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Laboratory-scale purification of microcystins using flash chromatography and reversed-phase high-performance liquid chromatography

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Abstract

Microcystins were extracted from 7 l (equivalent to 313 g dry weight) of cyanobacterial scum collected from Rutland Water in Leicestershire, UK in 1989. The resulting aqueous extract was rapidly concentrated on a C_{18} flash chromatography cartridge and microcystins were eluted using a step gradient. Fractions were collected manually and monitored by UV spectrophotometer and analytical HPLC. Fractions containing microcystins of similar polarity were pooled to give three fractions. Simple isocratic methods for separating each fraction were developed on an analytical column and scaled up to a 15×7.5 cm I.D. column. Closed-loop recycling was used to maximise yield and purity of two hydrophobic microcystins.

Keywords: Flash chromatography; Preparative chromatography; Peptides; Microcystins

1. Introduction

Microcystins are a group of hepatotoxic, cyclic heptapeptides which are produced by bloom-forming cyanobacteria (blue-green algae) in both freshwater and marine environments. These compounds have been responsible for numerous animal fatalities and several cases of human illness [1–3]. Microcystins act by inhibiting several eukaryotic protein phosphatases that are essential for many cell regulatory processes such as growth, protein synthesis, glycogen metabolism and muscle contraction [4,5]. This biological activity has implicated them as tumour promoters, which has now been substantiated by several researchers [6].

Microcystins possess the general structure cyclo-(D-Ala-L-*X*-erythro-β-D-methyl aspartic acid-L-*Y*-Adda-D-isoglutamic acid-*N*-methyldehyroalanine), where *X* and *Y* represent variable amino acids (Fig. 1). Adda is the abbreviation for 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-dienoic acid, which is unique to microcystins and related pentapeptides. Microcystin nomenclature is based on the variable amino acids, e.g. microcystin-LR (MC-LR) contains leucine and arginine [7]. As well as variation in amino acids there are a number of microcystins with minor chemical modifications, giving a current total in excess of fifty variants.

Increasing interest in these compounds (such as investigations into their toxicology, provision of analytical standards for environmental analysis and their use as biochemical tools) has greatly increased

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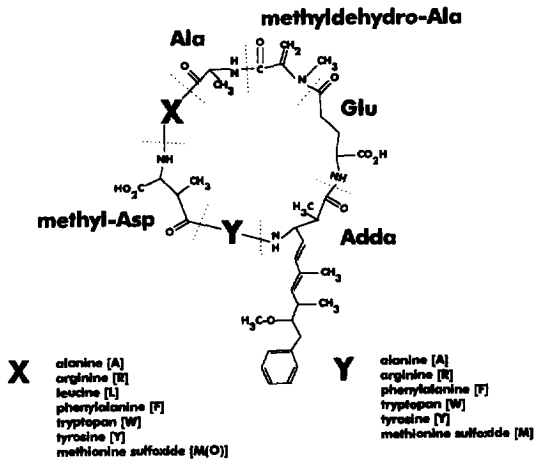

Fig. 1. General structure of microcystins where *X* and *Y* represent variable amino acids.

the demand for pure microcystins. Investigations into the chemical synthesis of these compounds is currently in progress; however, this has yet to be achieved. It is therefore important to develop methodology which can accommodate the purification of milligram to gram quantities of microcystins from natural bloom and/or cultured cells.

Early methods of microcystin purification involved the use of DEAE cellulose and Sephadex G50 with final purification using high-voltage paper electrophoresis [8]. HPTLC has also been used as a final purification after concentration and clean-up using C_{18} Sep-Pak cartridges; however, this methodology is only suitable for microgram amounts of material [9]. Most of the recent methods reported in the literature describe initial concentration of an aqueous extract on C_{18}. This may be either using commercially available solid-phase extraction cartridges or bulk C_{18} packed in a glass column. This is followed by two or more semi-preparative or preparative HPLC steps, often with final purification by analytical HPLC [10,11]. However, these methods are time-consuming, often resulting in poor yield due to the number of steps involved in the purification.

Recently we described simplified methodology for the purification of microcystins from laboratory cultures and bloom samples [12]. This involved a methanolic extraction, followed by concentration of diluted supernatant onto a C_{18} Sep-Pak cartridge (1 g). The cartridge was eluted with a stepwise gradient of methanol in water starting with 10% (v/v) methanol and increasing in 10% increments. Microcystin-containing fractions were pooled and separated by semi-preparative HPLC to facilitate purification of microcystins. Although this method was much simpler than those previously described, it was only suitable for microgram to milligram quantities of material.

This paper describes how the method outlined above was scaled up to enable purification of gram quantities of microcystins using reversed-phase flash chromatography and laboratory-scale preparative HPLC. The source material was scum collected from Rutland Water in 1989 which had previously been shown to contain several microcystins [12]. The sample represented a challenge since the total concentration of target microcystins represented approximately 0.7% of the dry weight of the algal cells and the microcystins were of a broad ranging polarity. The methods presented could be readily adapted to isolate microcystins from other cyanobacterial material.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and obtained from Fisons (Loughborough, UK) unless stated otherwise. HPLC-grade methanol and acetonitrile were also obtained from Fisons except where otherwise stated.

Microcystin standards were purified from cultured cells and bloom material as previously described [12].

2.2. Cyanobacterial material

Cells of *Microcystis aeroginosa* from a bloom at Rutland Water (Leicestershire, UK) in September 1989 were kindly provided by Anglian Water, Cambridge, UK. Cells were maintained at −20°C until thawed for extraction.

2.3. Extraction of microcystins

Approximately 7 l of concentrated scum equivalent to 313 g dry weight of cells was extracted in one volume of methanol (plus ammonium acetate [0.5%] to enhance pellet formation) for 30 min with regular agitation. Methanol was used for extraction since this had previously been shown to be the most practical solvent for extracting all of the microcystin variants [12]. The extract was centrifuged at 1500 *g* for 30 min, the supernatants were decanted and the pellets were re-extracted a further two times. The pooled supernatants were rotary-evaporated at 40°C to remove the methanol and the resulting oily residue was stored at −20°C until required for further processing.

The extract was prepared for flash chromatography by combining the concentrate with methanol and water at a ratio of 1:1:8 (extract–methanol–water). The aqueous extract (15.2 l) was then filtered through GF/C disks (Whatman, Maidstone, Kent, UK) prior to loading onto the flash cartridge.

2.4. Concentration and clean-up of microcystins: flash chromatography

Concentration and clean-up was achieved using a Biotage Flash 75 S system (Biotage, Charlottesville, PA, USA) which relies on the application of approx. $137.8 \cdot 10^7$ Pa (20 psi) pressure to a stainless steel solvent reservoir to drive sample/solvent through a cartridge housed in a radial compression module [13]. Two stationary phases, spherical Hyperprep C_{18} (30 μm particle size, 120 Å; Shandon, Runcorn, UK) and irregular Bondapak C_{18} (37–55 μm particle size, 125 Å; Waters, Watford UK) were packed in 9×7.5 cm I.D. cartridges (Biotage). The performance of each stationary phase was examined to determine if there was any advantage to using the smaller spherical Hyperprep for this type of application.

A volume of 8 l of the aqueous extract containing the microcystins was applied to a preconditioned cartridge packed with Shandon Hyperprep C_{18}. Radial compression was provided by nitrogen at $5.51 \cdot 10^{10}$ Pa (80 psi). The extract was applied at a flow-rate of 100 ml/min. The microcystins were eluted using a step gradient from 0 to 100% methanol in 10% increments (2 l solvent per step) and fractions (200 ml) were collected manually. The presence of microcystins was determined by monitoring fractions using a spectrophotometer (Phillips, York, UK) to determine absorbance at 238 nm (absorbance maxima for microcystins). Fractions with high absorbance at 238 nm were analysed by HPLC to identify and quantify microcystins.

This was repeated, loading the remaining 7.2 l of extract onto the cartridge packed with Bondapak C_{18}.

2.5. Analytical HPLC

Fractions were routinely monitored as described previously [14]. Purity of compounds was determined by HPLC with high resolution diode array detection using a Waters 996 detector. Samples were separated on a Symmetry C_{18} column (15×0.46 cm I.D.; 5 μm particle size; Waters). Eluents were Milli-Q water (Millipore, Watford, UK) and acetonitrile (Rathburn, Walkerburn, UK), both containing 0.1% trifluoroacetic acid (TFA). Separation of microcystins was achieved using a linear gradient starting at 30% (v/v) aqueous acetonitrile increasing to 35% over 5 min followed by an increase to 60% over the next 25 min. Detector resolution was set at 1.2 nm and data acquired from 200 to 350 nm.

2.6. Method development and load optimization

Flash chromatography fractions containing microcystins of similar polarity were pooled resulting in three major fractions. Separations of microcystins (different conditions depending on microcystins in the fraction, e.g. methods 1–3 below) were optimized at analytical scale on Shandon Hyperprep C_{18} column (15×0.46 cm I.D.; 12 μm particle size; 100 Å pore size) using a mobile phase of ammonium acetate (0.1%, w/v) (A) and acetonitrile (B) at a flow-rate of 1.5 ml/min. Eluent was monitored at 238, 214 and 254 nm using a Waters 490 detector.

Method 1: fractions eluted from the flash column which were found to contain more polar microcystins were pooled to give fraction 1. The components were separated with a mobile phase A–B (80:20).

Method 2: flash fractions containing mainly MC-LR (**5**) were pooled to give fraction 2. This was separated using a mobile phase A–B (78:22) with a

step increase to 25% B after 12 min to ensure elution of MC-LY (**8**).

Method 3: fractions containing the hydrophobic microcystins were pooled and separated using a mobile phase A–B (76:24) with a step increase to 30% B after 8 min.

Once these methods had been developed, increasing loads were injected onto the analytical column until resolution became unacceptable, thereby providing an indication of maximum sample load, i.e. amount of sample (mg) per gram of packing material which can be loaded without significant loss of resolution.

2.7. Preparative HPLC

Equipment included a Kiloprep 100 laboratory-scale HPLC with a KPCM 100 compression module (Biotage), a UV detector (Linear 205) and a Linear 1201 chart recorder (Thermoseparations, Stone, UK). Samples were injected via a loop (35 ml).

Samples were separated on a 15×7.5 cm I.D. cartridge packed with Shandon HS BDS C_{18} (12 μm particle size) with a mobile phase of ammonium acetate (0.1%, w/v) and acetonitrile as described in methods 1–3 at a flow-rate of 400 ml/min. Eluent was monitored at 214 and 238 nm and fractions were collected manually.

Fractions were analysed quantitatively and qualitatively by analytical HPLC as described.

2.8. Desalting and concentration

Fractions containing compounds of acceptable purity were pooled and diluted with 1 volume of

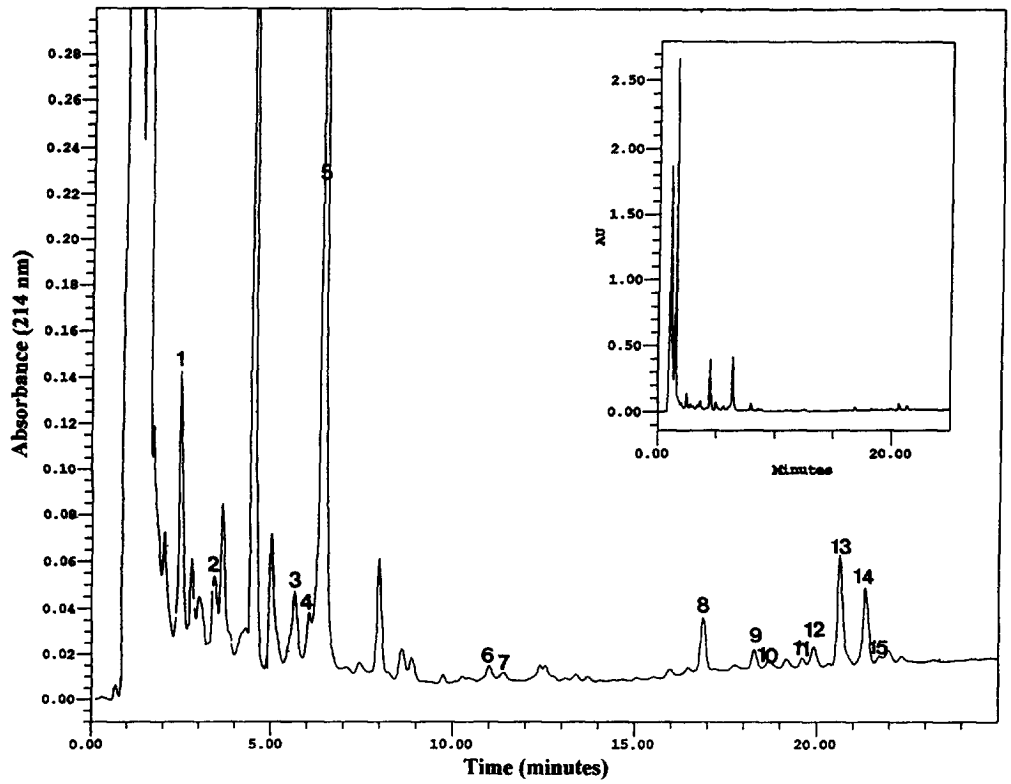

Fig. 2. Analysis of aqueous methanolic extract by reversed-phase HPLC with diode-array detection to determine the number of microcystin variants. Microcystins were identified by comparison of retention time and UV spectra to those of standards, or solely on the basis of UV spectra in the case of uncharacterised microcystins. Microcystins are numbered and those previously characterised include **5**=MC-LR, **8**=MC-LY, **13**=MC-LW and **14**=MC-LF.

Milli-Q water. The diluted fraction was then pumped onto the preparative HPLC cartridge via the main pump at a flow-rate of 200 ml/min. The cartridge was washed with 1.5 l of Milli-Q (approximately 3 column volumes) and microcystins were eluted with 100% methanol.

3. Results

3.1. Identification of microcystins

Fifteen microcystins were identified in the extract including MC-LR (**5**), MC-LY(**8**), MC-LW (**13**) and

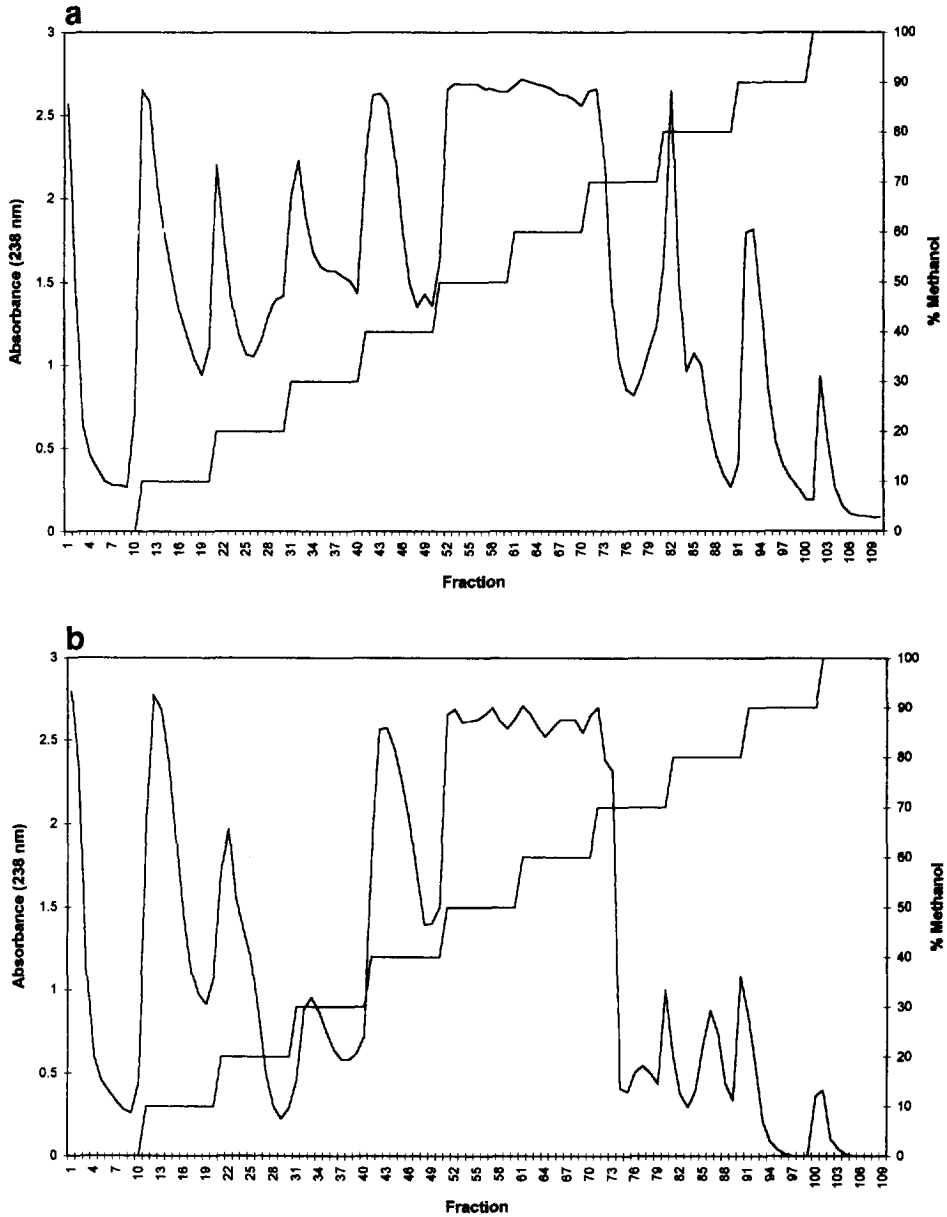

Fig. 3. Absorbance (238 nm) of fractions eluted from (a) Hyperprep C_{18} and (b) Bondapak C_{18} using a step gradient with increasing proportion of methanol in increments of 10% (2 l per step).

Table 1
Distribution of microcystins in fractions after reversed-phase flash chromatography using Hyperprep C_{18}

Aqueous methanol (%, v/v)	Yield of microcystin (mg)[a]														
	1[b]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Hyperprep C_{18}															
30	–	30	–	–	–	7	–	–	–	–	–	–	–	–	–
40	–	4	2	–	17	–	–	–	–	–	–	–	–	–	–
50	–	–	26	22	617	–	–	20	–	–	–	–	–	–	–
60	107	–	–	–	38	–	4	–	–	7	7	17	53	41	7
70	–	–	–	–	–	–	11	–	–	–	–	–	–	3	7
Bondapak C_{18}															
30	–	7	–	–	–	–	–	–	–	–	–	–	–	–	–
40	–	37	2	–	15	7	–	–	–	–	–	–	–	–	–
50	62	–	31	21	600	–	–	18	–	–	–	–	–	–	–
60	58	–	–	–	–	–	2	–	8	7	7	14	47	22	3
70	–	–	–	–	–	–	11	–	–	–	–	–	5	27	11

[a] As determined by reversed-phase HPLC where quantitation was by external standardisation where standards were available, and unknowns were quantified based on calibration obtained for microcystin-LR (**5**).
[b] Numbers refer to peaks shown in Fig. 2.

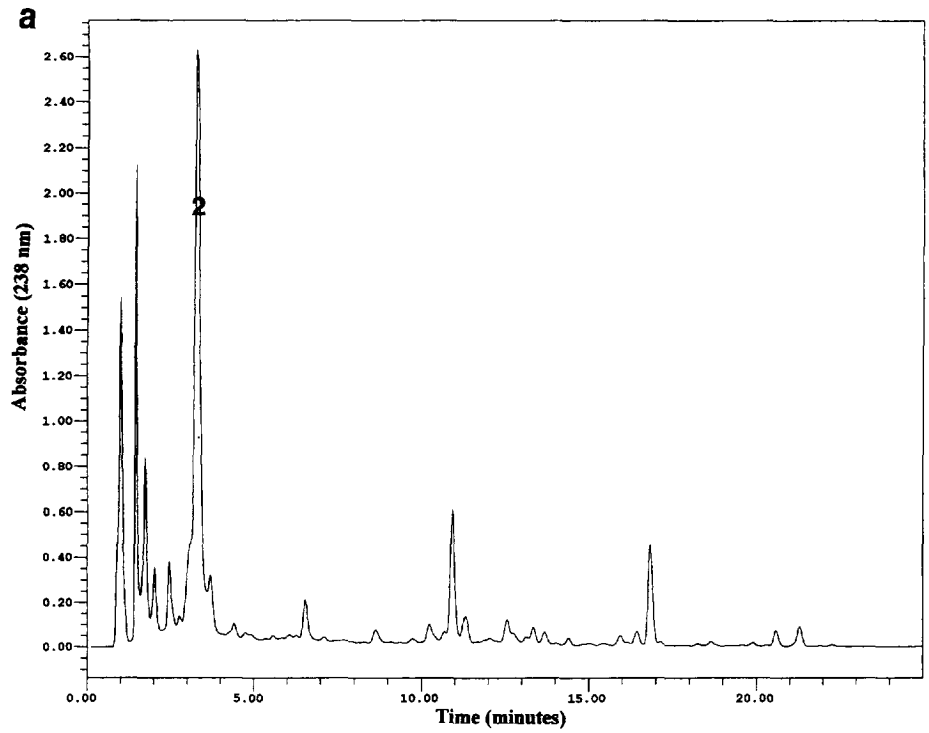

Fig. 4.

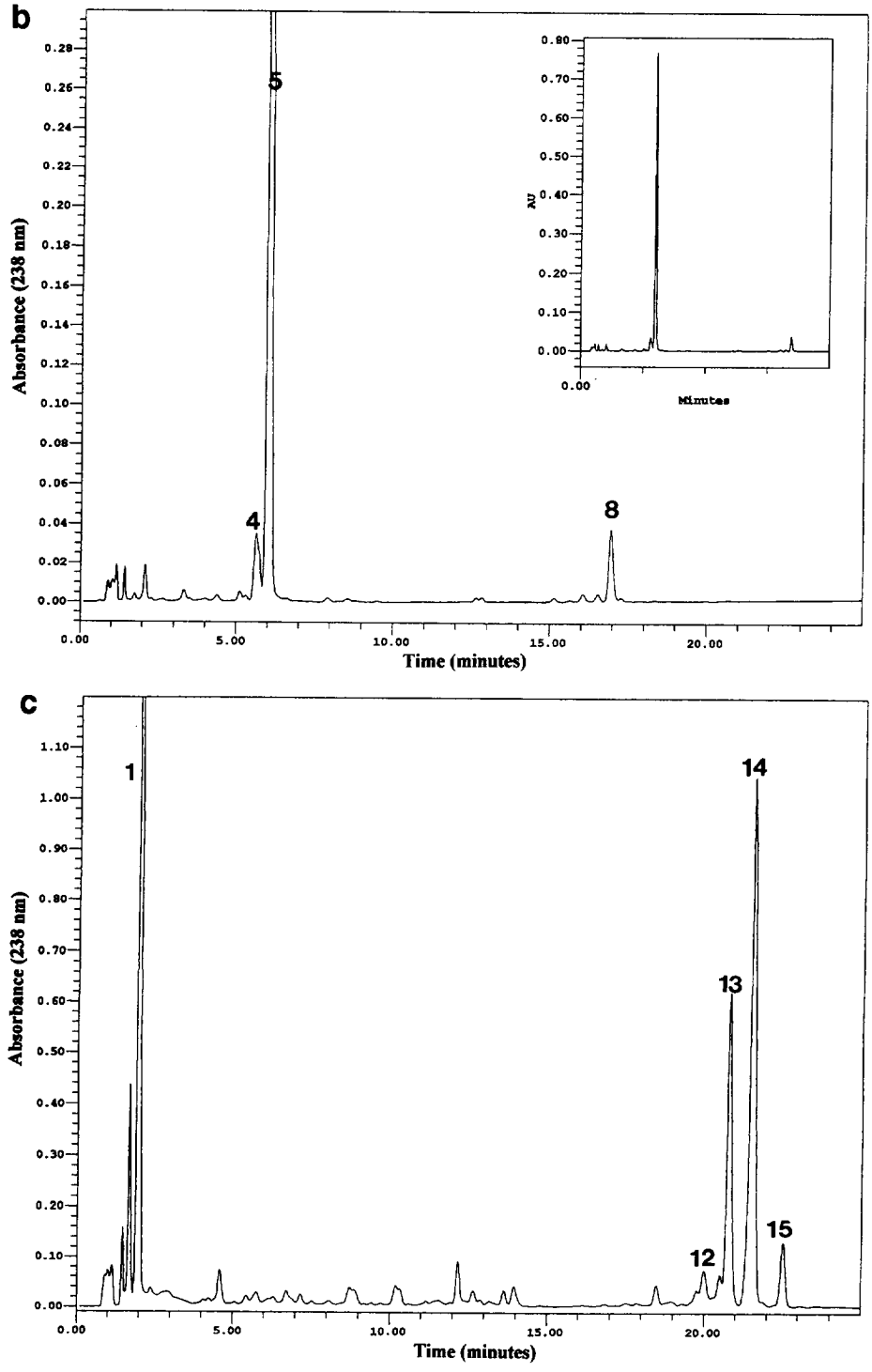

Fig. 4. Flash fractions were pooled to give three main fractions which were analysed by reversed-phase HPLC with diode-array detection: (a) fraction 1 containing predominantly an uncharacterised microcystin (**2**); (b) fraction 2 contained predominantly MC-LR (**5**) and MC-LY (**8**); (c) fraction 3 contained hydrophobic microcystins **12**, **13**, **14** and **15** and an uncharacterised microcystin (**1**).

MC-LF (**14**) along with eleven uncharacterised components having UV spectra indicative of microcystins (Fig. 2). The objective was to purify as many of these compounds as possible using flash chromatography followed by HPLC.

3.2. Flash chromatography

Concentration and step gradient elution of the aqueous extract (approximately 1 g microcystins per cartridge) was achieved in 3 h. Spectrophotometric absorbance profiles were very similar for the two cartridges, with major absorption (238 nm) occurring from fractions 40 to 75 (Fig. 3). Visual observation found extensive yellow/orange colour in fractions eluted in 0 and 10% methanol whilst those fractions eluted in 80, 90 and 100% methanol contained high concentrations of chlorophylls. Fractions eluted with 20–70% methanol where almost colourless.

Analysis of the fractions where there was high absorbance at 238 nm confirmed the presence of microcystins. Table 1 shows the distribution of microcystins across the gradient for both cartridges. The selectivity of the cartridges was very similar for the majority of microcystins although MC (**2**) was eluted from the Shandon cartridge in 30% methanol compared to 40% from the Waters Bondapak.

Fractions of similar composition were pooled to give three major fractions which could be more easily separated by HPLC (Fig. 4). From the first flash run (i.e. Shandon Hyperprep) fractions 21–30 were pooled to give fraction 1 (Fig. 4a) where the main microcystin was MC-**2**. Fractions 51–57 were pooled and contained MC-LR (**5**) and MC-LY (**8**) (Fig. 4b). Fractions 64–71 were pooled giving a sample containing the hydrophobic MC-LW (**13**) and MC-LF (**14**) along with several uncharacterised microcystins (Fig. 4c). Fractions from the second flash run were pooled in a similar manner (data not shown).

The effectiveness of this technique was particularly well illustrated for the major microcystin-LR (**5**) where approximately 0.6 g was recovered at a purity of 75% in each flash run (MC-LR (**5**) represented approximately 9% of the extract before flash chromatography).

3.3. Load optimisation

Fig. 5 shows chromatograms as increasing amounts of fraction 1 (0.05–0.25 mg per gram of packing material) were injected onto the column. Although a simple separation was developed, resolution deteriorated at low loading, this was possibly due to increased volume of methanol (50 μl for 0.25 mg/g injection) reducing retention of the more polar components. Due to limited material it was not possible to load increasing concentrations in identical volumes as is generally preferred. Further optimisation would be necessary before separation of fraction 1 could be satisfactorily scaled-up.

Fraction 2 containing MC-LR (**5**) and MC-LY (**8**) was separated using an isocratic system with a single step increase in the percentage of acetonitrile which was found necessary to elute MC-LY (**8**) within a reasonable timescale. Loads of increasing amount from 0.05 to 0.75 mg MC-LR (**5**) or a total load of approximately 0.1 to 1.5 mg/g packing material were injected onto the analytical column to determine maximum load attainable on scaling up to the 15×7.5 cm I.D. column (Fig. 6). At 1.5 mg/g the resolution of MC-LY (**8**) became unacceptable, therefore in order to obtain maximum yield of pure MC-LY (**8**) a maximum total load of 1 mg/g would be recommended.

Fig. 7 shows chromatograms of fraction 3 as load was increased. Resolution of MC-LW (**13**) and MC-

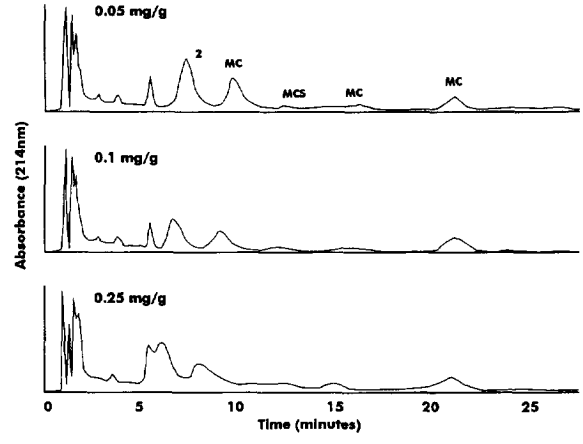

Fig. 5. Separation of fraction 1 and optimisation of load on Hyperprep HS BDS C_{18} (15 cm × 0.46 cm I.D.; 12 μm particle size).

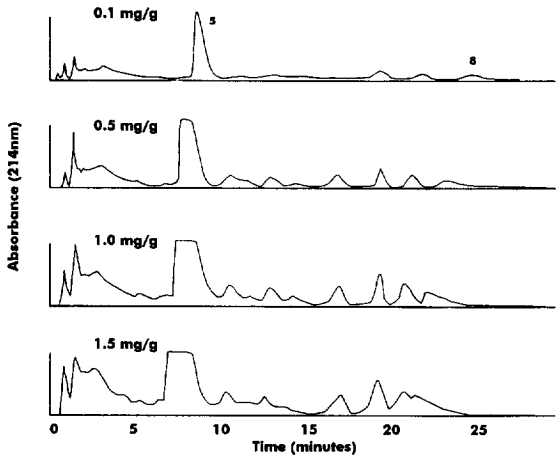

Fig. 6. Separation of fraction 2 and optimisation of load on Hyperprep HS BDS C_{18} (15 cm × 0.46 cm I.D.; 12 μm particle size).

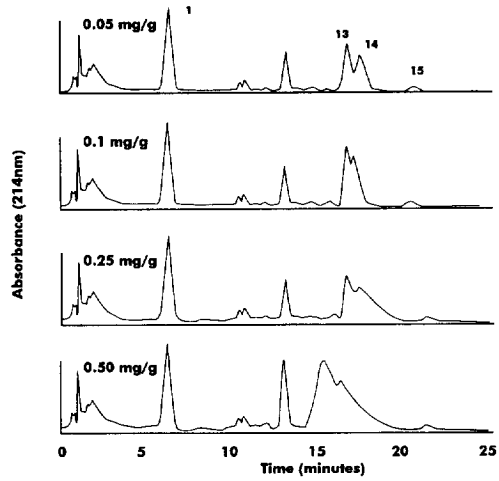

Fig. 7. Separation of fraction 3 and optimisation of load on Hyperprep HS BDS C_{18} (15 cm × 0.46 cm I.D.; 12 μm particle size).

LF (**14**) rapidly deteriorated, therefore it became necessary to investigate the use of closed-loop recycling when the separation was scaled-up to the 15×7.5 cm I.D. column. Closed-loop recycling is sometimes used to achieve better resolution and involves diverting the column effluent from the detector back onto the column via the pump. This may be repeated a number of times to achieve adequate separation of two similar compounds which would otherwise be very difficult. This technique has been used for the successful purification of enantiomers and diastereoisomers [15].

3.4. Preparative HPLC

Five microcystins were purified from both fractions 2 and 3 with purity greater than 90% (Table 2). Separation of fraction 2 was successfully scaled-up to the 7.5 cm I.D. column as shown in Fig. 8 where 352 mg of MC-LR (**5**) (a total load of approximately 0.5–0.6 g) was injected. Fig. 9 shows chromatogram of purified MC-LR (**5**) where the main contaminant was a related microcystin as indicated by its UV spectrum. Microcystin-LY (**8**) was also purified but found to contain closely eluting microcystins. Microcystins **3** and **4** were concentrated but still contained MC-LR (**5**), requiring further purification to obtain sufficient material for characterisation purposes.

Peak **1** was easily purified at the preparative scale, in sufficient quantities for characterisation studies (to

Table 2
Recovery and purity of microcystins from pooled flash fractions using laboratory-scale preparative HPLC

	Microcystins with purity >75%					
	1[a]	5	8	12	13	14
Fraction[b]	3	2	2	3	3	3
Total load (g)[c]	0.2	0.6	0.6	0.1	0.1	0.1
Recovery (%)	93	76	86	75	70	90
Purity (%)[d]	>95	>95	>80	>95	>90	>95

[a] Numbers refer to peaks shown in Fig. 2.
[b] Fraction from flash run as shown in Fig. 4.
[c] Total load is approximate since it was not possible to quantify unknown components which were not microcystins.
[d] Purity as assessed by reversed-phase HPLC with diode-array detection.

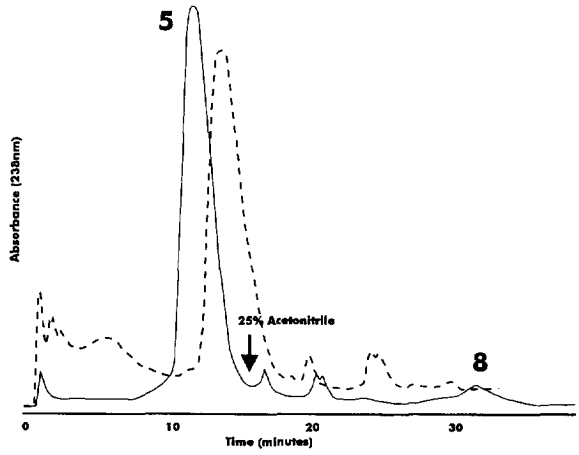

Fig. 8. Separation of fraction 2 on 15 cm × 7.5 cm I.D. column where total load was approximately 0.6 g. Broken line shows absorbance at 214 nm.

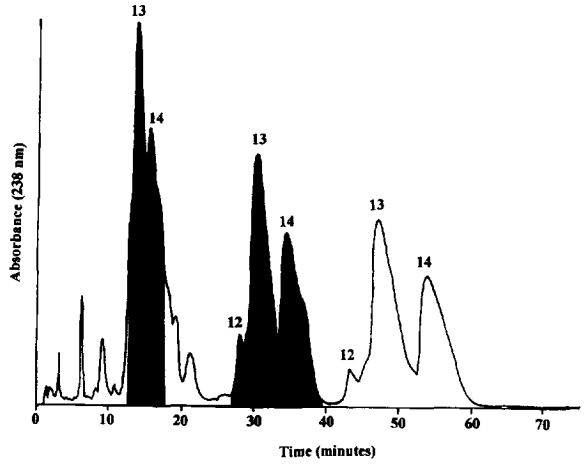

Fig. 10. Separation of MC-LW and MC-LF (**13** and **14**) and an unknown microcystin (**12**) which initially coeluted with MC-LW (**13**) using closed-loop recycling. The dark areas represent the effluent that was recycled.

be published at a later date). Peaks **13** and **14** were collected together, concentrated and reinjected where the mobile phase was 30% acetonitrile. Separation was achieved by using closed-loop recycling where the effluent from the column was diverted back though the column twice as indicated in Fig. 10. Without sacrificing a significant amount of MC-LW (**13**), the effluent recycled also contained peak **12**, which was sufficiently resolved by the final pass to enable collection, resulting in 0.5 mg of this uncharacterised microcystin (75% recovery with purity >95%). Purity of MC-LW (**13**) was lower than anticipated due to contamination of an unknown microcystin which was found to coelute. The effectiveness of using recycle was demonstrated by the yield (90%) and purity (>95%) obtained for MC-LF (**14**), not achievable using conventional batch elution methods to date.

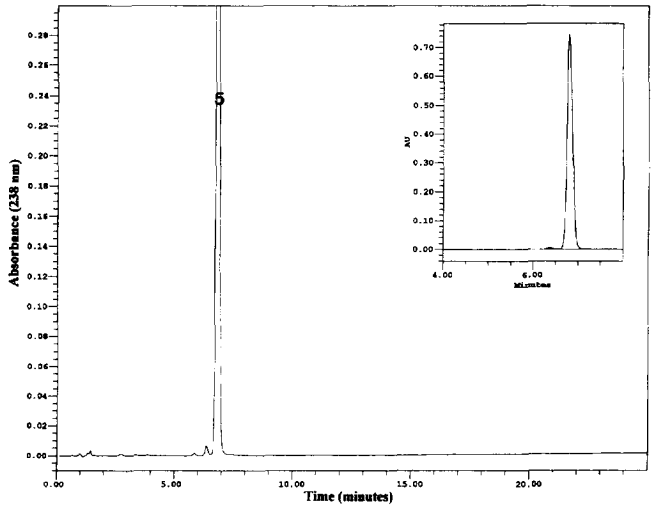

Fig. 9. Analysis of purified MC-LR (**5**) by reversed-phase HPLC with diode-array detection showed a purity of 98%.

4. Conclusions

Reversed-phase flash chromatography using prepacked cartridges was demonstrated to be a facile, rapid and reproducible way to concentrate and clean-up gram quantities of microcystins in the complex cyanobacterial extract. In previous studies, examining performance characteristics of the flash cartridges packed with Hyperprep C_{18} and Bondapak C_{18} using ethyl and methyl parabens, greater efficiency and resolution were obtained with the cartridge packed with Hyperprep (unpublished data). However, when used for concentration and clean-up of microcystins, both cartridges worked well, suggesting that there is no advantage in using high-performance stationary phases for applications of this nature.

This application demonstrates that the use of flash chromatography with prepacked cartridges offers a powerful tool for concentration and clean-up of natural products, resulting in simplified HPLC method development and scale-up.

Although post-flash samples were still complex compared to many preparative samples, 0.26 g MC-LR (**5**) was successfully purified to 98% purity when a total load of 0.6 g was injected onto the preparative column. It would be possible to increase the total load to 1 g and purify 0.5 g MC-LR (**5**) per run since the column was not overloaded. Thus, using the methods presented here 0.5 g MC-LR (98% purity) could easily be purified from the crude aqueous extract within a day. Both the flash chromatography and HPLC methods are simple and could easily be scaled-up as necessary.

An additional advantage of this methodology was the concentration of milligram quantities of minor microcystins; however, further purification by semi-preparative HPLC was required to obtain material of suitable purity for characterisation. Although it was difficult to separate MC-LW (**13**) and MC-LF (**14**), they were easily purified by using closed-loop recycling where they were passed through the column two additional times thereby enhancing resolution.

Desalting the microcystins in the HPLC fractions by using the same HPLC cartridge used for the separations was rapid and allowed the component(s) to be eluted in a small volume which could be readily dried down without exposing the compounds to prolonged harsh conditions which may affect stability.

In summary, we were able to develop complete methodology for purifying milligram–gram quantities of microcystins from cyanobacterial scum, a more complex matrix than extracts derived from cultured cyanobacteria. This approach could be used for scaling-up purification of many natural products.

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