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Short communication

Determination of fumonisins B_1, B_2, B_3 and B_4 by high-performance liquid chromatography with evaporative light-scattering detection

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Abstract

Fumonisins B_1, B_2, B_3 and B_4 (FB_1–FB_4), a group of mycotoxins produced by the fungus *Fusarium moniliforme*, were separated by HPLC using an analytical-scale, base-deactivated C_8 column and a gradient of trifluoroacetic acid buffer (pH 2.7) and acetonitrile. An evaporative light-scattering detector was used to detect the fumonisin peaks. A semi-preparative-scale, base-deactivated C_8 column with a 1:14 mobile phase split facilitated the purification of analytical standards of FB_1.

1. Introduction

The fumonisins are a group of aliphatic mycotoxins, produced by *Fusarium moniliforme* and a few other fungi [1–3], which have been found in many food products made from corn [4]. Seven different fumonisins have been described: FA_1, FA_2, FB_1, FB_2, FB_3, FB_4 and FC_1 [5–7]. The most abundant of these are the four B-type fumonisins (Fig. 1). Corn containing these toxins induces leukoencephalomalacia in horses, pulmonary edema in hogs, and hepatic carcinomas in rats [2,3,8]. FB_1 has also been associated with high incidences of esophageal cancer in humans in South Africa [9] and China [10].

Analysis of fumonisins by high-performance liquid chromatography (HPLC) normally requires derivatization with various reagents to

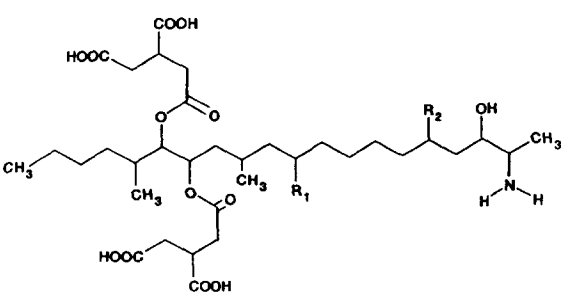

Fig. 1. Structures of fumonisins B_1–B_4. FB_1: $R_1 = R_2 = OH$; FB_2: $R_1 = H$, $R_2 = OH$; FB_3: $R_1 = OH$, $R_2 = H$; FB_4: $R_1 = R_2 = H$.

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allow detection. Maleic anhydride derivatization and a UV detector were used in the first method [11]. Other reagents requiring fluorescence detection have been used: *o*-phthaldialdehyde [4,12], fluorescamine [13–15], 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole [16]; naphthalene 2,3-dicarboxaldehyde [17–19] and 9-fluorenylmethyl chloroformate [20]. LC–mass spectrometry (MS) has also been used for analysis of FB_1 [21,22].

In this study we describe the use of HPLC for the determination of underivatized fumonisins with evaporative light-scattering detection (ELSD). This detection method facilitates both the purification of fumonisins for analytical standards and the collection of impurities for further, off-line analysis.

2. Experimental

FB_1 and FB_2 were obtained from the Council for Scientific and Industrial Research, Pretoria, South Africa. FB_3 and FB_4 were obtained from Dr. R.D. Plattner of the Agricultural Research Service, United States Department of Agriculture, Peoria, IL, USA.

For the analytical-scale experiments, a Shimadzu (Kyoto, Japan) liquid chromatograph was used with a 250 × 4.6 mm, 5 μm YMCbasic base-deactivated C_8 column (YMC, Wilmington, NC, USA). The column was maintained at 40°C. The mobile phase consisted of a binary, three-step, variable concentration gradient with a constant flow rate of 1.0 ml/min. Solution A was acetonitrile–water–trifluoroacetic acid (TFA) (5:95:0.025, v/v). The resulting solution had a pH of 2.7. Solution B was acetonitrile–water–TFA (90:10:0.025, v/v). The gradient comprised a linear program of 20 to 60% B in 30 min, followed by 60 to 80% B in 10 min, and then 80 to 100% B in 2 min.

Aqueous samples (20 μl) of FB_1, FB_2, FB_3 and FB_4 (nominal concentrations of 100, 100, 103 and 132 μg/ml, respectively) and of solvent blanks were injected. A Varex MK III evaporative light-scattering detector (Alltech, Deerfield, IL, USA) was used to detect fumonisins and other components. A 1:1 mobile phase split was achieved by directing the 1.0 ml/min flow into a ZT1 zero-dead-volume tee (Valco, Houston, TX, USA) with two identical outlet arms, each a 350 mm length of stainless-steel capillary tubing. This apparatus allowed simultaneous detection and collection of all low-volatility analytes. Detector response was registered by a Shimadzu Model C-R3A Chromatopac integrator.

For the semi-preparative-scale experiments, a 250 × 10 mm, 5 μm YMCbasic C_8 column and the same HPLC hardware were used. However, to achieve optimal separation of minor impurities from FB_1, the chromatographic conditions were modified: column temperature 50°C, HPLC flow-rate 5.0 ml/min, and solvents A and B (as defined above) with a mixture composition of 30% B for the first 20 min and 100% B thereafter. The detector plumbing configuration was modified to produce a 14:1 split, which was appropriate for the much larger mobile phase flow allowed by the larger column. The 14:1 split modification was achieved using two fused-silica capillaries as outlet legs from the ZT1 tee. The minor flow outlet (125 mm × 50 μm I.D.) was connected to the detector inlet, while the major flow outlet (250 mm × 100 μm I.D.) led the majority of the mobile phase to waste or, whenever a peak was detected, to collection. Linear flow-rate calculations for each leg confirmed that the asymmetric design led to a difference in exit time from the outlets of less than 0.5 s and that the detected portion of the sample exited before the collectable portion. Detector output for the Varex MK III ELSD is indicated in real time from a digital meter located, with other parameter readouts, on the front panel. The Shimadzu integrator response, when used in normal acquisition mode rather than plotting mode, typically lagged the real time signal by about 5 s. These details are significant because the purpose of the second instrumental configuration and associated chromatographic conditions was to enable collection of trace components in amounts sufficient for off-line identification by MS. By watching the detector output, rather than the integrator trace, accurate collection of separated components was possible.

The sample for the second set of experiments

consisted of partially purified FB_1 produced by growing *F. moniliforme* NRRL-13616 on corn for 28 days. After extraction and cleanup [15], it was dissolved in 100% water at a concentration of 25 mg/ml. Sample injection volumes were varied from 1 to 10 μl and the mobile phase composition was also varied. The objective was to find the maximum sample load consistent with quality separation of minor components. Good separation with reasonable sensitivity was obtained using a 4-μl injection at 30% B. In that case, 100 μg was the total sample load on the column.

3. Results and discussion

The analytical base-deactivated C_8 column, with the water–acetonitrile gradient, successfully separated the four fumonisins tested. The retention times of FB_1, FB_2, FB_3 and FB_4 in this gradient were 16.3, 21.4, 19.1 and 24.5 min, respectively (Fig. 2). No peaks were found after the injection of pure water.

Each of the fumonisin samples and solvent controls showed a peak, which has not yet been identified, at $t_R \approx 3.3$ min (Fig. 2). Most of this peak disappeared if the sample solutions were stored in plastic rather than Pyrex vials.

Fig. 2 demonstrates good separation, reasonable chromatographic peak shape and, with one exception, good detector sensitivity with low background noise. The greatly reduced signal for FB_4 (Fig. 2D, peak at $t_R = 24.5$ min) arises from uncertainty in the concentration of the standard available, rather than from a reduced ELSD sensitivity for this fumonisin.

Fig. 3 shows a separation, using the semi-preparative C_8 column, of FB_1 and several minor impurities extracted from cultures of *F. moniliforme* on corn. The approximate mass of the impurity at $t_R = 10.6$ min was less than 1 μg, but due to the flow split, only ca. 60 ng of this peak entered the detector. The experimental arrangement allowed collection of ca. 800 ng of this impurity and up to 90 μg of the FB_1 from a single injection of the crude extract. The recovery of each purified component in the semi-preparative-scale experiment was determined by the split ratio used. That is with a 14:1 split, 1/15 of each component went to the detector and was lost. The remaining 14/15 = 93% of each sample was recovered.

ELSD appeared to be sufficiently sensitive to detect ca. 60 ng of FB_1 per injection. Depending on the split used, the limits of detection for components in solution varied from 6 μg/ml for the analytical-scale system to 220 μg/ml for the semi-preparative-scale system. This amounted to ca. 120 ng of analyte injected for the analytical-scale column and ca. 900 ng for the semi-preparative column.

Because the analytical base-deactivated C_8 column separates all four of the B-type fumonisins by HPLC without precolumn derivatization, aliquots can be collected individually as they elute from the column. Larger-scale purification of each, if desired, is possible with the semi-preparative column. As mentioned above, other methods for the separation and purification of fumonisins require either sample derivatization [12,13,15–17,20] or the use of MS [21,22] for detection.

For preparation of analytical standards of FB_1, detection methods using derivatization require blind sampling of the chromatographic eluate. The fumonisin itself does not produce a fluorescence detector response and the fumonisin derivatives, which can be detected, are not the product desired. The derivatized fumonisin cannot be used as an internal chromatographic standard in reversed-phase chromatography to aid collection of the fumonisin itself because it elutes *after* free fumonisin (data not shown).

A similar practical difficulty is found with on-line MS detection methods, such as that of Young and Lafontaine [22], who assayed FB_1, FB_2 and FB_3 by LC–particle beam MS using tetramethyl ester derivatives. The methyl esters have sufficient volatility for efficient vaporization and ionization to occur in the particle beam MS source. Since this is not true for fumonisin as a free acid, without the methylation step, blind sampling would again be required. Since the fumonisin methyl ester derivative elutes later than the free fumonisin, it could only be used as

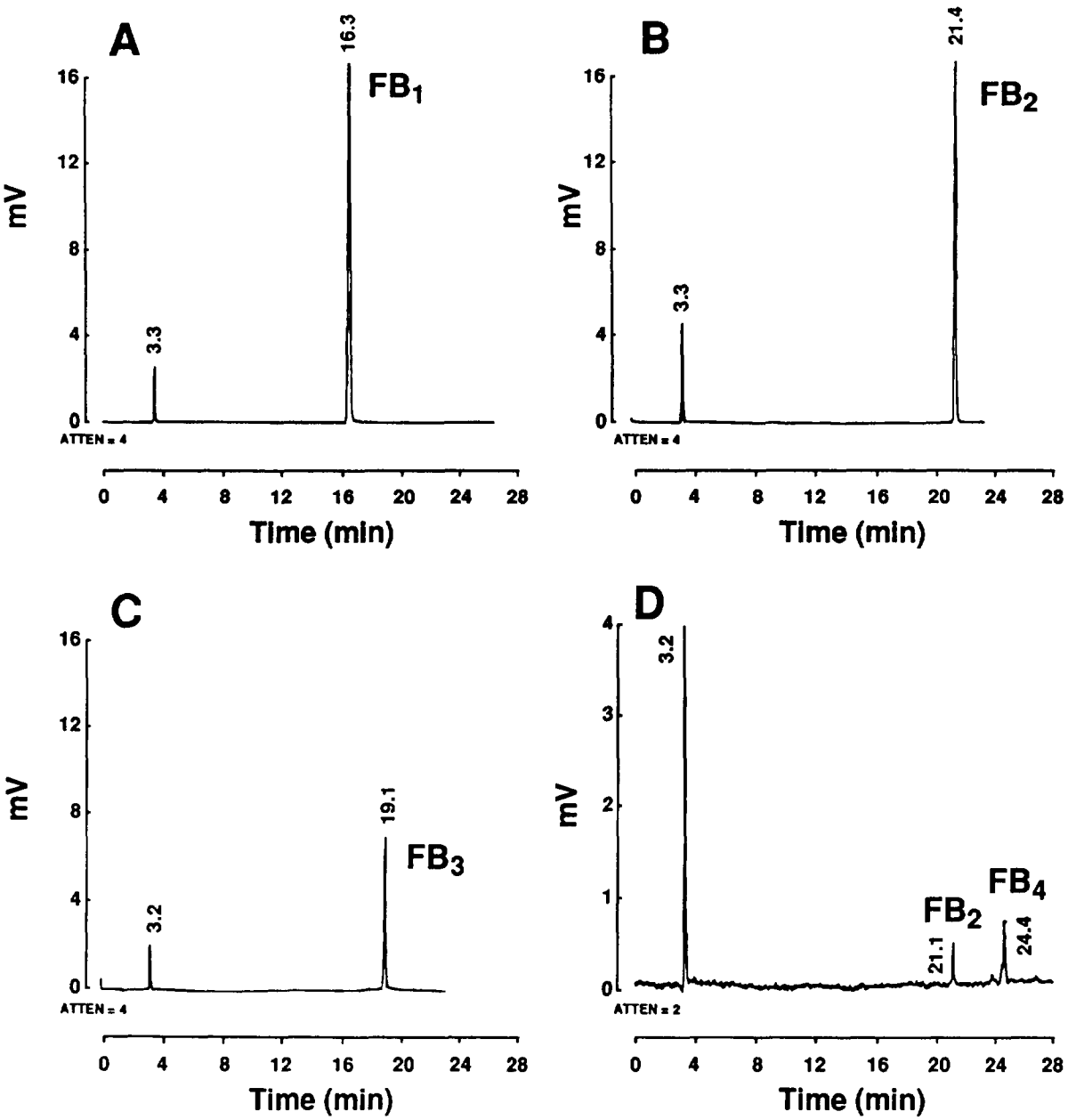

Fig. 2. HPLC elution profiles of FB_1–FB_4, produced with the analytical system. (A) FB_1, (B) FB_2, (C) FB_3, (D) FB_2 and FB_4. The experimental samples were nominally 2 μg each as injected on the column and 1 μg each as presented to the detector.

an external chromatographic standard. Such problems are resolved by the use of ELSD.

For safety, since analytes with low volatility are detected as an aerosol by ELSD, this aerosol must be contained and vented to a chemical fume hood. The integrity of plumbing connections must also be verified before working with toxic substances.

This work has demonstrated that reversed-phase HPLC–ELSD is feasible for the determi-

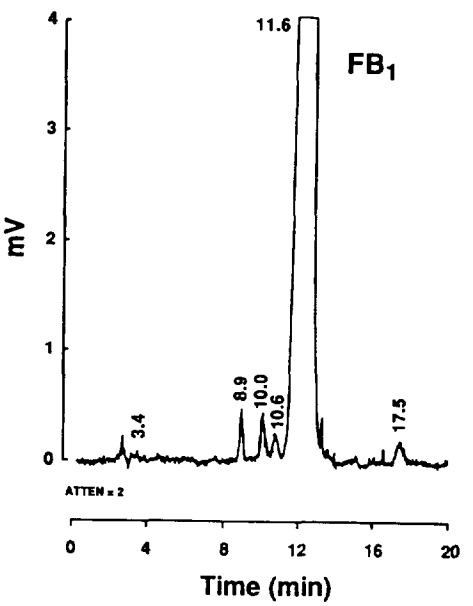

Fig. 3. Low-attenuation HPLC chromatogram of FB_1, showing trace impurities, produced with the semi-preparative system. From an experimental sample of 100 μg, about 7 μg reached the detector and the remainder was available for collection. This separation yielded almost 90 μg of pure FB_1 from an impure sample of 100 μg.

nation, without derivatization, of FB_1, FB_2, FB_3 and FB_4 as well as for the purification of FB_1.

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References

[1] P.E. Nelson, A.E. Desjardins and R.D. Plattner, *Annu. Rev. Phytopathol.*, 31 (1993) 233.
[2] W.P. Norred, *J. Toxicol. Environ. Health*, 38 (1993) 309.
[3] P.M. Scott, *Int. J. Food Microbiol.*, 18 (1993) 257.
[4] M.E. Stack and R.M. Eppley, *J. Assoc. Off. Anal. Chem. Int.*, 75 (1992) 834.
[5] S.C. Bezuidenhout, W.C.A. Gelderblom, C.P. Gorst-Allman, R.M. Horak, W.F.O. Marasas, G. Spiteller and R. Vleggaar, *J. Chem. Soc., Chem. Commun.*, (1988) 743.
[6] M.E. Cawood, W.C.A. Gelderblom, R. Vleggaar, Y. Behrend, P.G. Thiel and W.F.O. Marasas, *J. Agric. Food Chem.*, 39 (1991) 1958.
[7] B.E. Branham and R.D. Plattner, *J. Nat. Prod.*, 56 (1993) 1630.
[8] N.P.J. Kriek, T.S. Kellerman and W.F.O. Marasas, *Onderstepoort J. Vet. Res.*, 48 (1981) 129.
[9] J.P. Rheeder, W.F.O. Marasas, P.G. Thiel, E.W. Sydenham, G.S. Shephard and D.J. van Schalkwyk, *Phytopathology*, 82 (1992) 353.
[10] F.S. Chu and G.Y. Li, *Appl. Environ. Microbiol.*, 60 (1994) 847.
[11] W.C.A. Gelderblom, K. Jaskiewicz, W.F.O. Marasas, P.G. Thiel, R.M. Horak, R. Vleggaar and N.P.J. Kriek, *Appl. Environ. Microbiol.*, 54 (1988) 1806.
[12] G.S. Shephard, E.W. Sydenham, P.G. Thiel and W.C.A. Gelderblom, *J. Liq. Chromatogr.*, 13 (1990) 2077.
[13] E.W. Sydenham, W.C.A. Gelderblom, P.G. Thiel and W.F.O. Marasas, *J. Agric. Food Chem.*, 38 (1990) 285.
[14] T.M. Wilson, P.F. Ross, L.G. Rice, G.D. Osweiler, H.A. Nelson, D.L. Owens, R.D. Plattner, C. Reggiardo, T.H. Noon and J.W. Pickrell, *J. Vet. Diagn. Invest.*, 2 (1990) 213.
[15] M. Holcomb, J.B. Sutherland, M.P. Chiarelli, W.A. Korfmacher, H.C. Thompson, J.O. Lay, L.J. Hankins and C.E. Cerniglia, *J. Agric. Food Chem.*, 41 (1993) 357.
[16] P.M. Scott and G.A. Lawrence. *J. Assoc. Off. Anal. Chem. Int.*, 75 (1992) 829.
[17] R.J. Bothast, G.A. Bennett, J.E. Vancauwenberge and J.L. Richard, *Appl. Environ. Microbiol.*, 58 (1992) 233.
[18] G.M. Ware, O. Francis, S.S. Kuan, P. Umrigar, A. Carman, L. Carter and G.A. Bennett, *Anal. Lett.*, 26 (1993) 1751.
[19] G.A. Bennett and J.L. Richard, *J. Assoc. Off. Anal. Chem. Int.*, 77 (1994) 501.
[20] M. Holcomb, H.C. Thompson and L.J. Hankins, *J. Agric. Food Chem.*, 41 (1993) 764.
[21] J. Chen, C.J. Mirocha, W. Xie, L. Hogge and D. Olson, *Appl. Environ. Microbiol.*, 58 (1992) 3928.
[22] J.C. Young and P. Lafontaine, *Rapid Commun. Mass Spectrom.*, 7 (1993) 352.