Fusarin C Purification and Measurement in Submerged Cultures of *Fusarium moniliforme* by High-Performance Liquid Chromatography

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High-performance liquid chromatography (HPLC) was used to purify and measure fusarin C in ethyl acetate extracts of *Fusarium moniliforme* NRRL 13616 submerged cultures. Fusarin C was purified by semipreparation HPLC on a 21.4 × 250 mm silica column eluted with 8 mL/min chloroform/methanol (96:4) and detected by UV absorbance at 365 nm. Initial analytical HPLC of purified fusarin C produced a single peak, while subsequent analyses yielded as many as four peaks with retention times corresponding to known isomeric forms of the compound. Sample levels of fusarin C were determined by measuring the UV absorbance at the retention times of the fusarin C isomers and comparing these values to those obtained from purified fusarin C under identical conditions. Prior to HPLC analysis, fusarin C standard was evaluated for loss in UV sensitivity at 365 nm. Phenazine-1-carboxylic acid (PCA) or phenothiazine was used as internal standard when fusarin C derived from liquid culture samples was measured and was essential for reproducibility during automated HPLC analysis.

INTRODUCTION

Grains and feeds contaminated by the mold *Fusarium moniliforme* have been implicated in causing leukoencephalomalacia in horses, esophageal cancer in humans, and hepatocarcinomas in ducks and mice (Kriek et al., 1981; Marasas et al., 1981, 1984; Nelson and Wilson, 1986). Strains of *F. moniliforme* isolated from these feeds produce numerous toxins including the potent mutagen fusarin C (Cheng et al., 1985; Gelderblom et al., 1983). Fusarin C is a polyketide, secondary metabolite with a molecular formula of $C_{23}H_{29}NO_7$, as determined by high-resolution mass spectral analysis (Wiebe and Bjeldanes, 1981). The chemical structure (Figure 1) consists of a polyenic chromophore with a substituted 2-pyrrolidone moiety (Gelderblom et al., 1984c).

Fusarin C is a structurally unstable compound that deteriorates spontaneously and in response to UV radiation (Gelderblom et al., 1983; Scott et al., 1986). Additionally, UV radiation induces the formation of three fusarin C isomers through stereoisomerization in the polyene portion of the molecule. While the three UV-induced isomers retain their mutagenicity, two isomeric forms of fusarin C that arise due to spontaneous changes in the substituted 2-pyrrolidone moiety render the compound nonmutagenic (Gelderblom et al., 1984a,c). These results suggest that the 2-pyrrolidone moiety imparts mutagenicity to the compound. This theory is supported by the fact that fusarins A and D, compounds which differ from fusarin C by lacking the epoxide group in this moiety, are nonmutagenic (Gelderblom et al., 1984c). The formation of spontaneous and UV-induced fusarin C isomers is readily detected by HPLC analysis (Gelderblom et al., 1984a).

Thin-layer and liquid chromatographic methodologies have been described for the detection of fusarin C (Farber and Sanders, 1986; Gelderblom et al., 1983, 1984b; Wiebe & Bjeldanes, 1981). While thin-layer chromatography (TLC) provides a simple technique for the qualitative measurement of the compound, HPLC methods are capable of separating fusarin C from sample components and measuring the compound by UV absorbance at 365 nm. However, quantitative analysis of fusarin C using HPLC is hampered by the rapid formation of isomers which are separated and detected as multiple peaks during analysis.

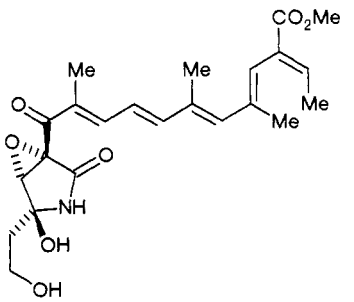

Figure 1. Molecular structure of fusarin C (Gelderblom et al., 1984c).

Our current investigation of the nutritional and environmental factors that influence fusarin C biosynthesis by *F. moniliforme* NRRL 13616 requires a quantitative method for measuring the compound. Reported is an HPLC method for determining fusarin C levels in liquid culture samples which relies on measuring the isomeric forms of the compound, evaluating the deterioration of the fusarin C standard prior to its use, and using an internal standard. Also presented is a rapid semipreparative HPLC method for purifying fusarin C.

MATERIALS AND METHODS

Culture Growth. Spores were produced on a V-8 agar plate from a single-spore isolate of *F. moniliforme* NRRL 13616. V-8 agar consists of 200 mL of V-8 juice/L, 3.0 g/L calcium carbonate, and 20 g/L agar. The V-8 agar plate containing spores was rinsed with deionized water, and the spores were dried on silica beads for long-term storage at 4 °C.

For submerged culture inocula, silica-dried spores were grown and sporulated on 25 mL of V-8 agar in 75-cm² tissue culture flasks (Corning 25116) at room temperature. Spores were harvested by rinsing the agar surface of the V-8 agar flasks with sterile-filtered, deionized water. A concentration of 5×10^6 spores/mL was used for submerged culture growth.

Pure fusarin C standard was obtained from 500 mL of *F. moniliforme* submerged cultures grown in 1-L, baffled Erlenmeyer flasks (Bellco Glass 2543-01000). The culture medium was composed of glucose, 30 g/L; $(NH_4)_2SO_4$, 1.4 g/L; KH_2PO_4, 2.0 g/L; $MgSO_4 \cdot 7H_2O$, 0.3 g/L; $CaCl_2 \cdot 2H_2O$, 0.4 g/L; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, and thiotic acid, 500 μg/L; and folic acid, biotin, and vitamin B_{12}, 50 μg/L in deionized water (Jackson et al., 1989). Cultures were grown for 9 days at 220 rpm and 28 °C in a rotary shaker–incubator (Farber and Sanders, 1986). A pH of 5.0 was maintained by the daily addition of 2 N NaOH or 2 N HCl. Samples for HPLC analysis were obtained from cultures grown under the foregoing conditions and in the presence of various trace metal concentrations, as previously described (Jackson et al., 1989).

Fusarin C Extraction and Sample Preparation. Due to the rapid photodeterioration of this compound, all fusarin C standard and sample preparations were carried out under "gold" fluorescent lighting (Westinghouse, Catalog No. 6YT-126D) in amber screw-cap vials (Scott et al., 1986). HPLC grade solvents were used during all sample extraction, preparation, and chromatographic procedures. Liquid culture samples were stored in the dark at −20 °C and extracted on the day HPLC analysis was conducted.

Fusarin C was extracted from whole culture broths with 3 volumes of ethyl acetate, as described previously (Farber and Sanders, 1986). For fusarin C standard purification, 500 mL of culture broth was extracted and the ethyl acetate evaporated with a rotary evaporator (Buchler flash evaporator). The dried extract was reconstituted in 3.5 mL of chloroform/methanol (96:4) for semipreparative HPLC analysis.

Sample preparation for fusarin C analysis consisted of extracting 3-mL culture samples and evaporating the ethyl acetate under nitrogen at room temperature. The dried extracts were reconstituted in chloroform and applied to a 3-mL, disposable, silica prep column (Baker, Catalog No. 7086) which had been conditioned with chloroform (Scott et al., 1986). The column was rinsed with 3 volumes of chloroform to remove nonpolar compounds and the fusarin C eluted with 1 mL of methanol. The methanol was evaporated under nitrogen and the dried extract reconstituted in chloroform/methanol (19:1) with an internal standard for HPLC analysis. Concentrations of 250 μg/mL phenothiazine (Sigma Chemical, Catalog No. P-4889) or 25 μg/mL phenazine-1-carboxylic acid (Gurusiddaiah et al., 1986) were used as internal standards.

Fusarin C Purification and Analysis. Fusarin C was purified by using semipreparative HPLC. The crude fusarin C extract was injected (Rheodyne 7125 injector) onto a 21.4 × 250 mm Dynamax silica column (Rainin Corp.) and eluted with chloroform/methanol (96:4) at 8 mL/min. Fusarin C was detected at 365 nm by using a variable-wavelength detector (ISCO, Lincoln, NE). Peaks were collected and analyzed for fusarin C by using TLC (Gelderblom et al., 1983). The fusarin C peak was rechromatographed and the center of the peak collected. Fusarin C purity was confirmed by electron impact mass spectroscopy, nuclear magnetic resonance (NMR), and analytical HPLC analyses (Gelderblom et al., 1983, 1984c; Weibe and Bjeldanes, 1981).

At the time of purification, the absorbance at 365 nm was determined for a concentration of 10 μg/mL fusarin C in chloroform/methanol (19:1). For use as HPLC standards, 100-μg aliquots of purified fusarin C were dried under nitrogen in amber vials and stored as gums in the dark at −20 °C. Prior to use, the amount of detectable fusarin C standard and its isomers was determined by comparing the A_{365} of 10 μg/mL fusarin C in chloroform/methanol (19:1) to original absorbance values.

Culture sample extracts were analyzed for fusarin C by using a modification of a HPLC technique described by Gelderblom et al. (1983). Twenty-microliter samples were injected (Waters, 710B WISP with 200-μL loop) onto a 4.6 × 250 mm silica column (Du Pont Zorbax Sil) and eluted with chloroform/methanol (19:1) at 1.5 mL/min. Fusarin C was detected by 365 nm with a variable-wavelength detector (Kratos Analytical Instruments, Spectroflow 757). HPLC of purified fusarin C was used to determine the retention times for the isomeric forms of the compound. Internal standard calibration was accomplished by comparing the sum of the peak areas for the isomeric forms of fusarin C standard to the peak area of the internal standard. Fusarin C was measured in samples as the sum of the peak areas for its isomeric forms.

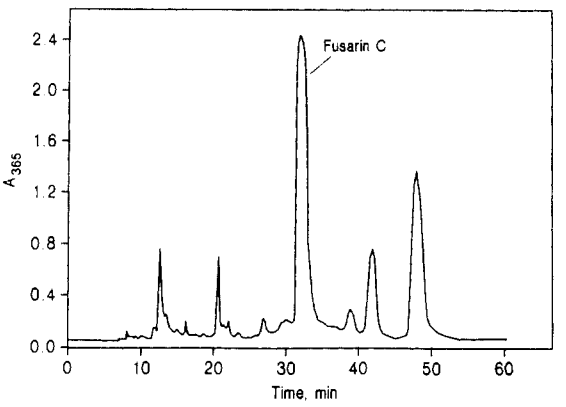

Figure 2. Semipreparative liquid chromatography of *F. moniliforme* ethyl acetate extract; 20-mg sample in 1.0 mL of chloroform/MeOH (96:4). Isocratic elution is with chloroform/methanol (96:4) at 8.0 mL/min, detection by UV absorbance at 365 nm (2 AUFS). Fusarin C, 1.9 mg, elutes at 31.4 min.

RESULTS AND DISCUSSION

Fusarin C Purification. Semipreparative HPLC of *F. moniliforme* culture broth extracts produced a relatively pure fusarin C fraction which eluted at a retention time of 31.4 min (Figure 2). Other fractions containing lesser amounts of fusarin C eluted at retention times of 39, 42, and 49 min and are presumed to be isomers of fusarin C. Purified fusarin C was obtained by rechromatographing the 31.4-min fraction and collecting the center of the fusarin C peak. By use of this HPLC purification technique, the extract (305 mg) derived from 500 mL of *F. moniliforme* culture broth yielded 29 mg of pure fusarin C. Semipreparative HPLC is a rapid and efficient method for purifying fusarin C from *F. moniliforme* submerged culture extracts. Extracts from *F. moniliforme* submerged cultures lack the interfering ethyl acetate extractable compounds found in corn-grown cultures.

Mass spectral data indicated that the fusarin C fraction collected was pure. In the CI mass spectral analysis, the protonated moelcule, m/z = 432, is the most intense ion observed. In the EI mass spectra, the parent ion, m/z = 431, and major fragment ions, at m/z = 281, 213, 185, and 133, were observed. A proton NMR spectrum of the pure fashion C fraction was in good agreement with that reported by Gelderblom et al. (1984a). All analytical techniques confirmed that fusarin C was present in a highly purified form.

Immediately following its purification, fusarin C produced a single peak by analytical HPLC (Figure 3). After storage for 8 days at −20 °C, HPLC analyses of the purified fusarin C produced chromatographic patterns that contained multiple peaks (Figure 3). These chromatographic patterns are comparable to those obtained by Gelderblom et al. (1983, 1984c) for the isomeric forms of fusarin C. While retention times were predictable for the presumed isomers of fusarin C, the amount of compound detected at each retention time was variable. These results agree with the findings of Gelderblom et al. (1983), which showed that isomeric changes in fusarin C form occur readily.

The rapid isomerization of fusarin C makes its quantification by measurement of absorbance at one retention time unreliable. Even though all sample preparations were performed under gold fluorescent lighting and samples stored in amber vials, the formation

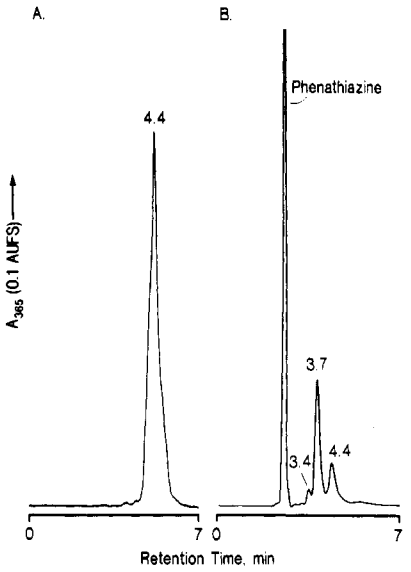

Figure 3. Liquid chromatography of purified fusarin C on an analytical silica column eluted with chloroform/methanol (19:1). Flow rate is 1.5 mL/min, UV absorbance at 365 nm, 0.1 AUFS, injection volume 20 μL. (A) Chromatograph of purified fusarin C (0.9 μg) analyzed immediately following purification. Retention time: 4.4 min. (B) Chromatograph with internal standard of the same purified fusarin C (0.4 μg) stored as a gum at −20 °C for 6 months. Retention times: phenothiazine, 2.5 min; fusarin C isomers, 3.4, 3.7, and 4.4 min.

Table I. Peak Area Response of Purified Fusarin C Exhibiting Multiple Retention Times during HPLC Analysis

injection time,[a] h	int std[b] peak area[c] (×1000)	fusarin C standard (153 μg/mL) peak area[c] (×1000) at retention time, min					peak area ratio (fusarin C/int std)
		3.4	3.7	4.1	4.4	total	
0	912	245	125		4711	5081	5.6
2	897	376	481	4140		4997	5.6
8	939		2099	2997		5096	5.4

[a] Time of HPLC analysis following the addition of purified fusarin C to chloroform/MeOH (96:4). Eight-day-old fusarin C standard was stored as a gum in the dark at −20 °C. [b] Phenazine-1-carboxylic acid, 25 μg/mL. [c] Area in response to absorbance at 365 nm.

of isomers was unavoidable. To overcome this problem, the sum of the peak areas for the isomeric forms of the compound was used as the peak area response for fusarin C. This approach, in conjunction with an internal standard, gave reproducible fusarin C measurements. Repeated HPLC analysis of a fusarin C standard showed that even though the amount of compound detected at any one retention time was variable, the total response was a reproducible measure of fusarin C (Table I).

Under the chromatographic conditions of this study, the isomeric forms of fusarin C had retention times of 3.4, 3.7, 4.1, and 4.4 min (±0.10 min). More recent HPLC analyses with new columns have shown that these retention times can vary considerably between several Zorbax Sil columns, although the same peak pattern is retained. The retention times for the isomeric forms of fusarin C should be determined empirically for each chromatographic system used.

In addition to the problem of isomerization, fusarin C is also subject to spontaneous and UV-induced deterioration to nonpolar compounds that are not detected at 365 nm. The loss in detectable fusarin C was determined for fusarin C standards prior to their use in HPLC analysis.

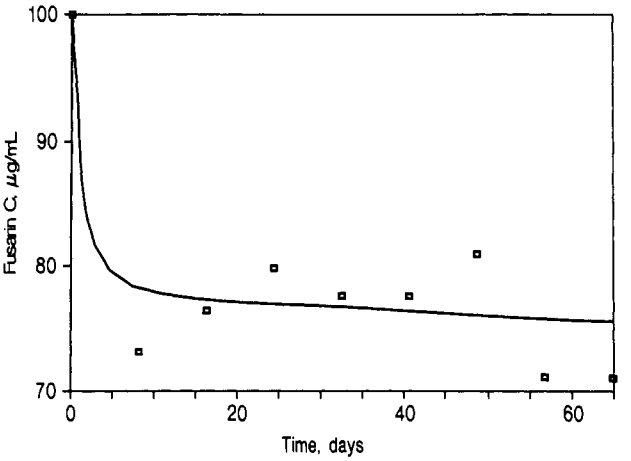

Figure 4. Effect of −20 °C storage on detectable fusarin C in purified 100-μg fusarin C samples stored as gums in amber vials.

One hundred microgram fusarin C samples stored in the dark as gums at −20 °C in amber vials lost approximately 25% absorbance following 10 days of storage with little additional loss following 65 days of storage (Figure 4). Analyses of fusarin C samples stored for over 1 year show a loss of greater than 50% in absorbance at 365 nm. Scott et al. (1986) showed a similar, but more rapid, deterioration profile for fusarin C. This loss in absorbance at 365 nm translates into a loss in detectable fusarin C which must be accounted for when stored fusarin C is used as standards.

The use of an internal standard overcomes the problem of sample solvent evaporation and makes the analysis volume independent. This is particularly critical when a volatile mobile-phase solvent is used with an autosampler. PCA and phenothiazine are adequate internal standards for sample extracts derived from liquid cultures. We initially used PCA, purified from *Pseudomonas fluorescens* culture supernatant, but phenothiazine works equally well and is available commercially. Analysis of our liquid culture extracts showed that interfering compound did not elute at the retention times of PCA and phenothiazine, 2.50 and 2.40 min, respectively. Sample cleanup with a silica preparative column aided in removing compounds that may have interfered with the internal standards. Extracts of *F. moniliforme* infected corn contained numerous compounds that eluted at the same time as these internal standards. Other internal standards such as anthracene, *O*-nitrophenylhydrazine, *p*-nitrophenylhydrazine, and 2-amino-5-nitrotoluene have been tried but were not suitable for use with *F. moniliforme* infected corn extracts. Additional work is needed to determine an appropriate internal standard for use with corn-extracted samples.

Detection response in relation to fusarin C concentration is linear from 5 to 150 μg/mL. The sensitivity of detection is ±0.5 μg/mL over this range. Over 100 duplicate injections of extracted culture broths containing fusarin C concentrations ranging from 0.1 to 115 μg/mL were analyzed for repeatability. The difference between duplicates was regressed on the average amount of fusarin C in the sample, and a slope of 0.0109 ± 0.0045 ($R^2 = 0.05$) was found, indicating that the absolute amount of fusarin C present did not influence the size of the difference between duplicates of the same sample.

Our analyses of extracts from liquid culture samples of *F. moniliforme* show that fusarin C can be detected and measured (Figure 5) by this method. HPLC analysis of liquid culture samples stored for 2 months in the dark at

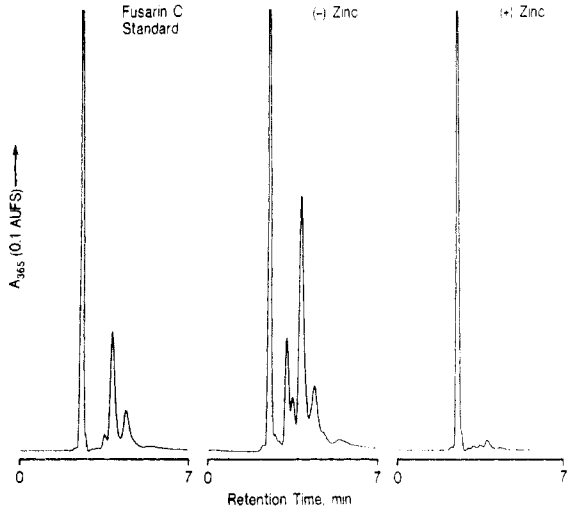

Figure 5. Liquid chromatography of fusarin C standard and *F. moniliforme* liquid culture sample on an analytical silica column eluted with chloroform/methanol (19:1). Samples include phenothiazine, 250 μg/mL, as an internal standard (retention time, = 2.5 min). Injection volume is 20 μL, absorbance at 365 nm, flow rate 1.5 mL/min. Three isomers were detected in the 25.1 μg/mL fusarin C standard at retention times of 3.4, 3.7, and 4.5 min. Extracts derived from cultures grown in the absence of zinc (A) produced high levels of fusarin C, 46.3 μg/mL, while those grown in the presence of zinc (B) produced very low levels, 3.9 μg/mL (Jackson et al., 1989).

−20 °C yielded fusarin C measurements comparable to those from identical samples analyzed immediately following sampling. Due to losses in detectable fusarin C in stored culture extracts, liquid culture samples were extracted on the day HPLC analysis was conducted.

The measurement of fusarin C at one retention time during HPLC analysis is unreliable due to rapid changes in fusarin C form which produce multiple retention times for the compound. Our results show that measuring the isomeric forms of fusarin C as a sum response for the compound provides an adequate method for determining fusarin C levels by HPLC. This method is most accurate when fusarin C standard deterioration is determined prior to sample analysis and an internal standard is used.

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