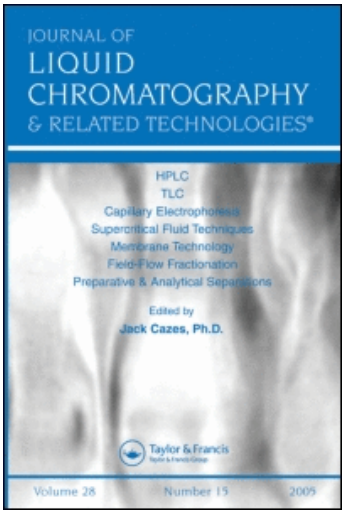

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Scytophycins in Cyanophyte Cultures by High Performance Liquid Chromatography

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To cite this Article Patterson, Gregory M. L. and Bolis, Christine M.(1993) 'Determination of Scytophycins in Cyanophyte Cultures by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 16: 2, 475 — 486
To link to this Article: DOI: 10.1080/10826079308020925
URL: http://dx.doi.org/10.1080/10826079308020925

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(2), 475-486 (1993)

DETERMINATION OF SCYTOPHYCINS IN CYANOPHYTE CULTURES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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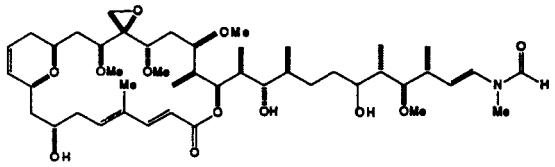
ABSTRACT

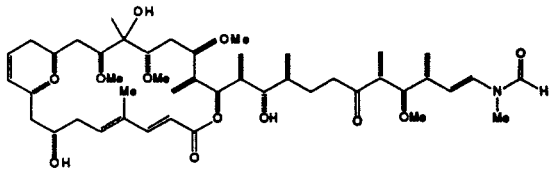
A high performance liquid chromatography method is described for the determination of scytophycin macrolides in algal extracts and culture filtrates. Samples of lyophilized algae were disrupted by ultrasonication in methanol. Algal extracts and culture filtrates were concentrated by adsorption onto C-18 solid-phase extraction columns and elution with methanol. Scytophycins were analyzed on a C-18 column with UV detection at 261 nm. The mobile phase consisted of acetonitrile (33%), methanol (33%), and water (33%). Overall recovery exceeded 93%.

INTRODUCTION

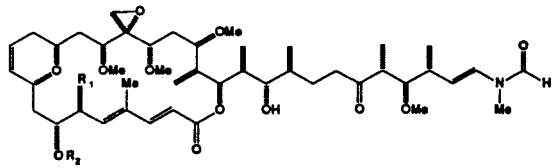
The scytophycins are congeneric macrolides produced by blue-green algae (cyanobacteria), especially species of the closely related genera Scytonema and Tolypothrix. At present, the scytophycin family consists of nine members (Figure 1). Nomenclature of the group is somewhat confused as the first congener isolated, tolytoxin, was not completely characterized at the time



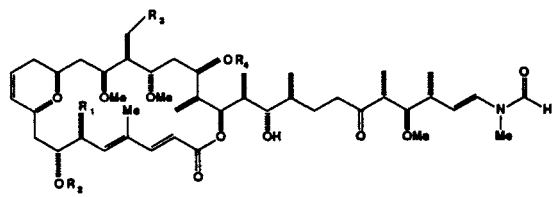
	Name
1	scytophycin A



	Name
2	scytophycin D



	R_1	R_2	Name
3	OH	Me	tolytoxin
4	OH	H	6-hydroxyscytophycin B
5	H	H	scytophycin B



	R_1	R_2	R_3	R_4	Name
6	H	H	H	Me	scytophycin C
7	H	H	H	H	19-O-demethylscytophycin C
8	OH	OH	Me	Me	6-hydroxy-7-O-methylscytophycin E
9	H	H	OH	Me	scytophycin E

Figure 1. Chemical structures of scytophycin compounds.

of isolation. The second macrolide isolated (Scytophycin B) was the first to be completely characterized [1] and thus became the parent compound of the group. No single algal strain has been found that expresses the entire range of scytophycins in culture [1-3].

The scytophycins, especially tolytoxin, are potent antifungal, anticancer agents [4,5] which act at nanomolar concentrations by disrupting microfilament organization, thus inhibiting cell division [6].

A quantitative method for the determination of scytophycins in algal cells and culture medium was required for an investigation of the physiology of scytophycin production in cyanophytes. The analytical scheme was particularly aimed at quantitation of tolytoxin, the major product of interest. This paper describes and evaluates extraction methods, recovery from solid-phase extraction columns, and a high-performance liquid chromatography (HPLC) assay for scytophycins using a reversed phase column with UV detection at 261 nm.

MATERIALS AND METHODS

Reagents

Acetonitrile and methanol were reagent grade (Fisher, Pittsburgh, PA) and were redistilled before use. Water was deionized and distilled. Scytophycin standards were the generous gifts of Drs. Shmuel Carmeli, Masami Ishibashi, and Richard E. Moore. [^{3}H]-6-hydroxy-7-O-methylscytophycin A (10.97 Ci/mmol) was synthesized by reduction of the ketone carbonyl of tolytoxin (6-

hydroxy-7-O-methylscytophycin B) with NaB^3H_3 [7]. Algal cultures were grown as previously described [8].

Instrumentation and Conditions

The HPLC system consisted of a Beckman model 334 liquid chromatograph with a 20 μL sample loop and a Hitachi model 100-10 spectrophotometer outfitted with an Altex flow cell attachment (Beckman, Fullerton, CA); a Hewlett-Packard model 3394 integrator (Hewlett-Packard, Avondale, PA); and a Whatman ODS-3 column, 4.6 mm x 25 cm, 5 μm particle size (Whatman LabSales, Hillsboro, OR). The mobile phase was 33% acetonitrile, 33% methanol, and 33% water. Flow rate was 1.0 mL min^{-1}.

Calibration Curve

A stock solution of tolytoxin prepared in methanol was determined by UV absorption (261 nm; ε 27,000) to contain 256 μg mL^{-1}. Working solutions of appropriate concentration were made by dilution of the stock solution with methanol. Each point on the calibration curve was the average of three replicate determinations.

Extraction & Sample Preparation

Unless otherwise specified, lyophilized algal cells (typically 15 to 25 mg) were suspended in 5 mL methanol in heavy-walled glass tubes and homogenized with a microprobe-equipped cell disrupter (Virsonic 300, VirTis Co, Gardiner, NY) at maximum power for 2 minutes. Sample tubes were suspended in an ice-water bath during

sonication. After steeping overnight at 4°C, the tubes were centrifuged at 1,750 x *g* for 5 minutes. A 2 mL aliquot of the supernatant fluid was diluted to 10 mL with water and adsorbed onto extraction columns as described above for the culture medium. After evaporation, the residue was redissolved in 0.4 mL of methanol prior to filtration.

To compare extraction methods, 25 mg samples of lyophilized cells from a single algal culture were weighed into individual tubes and suspended in 5 mL methanol. The tubes were subjected to the following treatments: a) Steeping, in which the sample tubes were held without agitation at 4° C; b) Shaking, in which the sample tubes were agitated continuously at slow speed on an orbital mixer, also at 4° C; c) Bath sonication, in which the tubes were partially submerged in an ultrasonic bath (160 watt, Fisher FS-9) and sonicated for 15 minutes; and d) Probe sonication, in which sample tubes were treated with a cell disrupter as described above. After sonication, the sample tubes were held without agitation at 4° C.

After 4 hours extraction time, all sample tubes were centrifuged at 1750 x *g* for five minutes. An aliquot (0.5 mL) of the supernatant fluid was removed for analysis before the marc was resuspended by gentle shaking. This procedure was repeated after 24 hours, with the additional step of replacing the extracting solvent with fresh methanol.

Aliquots of culture medium were adsorbed onto a disposable reversed phase extraction column (Prep-Sep C-18, Fisher). The column was preconditioned with 20 mL methanol and 20 mL water. The

sample solution was drawn through the column by vacuum, followed by 20 mL water. The scytophycin-containing fraction was eluted twice with 2 mL aliquots of methanol. This solution was evaporated under nitrogen gas. The residue was redissolved in 1 mL of methanol and filtered (Nylon Acrodisc 13, Gelman, Ann Arbor, MI) prior to injection.

To estimate efficiency and recovery from solid-phase extraction columns, a culture of Scytonema ocellatum, strain FF-66-3, was harvested by filtration. The cell mass was lyophilized (112 mg) and extracted with 25 mL methanol with sonication. Both the cell extract and culture medium were spiked with [^{3}H]-6-hydroxy-7-O-methylscytophycin A equivalent to 200,000 DPM and divided into 5 equal aliquots. Each aliquot was chromatographed as described for sample work-up.

Radioactivity Determination

Radioactivity was determined by liquid scintillation counting in a Beckman LS-230 scintillation counter. Samples were suspended in 5 mL ScintiVerse II (Fisher) and counted for a minimum of 20 minutes.

RESULTS AND DISCUSSION

Tolytoxin was extracted easily and rapidly from the lyophilized algal cells. The relative values shown in Table 1 represent tolytoxin content as measured after extraction. The highest value obtained was defined as 100%. Each value represents

TABLE 1

Comparison of Extraction Methods

	Extraction Time		
Method	4 hours	24 hours	48 hours*
Steep	79.9 ± 11.9**	96.4 ± 1.9	96.9 ± 1.0
Shaking	80.9 ± 6.9	90.8 ± 2.7	93.1 ± 2.6
Bath Sonicator	80.9 ± 4.1	94.5 ± 9.8	100.0 ± 6.4
Probe Sonicator	93.0 ± 5.4	95.5 ± 3.3	96.4 ± 3.3

* with solvent change
** values are relative percentages of extractable tolytoxin with highest value obtained equal to 100%.

the mean value for five samples ± standard deviation. Essentially all of the extractable tolytoxin was removed after 24 hours; more than 90% of the tolytoxin could be extracted within 4 hours by disrupting the cells by ultrasonication. The use of an ultrasonic bath, as suggested for extraction of hepatotoxins from blue-green algae [9], did not appear to accelerate extraction as efficiently as did the probe-type cell disrupter.

In order to concentrate the analytes and eliminate substances that might either interfere with detection or reduce column life, samples were adsorbed onto disposable reversed phase extraction columns using the procedure detailed above (Methods). Recovery of scytophycins was estimated by measuring recovery of a radioactive derivative of tolytoxin. As shown in Table 2, recovery of radioactivity was essentially quantitative.

TABLE 2

Recovery by Solid-Phase Extraction

Step	Culture Medium	Cell Homogenate
Filtered Extract	197623 ± 4093*	195837 ± 2830
Unbound Effluent	595 ± 2700	0 ± 1100
Methanol Eluate	201130 ± 743	194045 ± 1236
Second Methanol Wash	59 ± 100	116 ± 98
Overall Recovery	100.5 ± 0.4%	97.0 ± 0.6%

* DPM. Each value represents the mean of the five samples ± standard deviation.

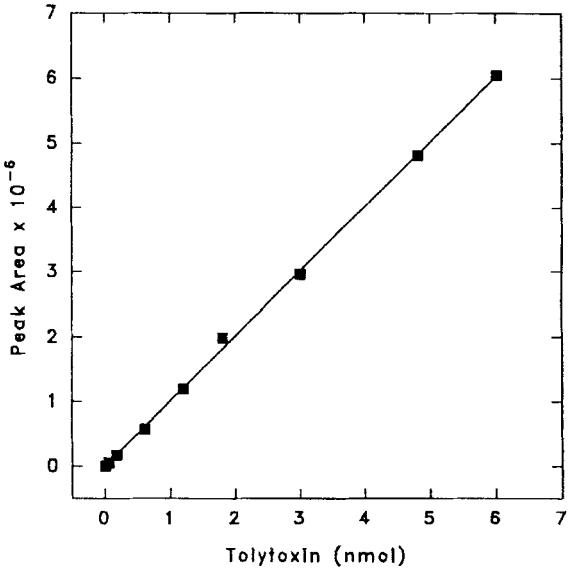


Figure 2. Calibration curve for tolytoxin.

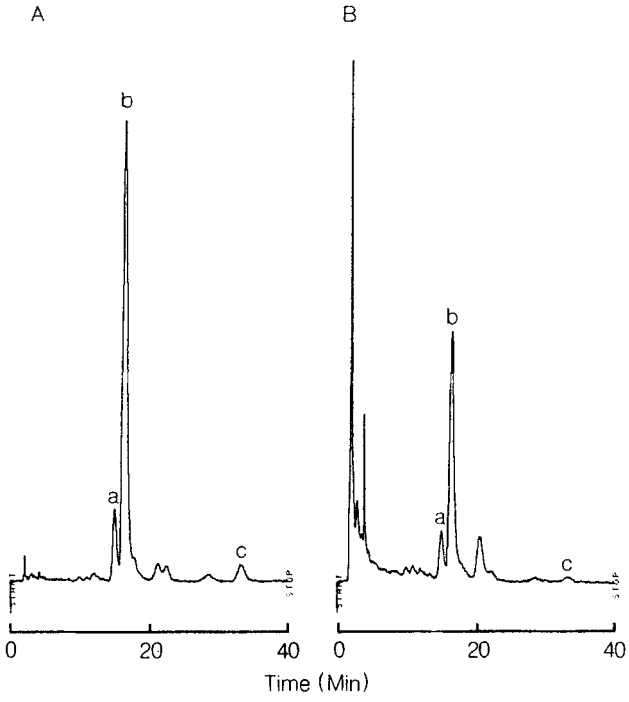


Figure 3. Chromatograms of (A) cell homogenate, (B) medium filtrate from a culture of Scytonema ocellatum strain FF-66-3. Peaks identified are (a) 6-hydroxy-7-*O*-methylscytophycin E, (b) tolytoxin, and (c) 19-*O*-demethylscytophycin C.

Although various mobile phase compositions of acetonitrile/water, methanol/water, and acetonitrile/triethylammonium acetate were tested, optimal separation was obtained using 33% acetonitrile, 33% methanol, and 33% water. The separation was performed at room temperature.

Detector linearity was demonstrated by chromatographing standard solutions containing 5.8 pmol to 5.0 nmol of tolytoxin per

TABLE 3

Capacity Factors for Scytophycin Compounds

Compound	Capacity Factor (k')
Tolytoxin	7.2
Scytophycin A	8.6
Scytophycin B	12.2
Scytophycin C	9.8
Scytophycin D	10.4
Scytophycin E	7.8
6-hydroxyscytophycin B	5.6
19-*O*-demethylscytophycin C	18.5
6-hydroxy-7-*O*-methylscytophycin E	6.5

20 μL injection. The plot of tolytoxin dose versus integrated peak area is shown in Figure 2. The regression equation was determined to be $Y=(1.00 \times 10^6)X + 8.99 \times 10^3$. The coefficient of determination was calculated to be > 0.999. Preparation of calibration curves for other scytophycin compounds was precluded by the limited amounts of material available.

Figure 3 shows typical chromatograms from the analysis of tolytoxin and other scytophycins in a cell extract and a medium filtrate from one of the producing organisms, Scytonema ocellatum strain FF-66-3. The major compound of interest (tolytoxin) elutes at a retention time of 16.4 minutes, and is well separated from other components.

Retention times of all of the known scytophycin compounds were determined by chromatography of authentic samples. Retention times ranged from 13.2 to 38.9 minutes (Table 3).

In summary, the reversed phase HPLC method described here is simple, rapid, and suitable for analysis of the effects of culture treatments on the scytophycin content of cyanophyte cultures.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. L.K. Larsen for editorial assistance and Dr. Apryll M. Stalcup for a critical review of the manuscript. This research has been funded by the Aquaculture Development Program of the State of Hawaii and by the University of Hawaii Sea Grant College Program (Project R/MP-4) under Institutional Grant NA89AA-D-SG063 from NOAA. This is Sea Grant publication UNIHI-SEAGRANT-JC-92-25.

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Received: May 22, 1992
Accepted: June 5, 1992