Derivation of Fumonisins B_1 and B_2 with 6-Aminoquinolyl *N*-Hydroxysuccinimidylcarbamate

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Fumonisins, a group of toxins produced mainly by strains of *Fusarium moniliforme* Sheldon, have been associated with several diseases in animals and with esophageal cancer in humans. Disadvantages of several derivatizing agents currently used for liquid chromatography include instability of the derivatives and formation of two peaks. Therefore, 6-aminoquinolyl *N*-hydroxysuccinimidylcarbamate, a new derivation reagent for amino acid analysis, was investigated. The derivatives formed are stable, and 2 ng of fumonisin B_1 or B_2 can be detected, with a linear response up to at least 800 ng injected. The method permits detection of fumonisins in ground corn using a described cleanup procedure. The HPLC detection limit for both toxins is 2 ng, which would give an apparent detection limit of 0.26 μg/g.

Keywords: *Fumonisin B_1; fumonisin B_2; mycotoxins; analysis; 6-aminoquinolyl N-hydroxysuccinimidylcarbamate; AccQ·Fluor reagent; HPLC; fluorescence detection*

INTRODUCTION

Fumonisins, a group of toxins produced mainly by *Fusarium moniliforme* strains, have been implicated in leukoencephalomacia in horses and with pulmonary edema in pigs (Gelderblom et al., 1992; Harrison et al., 1990). Their presence has also been associated with esophageal cancer in humans (Rheeder et al., 1992; Chu and Li, 1994). In studies carried out in the United States, a pattern emerged suggesting that concentrations of fumonisin B_1 (FB_1) greater than 10 μg/g in feed could cause the equine disease (Wilson et al., 1992; Ross, 1994). In addition, commercial corn-based human foodstuffs from retail outlets frequently contain fumonisins (Scott, 1993; Sanchis et al., 1994).

Owing to the high levels of fumonisins that can occur in foods and feeds, and the demonstrated ability of FB_1 to cause several diseases, these mycotoxins are a real cause for concern in both health and economic terms (Thiel et al., 1992). Consequently, the development of analytical methods is therefore important.

Analytical procedures for quantitative analysis of fumonisins include several HPLC methods using fluorescence detection (Sydenham et al., 1992; Shephard et al. 1990; Scott and Lawrence, 1992; Holcomb et al., 1993). However, some derivatives do not appear to be very stable or are only applied to the analysis of fumonisin B_1 in corn (Holcomb et al., 1993; Alberts et al., 1993).

Since Millipore has developed a sensitive and stable method for amino acid analysis (Cohen and Michaud, 1993), we have checked out its application to fumonisin B_1 and B_2 analysis.

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EXPERIMENTAL PROCEDURES

Safety Note: Fumonisins B_1 and B_2 are suspected carcinogens and should be handled with caution.

Apparatus. The HPLC system consisted of Applied Biosystem Series (ABI Analytical Kratos Division, Ramsey, NJ) Model 400 pumps, a Model 491 dynamic mixer/injector with a 20 μL loop, a Model 980 fluorescence detector (excitation at 395 nm and emission at 418 nm cutoff filter), and a Hewlett-Packard 3396 Series II integrator (Hewlett-Packard, Avondale, PA). The reversed-phase column was a Nova-Pak C_{18} (15 × 0.39 cm) preceded by a C_{18} guard column (Waters Chromatography Division, Millipore Corp., Milford, MA).

Chemical Standards. Fumonisin B_1 and B_2 standards were purchased from the Division of Food Science and Technology, CSIR, Pretoria, South Africa. A stock solution of 1 mg/mL of FB_1 and 1 mg/mL of FB_2 in water/acetonitrile (1 + 1) was prepared. From these, standard solutions of 100, 50, 30, 10, and 1 μg/mL of fumonisins B_1 and B_2 in water/acetonitrile (1 + 1) were prepared.

Reagents. Potassium phosphate buffer was prepared by dissolving 0.1 M potassium dihydrogen phosphate in ultrapure water (Milli-Q_{185} Plus, Millipore) and adjusting the pH to 7 with 0.1 M NaOH.

HPLC mobile phases were (A) phosphate buffer and (B) HPLC grade methanol (Romil, Germany). Each mobile phase was filtered and degassed.

AccQ·Fluor reagent kit was purchased from Waters (Millipore) and used according to the manufacturer's instructions.

Strong anion-exchange columns (Analytichem Bond-Elut, Varian, Harbor City, CA) were used for cleanup of corn sample extracts before derivation.

Derivation and Gradient HPLC. For the derivation, a 50 μL fraction of each of the cleaned up sample extracts and standards was added to separate 2 mL screw-cap vials. Then 60 μL of AccQ·Fluor borate buffer and 20 μL of AccQ·Fluor reagent were added to each vial. Each reaction mixture was allowed to stand for 1 min at room temperature and then heated for 10 min at 55 °C.

The gradient program was started at 50% of A and B, then ramped to 65% B in 11 min, and finally held for 2 min. The total analysis time was 15 min. The LC column was kept at room temperature with a flow rate of 1.25 mL/min.

Preparation of Standard Curve. Standards curves were constructed by injection of standard samples derivatized and

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prepared from the method described, using 100, 50, 30, 10, and 1 μg/mL of fumonisns B_1 and B_2 as initial concentrations.

These curves were constructed by plotting peaks heights, measured in millivolts, and amounts of fumonisins in nanograms.

Reproducibility and Stability Studies. Fourteen identical standard samples from 50 μg/mL fumonisin B_1 and B_2 standard solutions were prepared according to the procedure described. After periods of 0, 1, 4, 6, 24, and 48 h at room temperature, these samples were analyzed in groups of three and their peaks heights, measured in millivolts, compared against a fresh standard. All analyses were carried out using a full loop injection technique.

Toxin Extraction of Corn Samples. Corn samples were extracted and purified using a modification of the method of Shephard et al. (1990). Thus, kernels were ground, and 25 g of ground material was extracted by magnetic stirring for 30 min with 50 mL of methanol/water (3 + 1), followed by centrifugation at 500*g* at 4 °C for 10 min. The supernatant was filtered through a Whatman No. 4 filter paper, and a 10 mL portion was applied to a Bond-Elut SAX cartridge (Analytichem Bond-Elut) which had been conditioned with methanol (5 mL), followed by 5 mL of methanol/water (3 + 1). Subsequently, the cartridge was washed successively with 8 mL of methanol/water (3 + 1) and methanol (3 mL); finally the toxins were eluted with 21 mL of 0.5% acetic acid in methanol. The eluate was evaporated to dryness under vacuum, redissolved in 2 mL of methanol, transferred to a 4 mL screw-cap vial, and reevaporated to dryness under a nitrogen stream in a sandbath at 60 °C. Residue was dissolved in 0.2 mL of methanol. Samples were analyzed as described above for reproducibility and stability studies. Fumonisin concentrations of the corn samples were calculated from the chromatographic peak height as follows:

$$C\ (\mu g/g) = \mathrm{SVDT}/(\mathrm{WIAM} \times 10^3)$$

C is the concentration of the fumonisin present in the sample (μg/g); S is the amount of fumonisin present in the sample injected into the HPLC (ng) (this amount was obtained applying the fumonisin peak height in the sample to the equation for the regression line); V is the volume of solvent needed to carry out the corn extraction; D is the volume added to the residue from cleanup; T is the total volume of derivatized sample solution; W is the corn sample weight (g); I is the injection volume; A is the volume of the extracted sample cleaned up in the SAX column; M is the volume of the sample after cleanup employed for preparing the derivative (all volumes are in mL).

Statistical Analysis. The data were processed using the Statgraphics 2.0 program (Statistical Graphics Corp., Rockwell, MD).

Homogeneity between linear regressions calculated on differents days was analyzed by a test for homogeneity of slopes using the SAS system 6.03 version (SAS Institute, 1988).

RESULTS AND DISCUSSION

A fluorescent derivative can be obtained with fumonisins B_1 and B_2 by the reaction of the reagent with the primary amine of both fumonisins. Shephard et al. (1990) showed that a highly fluorescent derivative could be obtained by reacting the fumonisins with OPA reagent. However, the derivative is not stable. Scott and Lawrence (1992) used NBD-F derivatives with a half-lives of 20 min. Holcomb et al. (1993) described a new stable derivative but it is only used for analysis of samples spiked with fumonisin B_1. Our objective was to find another fluorescent reagent to react with the primary amine of fumonisins to give a stable derivative. The AccQ·Fluor reagent is a new product that seems to present this property and that has been used in the analysis of amino acids (Cohen and Michaud, 1993).

Table 1 shows the good linearity obtained for fumonisins B_1 and B_2 from 7.5 to 750 ng when the analyses were carried out in two different days. We suggest that calibration curves should be calculated every day because of the significant differences found within regression lines.

Table 1. Linearity of Fumonisin B_1 and B_2 Standards (7.5–750 ng)

	day	slope[a] (μV/ng)	*y*-intercept (μV)	df[b]	F[c]	R^2 [d]
FB_1	1	59.8	950	3	15228	0.999
	2	47.7	314	8	5928	0.998
FB_2	1	55.9	780	3	4803	0.999
	2	46.4	−1.08	8	914	0.991

[a] Significant differences were found between slopes for FB_1 and FB_2 ($P < 0.05$, test for homogeneity of slopes). [b] Degree of freedom. [c] *F* ratio. [d] Coefficient of determination.

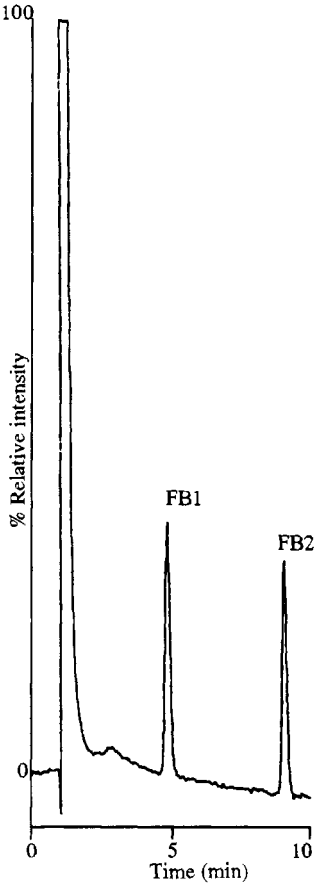

Figure 1. HPLC chromatogram of the AccQ·Fluor derivative of 75 ng of fumonisin B_1 and fumonisin B_2 standards.

On the basis of 14 replicates, the mean peak heights for FB_1 and FB_2 were 15.6 and 14.1 mV, with CV of 7.7% and 6.5%, respectively. The derivative was stable for at least 48 h (Table 2). The HPLC detection limit is 2 ng (signal:noise ratio = 3:1) which, when taking the prepurification procedure into account, would give an apparent detection limit of 0.26 μg/g on corn material.

The AccQ·Fluor derivation reaction was applied to the determination of fumonisins B_1 and B_2 in ground corn naturally contaminated (field-inoculated). The same samples were also analyzed using the Shephard method (Shephard et al., 1990). Both results are in good agreement (Table 3). Figure 1 shows the HPLC chromatogram of fumonisins B_1 and B_2 containing 75 ng of each, whereas Figure 2 shows the HPLC chromatogram of a cleaned up extract from a ground corn naturally contamined containing 1.3 and 0.3 μg/g of fumonisins B_1 and B_2, respectively.

In conclusion, AccQ·Fluor reagent is a useful alternative fluorescence derivatizing reagent for determination

Table 2. Stability of AccQ·Fluor Derivatives of Fumonisin B_1 and B_2 Standards (50 μg/mL)

	FB_1(peak height in mV)		FB_2 (peak height in mV)	
time, h	$X \pm$ SD	$X \pm$ SD, fresh standard	$X \pm$ SD	$X \pm$ SD, fresh standard
0	16.5 ± 1.3		13.3 ± 0.85	
1	17.6 ± 1.1	18.1 ± 0.73	13.1 ± 1.1	12.5 ± 0.81
4	18.1 ± 0.73	15.8 ± 2.1	13.2 ± 1.1	12.7 ± 0.56
6	17.5 ± 0.50	15.8 ± 2.1	13.2 ± 0.75	12.7 ± 0.56
24	16.4 ± 0.65	17.2 ± 0.65	13.0 ± 0.29	13.5 ± 0.40
48	17.5 ± 1.8	17.3 ± 1.5	13.6 ± 0.80	13.4 ± 0.65

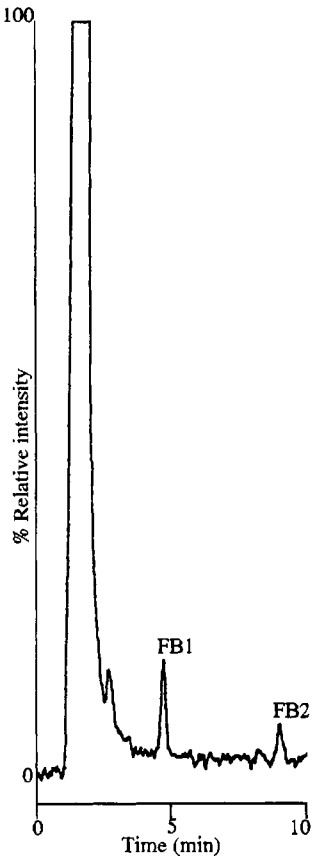

Figure 2. HPLC chromatogram of the AccQ·Fluor derivative of extract from "naturally" contamined corn, containing 1.3 and 0.3 μg/g of fumonisin B_1 and fumonisin B_2, respectively.

Table 3. Results of the Analysis of Fumonisins B_1 and B_2 in Two Corn Samples Using Shephard and AccQ·Fluor Methods

	FB_1 (μg/g)		FB_2 (μg/g)	
sample	Shephard method	AccQ·Fluor method	Shephard method	AccQ·Fluor method
1	1.0	1.3	0.5	0.3
2	1.3	2.0	0.7	0.6
3	10.3	13.8	5.8	4.2

of fumonisins B_1 and B_2 in corn. In addition, several studies are being carried out to increase the method sensitivity and to learn its applicability to different corn-based products.

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