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# Note

## Determination of sterigmatocystin in fermentation broths by reversed-phase high-performance liquid chromatography using post-column fluorescence enhancement

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Sterigmatocystin is a highly toxic [1] and carcinogenic [2] compound produced by certain species of *Aspergillus*, *Cheatomium*, *Bipolaris* and *Penicillium*. Sterigmatocystin is often found in foodstuffs [3–14] as a metabolite of the ubiquitous *A. versicolor* and has been observed as a contaminant in the fermentation process. Due to the toxicity of sterigmatocystin, the fermentation broth produced by these organisms may present handling and safety problems. As a result, a rugged method for the determination of sterigmatocystin in culture is a necessity. Thin-layer chromatography (TLC) has been widely utilized for the determination of sterigmatocystin [9,10,15–20], but is not easily automated for routine analysis. In addition, the fluorescence intensity of the aluminum chloride derivatized sterigmatocystin has bene observed to decay with time [17]. Reversed- and normal-phase high-performance liquid chromatography (HPLC) has also been utilized extensively [21–30], but the resolution of sterigmatocystin from matrix components is often difficult. This problem may be avoided with extensive sample cleanup or by converting the sterigmatocystin to a fluorescent derivative The commonly utilized acetyl derivative of sterigmatocystin may be prepared by the reaction of sterigmatocystin with acetic anhydride and pyridine. The utility of this approach, however, is limited by the relatively long reaction time, sensitivity of the reaction to water, and the gradual decomposition of the acetyl derivative [21]. In this paper, we report an HPLC method for the determination of sterigmatocystin in fermentation broths utilizing a post-column reaction to produce a fluorescent derivative. This method offers enhanced selectivity and sensitivity over UV detection and is easily automated for routine analysis.

## EXPERIMENTAL

### *Chemicals*

Sterigmatocystin was obtained from Sigma (St. Louis, MO, U.S.A.). Standards were prepared by dissolving the appropriate amount of material in HPLC-grade

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methanol. All solvents were of HPLC grade and were purchased from Fisher Scientific (Springfield, NJ, U.S.A.). All other chemicals were of reagent grade and were filtered prior to use.

*Apparatus*

A Perkin-Elmer Series 4 liquid chromatograph with a Perkin-Elmer LC-420 autosampler was used as the chromatographic system. A Brownlee Newguard RP-18 guard column (15 × 3.2 mm I.D., 7 μm) (Applied Biosystems, Foster City, CA, U.S.A.) was used prior to the Beckman Ultrasphere $C_{18}$ analytical column (Beckman Instruments, Fullerton, CA, U.S.A.). The particle size of the 25 cm × 4.6 mm I.D. analytical column was 5 μm. The aluminum chloride solution was delivered to the mixing tee by a Beckman 1108 solvent delivery module. The reaction coil, consisting of 0.010-in. diameter stainless-steel tubing, was heated by an FH40 heater controlled by a FH50 controller module from FIAtron (Oconomowoc, WI, U.S.A.). The fluorescence of the eluate was monitored with a Waters Model 420 fluorescence detector. The excitation filter was 254 nm and the emission filter was 455 nm. Alternatively, the absorbance was monitored with a Kratos Model 773 absorbance detector.

*Extraction of sterigmatocystin*

The fermentation broth was filtered with a Whatman No. 1 filter. A 5-g portion of the damp filter cake was extracted with 10 ml of methanol and homogenized. The supernatant solution was then filtered with a 0.45 μm LID/X filter obtained from Genex (Gaithersburg, MD, U.S.A.). The filtered solution was then used for analysis. Alternatively, 10 ml of methanol was added to a 3 g portion of whole broth. The suspension was homogenized and filtered as above.

*Chromatographic conditions*

The mobile phase consisted of methanol–water (88:12). The flow-rate of the mobile phase was 0.5 ml/min. For samples with late-eluting peaks, a cleaning gradient consisting of methanol was employed for 3 min after the elution of sterigmatocystin. A 5% (w/w) solution of anhydrous aluminum chloride in methanol was delivered at a flow-rate of 0.5 ml/min to the mixing tee. Unless otherwise specified, the injection volume was 50 μl. Optimization experiments were performed in the flow injection mode.

*Thin-layer chromatography*

Whatman LK-5D silica TLC plates were washed with methanol and dried in an oven at 60°C prior to use. The samples were spotted with 5-μl Drummond microcaps and developed with carbon tetrachloride–chloroform–acetic acid (49.5:49.5:1, v/v/v). This solvent composition was found to provide improved resolution over the AOAC method [31]. Otherwise, the method was unchanged. The TLC plates were scanned after derivatization with aluminum chloride with a CAMAG TLC Scanner II with mercury source ad a 460 nm emission cutoff filter.

## RESULTS AND DISCUSSION

Sterigmatocystin reacts with aluminum chloride in solution to give a fluorescent product, 1,3,8-trihydroxyxanthone [32]. The extent of this reaction was found to be

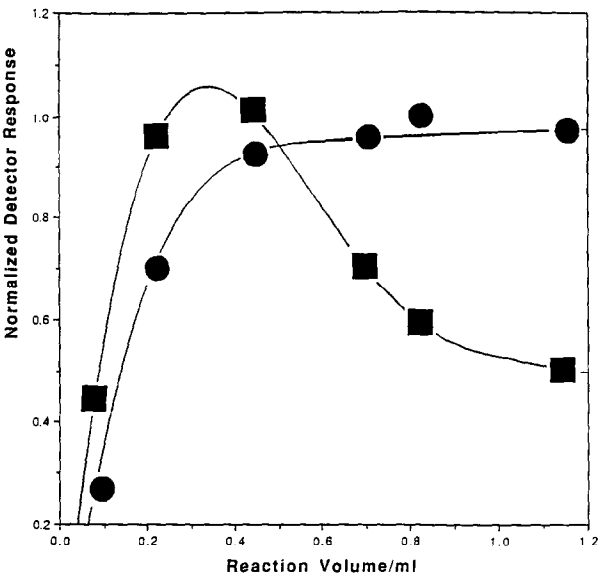


Fig. 1. Plot of normalized peak area (circles) and height (squares) as a function of post-column reactor volume in ml. The mobile phase and reagent solution flow-rates were held constant at 0.5 ml/min.

dependent on reaction time, temperature, $AlCl_3$ concentration and solvent composition. Fig. 1 shows a plot peak height and area as a function of reactor volume at 25°C. The area of the peak increased as the reactor volume was increased from 0.08 to 0.4 ml. With reactor volumes greater than 0.4 ml, no increase in peak area was observed. Over the domain 0.08 to 0.4 ml, the plot of peak height matched that of peak area. With greater reactor volumes, however, the peak height decreased, probably as a result of longitudinal diffusion in the post-column reactor.

The temperature of the reaction coil had a significant effect on the detector response. At 30 and 35°C, the fluorescence intensity was enhanced 20 and 31%, respectively, over the signal at 25°C. Further increase in temperature to 50°C did not result in any signal enhancement, suggesting that the reaction had reached completion.

Francis *et al.* [24] has observed that the fluorescence intensity of 1,3,8-trihydroxyxanthone on TLC plats decayed upon exposure to atmospheric water. Similarly, the intensity of the fluorescence signal in the flowstream was inversely proportional to the aqueous composition of the mobile phase. Table I lists the relative peak areas for sterigmatocystin as a function of the mobile phase composition obtained in the flow injection mode.

Fig. 2 shows a comparison of the fluorescence and absorbance chromatograms of a typical sample of fermentation extract. The fluorescence chromatogram displayed no interferences with the sterigmatocystin peak. In contrast, the absorbance chromatogram displayed poor resolution. Abramson and Thorsteinson [21] have also observed poor resolution of sterigmatocystin from barley extracts with a similar chromatographic system. The gradient pogram was designed to ensure the complete elution of matrix components but is not necessary for less complex samples.

TABLE I

EFFECT OF WATER ON SIGNAL INTENSITY

| Mobile phase composition: methanol–water | Relative response |
|---|---|
| 100:0 | 1.00 |
| 95:5 | 0.94 |
| 90:10 | 0.91 |
| 85:15 | 0.78 |
| 80:20 | 0.76 |
| 70:30 | 0.73 |
| 60:40 | 0.66 |
| 50:50 | 0.63 |

The retention time of the sterigmatocystin was highly reproducible. A series of 12 injections of a 0.82-ppm sample solution gave an average retention time of 9.78 min with a coefficient of variation (C.V.) of 0.2%. The C.V. of the area of the standards was 2.1%. The response of the detector was linear over the domain 0.1 to 15 ppm. At concentrations in excess of 30 ppm, the calibration plot bowed toward the concentration axis. A detection limit of 0.09 ppm was determined on the basis of signal-to-noise

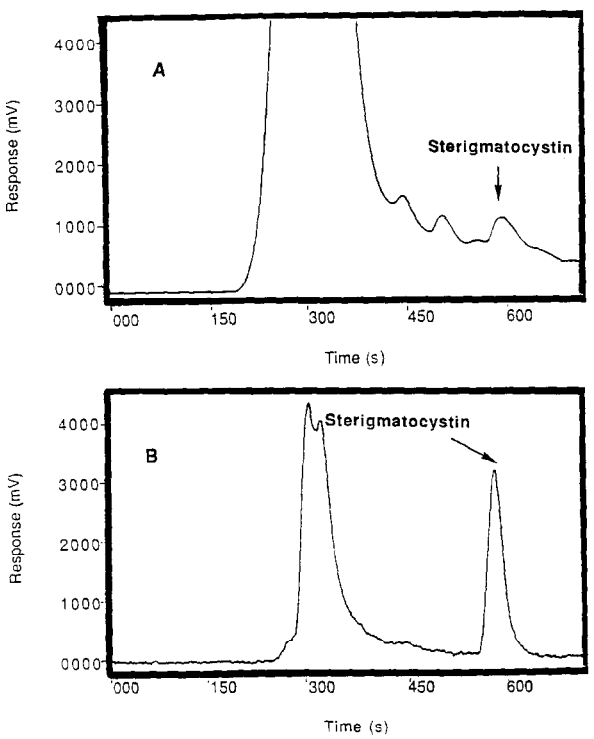


Fig. 2. Typical chromatograms of a fermentation extract containing 2 ppm sterigmatocystin. (A) Absorbance detection at 325 nm. (B) Post-column derivatization with fluorescence detection.

TABLE II

RECOVERY OF STERIGMATOCYSTIN FROM SPIKED BROTH SAMPLES

| Initial ($\mu$g)[a] | Added ($\mu$g) | Recovery (%) |
|---|---|---|
| 1.20 | 0.94 | 95 |
| 1.20 | 1.13 | 91 |
| 1.20 | 1.50 | 93 |
| 1.20 | 2.25 | 89 |
| 1.20 | 4.30 | 89 |
| 1.20 | 8.00 | 89 |

[a] Mass of sterigmatocystin initially present in a 1.5-ml aliquot of fermentation extract.

ratio. Table II lists the recovery of sterigmatocystin spiked into the sample solution. Over the domain of 0.94 to 8.00 $\mu$g spiked, the recovery ranged from 95 to 89%, indicating quantitative recovery. In addition, the quantitative recovery suggests that unresolved matrix components do not quench the fluorescence of the derivatized sterigmatocystin.

To further determine the specificity of the sterigmatocystin peak, several emission cutoff filters were substituted for the 455-nm filter normally employed. A standard and a "worst-case" sample, prepared by mixing broths obtained at the conclusion of several fermentations, were run with each filter. Table III lists the peak areas of the samples and standards as a fuction of cutoff wavelength. The ratio of the sample area to standard area was invariant with emission cutoff filter. Therefore, an interfering peak must possess the identical emission spectrum as well as retention time and reaction specificity to be mistaken for sterigmatocystin.

The HPLC method was validated by comparison to the AOAC standard TLC method [31]. About fifty samples of fermentation origin were analyzed by both methods. Fig. 3 depicts a scatter diagram of the HPLC data *versus* TLC data with a best-fit line. A near ideal slope of 1.01 was obtained by a simple regression. The intercept of the plot corresponded to 0.06 ppm, which is most likely an artifact of the densitometer. In all cases studied, the HPLC data agreed with the TLC data within experimental error of both methods.

TABLE III

PEAK AREA AS A FUNCTION OF EMISSION FILTER

| Emission filter (nm) | Standard area ($\times 10^3$) | Sample area ($\times 10^3$) | Ratio |
|---|---|---|---|
| 530 | 1320 | 662 | 0.50 |
| 495 | 2840 | 1330 | 0.47 |
| 455 | 3840 | 1870 | 0.49 |
| 525 | 4063 | 2039 | 0.50 |

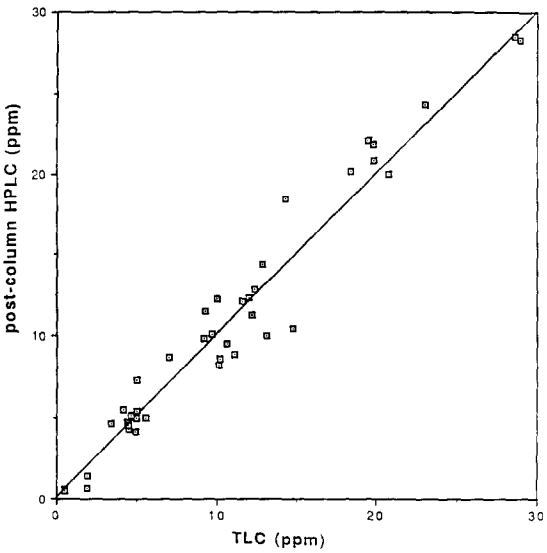


Fig. 3. Comparison of post-column HPLC method with AOAC TLC method. Data were obtained from samples of fermentation extract. Line: HPLC = 1.01 · TLC + 0.63; correlation coefficient = 0.96.

## CONCLUSIONS

A rapid and reliable chromatographic system has been demonstrated for the analysis of sterigmatocystin. Comparison of the HPLC method with the AOAC planar chromatographic method gave an excellent correlation, suggesting that no interferences were present in the HPLC assay. The use of post-column derivatization eliminates uncertainty resulting from incomplete derivatization and decomposition of the derivative. Although the assay was developed for samples of sterigmatocystin in culture, the post-column derivatization scheme may be easily adapted for other applications. Indeed, the water dependence of the fluorescence intensity suggests that aluminum chloride derivatization is well suited for normal-phase chromatography. This phenomenon is not a problem for reversed-phase applications because the sterigmatocystin elutes long after the void volume of the column and reactor.

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