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Reversed-phase liquid chromatographic–mass spectrometric determination of microcystin-LR in cyanobacteria blooms under alkaline conditions

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Abstract

Reversed-phase HPLC coupled to the atmospheric pressure ionization–electrospray ionization (API–ESI) MS was used for microcystin-LR detection and quantitation in samples of dried *Microcystis aeruginosa* cells. An alkaline linear gradient (20 mmol/l ammonium hydroxide–acetonitrile, pH 9.7) was used for elution of the toxic peptides. Limit of detection was 1 μg/ml (20 ng per injection) in the scan mode of MS and 0.1 μg/ml (2 ng per injection) in the case of selective ion monitoring.

Keywords: Cyanobacteria; Microcystin

1. Introduction

Toxic cyanobacteria, or blue-green algae, represent a serious problem in freshwater reservoirs in northern Thailand where all these reservoirs are contaminated with toxic cyanobacteria, *Microcystis aeruginosa* Kutz. [1]. Even when the toxins present (cyclic heptapeptides known as microcystins) do not exceed the WHO water guideline value (1 μg/l) for microcystin-LR [2], they still represent a serious health risk for the residents [3]. Owing to their hepatotoxic and tumor promoting activity [4–6], it was proposed that these compounds are responsible for the increased incidence of hepatocarcinoma in exposed populations, in particular in developing countries, where a high proportion of the population consumes untreated surface water [7,8].

All microcystins possess a common cyclic heptapeptide structure, contain unusual amino acids and their hepatotoxicity is based on their capability to inhibit liver phosphatases [9]. This effect is due to the presence of a hydrophobic amino acid, 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid (ADDA), in their structure, which binds to the same site on the enzymes as okadaic acid and may, therefore, act as a tumor promotor [2]. Over 50 microcystin analogues have been isolated and identified so far [10,11].

A number of analytical techniques are currently available for both the quantification and identification of microcystins in naturally occuring samples: enzyme based assays [12], immunoassays [13,14] (inclusive immunoaffinity purification [15]), thin-layer chromatography [16] and high-performance liquid chromatography techniques [17–20] (also in combination with mass spectrometry [21]) can be traced in the literature. Capillary electrophoresis (occasionally coupled to MS) represents another method of choice [22–24]. In all methods reported increasing of the sensitivity and the possibility to detect (and quantitate) minute amounts of the toxins was emphasized. Of particular interest is the quantitation of microcystin-LR which is the most abundant and most toxic member of the microcystin family of peptides. In the case of analyzing *Microcystis aeruginosa* cells, sensitivity of the method used is not of prime importance as the microcystin content in this material exceeds considerably the concentrations found in polluted water.

As far as the liquid chromatography methods for microcystin analysis is concerned, all of them used an acidic (acetic or formic acid) based mobile phase. The peptide or protein analysis is routinely made in mobile phases with

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trifluoroacetic acid as ion-pairing agent. Using trifluoroacetic acid in the HPLC–MS mode appears not to be the best choice as it acts as the "ion killer" in the case ESI ionization. On the other hand, because acetic and formic acid are both much poorer ion-pairing agents, omitting of trifluoroacetic acid is likely to result in lower separation efficiency in the reversed-phase operational mode. Consequently, it appeared challenging to use another approach for the reversed-phase chromatography separation of these toxic peptides. An interesting alternative appeared to be the separation in alkaline pH on a silica-gel column stable in alkaline mobile phase (Zorbax Extend column) [25]. Using an alkaline mobile phase has also the advantage of good sensitivity and good mass spectrum resolution when compared with the mobile phases using trifluoroacetic acid. This approach was, for example, successfully applied in the peptide mapping of eggshell proteins [26].

The objective of this communication was to develop RP-HPLC–MS separation of microcystin-LR peptides under alkaline conditions.

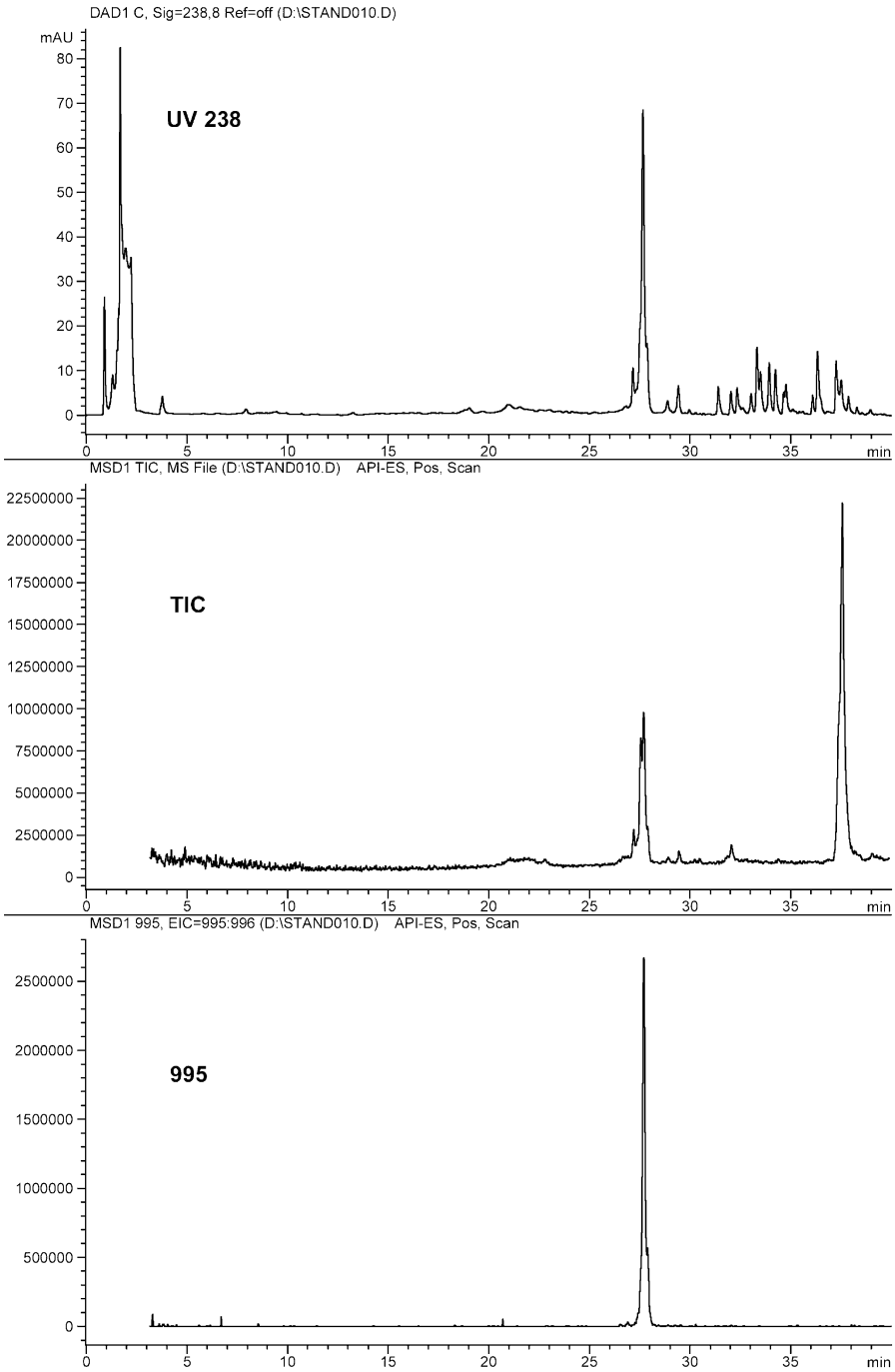

Fig. 1. HPLC profile of the cyanobacterial extract: UV detection at UV (238 nm); total ion current (TIC) 995; extracted ion analysis at *m*/*z* 995–996.

2. Experiment

2.1. Instruments

An HP 1100 LC/MSD system (formerly Hewlett-Packard, now Agilent, Palo Alto, CA, USA) consisting of a degasser, binary pump, an autosampler, a thermostatted column compartment, diode array detector and mass spectrometric detector (MSD) was used throughout this study. The apparatus was controlled, and data collected and managed by the ChemStation A.06.03 computer program.

2.2. Chemicals

Acetonitrile and methanol (HPLC gradient grade) were purchased from Merck (Darmstadt, Germany), ammonium hydroxide was from Lachema (Brno, Czech Republic), p.a. quality. All solutions were prepared in MilliQ water (Millipore, Bedford, MA, USA).

Microcystin standards, MC-YR (100 μg/ml), MC-LR (250 μg/ml), and MC-RR (250 μg/ml) were purchased from the Wako Pure Chemicals (Osaka, Japan). The reported purity of all three standards was 95%.

2.3. Obtaining and identifing cyanobacteria

Cyanobacterial scum at Huay Yuak water reservoir in Chiang Mai, Thailand, was collected using a plankton net mesh sized 10 μm in September, 2001, when blue-green algae bloomed. Microscopic examination and identification revealed that the water blooms were dominated by *M. aeruginosa* (over 95%). The algae sample was lyophilized and kept in a desiccator at 4 °C.

2.4. Extraction of cyanobacterial toxins

Lyophilized algae cells were extracted with methanol and vortex mixed at room temperature for 3 min and

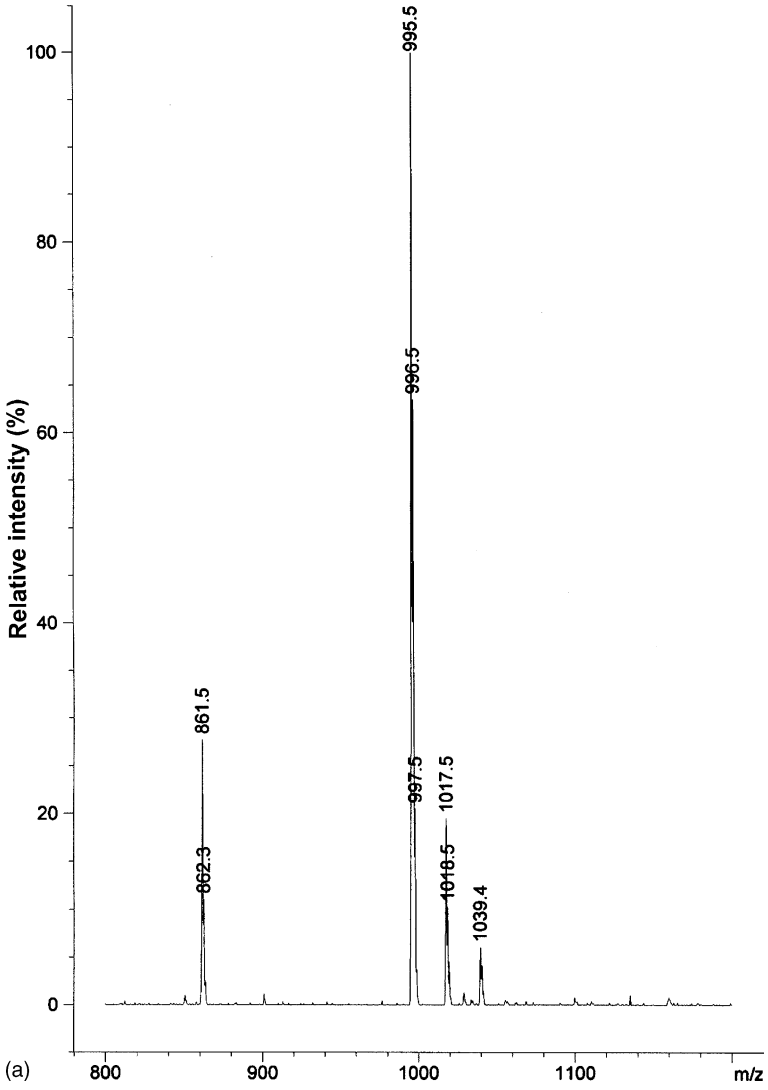

Fig. 2. Mass spectrum of: (a) the microcystin-LR identified in the *Microcystis aeruginosa* cells extract; and (b) the microcystin-LR standard.

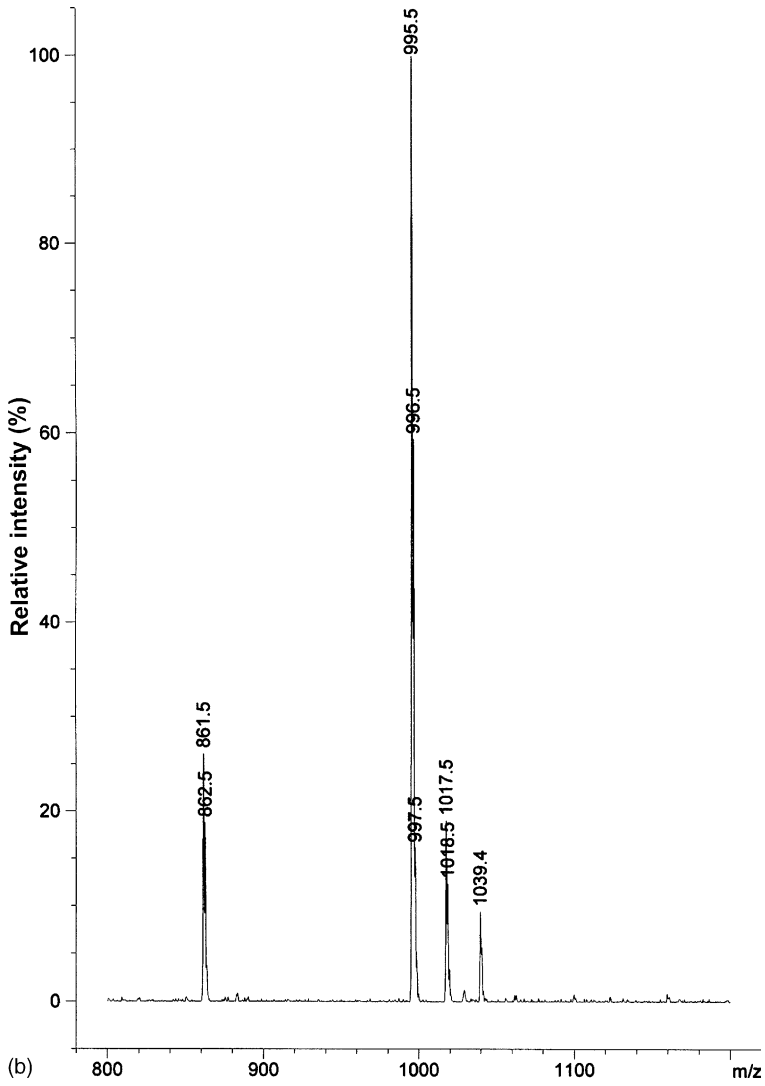

Fig. 2. (*Continued*).

sonicated for another 4 min period. The sample was then centrifuged at 2000 × *g* for 2 min and filtered through a 0.45 μm filter (Millex-HV; Millipore, Bedford, MA, USA). The supernatant was injected into the HPLC–MS system for identification and quantification of the microcystins.

2.5. Conditions for the HPLC–MS

Chromatographic separation was carried out on a Zorbax 300 Extend-C18 column (150 mm × 4.6 mm i.d., 3.5 μm, Rockland Technologies (Hewlett-Packard)). A 20 μl sample was injected. Elution was achieved by a linear gradient (A: water with 20 mmol/l NH_4OH, pH 9.7; and B: acetonitrile). Separation was started by running the system isocratically for 2 min with mobile phase A only, followed by a gradient elution to 35% B over 40 min. Finally, the column was eluted with 100% B for 10 min. Equilibration before the next run was achieved by 10 min washing with buffer A. The flow-rate was 1 ml/min, the column temperature held at 25 °C and the UV absorbance detection was set to 238 nm.

Atmospheric pressure ionization–electrospray ionization (API–ESI) in positive mode and mass spectrometry in full scanning mode were used. Operating conditions were as follows: drying gas (N_2), 13 l/min; drying gas temperature, 350 °C; nebulizer pressure, 3.8×10^5 Pa (55 psi); capillary voltage, 4500 V; in scan mode ions were observed over the mass range of *m*/*z* 800–1200, in SIM (selected ion monitoring) mode three ions were observed: 995, 1038 and 1045; and the fragmentor was set at 80 V.

3. Results and discussion

A typical chromatogram of the microcystins containing cyanobacterial extract is shown in Fig. 1. This chromatogram represents two records: (i) the total ion current; and (ii) the extracted ion at *m*/*z* 995 (characteristic ion for microcystin-LR; retention time, 27.2 min).

The mass spectrum of the microcystin-LR identified in the microcystic cyanobacteria extract revealed the molecular mass of 995 (Fig. 2(a)), which was fully compatible with the standard sample (Fig. 2(b)).

The quantity of microcystin-LR found in the dried cyanobacterial cells was quantitated as 1.86 mg/g of dried cells (S.D. = 0.18, n = 3). The respective validation data in scan mode are: quadratic calibration curve (five points in the range from 2.5 to 20 μg/ml): $y = 28975x^2 + 148767x + 43547$, correlation coefficient 0.99998. Limit of quantitation (LOQ) was 2 μg/ml (40 ng per injection) and limit of detection (LOD) was 1 μg/ml (20 ng per injection). In the case of the use of SIM mode, LOQ was 0.2 μg/ml (4 ng per injection) and LOD was 0.1 μg/ml (2 ng per injection). Other microcystins were studied as well (YR and RR: $[M+H]^+$ ions, 1045 and 1038; and the relevant retention times were 26.6 and 29.2 min, respectively). None of them was detected in the dried samples of *Microcystis aeruginosa* cells.

Reversed-phase chromatography using alkaline mobile phase with MS detection appears a simple and rugged method for microcystin-LR assay. Using this approach it was possible to show that the cyanobacterial cells collected from the Chiang Mai reservoir contained only the most toxic microcystin-LR.

4. Conclusion

Using an alkaline mobile phase in the RP-HPLC–MS system was found a suitable alternative to separating (and quantitating) microcystin-LR in natural samples under acidic elution conditions. This approach is fully compatible with the MS step: it abolishes the negative effect of trifluoroacetic acid and yields good resolution of microcystin-LR present in the naturally occurring samples (particularly from microcystins-YR and -RR). Quantitation of the samples revealed 1.86 mg/g (S.D. ±0.18) of microcystin-LR in dried cells.

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