Capillary Zone Electrophoresis and HPLC for the Analysis of Fluorescein Isothiocyanate-Labeled Fumonisin B_1

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Because of the presence of charged tricarballylic acid groups, the fumonisins can be separated by electrophoretic techniques. Fluorescein isothiocyanate derivatives of fumonisins B_1 (FB_1) and B_2 (FB_2) and hydrolyzed fumonisin B_1 were prepared and analyzed by capillary zone electrophoresis (CZE) and reverse-phase high-performance liquid chromatography (HPLC). The limit of detection for FB_1 was 2.5×10^{-14} g (0.025 pg) by CZE and 8×10^{-10} g (0.8 ng) by HPLC. As little as 4 ng and as much as 20 μg of FB_1 were derivatized for analysis by CZE. Derivatization of amounts of FB_1 between 0.2 and 20 μg yielded excellent agreement when measured by both HPLC and CZE (r^2 = 0.9996). FB_1 added to equine serum at a level of 25 ng/mL could be detected after cleanup with an affinity column and analysis by CZE. This is the first reported application of capillary electrophoresis to the analysis of fumonisins.

Keywords: *Capillary electrophoresis; fumonisins; fluorescein isothiocyanate; serum*

INTRODUCTION

Exposure to fumonisins, mycotoxins produced by *Fusarium moniliforme* and *Fusarium proliferatum* (Bezuidenhout et al., 1988; Ross et al., 1990; Thiel et al., 1991; Nelson et al., 1992), can cause leukoencephalomalacia in horses (Wilson et al., 1992) and pulmonary edema in swine (Colvin and Harrison, 1992). The common occurrence of fumonisins in corn products has driven the development of new analytical techniques for these toxins. The majority of methods for the analysis of the fumonisins employ high-performance liquid chromatography (HPLC) of a fluorescent derivative: either *o*-phthaldialdehyde (OPA; Shephard et al., 1992) or naphthalene dicarboxaldehyde (NDA; Bennett and Richard, 1994). Because of the presence of two tricarballylic acid groups, the fumonisins have the potential to be separated by electrophoretic techniques.

Despite the broad potential of capillary electrophoresis, application to the analysis of mycotoxins, such as fumonisins, has until now remained unexplored. The present research describes the first application of capillary zone electrophoresis (CZE) for fumonisin analysis. Fluorescein isothiocyanate derivatives of fumonisins B_1 (FB_1) and B_2 (FB_2) and hydrolyzed fumonisin B_1 (HFB_1) were prepared and analyzed by CZE with laser-induced fluorescence (LIF) detection. The CZE method was compared to HPLC methods incorporating either FITC or NDA derivatives of the fumonisins, and was applied to the analysis of FB_1 in horse serum.

MATERIALS AND METHODS

Safety Note. Fumonisins B_1 and B_2 reportedly promote cancer in rats (Gelderblom et al., 1988) and should be handled with appropriate caution.

Materials. All inorganic chemicals and organic solvents were of reagent grade or better. Fumonisins B_1 and B_2 and horse serum were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrolyzed fumonisin B_1 (HFB_1) was a gift from Ronald Plattner (USDA/ARS/NCAUR, Peoria, IL). Fluorescein 5-isothiocyanate (FITC "isomer I") and naphthalene-2,3-dicarboxaldehyde (NDA) were purchased from Molecular Probes, Inc. (Eugene, OR). Affinity columns for isolation of fumonisin (FumoniTest) were purchased from Vicam (Watertown, MA). Strong anion exchange (SAX) columns were 2.8 mL (500 mg of sorbent mass) obtained from Varian (Harbor City, CA).

Derivatization with FITC or NDA. For experiments describing the affect of buffer composition on fumonisin separation, retention, and reproducibility by CZE and HPLC, solutions of purified fumonisins in acetonitrile/water (1:1) were used. After dilution in water, aliquots of 0.2 to 20 μg of FB_1 were apportioned into vials. Methanol, containing 2% (v/v) acetic acid, was passed through a SAX column, collected, and 6 mL added to each sample. The original purpose of the methanol was to provide conditions similar to those for potential corn or serum samples that would be eluted with methanol or methanol/acetic acid. Samples were then heated to 60 °C and dried under a gentle stream of nitrogen. The use of treated methanol (passed through a SAX column) rather than untreated methanol was found to enhance the conversion of 10 μg of FB_1 to FB_1-FL 4-fold. Subsequent experiments indicated the reaction could be enhanced even if the SAX-treated methanol was dried onto vials before the addition of reactants. The treatment apparently adds a cofactor, or removes an inhibitor, of the derivatization. This aspect is currently under investigation.

The dried samples were dissolved in 500 μL of DMSO/BB (dimethyl sulfoxide and 50 mM borate buffer, pH 9.5, in the proportion 9+1). Fluorescein isothiocyanate (FITC, 75 μL of 1.3 mM in acetone) was added and the mixture incubated at 60 °C for 90 min. After the mixture was cooled to room temperature, 4425 μL of MeOH/BB (methanol and 50 mM borate buffer, pH 9.5, in the proportion 3+2) was added. This mixture was either used without dilution (HPLC analyses) or was diluted 1:20 in electrophoresis buffer.

For experiments testing the sensitivity of CZE, a modification of the above procedure was used. Vials, containing the equivalent of 4 mL of methanol which had been passed through a strong anion exchange column and dried under nitrogen, were prepared. Extracts of standard (for sensitivity and recovery experiments) containing 0.02 μg (20 ng) to 0.2 μg of FB_1 were added to the vials and dried under nitrogen at 60 °C. The volumes of DMSO/BB and FITC were reduced to 250 and 25 μL, respectively. The mixture was incubated as described above and brought to a final volume of 4.70 mL with CZE buffer. Samples prepared in this manner were not diluted further before analysis. For experiments with horse serum, improved derivatization was obtained by further reducing the volumes of reactants (120 μL of DMSO/BB, 30 μL of FITC), incubating at room temperature (24 °C), and increasing the reaction time to 3 h.

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For comparison with the FITC method, 10–40 ng of FB_1 was derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) as described for analysis of milk (Maragos and Richard, 1994), and 0.1–0.4 ng was injected.

HPLC of NDA and FITC Derivatives. The NDA derivative of FB_1 was quantitated by reverse-phase HPLC as described by Maragos and Richard (1994) using fluorescence detection (250 nm excitation and 470 nm emission). For FITC derivatives an additional method was developed for the reverse-phase HPLC separation of FB_1 from other fluorescent products. The chromatographic system consisted of a SpectraSystem P4000 solvent delivery module, SCM400 solvent degasser (Thermo Separation Products), Rheodyne model 9125 injector with a 20 μL sample loop, C18 NewGuard column (Brownlee Labs, #12035A), and a Zorbax C8 4.6 mm × 25 cm analytical column (DuPont Instruments, P.N. 880952-706). Solvent assignments were as follows: A, methanol; and B, phosphate buffer (10 mM, pH 7.5). The column was equilibrated with 30% A and 70% B. After sample injection solvent A was increased to 42% over 7.2 min, and held at this level until 12 min. The column was then washed with 90% A for 4 min after which the mobile phase was returned to the starting concentration of 30% A. Fluorescence of FITC derivatives was measured with a Spectra Physics FL2000 detector (excitation 488 nm, emission 520 nm), and data was acquired with a SP4270 integrator.

Capillary Zone Electrophoresis. FITC derivatives of the fumonisins were quantitated using a Beckman P/ACE System 5000 capillary electrophoresis unit, with argon ion laser-induced fluorescence (LIF) detection (laser module 488). The capillary (50 cm × 75 μm i.d.) was housed in a cartridge configured for LIF detection (Beckman #360604) and maintained at 35 °C. Data were acquired with a Gateway 2000 486DX2/50 computer and archived onto magnetic tape (Trakker, 250 MB, Colorado Memory Systems, Inc.).

Buffer composition (borate content, pH, and methanol content) was varied to determine the effect upon FB_1 migration time and peak height. Before each sample was injected, the capillary was rinsed 5 min with run buffer. Sample was injected under 0.5 psi (hydrodynamic injection) for 5.0 s, equivalent to a volume of 30 nL. After immersion in run buffer, voltage was applied and the current was maintained at 250 μA. At the conclusion of the run the capillary was rinsed once with 0.1 N NaOH (2.0 min, 0.5 psi) and once with deionized water (2.0 min, 0.5 psi). The composition of the run buffer for experiments describing the sensitivity and dynamic range of the method was 7% methanol (v/v) in 50 mM borate buffer (pH 9.5).

Analysis of Horse Serum. Fumonisin B_1 was added to horse serum at levels greater than or equal to 25 ng/mL and isolated using a commercially available affinity column. For each sample, serum (1.5 mL) was diluted to 10 mL with deionized water and passed through the affinity column at a flow rate of 1.5 mL/min. The column was washed with 5 mL of 0.01 M phosphate buffer (pH 7.5) and 5 mL of deionized water. Fumonisin B_1 was eluted with 1.6 mL of methanol into an 11 × 32 mm amber vial and dried at 60 °C under nitrogen. The dried extract was derivatized with FITC as described above. Fumonisin B_1 was separated by CZE using the conditions described above, with the exception that 60 mM borate buffer (pH 9.50) was used. Fumonisin B_1 recovery was calculated relative to purified FB_1 added to amber vials, dried down, and derivatized using the same method as serum samples.

RESULTS AND DISCUSSION

Derivatization with FITC. Fluorescein isothiocyanate (FITC) derivatives are less commonly used for labeling small molecular weight compounds than for larger substances such as peptides and proteins. This may be due to the additional reagent peaks and the extended reaction times required for adequate derivatization. For many small molecules the OPA or NDA derivatives are preferable because of the ease of derivatization and the presence of fewer reagent peaks. Commercial CE instrumentation with laser-induced fluorescence is currently limited to the argon ion laser, which is ideally suited for fluorescein derivatives. For this reason the FITC derivatives of fumonisins were prepared, rather than NDA or OPA derivatives, for analysis by CZE. Derivatization of purified FB_1 with FITC and analysis by reverse-phase HPLC was reproducible, with a coefficient of variation of 3.2% for eight separate samples (Table 1).

Table 1. Reproducibility of Derivatization of FB_1 with FITC

replicate	FB_1 peak area[a]
1	337 009
2	363 973
3	330 326
4	333 999
5	334 389
6	347 144
7	333 494
8	342 216
mean	340 319
SD	10 969
CV	3.2%

[a] Integrated area under the major FB_1 peak after separation by reverse-phase HPLC.

Initial studies, using a reaction volume of 575 μL and 0.2–200 μg of FB_1, gave consistent and predictable derivatization of FB_1 with FITC. The relationship between the amount of FB_1 added and the amount of derivative produced (FB_1-FL) was sigmoidal, regardless of whether HPLC or CZE was used for analysis (Figure 1). The sigmoid model fit the data over this range very well with $r^2 = 0.99998$ for the CZE method and $r^2 = 0.99996$ for the HPLC method. The range of FB_1 used was similar to the levels derivatized with the NDA method for fumonisins in corn (Bennett and Richard, 1994). Because of this, we are currently testing application of the CZE method to the analysis of corn.

Conditions Influencing CZE of Fumonisins. The anionic nature of the fumonisins would imply electrophoretic migration toward the anode (positive electrode); however, with fused silica capillaries, the electroosmotic flow of buffer toward the cathode (negative electrode) is sufficient to transport the fumonisins in this direction. The composition of buffer (pH, buffer type, concentration, and additives) dramatically influences both the electrophoretic separation and the electroosmotic flow. As a result, slight changes in buffer composition have dramatic effects upon the separation and migration of the fumonisins by CZE.

Buffer pH influences the charge on the fumonisin derivatives, the charge on the silica capillary, and the voltage required to maintain an established current. Figure 2 illustrates the effect small changes in pH have upon migration of the FB_1-FL derivative. Raising the pH by less than one unit more than doubled the migration time. Because of this, accurate calibration of the buffer pH was essential to obtain reproducible migration times.

Changes in buffer concentration strongly influence the ionic strength of the solution and therefore the voltage required to attain a given current. The extent to which the migration time was dependent upon the buffer concentration was considerable and was independent of pH (Figure 3). All buffers used to measure migration times reported in Figure 3 were adjusted to pH 9.50 with 5 N sodium hydroxide. The FB_1-FL migration time

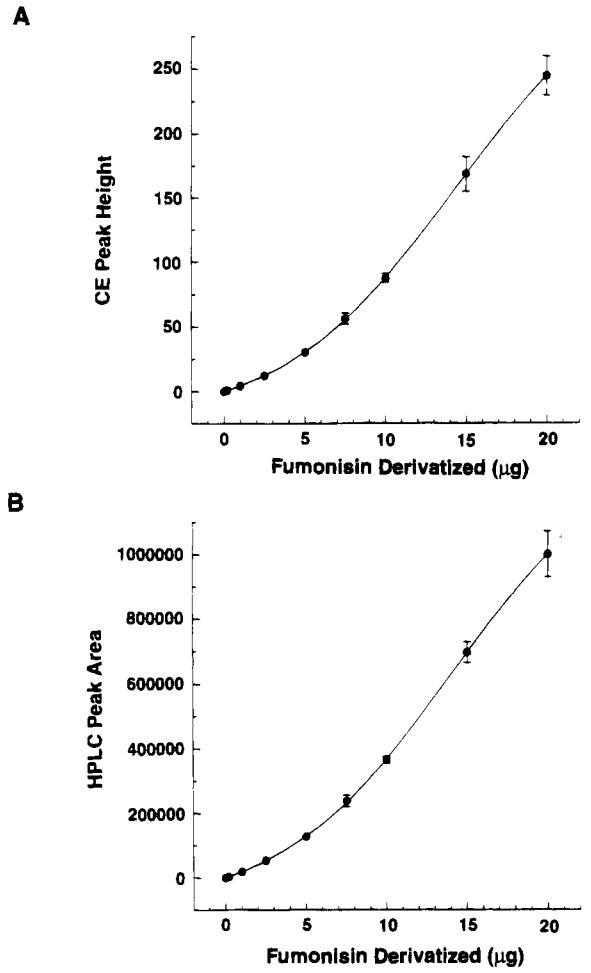

Figure 1. FB_1 derivatized with FITC and analyzed by (A) capillary zone electrophoresis or (B) reverse-phase HPLC. The amounts of FB_1 injected for CZE-LIF analysis ranged from 0.06 to 6.0 pg. The amounts of FB_1 injected for HPLC analysis ranged from 0.8 to 80 ng. Each point represents the average of triplicate determinations (±1 standard deviation).

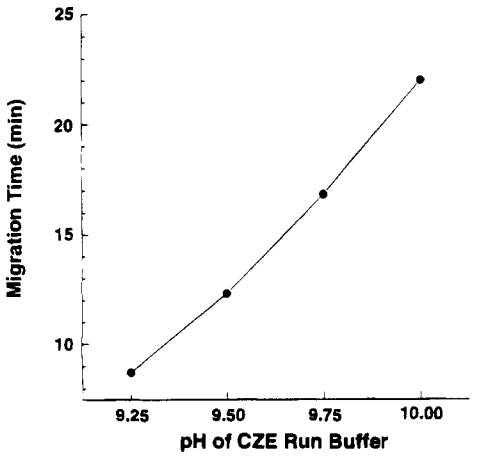

Figure 2. Effect of pH on the migration time of FITC-derivatized fumonisin B_1. All buffers were 50 mM borate; current and temperature were maintained at 250 μA and 35 °C, respectively.

increased logarithmically with borate concentration. At borate concentrations below 40 mM the limit of 30 kV was required in order to attain 250 μA, while above 90 mM the migration time exceeded the capacity of the instrument to acquire data (100 min). Within this range the voltage applied in order to provide a current of 250

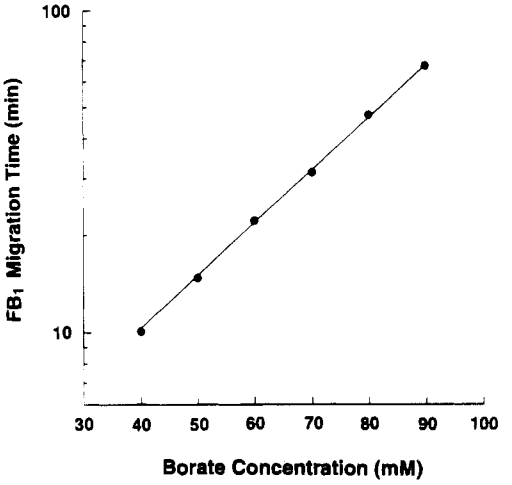

Figure 3. Effect of buffer concentration upon the migration time of FITC derivatized FB_1. All buffers were adjusted to pH 9.50 with 5 N sodium hydroxide. Current and temperature were maintained at 250 μA and 35 °C, respectively. The line represents the fit $\ln(y) = a + bx$ to a single experiment, $r^2 = 0.9993$.

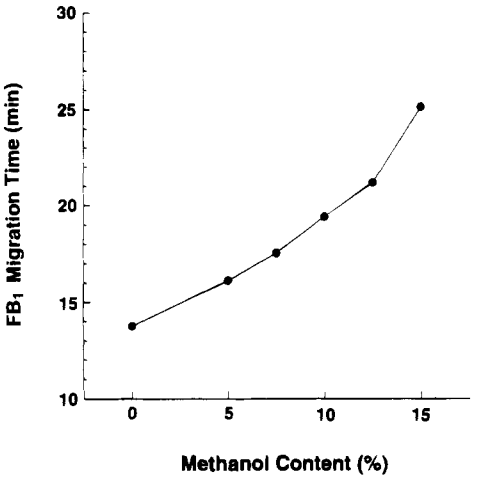

Figure 4. Effect of methanol concentration upon the migration time of FITC derivatized FB_1. All buffers contained a balance of 50 mM borate. Temperature was maintained at 35 °C. The current was maintained at 250 μA, except at 15% methanol (234 μA, 30 kV).

μA was 16.8 kV with 90 mM borate to 27.1 kV with 40 mM borate.

The migration time was also increased by the addition of methanol to the run buffer (Figure 4). Addition of 15% methanol roughly doubled the migration of FB_1-FL. Additives such as methanol affect the viscosity and ionic strength of the buffer and therefore the strength of the electroosmotic flow. Methanol can be used to lower the EOF by up to 30% (Kuhn and Hoffstetter-Kuhn, 1993).

Because of the dramatic effects of pH, borate concentration, ionic strength, temperature, and methanol concentration, these factors must be well controlled before attempting to quantitate fumonisins by CZE. With appropriate control of these variables CZE with LIF detection of the FITC derivatives was reproducible, with coefficients of variation (CV) of 3.4% and 2.8% for FB_1 and FB_2, respectively (Table 2). Peak height was found to be more reproducible than peak area, which needed to be corrected for electroosmotic flow (data not shown). This results because sample components that

Table 2. Reproducibility of CZE Injection

	fumonisin B_1		fumonisin B_2	
replicate	migration time, min	peak ht, RFU	migration time, min	peak ht RFU
1	15.34	20.68	16.44	22.07
2	15.42	19.81	16.53	21.27
3	15.35	19.28	16.46	20.96
4	15.28	21.22	16.37	22.68
5	15.29	19.97	16.39	22.10
6	15.06	21.17	16.13	22.87
7	15.15	20.62	16.22	21.91
8	15.04	21.27	16.09	22.45
9	14.93	20.90	15.97	22.06
mean	15.20	20.54	16.28	22.04
SD	0.16	0.70	0.19	0.61
CV	1.1%	3.4%	1.1%	2.8%

migrate slowly remain in the detector window for a greater length of time than components that migrate quickly.

Because of the large difference in charge, the fumonisin backbone (hydrolyzed FB_1 or HFB_1) was easily separated from FB_1. FB_1 and FB_2 differ by a single hydroxyl group (present at C10 in FB_1 and absent in FB_2), and separation required manipulating the buffer composition. Figure 5 demonstrates the separation of HFB_1, FB_1, and FB_2 from each other and from other fluorescein-derived reagent peaks. Separation of FB_1 from FB_2 was aided by the addition of methanol or by increasing buffer strength. Adequate separation was achieved with 65 mM borate, pH 9.50 (Figure 5). Except for the data in Figure 3, the borate concentration was generally kept at or below 60 mM to shorten analysis time. The derivatization of HFB_1 was less efficient than with FB_1 or FB_2 (Figure 5).

Comparison of HPLC and CZE Methods. The HPLC and CZE methods with FITC agree well for FB_1 derivatized in amounts between 0.2 and 20 μg (Figure 6, $r^2 = 0.9996$). The CZE method, however, is more sensitive. When amounts of FB_1 below 0.2 μg were derivatized (corresponding to less than 0.8 ng of derivative injected), the derivative could not be quantitated by HPLC. However, as little as 4 ng of FB_1 standard could be derivatized and as little as 0.025 pg detected by CZE-LIF (signal to noise ratio of 8). This indicates that for the FITC derivative the CZE method is roughly 50-fold more sensitive than the HPLC method for both the amount of FB_1 derivatized and the concentration of the solution injected.

For comparison, FB_1 was also derivatized with NDA and quantitated by HPLC (Maragos and Richard, 1994). By using HPLC for separation, the NDA derivative could be detected at lower levels than the FITC derivative. As little as 40 ng of FB_1 could be derivatized and detected using the NDA method, with injection of 0.4 ng giving a signal to noise ratio of 5. When HPLC was used for separation and detection, and using the smallest amount of FB_1 that could be derivatized as the criterion, the NDA method was roughly 5-fold more sensitive than the FITC method (40 ng vs 200 ng derivatized). However, the NDA method with HPLC detection was 10-fold less sensitive than the FITC method with CZE-LIF detection (40 ng vs 4 ng derivatized).

While the FITC method with CZE-LIF detection is 10-fold more sensitive with regard to the amount derivatized than HPLC of the NDA derivative (NDA-HPLC), the absolute amounts that were detected were vastly different: 0.4 ng by NDA-HPLC and 0.025 pg by

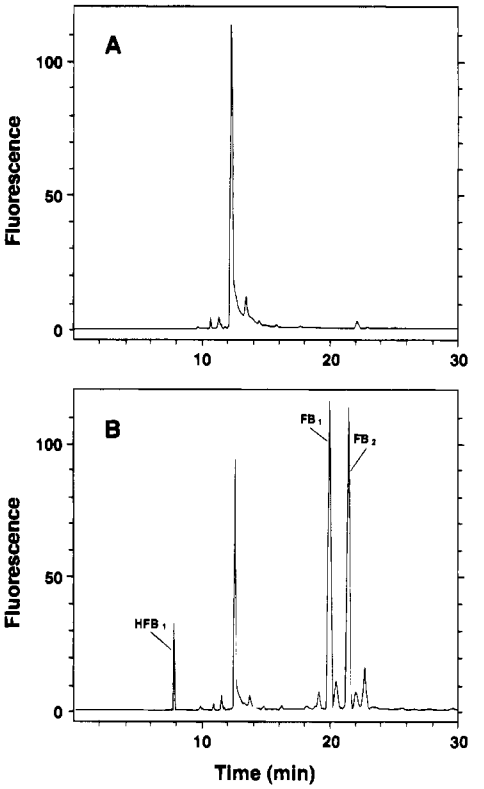

Figure 5. Separation of HFB_1, FB_1, and FB_2 by capillary zone electrophoresis: (A) reagent blank and (B) equal amounts of HFB_1, FB_1, and FB_2, derivatized with FITC and 30 nL injected (3.0 pg of each toxin assuming 100% was derivatized). Run buffer was composed of 65 mM sodium borate, pH 9.50. Current was maintained at 250 μA.

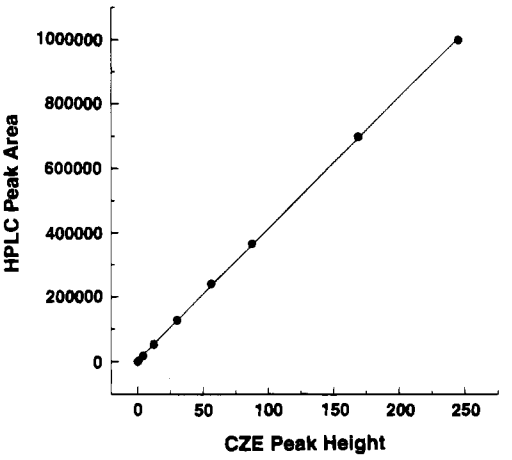

Figure 6. Comparison of HPLC and CZE methods for FITC derivatized fumonisin B_1.

CZE-LIF. By this criterion the CZE-LIF method is roughly 16000-fold more sensitive. The major reason for the large difference between comparisons of sensitivity based upon the amount derivatized or amount detected is the large difference in the volumes injected into the systems; roughly 1000-fold lower with CZE than HPLC. That is, although the CZE-LIF method was 16000-fold better at detecting the amount of derivative, the volume injected is also 1000-fold lower, this reduces the 16000-fold advantage by a factor of 1000 leaving a roughly 10–16-fold advantage by CZE-LIF.

In addition to injecting smaller volumes of sample by CZE, the volume of waste solvent generated was substantially less. The waste generated from CZE was generally less than 5 mL over the course of an 8 h day,

compared to roughly 500 mL when HPLC was used. This approximately 100-fold reduction in the amount of waste generated is a major benefit over current HPLC methods.

Detection of FB_1 in Horse Serum. Because of the greater sensitivity afforded by CZE-LIF, the possibility exists for more sensitive detection of FB_1 in complex matrices such as biological fluids and food products. For this reason FB_1 was added to horse serum, subjected to a commercial affinity column for cleanup, and then derivatized and analyzed by CZE-LIF. Recovery of 100 ng of FB_1/mL from horse serum ranged from 57% to 104% if the derivatization reaction was held at 60 °C for 90 min. However, using a longer incubation (3 h) and a lower temperature (24 °C) reduced the variability; recovery from four samples spiked at 100 ng/mL and analyzed concurrently was 76.0% with a coefficient of variation of 10.6% (range 67.0–85.6%). Fumonisin B_1 added to serum at 25 ng/mL could be detected qualitatively. Quantitation of FB_1 below 100 ng/mL was not limited by detector sensitivity, but rather by the presence of a component in the unspiked serum with a similar migration time as FB_1-FL. The identity of this component is currently being investigated. The sensitivity attained by CZE-LIF is similar to that reported previously for HPLC analysis of the OPA derivative of FB_1 in rat plasma (50 ng/mL; Shephard et al., 1992), and poorer than that achieved with FB_1 and FB_2 in milk (5 ng/mL; Maragos and Richard, 1994). Improved sensitivity with CZE-LIF may result when FB_1-FL is separated more efficiently from other serum components.

CONCLUSIONS

Fumonisins can be separated and reliably quantitated by capillary electrophoresis with LIF detection. The methods described here are in general comparable in sensitivity to those currently achievable with HPLC. The advantages of CZE include the use of much smaller sample volumes and the generation of substantially smaller volumes of hazardous waste. The potential also exists for further improvements in sensitivity. Obtaining adequate reproducibility required stringent control of a large number of experimental variables. For this reason quantitation of fumonisins by CZE-LIF has not yet reached the stage where it can rival HPLC for ease of use. Nevertheless, the use of capillary electrophoresis for mycotoxin analysis can be expected to grow as instrumentation and methodologies continue to improve.

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LITERATURE CITED

Bennett, G. A.; Richard, J. L. Liquid chromatographic method for analysis of the naphthalene dicarboxaldehyde derivative of fumonisins. *J. AOAC Int.* **1994**, *77*, 501–506.

Bezuidenhout, S. C.; Gelderblom, W. C. A.; Gorst-Allman, C. P.; Horak, R. M.; Marasas, W. F. O.; Spiteller, G; Vleggaar, R. Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. *J. Chem. Soc., Chem. Commun.* **1988**, 743–745.

Colvin, B. M.; Harrison, L. R. Fumonisin-induced pulmonary edema and hydrothorax in swine. *Mycopathologia* **1992**, *117*, 79–82.

Gelderblom, W. C. A.; Jaskewwicz, K.; Marasas, W. F. O.; Thiel, P. G.; Horak, R. M.; Vleggaar, R.; Kriek, N. P. J. Fumonisins-novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl. Environ. Microbiol.* **1988**, *54*, 1806–1811.

Kuhn, R.; Hoffstetter-Kuhn, S. In *Capillary Electrophoresis: Principles and Practice*; Springer-Verlag: New York, 1993.

Maragos, C. M.; Richard, J. L. Quantitation and stability of fumonisins B_1 and B_2 in milk. *J. AOAC Int.* **1994**, *77*, 1162–1167.

Nelson, P. E.; Plattner, R. D.; Shackelford, D. D.; Desjardins, A. E. Fumonisin B_1 production by Fusarium species other than *F. moniliforme* in section Liseola and by some related species. *Appl. Environ. Microbiol.* **1992**, *58*, 984–989.

Ross, P. F.; Nelson, P. E.; Richard, J. L.; Osweiler, G. D.; Rice, L. G.; Plattner, R. D.; Wilson, T. M. Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. *Appl. Environ. Microbiol.* **1990**, *56*, 3225–3226.

Shephard, G. S.; Thiel, P. G.; Sydenham, E. W. Determination of fumonisin B_1 in plasma and urine by high-performance liquid chromatography. *J. Chromatogr.* **1992**, *574*, 299–304.

Thiel, P. G.; Marasas, W. F. O.; Sydenham, E. W.; Shephard, G. S.; Gelderblom, W. C. A.; Nieuwenhuis, J. J. Survey of fumonisin production by Fusarium species. *Appl. Environ. Microbiol.* **1991**, *57*, 1089–1093.

Wilson, T. M.; Ross, P. F.; Owens, D. L.; Rice, L. G.; Green, S. A.; Jenkins, S. J.; Nelson, H. A. Experimental reproduction of ELEM. *Mycopathologia* **1992**, *117*, 115–120.

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