extend into this cage. This model is consistent with the observed and reported importance of the C14 and C15 side chains, the less-than-expected contribution of C5 and C10 hydroxyls to antigenic individuality among fumonisins, the lack of cross-reactivity with intact FB_1 by antisera prepared against either the C1−C14 fragment (above) or hFB_1 (Maragos et al., 1996), and the importance of conformational epitopes in the recognition of fumonisins by antibodies.

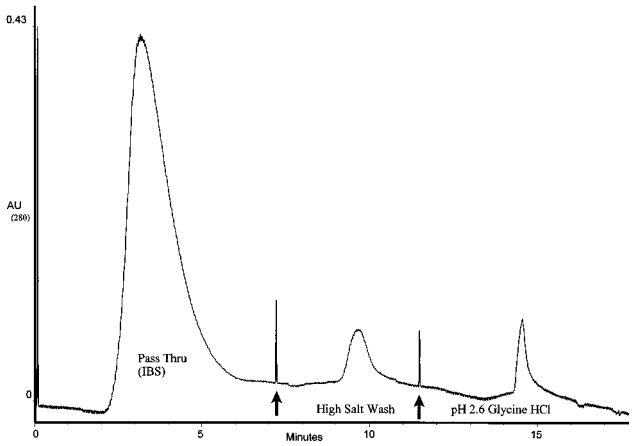

Figure 5. Affinity purification of anti-FB_1 antisera using Poros NH_2 media derivatized with Fmoc-protected FB_1 coupled through the tricarballylic carboxylic group and then deprotected to leave the C1−C10 antigenic domain of FB_1 distal to the support. Affinity purification resulted in a 10^6-fold enrichment of anti-FB_1 antibodies in the low pH eluted fraction relative to the pass-through. Affinity-purified anti-(C1−C10)-FB_1 was used to prepare immunoaffinity columns used in the on-line IAC/LC/ESI-MS procedure.

Although immunoassays to detect fumonisins have great sensitivity (Yu et al., 1996; Yeung et al., 1996) and are useful to screen food products for fumonisin contamination (Sydenham et al., 1996b), major disadvantages have been the inability to distinguish among fumonisins (Azcona-Olivera et al., 1992; Tejadasimon et al., 1995; Yeung et al., 1996) and the tendency to overestimate levels of fumonisins due to cross-reactivity with compounds in biological matrixes that share antigenic determinants with fumonisins (Tejadasimon et al., 1995). The consequence of cross-reactivity among fumonisins and other substances is that the results are actually "FB_1 equivalents", and it is not apparent without further analytical determination whether the result is due to a small amount of FB_1, a mixture of fumonisins, or a larger amount of a cross-reacting substance. Since the objective was to develop a method capable of determining individual fumonisins in feed, and we had developed a "pan-specific" anti-fumonisin antibody capable of detecting FB_1, FB_2, FB_3, and hFB_1, the focus of work shifted to the use of antibodies for affinity concentration/cleanup in tandem with a determinative analytical method.

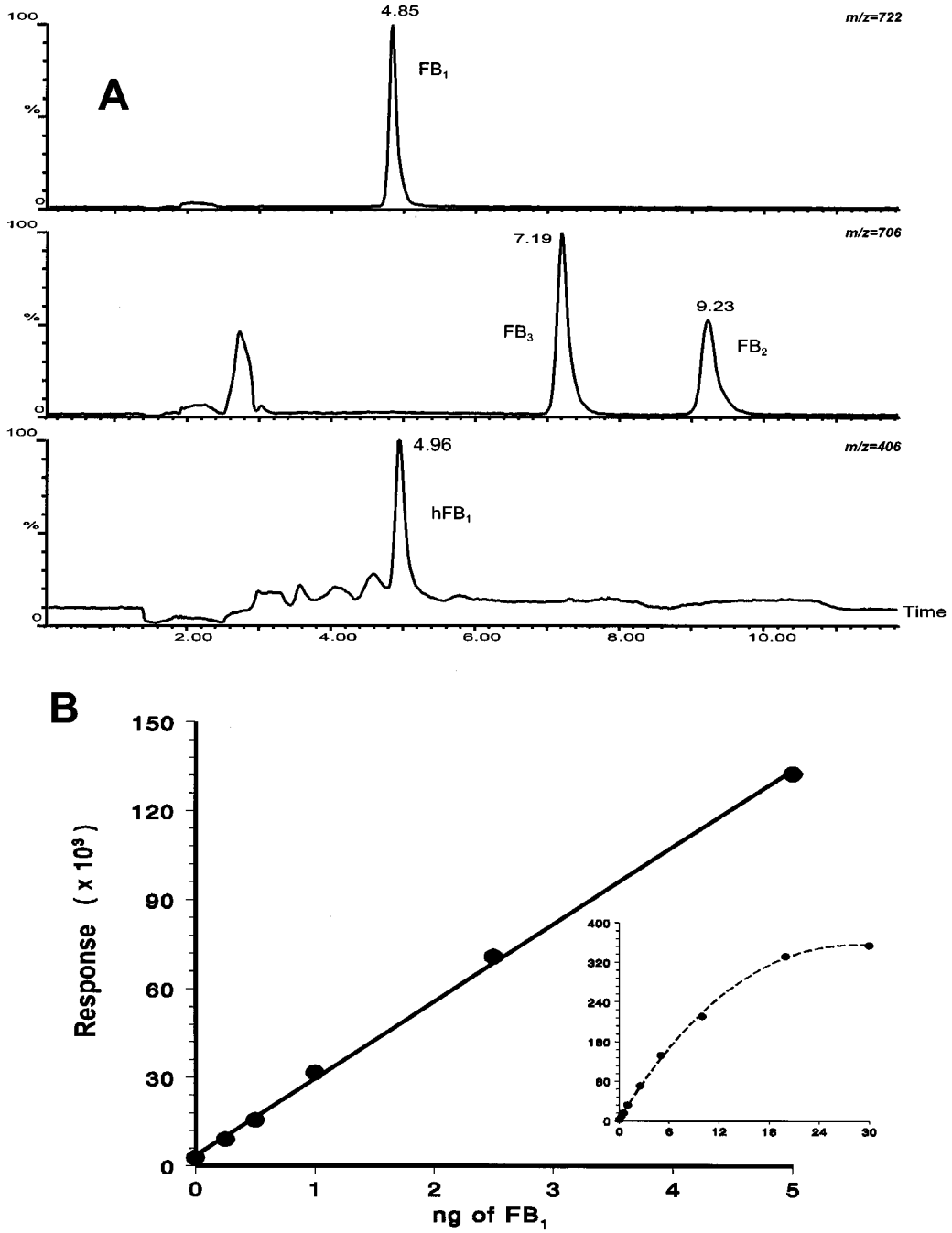

Figure 6. (A) Selected ion monitoring ESI-MS chromatrogram for the on-line HPLC separation of FB_1, FB_2, FB_3, and hFB_1. Identification is based on retention time and mass of the protonated molecule (*m/z*). FB_2 and FB_3 have the same mass (*m/z* 706) but are resolved on the basis of their retention times of 9.23 and 7.19 min, respectively. FB_1 and hFB_1 had retention times of 4.85 and 4.96 min and *m/z* of 722 and 406, respectively. (B) Calibration curve for the FB_1 immunoaffinity column. For a 100 μL volume IAC, the standard curve for on-line IAC/LC/ESI-MS determination of FB_1 (serial 2-fold dilutions of a 0.3 μg/mL solution of FB_1, 100 μL injections), was linear from 0 to 5 ng of FB_1 ($R = 0.999$), and 5 ng of FB_1 was considered the column capacity. The inset shows the relationship of the amount injected versus MS signal for 0−30 ng of FB_1.

Affinity purification of antisera prepared against the A-domain of FB_1 resulted in a 10^6-fold enrichment of anti-FB_1 antibodies measured by noncompetitive ELISA, relative to the pass through (Figure 5). Affinity-purified antibody was covalently coupled to the protein A using DMP. The resultant anti-FB_1 antibody-modified affinity matrix was slurry-packed in 2.1 × 30 mm HPLC columns, and these anti-FB_1 immunoaffinity cartridges were the IAC component of an on-line IAC/LC/ESI-MS assay for fumonisins in rodent feed. Previous work in our laboratory had established methodology for LC/ESI-MS detection of fumonisins (Churchwell et al., 1997)

The configuration of the IAC/LC/ESI-MS is summarized in Figure 3. The autosampler, pumps, switching valves, and mass spectrometer are computer controlled during a continuous sequence, which can be divided into four major phases: (A) load IAC, (B) elute IAC to trap, (C) elute trap column to the analytic column, and (D) bypass IAC/wash trap to regenerate system. Immunoaffinity capture and elution to the trapping column are accomplished using the aqueous mobile phases IBS and 2% formic acid, whereas backflushing the trapping column, separation of fumonisins on the analytical column, and detection in the mass

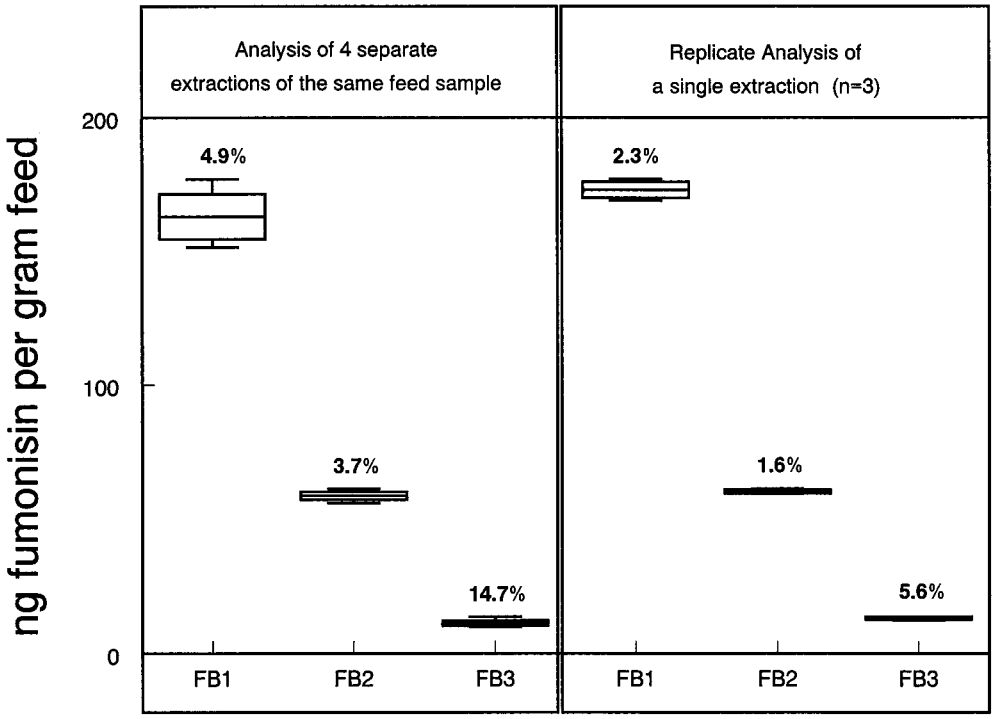

Figure 7. Variability associated with analysis of four separate extractions of the same feed sample (219) was greater than the variability associated with three replicate analyses of the same extract. Each 100 μL injection corresponds to 10 mg of feed equivalent. The horizontal lines through the boxes represent the sample means, the lower and upper edges of the boxes represent the 25th to the 75th percentiles of the data, and the error bars represent the 5th and 95th percentiles. Values are %CV.

spectrometer are accomplished using a volatile mobile phase comprised of 55% formic acid (0.1%) and 45% acetonitrile. The application's software is programmed to control sample injection, mobile phase changes, and column switching in a sequence that includes equilibration of columns, loading sample on the anti-FB_1 IAC, washing the IAC, elution of the IAC to the trapping column, back-flushing the trapping column, separation on the analytical column, and ESI-MS detection (see Table 1 for details).

Using this system, analytes that are retained by the IAC are washed to remove contaminants. These affinity-concentrated fumonisins are then separated on a C_{18} analytical column and detected by selected ion monitoring ESI-MS (see Figure 6A). FB_1 standard was used to determine column capacity and establish the linearity of the response curve. The correlation coefficient (R) for a second-degree polynomial (linear) fit of the MS signal versus FB_1 concentration from the detection limit up to 5 ng was 0.999 (see Figure 6B). Calibration curves were also determined for FB_1, FB_2, FB_3, and hFB_1 using 100, 50, and 25 μL injections of a mixture containing 20 ng/mL FB_1, 6 ng/mL FB_2, 3 ng/mL FB_3, and 3 ng/mL hFB_1. The slope (MS signal/concentration) for FB_1 injected as a pure standard and the slope for FB_1 taken individually from the mixture of fumonisins were similar (<4.2% change in slope). For each of the individual components in the mixture, the calibration curves were linear and correlation coefficients (R) were $\geq$0.99 (data not presented). The quantitation limit (s/n = 10) for FB_1 standard was estimated to be 250 pg using the protonated molecule signal (m/z 722). Similar sensitivity was observed for FB_2 and FB_3 (m/z 706) and hFB_1 (m/z 406). The calibration curves were based on a 2.1 $\times$ 30 mm IAC with 100 μL column volume and immobilized affinity-purified antibodies representing 0.5 mL of neat serum. The procedure is not restricted with regard to sample size. If the fumonisin concentration in the sample is low, a large sample volume can be loaded on the IAC. Conversely, if the concentration of the analyte is high, a smaller sample volume can be injected. The potential dynamic range of this assay system can easily be expanded by 2 orders of magnitude by adjusting injection volumes from 10 to 1000 μL of sample. The flow-through format during IAC loading allows for highly effective washing and removal of nontarget components of the sample, thus minimizing interference from the sample matrix, enhancing analytical precision, and allowing robust method performance.

The IAC-HPLC/ESI-MS system described was used to determine the fumonisin content in commercial rodent feed. In this set of samples the 100 μL injection volumes of feed extract represented 10 mg of corn-based rodent feed. No hFB_1 was detected in these samples (LOD $\sim$2.5 ppb). The selected ion monitoring MS chromatograms of feed sample 219 (corresponds to right panel of Figure 7) are presented in Figure 8A, and these results correspond to 173 ng/g FB_1, 60 ng/g FB_2, and 13.1 ng/g FB_3 in this sample. Similar results (159 ng/g FB_1, 62 ng/g FB_2, and 16 ng/g FB_3) were obtained when the same sample extract was analyzed using off-line cleanup with a commercial IAC column for sample cleanup prior to LC/ESI-MS quantitation as we recently described (Churchwell et al., 1997). The method variability associated with the analysis of multiple extracts prepared from the same ground feed sample (4.9% RSD) was greater than the variability associated with replicate analysis of aliquots of the same sample extract (2.3%; Figure 7). The variability associated with multiple injections of a single sample through the on-line IAC-LC/MS analysis (2.3% for FB_1) was comparable to that observed in our previous study observed with multiple injections of an FB_1 standard (2.2%). The

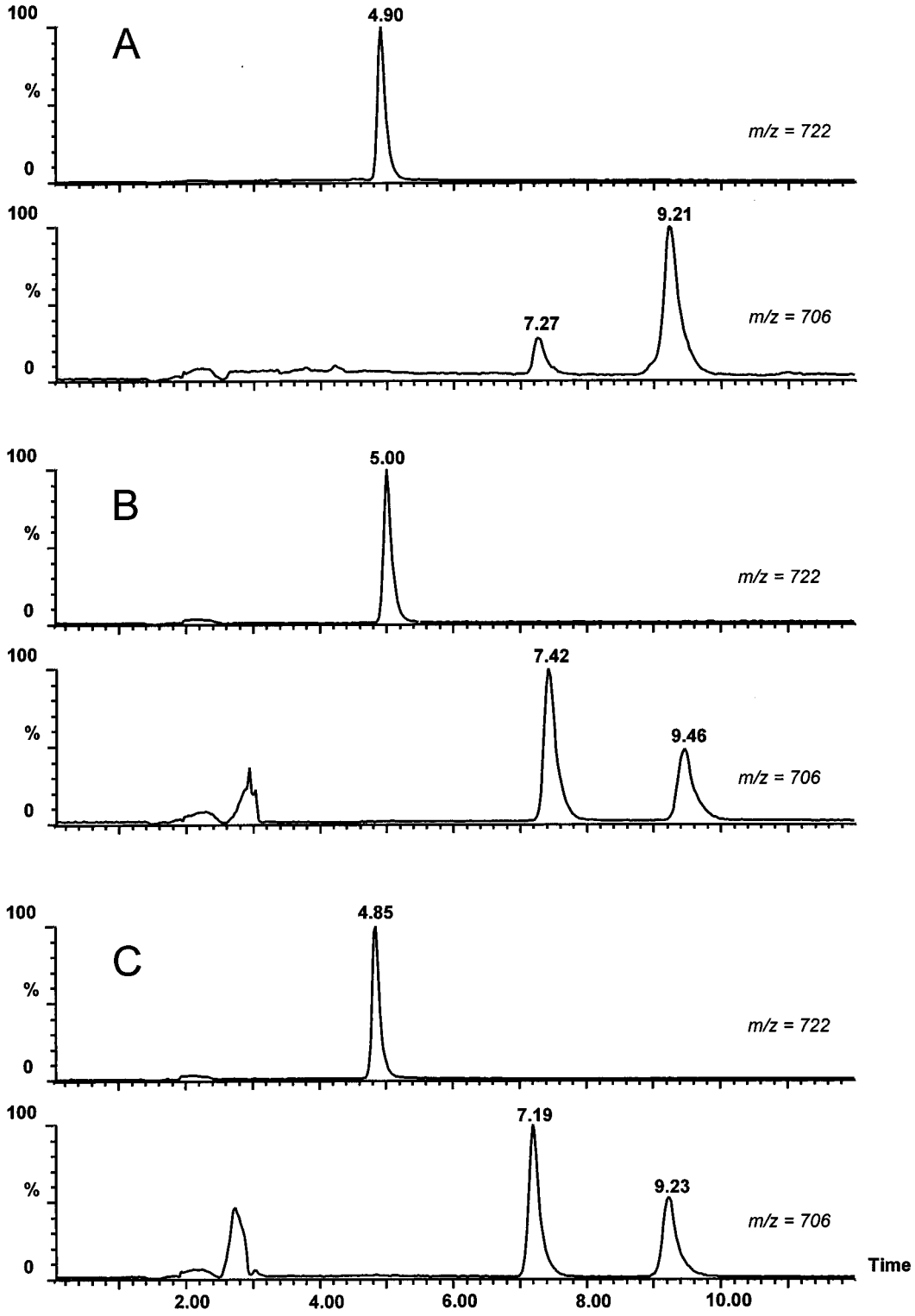

Figure 8. (A) Selected ion monitoring MS chromatograms of feed sample 219 (corresponds to right panel of Figure 7). Results of on-line IAC/LC/ESI-MS analysis of feed sample 219 were 173 ng/g FB_1, 60 ng/g FB_2, and 13.1 ng/g FB_3. The IAC was rugged and produced virtually identical selected ion monitoring MS chromatograms of a standard containing 1 ng/mL FB_1, 1 ng/mL FB_2, and 1 ng/mL FB_3 at injection 16 (B) and after 60 loading and elution buffer cycles, injection 74 (C).

variability associated with separate extractions and on-line IAC-LC/ESI-MS detection was lower than that observed in our previous study in which an off-line IAC cleanup procedure was used (intra-assay precision for FB_1 was 5.8−16.2% depending on spike level). These results show that the on-line method yields similar analytical results for fumonisins as produced by the previously validated off-line IAC procedure and suggest that the on-line procedure has greater reproducibility than the previously described off-line method.

An important feature of the IAC/LC/ESI-MS analysis method described here is the reusability of the IAC column and its durability. The IAC column was rugged and produced consistent results after being used for multiple feed samples and being subjected to multiple loading and elution cycles. Panels B and C of Figure 8

show the selected ion monitoring MS chromatograms of a standard containing 1 ng/mL FB_1, 1 ng/mL FB_2, and 1 ng/mL FB_3 at injections 16 and 74, respectively. In this example, 60 loading and elution buffer cycles had a negligible effect on system performance.

The *on-line* tandem IAC/LC/ESI-MS assay determines FB_1, FB_2, FB_3, and hFB_1 in a single automated procedure. The assay is based on a durable IAC containing affinity-purified antibody with specificity for epitopes common to the C1−C10 domains of FB_1, FB_2, FB_3, and hFB. The relevant analytes from fumonisin-contaminated feed samples are selectively retained on the IA column, eluted to a Poros 10R2 trap column, separated on a C_{18} analytical column, and detected with high sensitivity using electrospray ionization MS and selected ion monitoring. The on-line IAC/LC/ESI-MS method described here analyzes complex mixtures and provides structural confirmation. This methodology also has several advantages over the commercially available FumoniTest: the column is reusable; capability to detect hFB_1; no requirement for derivatization; and increased sensitivity. Column and mobile phase switching are automated, and all four analytes are determined from a single 100 μL injection of an acetonitrile/water extract of standard or fumonisin-contaminated corn and feed samples without a requirement for additional cleanup or derivatization. In automated runs of multiple feed samples, a quality control standard containing known levels of FB_1, FB_2, FB_3, and hFB_1 is analyzed at intervals between unknowns to detect and control for changes in mass spectrometry. As deuterium-labeled fumonisins become commercially available for use as internal standards, an obvious improvement will be the use of isotope dilution to further maximize method performance and ruggedness. Sensitivity is more than adequate (<1 ppb) for monitoring fumonisins in rodent feed for toxicological tests but could be increased further by increasing the sample injection volume. The procedure should allow more cost-effective analysis of corn-based food and animal feed for determination of low-level fumonisin contamination because of its sensitivity, reproducibility, and ease of automation.

ABBREVIATIONS USED

Dimethyl pimelimidate-HCl, DMP; enzyme-linked immunosorbent assay, ELISA; dimethyl sulfoxide, DMSO; 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride, EDC; electrospray ionization mass spectrometry, ESI-MS; fumonisin B_1, FB_1; fumonisin B_2, FB_2; fumonisin B_3, FB_3; hydrolyzed FB_1, hFB_1; FB_1 coupled through the tricarballylic carboxyl groups of the B domain to protein, FB_1-c-protein; 9-fluorenylmethylchloroformate, Fmoc; immunoaffinity columns, IAC; isotonic buffered saline, IBS; inhibitor concentration required to reduce binding of primary antibody by 50%, IC_{50}; keyhole limpet hemocyanin, KLH; poly ether ether ketone, PEEK; rabbit serum albumin, RSA.

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