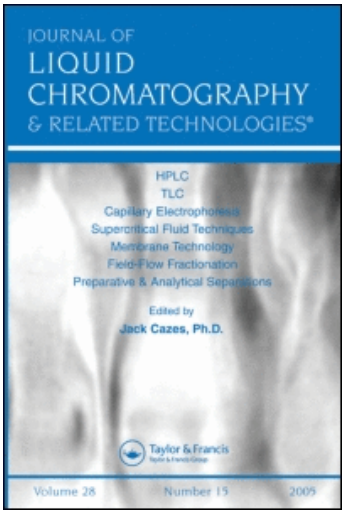

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RAPID AND EFFICIENT METHOD FOR EXTRACTION AND ISOLATION OF MICROCYSTIN RR FROM THE CYANOBACTERIUM

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RAPID AND EFFICIENT METHOD FOR EXTRACTION AND ISOLATION OF MICROCYSTIN RR FROM THE CYANOBACTERIUM

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ABSTRACT

A rapid and efficient method for extraction and isolation of microcystin RR from the cyanobacrerium is described. The method involves supercritical fluid extraction (SFE) for the fast extraction and silica gel column chromatography for the isolation of the microcystin RR. The procedure results in a purity of up to 96-98% microcystin RR without the need for a preparative HPLC. The advantage of the method developed here is that the sample handling steps are minimized, thus reducing possible losses of microcystin RR and saving extraction and isolation time.

INTRODUCTION

Bloom-forming cyanobacteria (blue-green algae) are commonly observed in eutrophic water bodies and pose a serious water quality problem because of the

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potent toxins that they often produce.(1,2) The most commonly reported toxins are the hepatotoxins, the largest group being heptapeptides, known as microcystins.

Over 65 different microcystins(3) have been isolated from cyanobacteria.(4,5) While *Microcystis* is the most studied genus, species in the genera *Anabaena, Nodularia, Nostoc* and *Oscillatoria* also contain these toxins.(6-8)

Microcystin RR (Figure 1) is characterized as a monocyclic heptapeptide containing a common moiety comprising 3-amino-9-methoxy-10-phenyl-2,3,8-trimethyldeca-4,6-dienoic acid (Adda), N-methyldehydroalanine (Mdha), D-alanine, β-linked D-erythro-β–methylaspartic acid and γ–linked D-glutamic acid, plus two L-amino acids as variants.(9,10)

Toxic cyanobacterial blooms have been reported in many countries.(11) Toxic waterblooms cause death of domestic animals and wildlife, and human illness. Cyanobacterial toxins are toxic to zooplankton and fish(12) and can be accumulated in fish and aquatic animals.(13)

The structures and function of toxin are classified into three groups, neurotoxin, hepatotoxins, and lipopolysaccharide. *Microcystis aeruginosa*, that is the most common toxin-producing cyanobacteria found worldwide, produces microcystins.

Many studies showed that these microcystins and nodularin inhibit in vitro activity of protein phosphatase in a cytosolic fraction of mouse liver.(14,15) Liver is reported as the target organ that shows the greatest degree of histopathological change when animals are poisoned by these cyclic peptide. The cause of death in mice is at least partially known and is concluded to be hypovolemic shock caused by interstitial hemorrhage.(16)

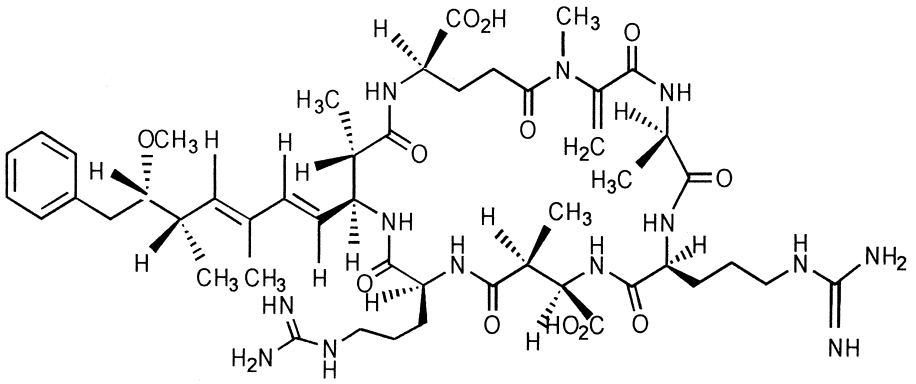


Figure 1. Structure of microcystin RR.

For the isolation of microcystins from cyanobacterial cells, the most widely used procedures(17,18) are as follows: the lyophilized cyanobacterial cells, which contain microcystins are extracted with organic solvents several times and then the extracts are applied to multi-step column chromatographies. For example, Harada et. al.(17) used 5% aqueous acetic acid solution as a extracting solvent and isolated microcystins using ODS column chromatography, silica gel column chromatography and gel permeation chromatography.

In this paper we describe a rapid and efficient method for extraction and isolation of microcystin RR from cyanobacterial cells. The unique feature of the method is that it uses one-step extraction (supercritical fluid extraction) and one-step single column chromatography (silica gel chromatography), instead of multiple extractions with organic solvents and multi-step column chromatographies.

The procedure results in a purity of up to 96-98% microcystin RR without the need for a further preparative HPLC. The suggested simple and rapid procedure for extraction and isolation of microcystin RR might be very useful for scientists in the field of cyanobacterial toxins.

EXPERIMENTAL

Reagents and Chemicals

Supercritical fluid extractions were performed with carbon dioxide, SFC grade (Scott Specialty Gases, Plumsteadville, PA). All solvents were HPLC grade from Aldrich (Milwaukee, WI). The microcystin RR standard was purchased from Sigma (St. Louis, MO).

Extraction of Microcystins

Microcystins were extracted from 5g of lyophilized water bloom material by supercritical fluid extraction. Supercritical fluid extractions of microcystins from cyanobacteria were performed using a Jasco (Tokyo, Japan) LC-900 SFE system. The schematic diagram of the system is shown in Figure 2. This system consisted of three sections: fluid delivery, extraction, and collection. The fluid delivery section included two pumps, which delivered liquid carbon dioxide and a modifier solvent separately. In the extraction section, supercritical fluid extractions were performed with carbon dioxide modified aqueous methanol. The collection section included a back-pressure regulator, which kept the pressure of an extraction vessel at the desired value.

The effluent flowing through the back-pressure regulator reduced its pressure to atmospheric and, thereby, solutes in the effluent reduced their solubility to

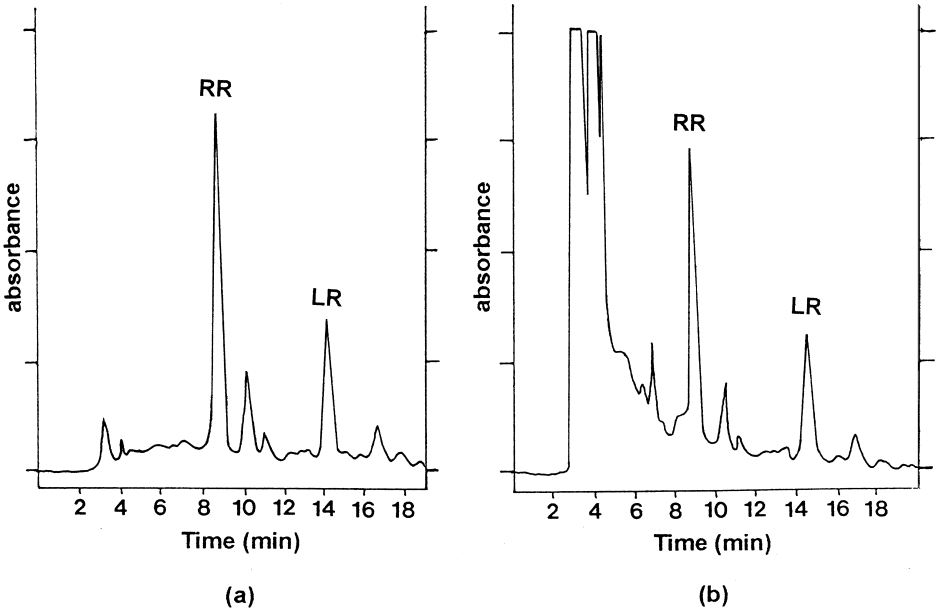


Figure 2. High performance liquid chromatogram of extracted microcystins (a) SPE (solid-phase extraction[22] with ODS cartridge); and (b) SFE (with ternary mixed fluid (90% CO_2, 9.0% methanol, 1.0% water) 40°C and 250 atm), HPLC conditions; MeOH : 0.05M phosphate buffer (pH=3) (52:48), 2.0mLmin^{-1}, 235nm.

virtually zero. In this way, the solutes were deposited and collected in a collection vessel. Since we used aqueous methanol modified CO_2 as an extracting solvent, the extracts were collected in a liquid solvent in the collection vessel. The detailed list of components of the system is given in the Figure 2 caption.

Isolation of Microcystin RR

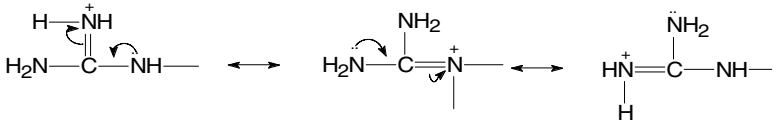
The extracts collected in the SFE collection vessel were evaporated under reduced pressure and then the residue was dissolved in 20mL of methanol and applied to a C_{18} cartridge. The cartridge, which contained microcystins was rinsed with 14 mL of a mixture of methanol and 0.05 M phosphate buffer solution (pH=2.4), followed by 20 mL of water. Microcystins were finally eluted from the C_{18} cartridge with 30 mL of methanol. The elute was evaporated and the residue was dissolved in 2 mL of methanol. The solution was then applied to a silica gel column chromatography. A silica gel column (10×330 mm, Kieselgel 60, E.

Merck, Darmstadt, Germany) was used with a mobile phase of ethyl acetate:isopropanol:water (30:45:25) and a flow rate of 2 mL/min.

Nineteen fractions, numbered 34-38, contained microcystin RR. Each fraction was developed on a TLC plate (Kieselgel K60, E. Merck, Darmstadt, Germany) with ethyl acetate:isopropanol:water (30:45:25). Purity of microcystin RR was monitered by a Beckman 116 pump (System Gold programmable solvent module 126), a 10 mm×15 cm ODS column, and Hewlett-Packard HPLC 1100 series diode array detector coupled in series. Methanol/0.05 M phosphate buffer pH 3 (52:48) was used as a mobile phase at a flow rate of 2 mL/min.

RESULTS AND DISCUSSION

For the extraction of microcystins, we used the same supercritical fluid procedure as the one used in our previous work.(19) Since the microcystins are sparsely soluble, when neat CO_2 was used as the extraction fluid, no microcystins could be extracted from freeze-dried cyanobacterial cells. However, with aqueous methanol modified CO_2, the extraction of microcystins was successful. In supercritical fluid extraction, the extraction of an analyte depends on its distribution between the supercritical fluid and the sorptive sites in the sample matrix. In general, for predicting optimal extraction conditions, one must have two considerations in mind: the ability of the supercritical fluid to compete with the analytes for the sorptive sites and the solubility of the analytes in the supercritical fluid. The latter usually appears to be more important. From Figure 1, microcystin RR contains a strong basic functional group (NHCNH-NH_2), i.e., a guanidine moiety. Microcystin LR contains one guanidine moiety and microcystin RR contains two guanidine moieties. These microcystins would be present mainly in the following cationic form:



The poor extraction result with neat CO_2 is probably caused by the fact that microcystins consist of fairly polar functional groups. The use of co-solvents can have a profound effect on increasing the solubility levels of polar solutes in supercritical fluids. In this study, our experimental results have indicated that 90% aqueous methanol (90% methanol + 10% water) was the most suitable cosolvent for the supercritical fluid extraction of microcystins from dried cyanobacterial cells. The cosolvent flow rate was 0.2 mL/min, and the supercritical CO_2 fluid flow rate was 2.0 mL/min. Therefore, the most suitable medium for

the supercritical fluid extraction of microcystins was a ternary mixed fluid (90% CO_2, 9.0% methanol, and 1.0% water). When 90% aqueous methanol was used as a cosolvent at 40°C and 250 atm, 95% of microcystin RR and 94% of microcystin LR were extracted. It is important to find the optimum SFE operating conditions that would result in the most efficient extraction of microcystins from cyanobacterial cells. In particular, the pressure and temperature of the supercritical fluid are the two most important parameters to be optimized for the most SFE experiments.

To find the optimum extraction temperature, the temperature of the extraction vessel was varied from 40 to 70°C (Table 1). The best extraction efficiency was shown at 40°C; therefore, this temperature was used in this study. The solvating strength of a supercritical fluid is related to its density, a parameter primarily dependent upon pressure. For this study, the extraction pressure was increased from 210 to 290 atm at intervals of 20 atm (Table 2). The extraction efficiency increases with increasing pressure of extracting fluid until the pressure reaches 250 atm. At pressures higher than 250 atm, the extraction efficiency decreases. Other compositions of methanol and water were also tried to find the best extraction condition (Table 3).

A very interesting finding in this experiment was the effect of water on the extraction of microcystins from cyanobacteria. From Table 3, it could be seen that when pure methanol was used as a cosolvent, only 7% of microcystin RR and 4% microcystin LR were extracted. A poor extraction efficiency with no water, indicates that water plays an important role in the supercritical fluid extraction of microcystins. Water has a much greater dielectric constant than methanol (water, 78.4; methanol, 32.7). When water is added, two functional groups of opposite charge in the microcystins can be separated easily. Microcystin RR has a basic functional group ($NHCNH\text{-}N^{+}H_2$) and an acidic functional group (COO^{-}). The work required to separate these two functional groups

Table 1. Supercritical Fluid Extraction of Microcystins RR and LR Using Various Temperatures

Temperature (°C)	Microcystin RR (% Extraction)	Microcystin LR (% Extraction)
70	12 ± 2	14 ± 3
60	17 ± 2	23 ± 2
50	94 ± 1	85 ± 2
40	98 ± 3	90 ± 1

Experimental conditions: 90-min extraction at 40°C and 250 atm, CO_2 flow 2.0 mL/min, and modifier flow 0.2mL/min. RSDs based on triplicate extractions under each condition.

Table 2. Supercritical Fluid Extraction of Microcystins RR and LR Using Various Pressures

Pressure (atm)	Microcystin RR (% Extraction)	Microcystin LR (% Extraction)
290	8 ± 3	10 ± 2
270	81 ± 2	83 ± 3
250	95 ± 2	94 ± 2
230	73 ± 1	67 ± 3
210	71 ± 3	57 ± 3

Experimental conditions: 90-min extraction at 40°C, CO_2 flow rate of 2.0 mL/min, and modifier flow rate of 0.2mL/min. RSDs are based on triplicate extractions at each condition.

is rapidly decreased by adding water, a cosolvent with a high dielectric constant. The extent of separation of two functional groups of opposite charge in the microcystins can affect the solubility of microcystins in methanol modified supercritical fluid CO_2.

This supercritical fluid extraction was compared to solid-phase extraction, the most widely used method for the extraction of microcystins from cyanobacterial cells (Figure 3). From Figure 3, it is noted that when the samples are extracted by SFE, more small compounds are also extracted compared to when SPE is used. However, since these small compounds elute earlier than microcystin RR and LR, the peaks of those compounds do not overlap with the peaks

Table 3. Supercritical Fluid Extraction of Microcystins RR and LR Using Different Compositions of Aqueous Methanol as a Cosolvent

Fluid Phase	Microcystin RR (% Extraction)	Microcystin LR (% Extraction)
90% CO_2 + 10% methanol	7 ± 2	4 ± 3
90% CO_2 + 9.5% methanol + 0.5% water	19 ± 2	21 ± 2
90% CO_2 + 9.0% methanol + 1.0% water	95 ± 1	94 ± 2
90% CO_2 + 8.5% methanol + 1.5% water	93 ± 2	92 ± 2
90% CO_2 + 8.0% methanol + 2.0% water	81 ± 2	88 ± 2
90% CO_2 + 7.5% methanol + 2.5% water	81 ± 4	85 ± 3

Experimental conditions: 90-min extraction at 40°C and 250 atm, CO_2 flow of 2.0 mL/min, and modifier flow of 0.2 mL/min. RSDs based on triplicate extractions under each condition.

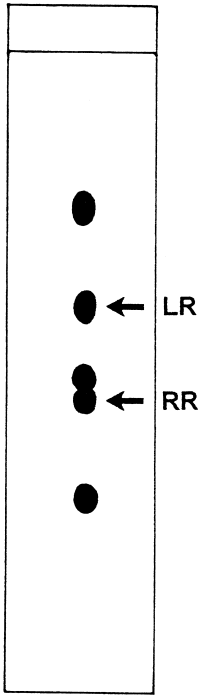


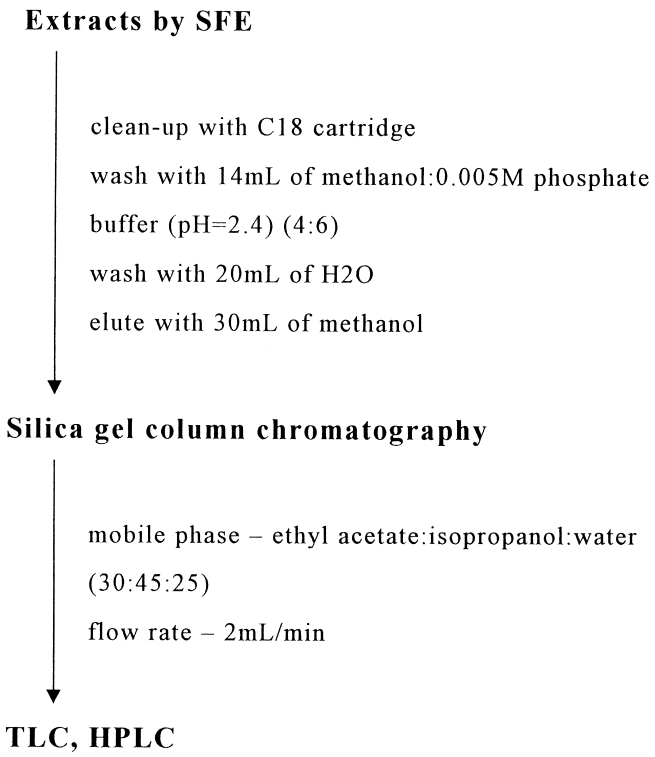
Figure 3. Thin layer chromatograms of Microcystin RR and LR. Elution solvent: ethyl acetate:isopropanol:water (30:45:25); UV detector.

of microcystin RR and LR in the HPLC chromatogram. The SFE procedure has a unique advantage over the SPE procedure in terms of analysis time; the former takes about 100 min., which includes 90-min extraction time and 10-min modifier evaporating time, and the latter takes about 4h.

The extracts collected from supercritical fluid extraction were treated according to our isolation procedures, which are shown in Scheme 1.

A typical elution table of microcystins, obtained by a silica gel column chromatography (10×330 mm, Kieselgel 60, E. Merck, Darmstadt, Germany) is shown in Table 4. Each fraction obtained from a silica gel column chromatography was developed on TLC plate. The typical TLC chromatogram is shown in Figure 3. Through a silica gel column chromatography and TLC developments of each fraction, both toxic fractions of microcystins LR and RR were obtained in the fraction numbers of 6-7 and 34-38.

Figure 4 shows the high performance liquid chromatograms of toxic fractions after silica gel column chromatography. The toxin peaks of microcystins



Scheme 1. Isolation procedure for microcystin RR.

Table 4. The Typical Pattern of Toxic Fractions from Silica Gel Column Chromatography (10 × 330 mm, Kieselgel 60, E. Merck, Darmstadt, Germany)

1	2	3	4	5	**6**	**7**	8	9	10
11	12	13	14	15	16	17	18	19	20
21	22	23	24	25	26	27	28	29	30
31	32	33	**34**	**35**	**36**	**37**	**38**	39	40
41	42	43	44	45	46	47	48	49	50
51	52	53	54	55	56	57	58	59	60

Experimental conditions: mobile phase, ethyl acetate + isopropanol + water (30:45:25), flow rate of 2mL/min, each fraction was collected for 5 minutes.

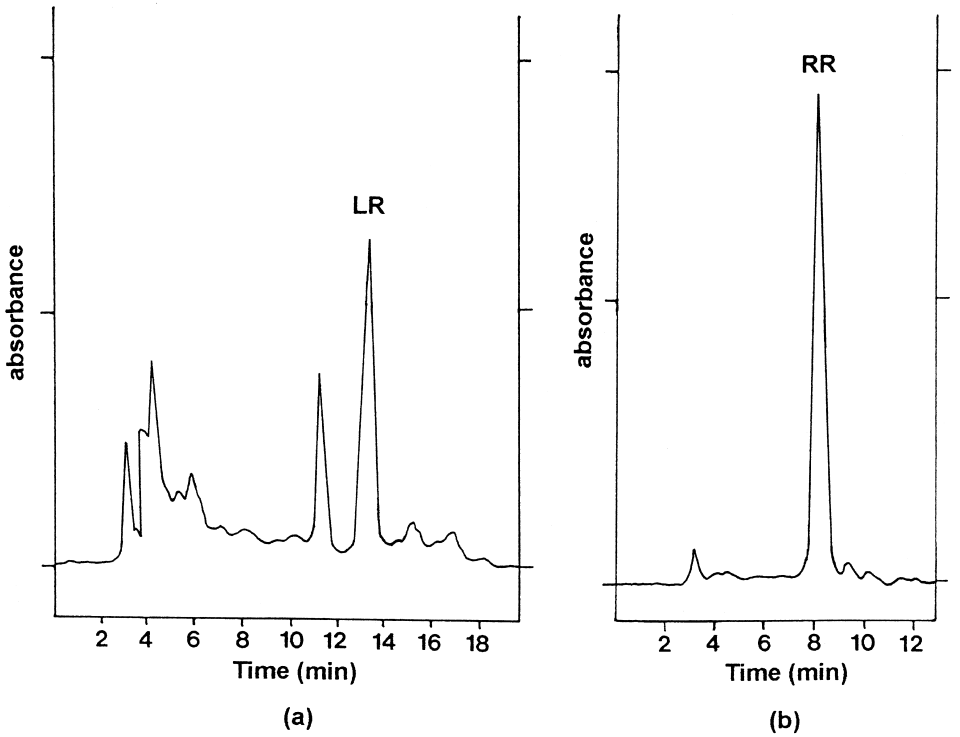


Figure 4. HPLC chromatogram of toxic fractions after silica gel column chromatography. (a) fractions 6,7; (b) fractions 34-38.

LR and RR were readily identified by comparison with standard samples. The results of our isolation experiments were the following; microcystin RR was well isolated by a silica gel column chromatography (Fig. 4(b)), however, microcystin LR was coeluted with other compounds (Fig. 4(a)). Therefore, the systems developed here would be a rapid and efficient method for the isolation of microcystin RR, and should be applicable to other microcystins if further clean-up procedures are developed.

According to the procedures mentioned above, five experiments of extraction and isolation of microcystin RR from 5 g of freeze-dried cyanobacterial cells were made. We could isolate the microcystin RR in the average amount of 4.75 mg ($\pm$ 300 μg), and in a purity of 96-98%.

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