Extraction of Microcystins from Cyanobacteria by Acetic-Acid Modified Supercritical CO₂

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Summary
A new method for the fast extraction of microcystins RR and LR in cyanobacterium was developed using supercritical CO₂. The microcystins were successfully extracted with a ternary mixture (90 % CO₂, 9.5 % acetic acid, 0.5 % water). The method developed here has several advantages over solid-phase extraction sample preparation for the analysis of microcystins. Sample handling steps are minimized, thus reducing possible losses of analytes and saving analysis time. No organic solvent extractions are involved in this method and no clean-up steps are employed.

Introduction
Algal blooms, in particular cyanobacterial blooms, are a major issue for water authorities causing significant taste and odour problems. The knowledge that many of these blooms are toxic has changed the concern from a purely aesthetic problem to one that affects human health.

Over 20 different cyclic peptide hepatotoxins termed microcystins [1] have been isolated from cyanobacteria (blue-green algae) [2, 3]. While Microcystis is the most studied genus, species in the genera Anabaena, Nodularia, Nostoc and Oscillatoria also contain these toxins [4-6].

Microcystins (Figure 1) are characterized as monocyclic heptapeptides 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 [7, 8].

Extraction with supercritical fluids as solvents have received wide attention recently. A number of potential advantages including more rapid extraction rates, more efficient extractions, increased selectivity, potential for combined analyte fractionation in conjunction with extraction are possible with supercritical fluid extraction (SFE).

These advantages of SFE accrue from the properties of a solvent at temperatures and pressures above its critical point. At elevated pressure this single phase will have properties which are intermediate between those of the gas and the liquid phases and are dependent on the fluid composition, pressure, and temperature. The compressibility of supercritical fluids is large just above the critical temperature and small changes in pressure result in large changes in density of the fluid [9]. The density of a supercritical fluid is typically 102–103 times that of the gas. Consequently, molecular interactions increase due to shorter intermolecular distances. However, the diffusion coefficients and viscosity of the fluid, although density dependent, remain more like that of a gas [9]. The "liquidlike" behaviour of a supercritical fluid results in greatly enhanced solubilizing capabilities compared to the corresponding liquid. These properties allow similar solvent strengths to liquids but with greatly improved mass-transfer properties which provide the potential for more rapid extraction rates and more efficient extraction due to better penetration of the matrix.

Supercritical CO₂ extraction has been commercially applied to caffeine removal from green coffee beans [10] and determination of PCBs in an industrial soil [11]. Other applications have been described in many articles [12, 13].

This study describes an investigation conducted to evaluate the applicability and efficiency of SFE methods for the extraction of blue-green algal hepatotoxins, microcystins from cyanobacteria. Supercritical fluid extractions using modified CO₂ were evaluated and compared to solid phase extraction (SPE).
extracts were centrifuged at 9000g for 10 min, and the supernate collected and applied to a Baker C18 cartridge. The cartridge was washed with 10mL water and 15mL 20 % aqueous methanol. Microcystins RR and LR were eluted from the cartridge with 15mL 90 % aqueous methanol and the eluate taken to dryness. For SFE, dried cells (0.1 g) were directly used for extraction. An extraction cell with an internal volume of 10 mL (JASCO, Tokyo, Japan) was used. The extraction cell was kept in a thermostatic chamber maintained at 40 °C with circulating air. Supercritical CO₂ at as 250 atm was used as extracting solvent. The CO₂ flow rate was 2.0 mL min⁻¹ and the aqueous acetic acid flow rate was 0.2 mL min⁻¹. Extraction time was 30 min.

**Instrumentation**

SFEs of microcystins from cyanobacteria were performed using a JASCO (Tokyo, Japan) LC-900 SFE system. A schematic diagram of the system is shown in Figure 2. The JASCO pumps are reciprocating, and do not compensate for the compressibility of the CO₂; they only measure the volume of flow. The rate of the CO₂ pump was 2.0 mL min⁻¹ and that of the modifier pump 0.2 mL min⁻¹. In Figure 2, the collection section included a back-pressure regulator, which kept the pressure of an extraction vessel at a desired value [14]. Effluent flowing through the back-pressure regulator reduced solubility of pressure to atmospheric and hence solutes in the effluent to virtually zero. Thus solutes were deposited and collected with liquid modifier (water and acetic acid) in a suitable vessel which was a glass tube, volume of 50 mL. The liquid modifier was removed with a rotary evaporator, and the residue dissolved with 1 mL methanol and then injected into the HPLC. A detailed list of components of the system are given in the caption to Figure 2.

Determination of microcystin RR and LR was performed with an apparatus comprising a Beckmann 116 pump (System Gold Programmable Solvent Module 126), a 150 x 10 mm ODS column and Hewlett Packard HPLC 1100 series diode array detector coupled in series. Methanol [0.05 M phosphate buffer pH 3 (55:45)] was used as mobile phase at 2 mL min⁻¹.

**Results and Discussion**

The microcystins included in this study are sparsely soluble in neat CO₂. For example it was used as the extraction fluid at 40 °C and 250 atm, no microcystins could be extracted from freeze-dried cyanobacterial cells. However, with aqueous acetic acid-modified CO₂, the extraction of microcystins was successful. In SFE, extraction of an analyte depends on its distribution between the supercritical fluid and the sorption sites in the sample matrix. In general, for predicting optimum extraction conditions one must have two considerations in mind: the ability of the supercritical fluid to compete with the analytes for sorption sites and the solubility of

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**Experimental**

**Reagents and Chemicals**

SFEs were performed with CO₂, SFC grade (Scott Specialty Gases, Plumsheadville, PA, USA). All solvents were HPLC grade from Aldrich (Milwaukee, USA). The microcystin RR, LR standards were supplied from Dr. Ken-ichi Harada, Meijo University.

**Sample Preparation**

Cyanobacterial cells, water blooms of Microcystis, were collected from several Korean lakes. Cells were lyophilized using LABCONCO FREEZONE 4.5 (Kansas city, USA) for 30h. For SPE, dried cells (0.1 g) were extracted with 5 % acetic acid (5 % acetic acid, 95 % water) three times for 30 min while stirring. The combined