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Isolation and preparative purification of microcystin variants

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Abstract

Preparative reversed-phase liquid chromatography was successfully used to purify two microcystins (microcystin LR and microcystin LA) from a cyanobacterial process waste. The separation protocol involved extraction of lyophilized cells by methanol, isolation and concentration by solid-phase extraction, and purification by reversed-phase HPLC. Milligram-level loading of microcystins was obtained on a solid-phase extraction cartridge packed with 0.5 g of C_{18} stationary phase. The separations were first carried out on an analytical column and then scaled-up to a preparative column. The microcystins were quantified by HPLC and enzyme-linked immunosorbent assay. A method to remove microcystins rapidly and economically from the cyanobacterial process waste is also described. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Preparative chromatography; Microcystins; Toxins; Peptides

1. Introduction

Microcystis, a cyanobacterial species, is known to produce a class of cyclic heptapeptides called microcystins [1–3]. Microcystins have been shown to inhibit protein phosphatases [4] and promote tumors [5], and have been reported to be the cause of numerous animal fatalities [6–8]. There has been an increasing need for highly pure microcystin standards in recent years [5] to serve the demands of chemists and toxicologists for structural and toxicological studies and enzyme-based assay development. It is therefore of continuing importance to develop preparative separation methods for these compounds.

Microcystins are a family of monocyclic heptapeptides, consisting of D-alanine, β-linked erythro-β-methylaspartic acid, γ-linked glutamic acid, the two unusual amino acids *N*-methyldehydroalanine (Mdha) and 3-amino-9-methoxy-10-phenyl-2,6,7-trimethyldeca-4, 6-dienoic acid (Adda), and a pair of variable L-amino acids. The multitude of variable L-amino acids coupled with slight variations in the side chains of the other amino acids has given rise to about 50 known microcystins so far [2]. Hence, a desired microcystin must be separated not only from other classes of compounds such as nodularins [9] that also have the hydrophobic Adda moiety (and hence may exhibit similar chromatographic properties), but also from closely retaining microcystins.

The focus of many articles on microcystins thus far has been the discovery of a new microcystin and its structural characterization [2,10]. There have been fewer reports on efficient methods for the preparative purification of microcystins [11,12]. In this paper, we report a method to isolate both microcystin LR

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(MC-LR) and microcystin (MC-LA) from a cyanobacterial process waste stream. The isolated microcystins were identified by electrospray mass spectrometry, and quantified by analytical HPLC and enzyme-linked immunosorbent assay (ELISA). Finally a simple method for the economical removal of microcystins is described.

2. Experimental

2.1. Materials

HPLC-grade acetonitrile (ACN) was obtained from EM Science (Gibson, NJ, USA) and sequanal-grade trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA). Deionized-distilled water was obtained using Milli-Q ultra-pure water system (Millipore, Bedford, MA, USA). Sep-Pak C_{18} (0.5 g) cartridges were obtained from Waters (Milford, MA, USA). Activated carbon was purchased from Westvaco (Covington, VA, USA). Microcystin standards were purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). The enzyme immunoassay kit for microcystin-LR was purchased from Strategic Diagnostics (Newark, DE, USA).

2.2. Apparatus

The HPLC system consisting of a quaternary pump (Model 600), UV detector (Model 486), and an autosampler (Model 717 plus), was controlled by a DEC (Nashua, NH, USA) personal computer using Waters Millennium software. A Rheodyne Model 7125i injector (Cotati, CA, USA) was used to inject preparative samples. Preparative column (25×2 cm I.D.) and a guard column (5×2 cm I.D.) was from Kromasil (Bohus, Sweden). Analyses were made using both Kromasil and Alltech (Deerfield, IL, USA) Alltima C_{18} column (25×0.46 cm I.D.). A Buchi RE 111 rotary evaporator (Flawil, Switzerland) was used to concentrate the liquid samples. The molecular mass of the samples was determined using a Perkin-Elmer Sciex API III mass spectrophotometer (Norwalk, CT, USA).

2.3. Procedures

2.3.1. Extraction

A.F.A. (Klamath Falls, OR, USA) supplied us with various batches of wet (slurry) and dry (lyophilized) process waste streams containing microcystins. The dry cells (20 g) were extracted with 600 ml of MeOH–water (75:25) by continuous stirring using an orbital shaker set at 400 rpm for 1 h at room temperature. After 1 h, the sample was centrifuged at 9650 *g* for 15 min. The extraction procedure was repeated once with 400 ml of the same solvent. The supernatants resulting from both the steps were pooled and reduced to approximately one-tenth of its initial volume by rotary evaporation at 35°C. The liquid concentrate was stored at 4°C. The effect of sonication on microcystin extraction was studied on slurry samples using a Branson sonifier model 250 set at 50% cycle time and an intensity of 5, for 10 min.

2.3.2. Isolation

The concentrate from the extraction step was brought to room temperature and centrifuged at 11 300 *g* for 10 min. A 25-ml aliquot of the microcystin-rich concentrate was applied to a preconditioned Sep-Pak C_{18} cartridge (0.5 g) at 1 ml/min. The preconditioning step included washing with 20 ml each of neat methanol and by 20% aqueous methanol. The loaded cartridge was washed with 20% aqueous methanol, after which the microcystins were eluted using 5 ml of 80% methanol at 1 ml/min. The cartridges were regenerated by washing with 20 ml each of methanol, chloroform and hexane. Liquid–liquid extraction (LLE) was also studied as an isolation step. The microcystin extract was contacted with an organic solvent [hexane–(*n*-butanol or methyl–ethyl ketone), 1:1], and shaken vigorously for 2 min using a vortexer. Centrifugation of the liquid mixture at 805 *g* for 15 min separated the organic layer from the aqueous layer. Samples from both layers were analyzed for microcystin content by HPLC.

2.3.3. Chromatography

The concentrated microcystin extract from the solid-phase extraction (SPE) was injected into either the Alltech or the Kromasil column. The chromato-

graphic profile and the retention times for the microcystins and their impurities were very similar on these two analytical columns, and hence both were used interchangeably for microcystin analysis. The methods developed on the analytical column were scaled-up to a preparative column (Kromasil, 25×2 cm I.D.). The MC-LR and MC-LA fractions were collected and analyzed as described below.

2.3.4. Analysis

The microcystins MC-LR and MC-LA were both analyzed quantitatively by HPLC based on a calibration curve constructed by analyzing commercially available MC-LR standards. The purity of the MC-LR fractions was assessed by quantifying the closely retaining impurities by fitting them to the MC-LR calibration curve. A similar procedure was adopted for assessing MC-LA's purity. In addition to HPLC, the samples were analyzed by an enzyme-based assay kit from Strategic Diagnostics. Typically, a dilution factor of 200 000:1 was essential to bring the sample concentration within the calibration curve. Further, the purified microcystins were identified by a Sciex API III+ triple quadrupole ionspray mass spectrometer.

2.3.5. Removal of microcystins

The process waste (1000 ml) was brought to room temperature and contacted with 20 g of activated charcoal. The resulting mixture was stirred, and samples were collected at 30-min intervals for residual microcystin analysis by HPLC.

3. Results and discussion

3.1. Extraction

Refrigerated slurry samples (non-lyophilized) was used to study the effect of sonication on cell rupture, and hence microcystin extraction. No significant improvement in microcystin content of the sonicated sample was found. Further, independent experiments on the sonication of commercial MC-LR standard solutions showed degradation. Hence, its possible that sonication yielded higher amounts of MC-LR through cell disruption but also degraded some of the MC-LR. These effects could offset each other, resulting in comparable amounts in both sonicated and un-sonicated samples. Consequently, cell disruption technique was not incorporated in the extraction protocol.

The extraction efficacy plot (Fig. 1) shows comparable extraction of MC-LR by most solvents, while 75% methanol in water was somewhat more efficient than the others. MC-LA, however, was less efficiently extracted by solvents containing acetic acid. We also found that no one solvent was superior in reducing the co-extraction of impurities. While Harada et al. [13] reported near-complete extraction of MC-LR using 5% acetic acid, more hydrophobic microcystins could not be extracted using this method. A more non-polar solvent mixture (butanol–methanol–water, 5:20:75) used by Krishnamurthy et al. [14] and Brooks and Codd [15] resulted in a better extraction of several microcystins. However, Lawton et al. [16] found methanol to be superior to both acetic acid and butanol-containing solvent mixtures for extracting most microcystin variants. We found 75% methanol to be modestly superior to both acetic acid and butanol–methanol–water (5:20:75) for MC-LR and MC-LA extraction, which

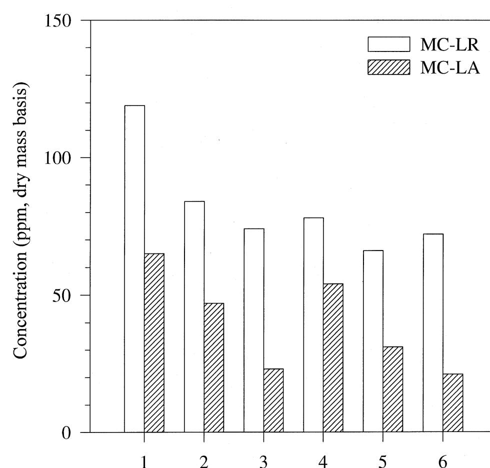

Fig. 1. Efficacy of extraction of microcystins (MC-LR, lines; MC-LA, no lines) by various solvent systems. The *x*-axis corresponds to the solvent compositions used in the extraction, and are: (1) MeOH–water (75:25); (2) butanol–MeOH–water (5:20:75); (3) butanol–MeOH–acetic acid–water (5:20:1:74); (4) MeOH–water (25:75); (5) MeOH–acetic acid–water (25:1:74); (6) acetic acid–water (5:95). The samples were quantified by HPLC as per conditions given in Fig. 2.

was in agreement with previous reports. This choice (75% methanol) had the added advantage of lower time consumption during the rotary evaporation step, because of higher organic content.

3.2. Isolation

SPE cartridge capacity was estimated by increasing feed loading, as shown in Table 1. The analysis of eluent from the various steps, for increasing loading, showed the presence of microcystins (both MC-LR and MC-LA) primarily (>98%) in the MeOH–water (80:20) step. The 85% methanol step contained insignificant amounts of MC-LR and MC-LA (<2% of the total amount extracted). The purity of the microcystins in the SPE effluent was found to decrease slightly (from 85 to 80%) when the sample was enriched from 2 to 5 times. However, we were able to load up to ca. 1 mg each of MC-LR and MC-LA on this cartridge (Sep-Pak C_{18}, 0.5 g). Our loading capacity for MC-LR (2 mg/ml, empty cartridge volume) was comparable to our estimate for Edwards et al. [11] (1.7 mg/ml, empty column volume), where they used a flash column.

Since our objective in preparative chromatography was to maximize the combined productivity, obtaining MC-LR and MC-LA in a single SPE fraction proved to be of significant advantage. While many reports [17–19] suggest 100% methanol for microcystins elution, we found that 80% methanol to be sufficient for eluting both MC-LR and MC-LA (Table 1) in a single step. Other stepwise elution sequences (e.g., loading step at 20% methanol, followed by elution using 35, 45 and 55% methanol, respectively) were tried, which not only had the disadvantage of not recovering all the MC-LR and MC-LA in a single step, but also did not remove additional impurities.

LLE experiments with solvents that are partially miscible with water (*n*-butanol and methyl-ethyl ketone) resulted in the formation of an intermediate layer. The microcystins were often present in more than one phase, thereby decreasing the efficiency of extraction. For example, in the butanol–water experiment, the MC-LR distributed as follows: 7% in the butanol phase, 43% in the intermediate layer, and 50% in the water phase. Similar intermediate layer formation was reported by Birk et al. [20] when they used diethyl ether as the extraction solvent. We found hexane to be the most efficient in terms of easy phase separation and removal of highly non-polar impurities. No intermediate layer was formed in this case. However, the microcystins were not concentrated (unlike SPE) because they remained in the aqueous phase, whose volume does not change appreciably in LLE.

The efficacy of SPE and LLE was compared by quantifying the neighboring impurities (peaks between 7 and 13 min for MC-LR, and between 23 and 30 min for MC-LA). A 5-ml feed volume was used in both SPE and LLE runs. Both methods resulted in comparable purity levels for MC-LR (77±1% by SPE and 71±0.2% by LLE) and MC-LA (83±2% by SPE and 82±4% by LLE). Albeit the processing times for both SPE and LLE were comparable, SPE was preferred as the isolation step since it not only resulted in the removal of strongly hydrophobic impurities (achieved by LLE), but was also able to concentrate the microcystins.

Table 1
The loading capacity for microcystins on the SPE cartridge[a]

Sample	Loading volume (ml)	Elution volume (ml)	MC-LR (μg)	MC-LA (μg)
Feed	10	n/a	535 (±22)	415 (±15)
Loading step	n/a	10	0	0
20% MeOH	n/a	5	0	0
80% MeOH	n/a	5	558 (±25)	389 (±19)
85% MeOH	n/a	5	0	0
Feed	25	n/a	1338 (±54)	1038 (±38)
Loading step	n/a	25	0	0
20% MeOH	n/a	5	0	0
80% MeOH	n/a	5	1448 (±51)	1157 (±53)
85% MeOH	n/a	5	5.0 (±0.2)	3.0 (±0.5)

[a] The feed represented the aqueous methanol extract of the cyanobacterial sample concentrated by ca. 10 times (full details on the feed and the extraction in the text). The effluent from the SPE cartridge was sampled during the feed loading step as well as the subsequent steps of 25, 80 and 85% MeOH. The microcystin concentration in each step was obtained from HPLC, using the conditions given in Fig. 2.

3.3. Purification

Fig. 2 shows the separation of MC-LR and MC-

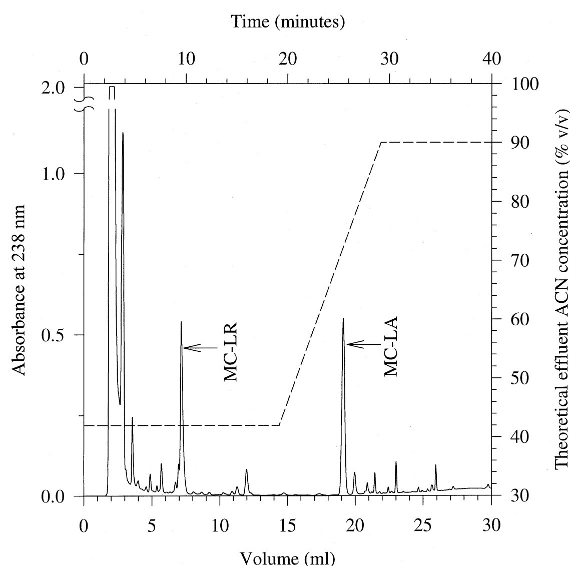

Fig. 2. Separation of MC-LR and MC-LA on an analytical column (25 cm×0.46 cm I.D.). The feed volume was 50 μl. The chromatographic conditions are as follows: isocratic ACN–water–TFA (42:58:0.05) for 15 min followed by a gradient of ACN–water–TFA (42:58:0.05) to ACN–water–TFA (95:5:0.05) in 10 min. The flow-rate was 0.75 ml/min. The gradient delay in our system was 5.6 ml. The theoretical effluent ACN accounts for system delay but does not account for ACN retention on the sorbent.

LA from their respective impurities on an analytical column. For preparative work (Fig. 3a), this modulator schedule was slightly altered: the gradient was made less steep in order to prevent the potential contamination of MC-LA. This method resulted in milligram quantities of purified MC-LR and MC-LA. Microcystin fractions collected from the preparative run were quantified by analytical HPLC, and the reconstructed chromatograms are shown in Fig. 3b,c. Each microcystin had several closely retaining impurities. The chromatographic profile for MC-LR (Fig. 3b) shows a non-Gaussian peak with a dip in the middle. The tailing effect is attributed to non-linear adsorption, while the increase in concentration after the dip is due to its desorption induced by the adjacent higher-retaining impurity. Fig. 3c shows enrichment of MC-LA, which is attributed to the focussing effect of the gradient [21,22].

A convenient measure of the efficacy of a preparative run is productivity, which is defined as

$$\text{Productivity} = \frac{\text{Product at specified purity}}{\text{Overall run time} \times \text{Empty column volume}}$$

where the overall run time is the sum of the time required for the elution of desired peak and the time required to regenerate the column.

Since MC-LR and MC-LA have comparable toxicity and commercial value, our objective was to optimize the conditions that resulted in the maximum combined productivity. We recovered 3 mg of MC-LR at 90% purity and 4 mg of MC-LA at greater than 98% purity in a single run, which resulted in a combined productivity of 0.09 mg/ml h. If the purity requirement for MC-LR was 95%, the combined productivity dropped to 0.06 mg/ml h (0.9 mg of MC-LR and 3.9 mg of MC-LA was recovered). These values were comparable to many found in the literature (see Table 2 for comparison). Experiments carried out with a lower loading (1 mg each of MC-LR and MC-LA) run under the same conditions resulted in lower productivity (0.03 mg/ml h). Other gradient schedule at a loading of 2 mg each of MC-LR and MC-LA also resulted in a lower productivity (0.04 mg/ml h). Although the runs with lower loading resulted in higher yields, their productivity values were lower, while significantly overloaded run gave higher productivity.

The comparison of our post-SPE (80% methanol fraction) chromatogram with the analysis of the post-flash MC-LR-rich fraction of Edwards et al. [11] shows fewer peaks for the latter. Since methanol was used as the extraction solvent in both cases it appears that our starting sample had significantly more components than that of Edwards et al. However, the microcystin content in our dry samples was estimated to be lower by a factor of 10 than many reported values (see Table 2). Hence a more equitable comparison of the effectiveness of microcystin purification method is obtained by taking the ratio of obtained productivity (P) to the initial level (I.L., concentration on dry mass basis). Despite the lower microcystin levels in the starting material and a complex feed, we achieved P/I.L. values better than or comparable to many results, as seen in Table 2. Hence, it is quite possible that our method could potentially yield much higher productivities when used on samples with higher microcystin content.

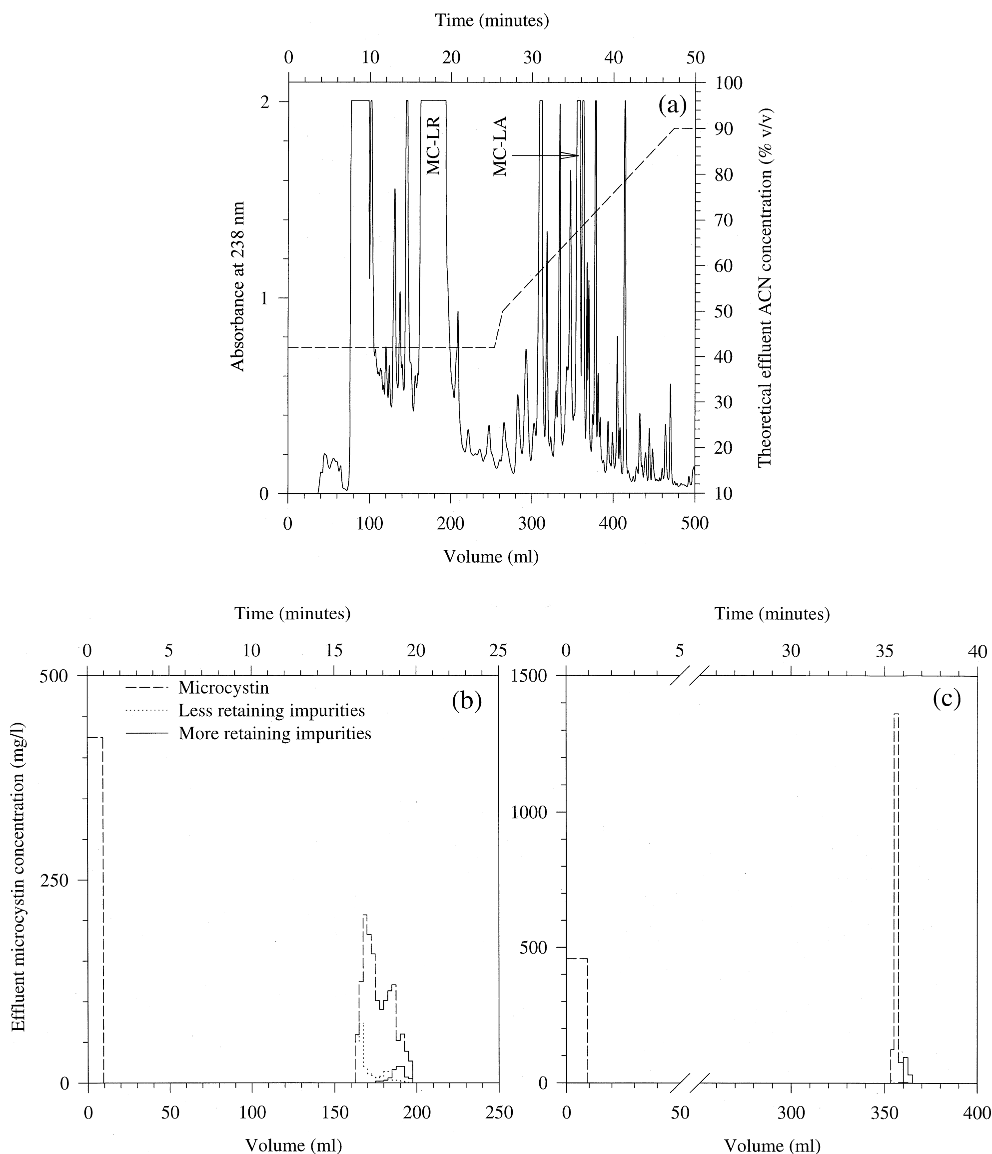

Fig. 3. Separation of MC-LR and MC-LA on a preparative column (25 cm×2 cm I.D.). The trace from the preparative run is given in (a). The column conditions immediately after sample injection was: isocratic ACN–water–TFA (42:58:0.05) for 25 min followed by a step gradient of ACN–water–TFA (50:50:0.05) in 1.0 min and then to ACN–water–TFA (90:10:0.05) in 21 min. The flow-rate was 10 ml/min. The loading was 4.0 mg of MC-LR and 4.3 MC-LA. Panels (b) and (c) are the reconstructed chromatograms of MC-LR and MC-LA, respectively in the preparative run, analyzed as per conditions given in Fig. 2.

3.4. Identification and analysis of microcystins

Fig. 4a,b shows the mass spectra of purified MC-LR and MC-LA fractions. The published m/z values for MC-LR and MC-LA are 995 and 910, respectively [2]. In Fig. 4a, besides the m/z peak at 995 we observed an additional m/z peak of 498.4. This is due to the association of two protons with MC-LR.

Table 2
Productivity of microcystins

Component	I.L.[a] (ppm)	Column L (cm)×I.D. (cm)	Recovery (mg)	*P* (mg/ml h)	*P*/I.L.[b] $\times 10^{-4}$	Refs.
MC-LR	2147	Shandon HS BDS C_{18} 15×7.5	260	0.67	3.12	[11]
MC-LR	4841	Hyperprep HS C_{18} 15×7.5	416	1.08	2.20	[12]
MC-LR	7030	Novapak C_{18} 10×2.5	70.3	0.38	0.54	[16]
5 Methyl ester derivatives of MC-LR	700	µ-Bondpak 15×1.9	0.35	0.01	0.20	[27]
MC-LR	240	µ-Bondapak 15×1.9, 30×1.9 Alltech ODS (25×0.46)×2	1.20	0.004	0.18	[26]
Mixture of 7 MC-LRs	3054	µ-Bondapak 15×1.9	15.3	0.06	0.18	[29]
3-Demethyl MC-LR	230	Chromatex ODS 94×1.1	2.80	0.05	2.34	[18]
7-Demethyl MC-LR	3333	Chromatex ODS 91×1.1 Toyopearl HW-40 91×1.2	14.3	0.09	0.27	[28]
3,7-Didesmethyl MC-LR	350	Cosmosil 5C_{18}-P 25×1	1.50	0.02	0.49	[28]
MC-LR	1000–4000	Altex C_{18} 25×0.94	0.75–3.00	0.07–0.30	0.70–0.75	[14]
MC-LR and MC-LA	210	Kromasil C_{18} 25×2	4.80	0.06	1.60	Present work

[a] µg/g of dry biomass.
[b] g of dry biomass/ml h. I.L., initial level; *P*, productivity.

For MC-LA we noticed two peaks, one at 910 and the other at 932. While the former is the signature of MC-LA, the latter could be due to the presence of trace amounts of sodium salt of MC-LA, possibly formed from the sodium hydroxide added to adjust the pH of the HPLC mobile phase. The association of alkali metals with microcystins has been previously reported by Dale et al. [23] and by Yuan et al. [24]. The chromatograms of purified MC-LR and MC-LA fractions are given in Fig. 5a,b. The increase in baseline after MC-LA elution is due to the UV absorption of ACN.

ELISA analysis of purified MC-LR fractions resulted in a good correlation with HPLC analysis only when the samples were free of HPLC solvents (ACN and TFA) and limited dilution. Significant gain in correlation was achieved only when the required dilutions were lower.

3.5. Removal of microcystins

The use of activated carbon to remove microcystins was studied. The liquid filter-waste sample with microcystin concentration of 18 (±3) ppm (MC-LR and MC-LA combined) was reduced to undetectable levels within an hour. Rate experiments reported by Donati et al. [25] with starting MC-LR concentrations of 2.2 ppm showed presence of MC-LR even after 72 h. This was due to lower amounts of activated carbon used in comparison to that of ours [10–30 mg/l (water) vs. 20 g/l (slurry process stream)]. We used large amounts of activated carbon

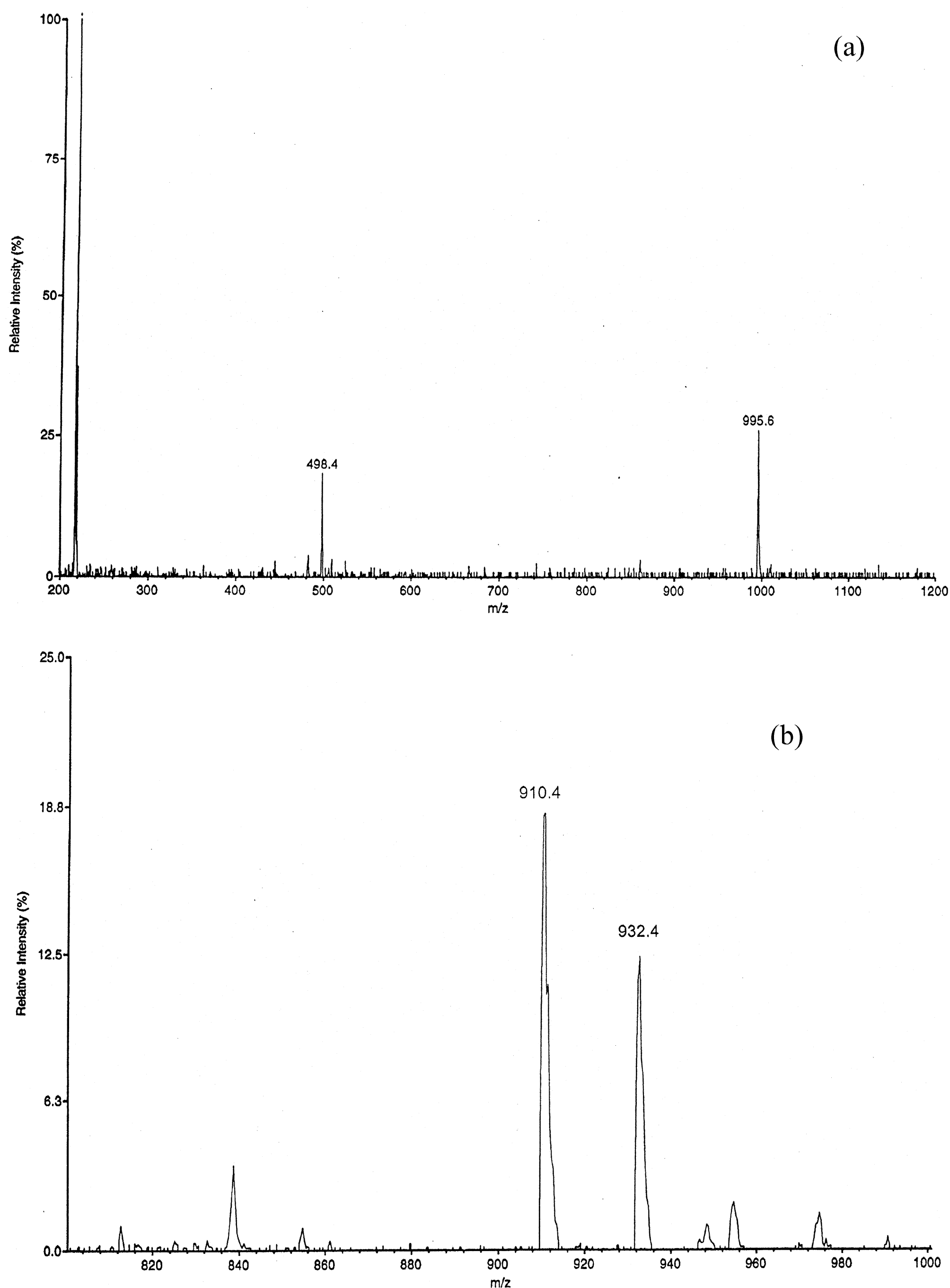

Fig. 4. Electrospray mass spectrometry analysis of microcystin fractions from chromatography, (a) MC-LR (D-Ala–L-Leu–erythro-β-Me-D-isoAsp–L-Arg–Adda–D-isoGlu–*N*-Me-dehydroAla) and (b) MC-LA (D-Ala–L-Leu–erythro-β-Me-D-isoAsp–L-Ala–Adda–D-isoGlu–*N*-Me-dehydroAla). The concentration of samples was ca. 25 μg/ml. The samples were suspended in ACN–water–TFA. The potential of the ionspray needle was placed at +4700 V to produce positive ions and −4500 V for negative ions, the potential of the orifice leading into the mass analyzer was set at 80 V. The nebulizer gas (air) was set to 42 p.s.i. and the curtain gas (nitrogen) was set to 0.6 l/min (1 p.s.i. = 6894.76 Pa).

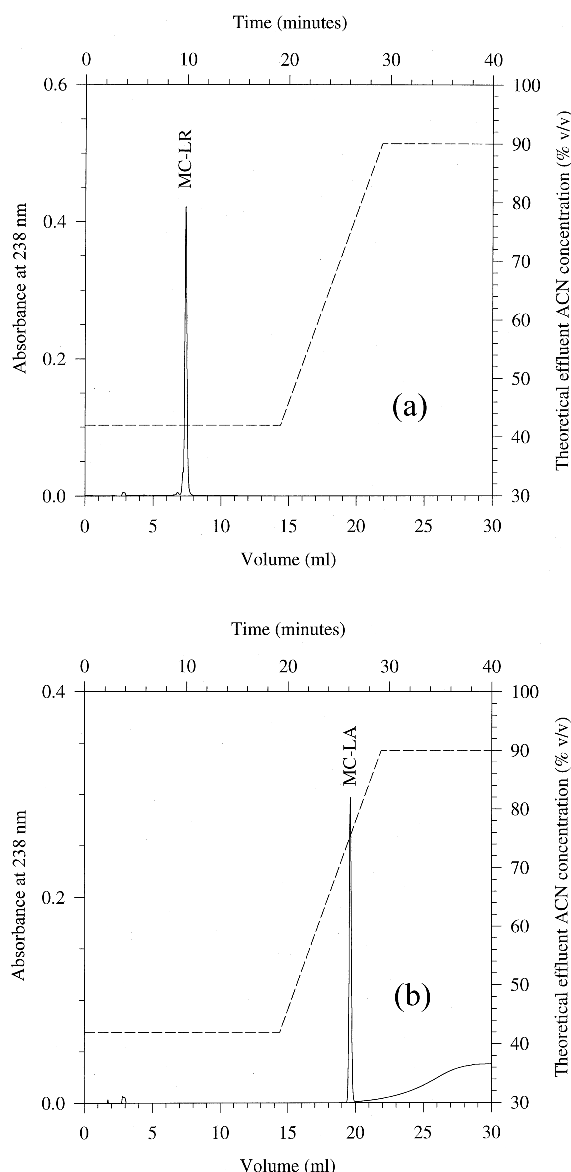

Fig. 5. HPLC analysis of the purified sample. (a) MC-LR, (b) MC-LA. All experimental conditions as in Fig. 2.

in order to reduce the competition between microcystins and other biomolecules for the binding sites. Since activated carbon is inexpensive, large quantities of liquid sample can be stripped of microcystins economically. The agreement of our results with those in the literature for different feed stocks indicates that this is likely to be an economical large-scale method for the removal of microcystins.

4. Conclusions

Microcystins LR and LA were purified from processed natural samples by preparative reversed-phase chromatography. Amongst the many organic solvents tried, methanol was found to be the best extracting agent, which is in agreement with several previous reports. Very high loading of microcystins on the solid-phase extraction cartridge was achieved. The analytical HPLC method was scaled-up to a preparative column, with substantial overloading, where milligram quantities of MC-LR and MC-LA at higher than 95% purity was obtained in a single step.

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