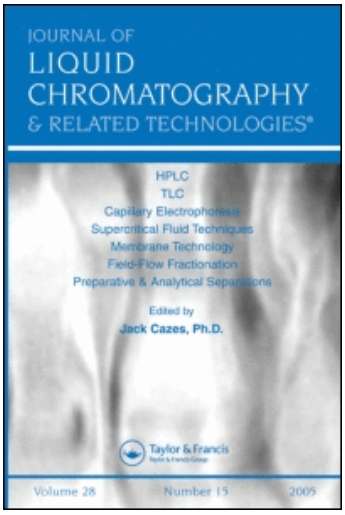

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LIQUID CHROMATOGRAPHY DETERMINATION AND LC-ESI-MS ANALYSIS OF MICROCYSTIN-LR FROM PCC STRAINS OF *MICROCYSTIS AERUGINOSA*

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ABSTRACT

Cyanobacteria (blue-green algae) have been shown to produce unique biologically active peptides such as microcystins, mainly from different species of *Microcystis aeruginosa*. Presence of these toxic peptides (hepatotoxin) in fresh water supplies are of increasing concern and has emphasized the need for a suitable analytical method. Amounts of microcystin-LR were estimated in the laboratory cultures of PCC strains viz. PCC-7806, 7820, and PCC-7941, using high performance liquid chromatography. The identity of microcystin-LR was confirmed by LC-ESI-MS. The lower detection limit of quantification is 1.0 μg/L.

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INTRODUCTION

The occurrence of toxic fresh water blooms of cyanobacteria (blue-green algae) has been reported in many countries (1–3). These toxic blooms have caused death of domestic animals and wildlife. The cases of human illness are also reported (4–6). Cyanobacteria produce several types of toxins that can be harmful to humans. The more frequently occurring are the microcystins, a group of at least 60 heptapeptides, which have the common structure of cyclo (-D-Ala-L-X-D-Erythro-methyl-ASP-L-Y-Adda-D-Glu-N-methyldehydro-Ala), where X and Y are the variable L-amino acids, and Adda is a unique twenty carbon β-amino acid (3-amino-9-methoxy 2,6,8-trimethyl-10-phenyldeca-4, 6-dienoic acid). Microcystins are named according to the variable amino acids, that they contain. Microcystin-LR (MC-LR) (Figure 1) is one of the most commonly occurring and frequently studied microcystin; it contains leucine (L) and arginine (R) in the variable position. Several methods are reported in the literature for the analysis of microcystin-LR from different strains of *Microcystis aeruginosa*, which describes mainly purification (7) with an ODS silica gel cartridge and separation on HPLC. In the present study we have examined the microcystin-LR contained in three PCC strains of *Microcystis aeruginosa* using liquid chromatography, and confirmation of the presence of microcystin-LR was made by LC-ESI-MS method.

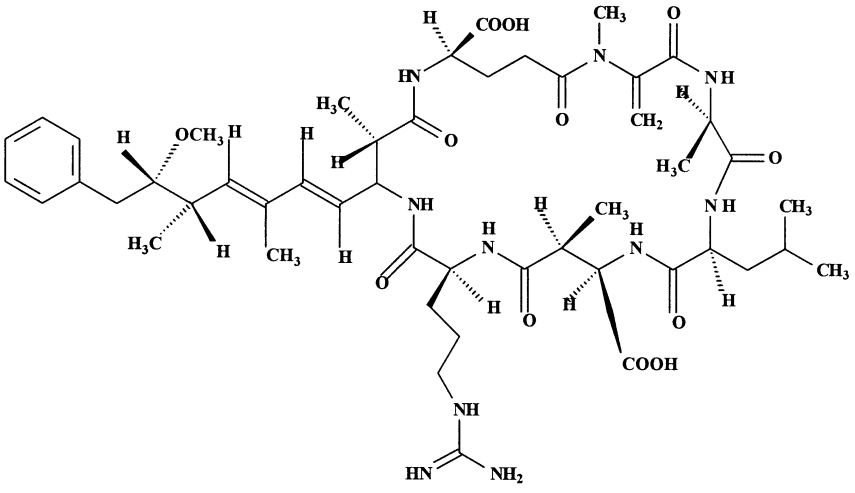

Figure 1. Microcystin-LR.

EXPERIMENTAL

Materials

Methanol, butanol, acetonitrile were of HPLC grade; trifluoroacetic acid and glycerol were of spectro grade from Merck Germany. Deionised distilled water was obtained by using Mill-Q Ultra pure water system (Millipore, Bedford, MA, USA). Standard sample of microcystin-LR was isolated from freeze dried culture of *Microcystis aeruginosa* and compared with the authentic sample received as a gift from Prof. Carmichael of Wright State University Dayton, Ohio, USA.

Organism and Cultivation

The *Microcystis aeruginosa* strains i.e. PCC-7806, 7820, and PCC-7941 were received from Pasture Culture Collection Centre, France. The strains were cultured in glass carboys containing one litre of CB (8) medium in static bath cultures. Cultures were maintained at a controlled temperature of 25°C at 1000-lux intensity and a 12 h photoperiod. Replicate cultures were kept in a static condition without aeration. At the exponential phase of growth, cells were harvested by centrifugation, lyophilises, and stored at −20°C until analysis.

Analysis Procedure

Dried cells (1 g) were extracted three times with 100 mL of solvent water: methanol, (80 : 20) for thirty minutes while stirring. The extract was centrifuged at 11 000 g and the supernatant was applied directly to Waters C-18 cartridge, preconditioned with methanol and water. The cartridge was rinsed with water (10 mL), followed by a mixture of water : methanol, (10 mL) (80 : 20) and the toxin was eluted with methanol (10 mL). The methanol fraction was dissolved in one mL of methanol and 5 µL of the solution was injected into high performance liquid chromatography, equipped with a constant flow pump (Waters 600, USA) and a variable wavelength U.V. detector (Waters 486, USA) operated at 230 nm.

The separation was performed on Nucleosil ODS C-18 5 µm (Esgee, 250 × 4.6 mm) using a mobile phase of methanol : water (61 : 39), containing 0.01% of trifluoroacetic acid and 0.8% of glycerol at a flow rate of 1 mL/min. The pH of the mobile phase was kept at 3.5. The injector volume was 5 µL for analytical analysis and a loop of 200 µL was used for the preparative purposes. Both the column and the precolumn were maintained at room temperature.

Peak area was calculated using a data processor of Millennium (32) Chromatography Manager software, version 3.05 (Waters, USA). The concentrations in cultured samples were determined by using the software Sigma Stat 2.0.

Purification of Microcystin-LR From PCC Strains

Dried cells (1 g) of PCC-7806, 7820, and PCC-7941 were extracted three times with 150 mL of solvent (water : methanol, 80 : 20) for 30 min while stirring. The extract was centrifuged at 11 000 g and the supernatant was applied on C-18 cartridge. The toxins containing the fraction obtained from the cartridge were successively subjected to preparative high performance liquid chromatography under the conditions mentioned above.

LC-ESI-MS

A Hewlett-Packard (Paloallo, CA, USA) HP-1100 series LC-ESI-MS system, equipped with pump, U.V. detector, an auto sampler, and an MS system coupled with an analytical work station were used. The analytical column used was Nucleosil ODS C-18, 5 μm (Esgee 250 × 4.6 mm) and LC conditions were the same, as has been described under analysis procedure. The MS system consisted of a standard atmospheric pressure ionisation (API) source configured as ESI. The ESI-MS (Micromass QuattroLC-MSMS) interface in positive mode was operated at 300°C gas temperature, 15.0 l/min drying gas flow, 30 psi neublizer gas pressure, and 3850 V of capillary voltage. Ion monitoring of the most abundant ion was used for detection and confirmation of microcystin-LR in the cultured, as well as in the standard sample.

Quantitative Determination

Solutions of various concentrations in the range of 0.1 μg to 1.0 μg/mL were prepared by taking a known volume of stock solution (200 μg/mL) and diluting it further with mobile phase. Five μL of each concentration was injected onto the HPLC system and the peak area was recorded. Average peak areas from three replicates were plotted against concentration of microcystin-LR for calibration curve. The extracted fractions (1 mL) from the different PCC strains (viz PCC-7806, 7820 and PCC-7941) were diluted in the known volume of mobile phase and injected onto the HPLC column, under the conditions at which the calibration curve of the standard microcystin-LR was drawn. In each case, the concentration of microcystin-LR in the cultured samples was determined from the

calibration curve of standard reference. The calibration curve follows the straight-line equation i.e., $Y = Yo + ax$, where Y is the peak area and X is the concentration. Reproducibility of the method was checked in different batch cultures of various strains of cyanobacteria.

RESULTS AND DISCUSSION

Microcystin-LR (Figure 1) is a toxic peptide of varying hydrophobicity. Hydrophobicity is mainly imparted by the presence of Adda moiety and other hydrophobic substituents present as variable amino acid like arginine. Hence, it can be easily chromatographed by reversed phase high performance liquid chromatography using acidic mobile phase. Addition of trifluoroacetic acid improves the chromatographic efficiency by maintaining the low pH and it protonates the carboxylic acid present in microcystin-LR and, thereby, acts as an ion-pairing agent, that in turn increases the retention time of poorly retained peptides. Figure 2 shows the high performance liquid chromatogram of PCC-7941, 7806, and PCC-7820 of *Microcystis areuginosa*, respectively. The peak appearing at 14.9, 15.0, and 15.0 min. for PCC-7941, 7806, and PCC-7820, respectively, indicated the presence of microcystin-LR, which had an excellent match with that of a standard sample of MC-LR (Figure 3). Other peaks also appeared along with microcystin-LR in the cultured sample, which could not be identified, because of non-availability of authentic samples of other toxins. The peak shape is relatively broad in different laboratory cultures, essentially because of the pH of the mobile phase, which was maintained at 3.5 by using 0.01% TFA that would have left few carboxylic acids unprotonated, and this would have resulted in differences in peak shapes. This is partly due to the isocratic conditions also used for the analysis of cultured samples.

Figure 4 shows the calibration curve of standard microcystin-LR as well as the laboratory-cultured sample. By using the peak area, excellent linearity for concentration of microcystin-LR between 0.1 μg/mL and 1.0 μg/mL was obtained with a correlation coefficient of 0.999.

The least square straight-line equation with the standard deviation of the intercept and the slope was $Y = -140.1219 + 10733979x$, where x is expressed in μg/mL of microcystin-LR in standards, as well as in cultured samples. The linear working range of the cultured samples of PCC strains extended between 0.15 μg to 0.64 μg using peak area ratios. From the calibration curve, the amount of microcystin-LR produced by PCC-7806, 7820, and PCC-7941 was found to be 0.63 μg, 0.64 μg and 0.15 μg of the dry cell, respectively.

The final confirmation of the peak identities was established by LC-ESI-MS analysis. For standard microcystin-LR the retention time 15–17 min and the principal peaks occurred at m/z 996. It has already been reported (9) in the

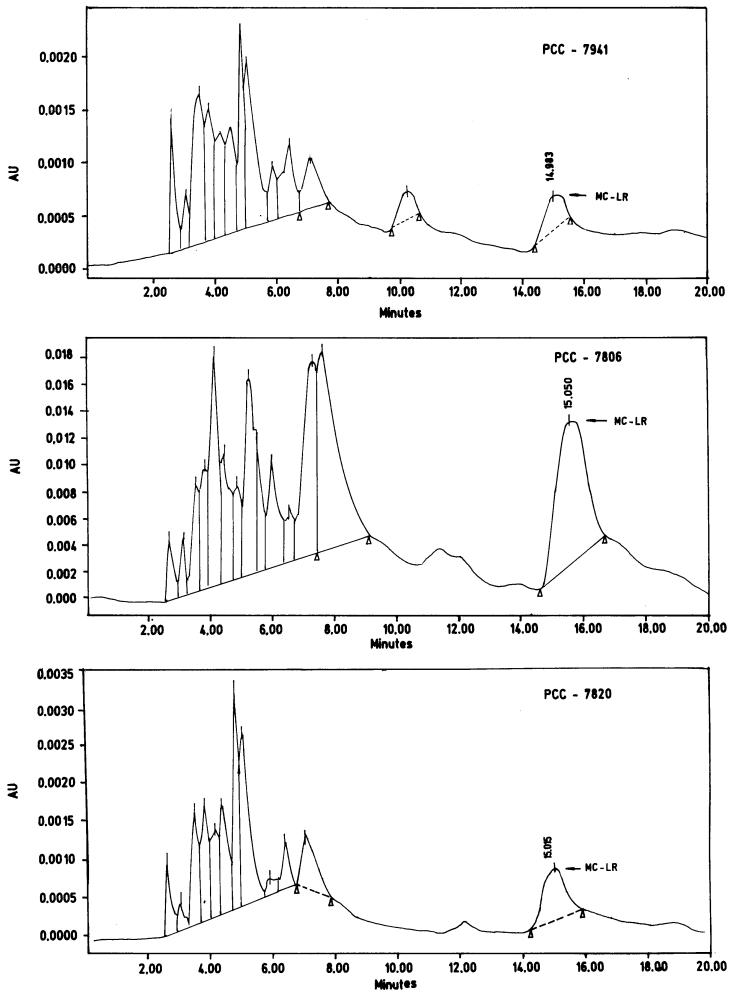

Figure 2. HPLC profile of PCC 7941, 7806, and 7820 (algal extract).

literature, that when peptides are analyzed by ESI-MS, compounds having higher molecular weight are more likely to give strong doubly charged protonated molecules $[M + 2H]^{2+}$. In the case of microcystin-LR (9), other than the molecular ion peak at 996, the natriated ($[M + H + Na]^{2+}$) at m/z 509 and kaliated ($[M + H + K]^{2+}$) at m/z 517 adducts (10) of the doubly charged ion were also observed in the mass spectrum of both the standard, as well as in the cultured

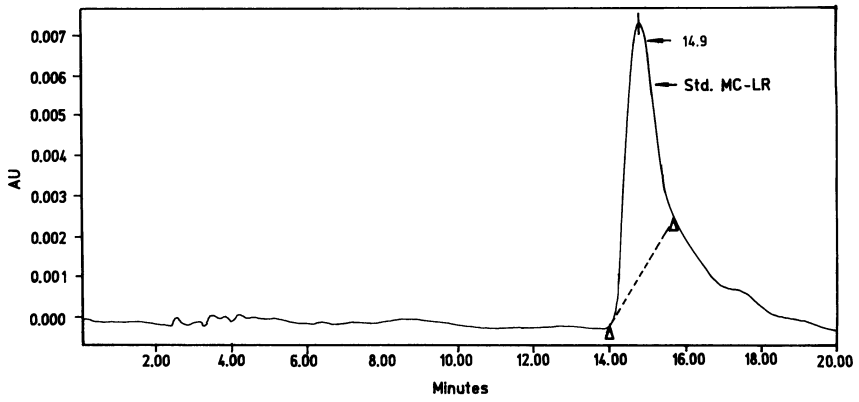

Figure 3. HPLC profile of standard Microcystin-LR.

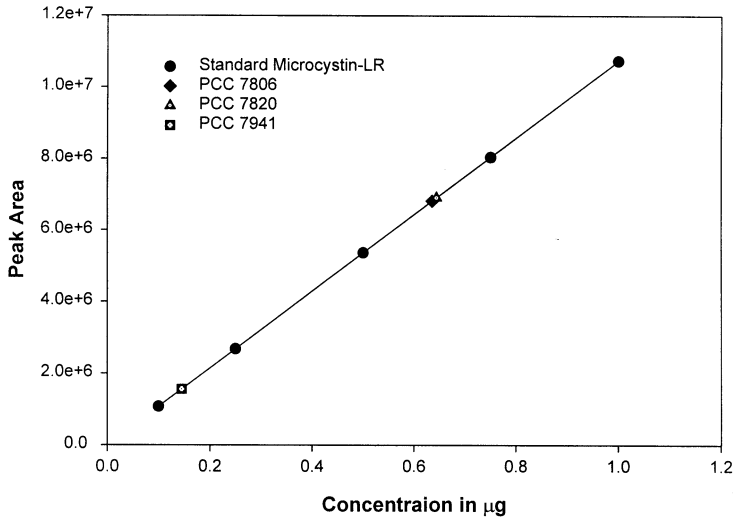

Figure 4. Calibration curve for Microcystin LR in standard sample and in laboratory cultured samples.

samples. The microcystin-LR isolated from PCC-7806 and PCC-7941 strains also showed excellent matches with those of PCC-7820 and the standard sample (Figure 5). Selected ion monitoring also confirms the presence of microcystin-LR in both standard and PCC strains (Figure 6). Approximately 100 fentomoles of the sample was consumed to obtain a full scan mass spectrum with signal to noise ratio of

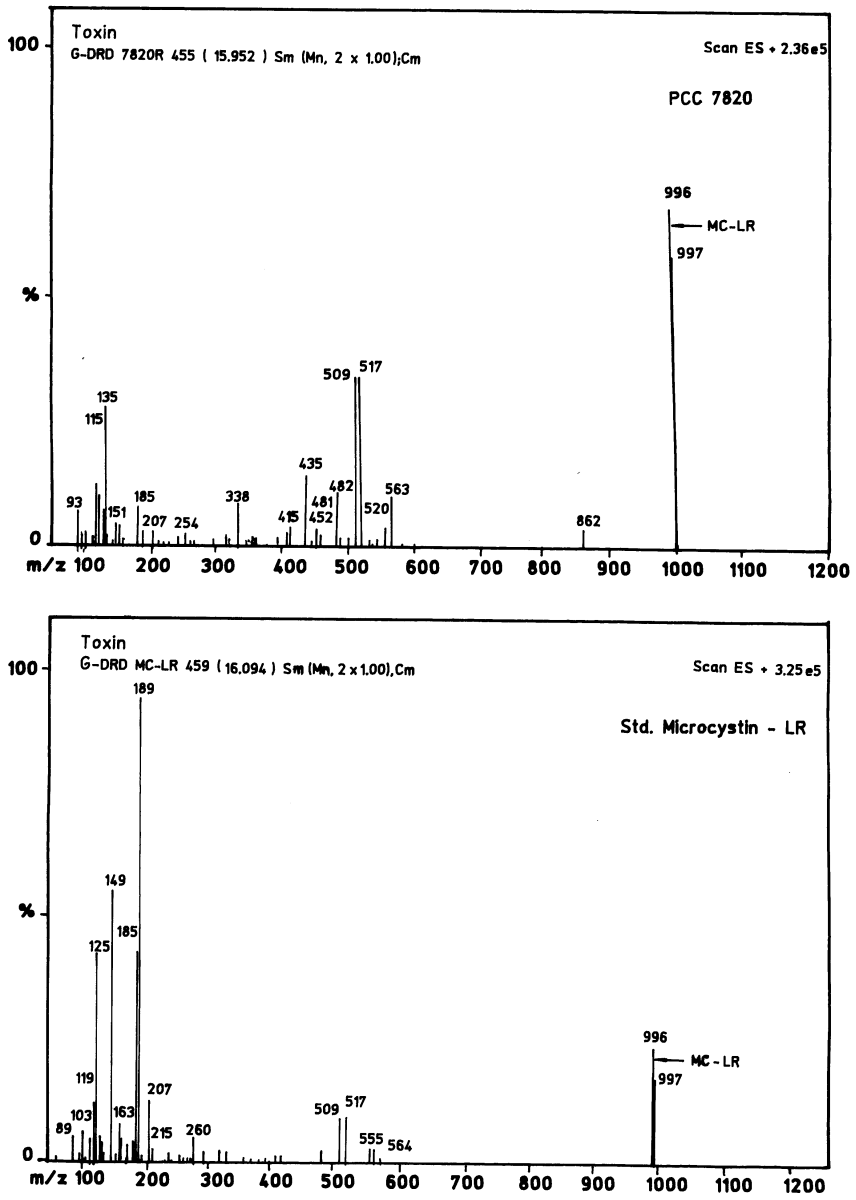

Figure 5. Electrospray mass spectra of Microcystin-LR showing molecular weight, at 996 $(M+H)^+$ in PCC 7820 and in standard Microcystin-LR.

20 : 1. For the LC-ESI-MS system, a split ratio of 11 : 1 was applied and 5 μL of the LC-eluent was directed into the mass spectrometer. The rest of the flow cell was diverted to the U.V detector fitted with a 2.5 μL flow cell. Since the capillary flow cell (90 nl) suitable to monitor a low LC flow rate was not available, the peak on the chromatogram tended to be rather broad. Although the compounds did not have

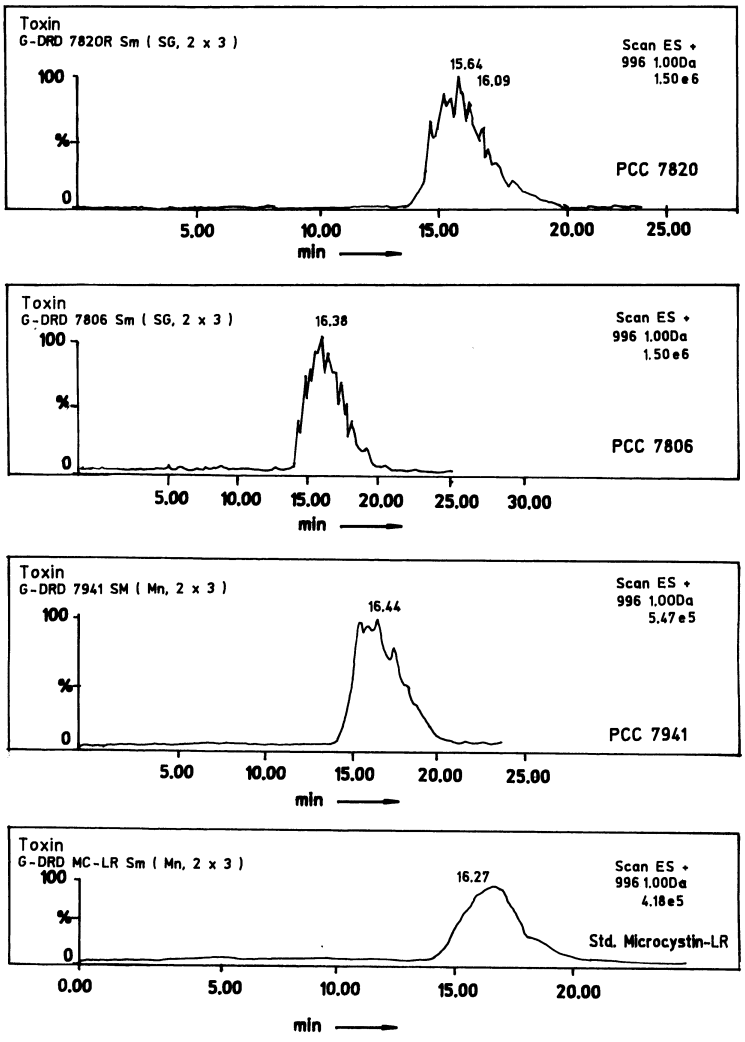

Figure 6. Selected ion chromatograms (m/z 996) of Microcystin-LR in PCC 7820, 7806, 7941, and standard Microcystin-LR.

base line separation on a UV chromatogram, nevertheless, the ion monitoring shows the compounds to be equally resolved.

CONCLUSION

The proposed RP-HPLC method for estimation of toxins in the cultured samples is sensitive, precise, robust, and accurate, and allows the determination of low levels, approximately 1 μg/L of microcystin-LR in the mixture. The method has also demonstrated to be suitable for purification of microcystin-LR from other compounds in algal material and has been applied to the purification and subsequent determination of microcystin-LR in algal material from environmental samples. The method presented in this paper is a combination of LC & LC-ESI-MS for the identification of peptide toxins, which has the separation capacity of liquid chromatography with the molecular weight information provided by the mass spectrometer and offers a much wider scope in the analysis of different toxins.

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REFERENCES

1. Skulberg, O.M.; Codd, G.A.; Charmichael, W.W. Ambio **1984**, *13*, 244–248.
2. Charmichael, W.W. Toxins of Fresh Water Algae. In *Handbook of Natural Toxins*; Tu, A.T., Ed.; Marcel Dekker, Inc.: New York, 1988; 121–147.
3. Gorham, P.R.; Charmichael, W.W. Hazards of Freshwater Blue–Green Algae (Cyanobacteria). In *Algae and Human Affairs*; Cambridge University Press: 1988; 403–431.
4. Billings, W.H.; Water Associated Human Illness in North Pennsylvania and Its Suspected Association with Blue–Green Algae Blooms. In *The Water Environment: Algal Toxins and Health*; Charmichael, W.W., Ed.; Plenum Press: New York, 1981; 243–250.
5. Falconer, I.R. Toxicity Assess **1989**, *4*, 175–184.

6. Watanabe, M.F.; Oishi, S. J. Jpn., J. Limnol. **1980**, *41*, 5–9.
7. Lawton, L.A.; Edwards, C. J. Chromatogr. A **2001**, *912*, 191–209.
8. Sherai, M.; Matsumara, K.; Ohotake, A.; Takamura, Y.; Aida, T.; Nakano, M. Appl. Environ. Microbiol. **1989**, *55*, 2569.
9. Spoof, L.; Karlsson, K.; Meriluoto, J. J. Chromatogr. A **2001**, *909*, 225–236.
10. Poon, G.K.; Griggs, L.J. J. Chromatogr. **1993**, *628*, 215–233.

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