Automated purification of microcystins

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Abstract

A simplified method for the concentration and purification of microcystins was developed. A 3-l volume of cyanobacterial scum (equivalent to 124 g dry mass of cells) collected from Rutland Water, Leicestershire, in 1989 was extracted in methanol and centrifuged and the supernatant was retained. This was diluted to ca. 20% aqueous methanol and applied to a reversed-phase flash chromatography cartridge. Microcystins were eluted with 70% aqueous methanol then reduced in vacuo. Gradient separation of the extract was optimized on an analytical column and scaled up to a 15 cm × 7.5 cm I.D. column with subsequent purification of individual compounds.

Keywords: Preparative chromatography; Flash chromatography; Sample preparation; Microcystins; Toxins

1. Introduction

Several genera of cyanobacteria (blue-green algae) produce toxic secondary metabolites which fall into two main categories: (1) neurotoxins, including the sodium channel blocker saxitoxin and its analogues, postsynaptic neuromuscular blocking agents [1,2], anatoxin-a and homoanatoxin-a [3,4] and the organophosphorus cholinesterase inhibitor anatoxin-a(s) [5]; and (2) peptide hepatotoxins, which include the heptapeptides, microcystins [6,7] and the pentapeptides known as nodularins [8] and motopurin [9]. These peptide toxins inhibit protein phosphatases 1 and 2A in eukaryotes in a specific and irreversible manner, resulting in the disruption of many cellular control mechanisms [10,11]. Owing to their biochemical mode of action, these com-

pounds have subsequently been demonstrated to exhibit tumour-promoting activity [12,13].

In several European surveys on the occurrence of toxic cyanobacterial blooms, peptide hepatotoxins, in particular microcystins, were shown to occur most frequently [14] and have been responsible for animal deaths and human illness [15]. In view of these incidents, along with the recent discovery that these toxins exhibit the same degree of toxicity when introduced intranasally as they do when injected intraperitoneally, there is increasing requirement for in-depth toxicological studies [16]. Lack of information regarding toxicology is largely due to lack of availability of sufficient amounts of purified microcystins. Purification methods reported in the literature could only be used to purify microgram to milligram amounts of microcystins. We recently presented a scaleable method for producing milligram to multigram amounts of microcystins

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where an aqueous methanol extract was concentrated on a reversed-phase flash cartridge, microcytins were eluted using a step gradient from 0 to 100% methanol in 10% increments [17]. Fractions were analysed by HPLC and those containing microcystins of similar polarity were pooled, resulting in three major fractions. These simplified fractions were separated by isocratic methods. Although successful, the process of purification was lengthy, with a requirement to assay flash fractions before pooling those of similar composition. This paper presents an alternative approach where the extraction/concentration procedure was simplified and the resulting extract containing all the microcytsins was separated using a gradient.

2. Experimental

2.1. Chemicals

Chemicals were of analytical-reagent grade and obtained from Fisons (Loughborough, UK). HPLC-grade methanol and acetonitrile were also obtained from Fisons. Pure water was obtained from a Milli-Q system (Millipore, Watford, UK). Microcystin standards were purified from cultured cells and bloom material as described previously [18].

2.2. Cyanobacterial material

As in previous work, the source of microcystins was *Microcystis aeruginosa* collected from Rutland Water (Leicestershire, UK) in September 1989. The cells were maintained at $-20°C$ until required.

2.3. Extraction of cyanobacterial cells

Cyanobacterial cells were extracted in methanol and spun at 1500 g for 20 min as described previously. In previous experiments, the supernatant was rotary evaporated to an oily residue, which was then combined with methanol and water at a ratio of 1:1:8 (extract–methanol–

water) and filtered through GF/C discs prior to loading on to a reversed-phase flash chromatography cartridge. Rotary evaporation of the methanol extract took almost 1 week and therefore, in order to reduce the processing time, the supernatant was diluted to 20% (v/v) with glass-distilled water and passed through GF/C filters (Whatman, Maidstone, UK) to remove remaining particulates. The aqueous extract (80 l) was applied directly to a preconditioned Bondapak C_{18} cartridge (9 cm × 7.5 cm I.D., 37–55 μm particle size) housed on a compression module (Biotage, Charlottesville, VA), USA) at a flow-rate of 200 ml/min. The eluent was monitored for breakthrough using analytical HPLC as described. The cartridge was washed with 0, 10 and 20% methanol (2 l each) and microcystins were eluted with 70% methanol (2 l). This sample was concentrated to 200 ml in vacuo at 40°C.

2.4. Analytical HPLC

Microcystins were identified in the cyanobacterial extract by analytical HPLC with diode-array detection as described previously [17].

2.5. Stationary phase selection

Several stationary phases frequently used for large-scale peptide separations were packed into analytical scouting columns and evaluated: (1) YMC ODS (25 cm × 0.46 cm I.D., 15 μm particle size, 120 Å pore size; YMC, Wilmington, DE, USA); (2) Vydac C_{18} (25 cm × 0.46 I.D., 15–20 μm particle size, 300 Å pore size; Vydac, Hesperia, CA, USA); (3) Shandon Hyperprep HS BDS C_{18} (15 cm × 0.46 cm I.D., 12 μm particle size, 100 Å pore size; Shandon HPLC, Runcorn, UK); and (4) Shandon Hyperprep HS BDS C_{18} (15 cm × 0.46 cm I.D., 8 μm particle size, 100 Å pore size).

The microcystin extract (0.1 mg) was separated on all four columns using a mobile phase consisting of ammonium acetate (0.1%, w/v) (A) and acetonitrile (B), where solvent B was maintained at 22% for the first 4 min followed by an

Channel Descr.: PDA 238.0 nm

A

(Chromatogram with y-axis AU from 0.00 to 2.40, x-axis Minutes from 0.00 to 20.00+; labeled peaks 1, 2, 3, 4, 5, 6, 7)

Channel Descr.: PDA 214.0 nm

B

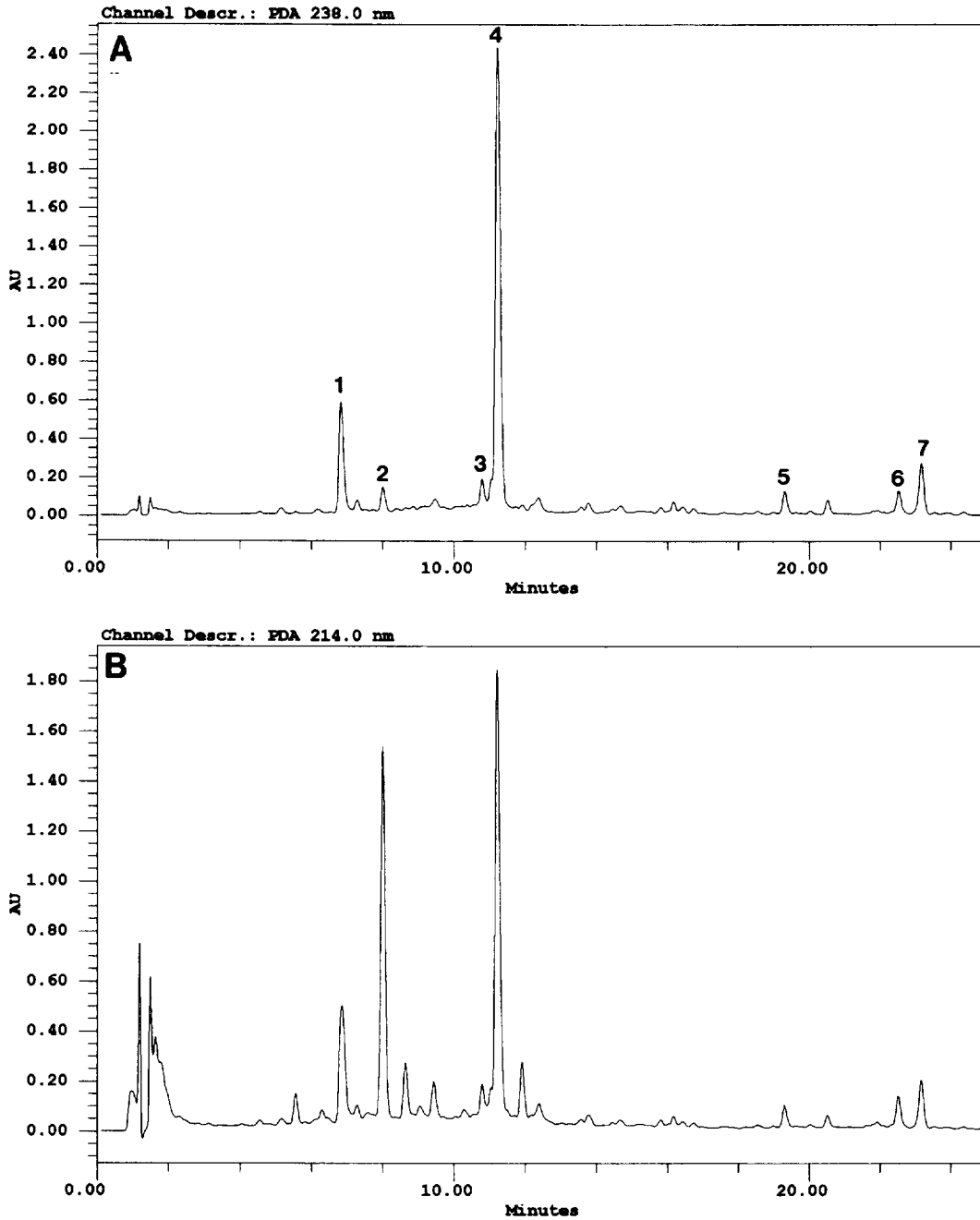(Chromatogram with y-axis AU from 0.00 to 1.80, x-axis Minutes from 0.00 to 20.00+)

Fig. 1. Identification of microcystins in post-flash extract by reversed-phase HPLC with diode-array detection. The extract was separated on a Symmetry C_{18} column (15 cm × 0.46 cm I.D., 5 µm particle size; Waters). Eluents were Milli-Q water and acetonitrile, 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 were acquired from 200 to 350 nm. Seven major microcystins were identified based on their retention times and UV spectra compared with those of standards or on characteristic UV spectra for identification of unknowns.

increase to 35% over the next 46 min (76 min for the 25-cm columns) at a flow-rate of 1.5 ml/min. The eluate was monitored at 238, 214 and 254 nm (Model 490 detector; Waters, Watford, UK).

2.6. Load optimization

Once the stationary phase had been selected and the gradient optimized, increasing loads were injected on to the column to determine the maximum load, that is, the amount of sample (mg/g packing material) that could be loaded without a significant loss of resolution.

2.7. Preparative HPLC

The equipment included a Kiloprep 100A controlled by Prepview software and a KPCM 100 compression module (Biotage). Samples were loaded on to a cartridge packed with Hyperprep HS BDS C_{18} (15 cm × 7.5 cm I.D., μm particle size) using a sample pump at a flow-rate of 100 ml/min and separated using a gradient starting at 20% B, increasing to 22% B over 4 min, followed by an increase to 32% over the next 36 min. The flow-rate was 400 ml/min and the eluate was monitored at 238 and 214 nm. Fraction collection was based on time and fractions were analysed by HPLC with diode-array detection to determine their purity.

2.8. Desalting and concentration

The purified components were desalted employing the Biotage flash apparatus used for concentration and clean-up. Fractions were diluted with one volume of Milli-Q water and passed through a new preconditioned C_{18} flash cartridge (9 cm × 7.5 cm I.D., Bondapak C_{18}, 37–55 μm particle size) at a flow-rate of 250 ml/min. The cartridge was washed with 1.5 l of Mill-Q water (ca. six column volumes) and purified microcystins were eluted in 500 ml aqueous methanol (80%, v/v).
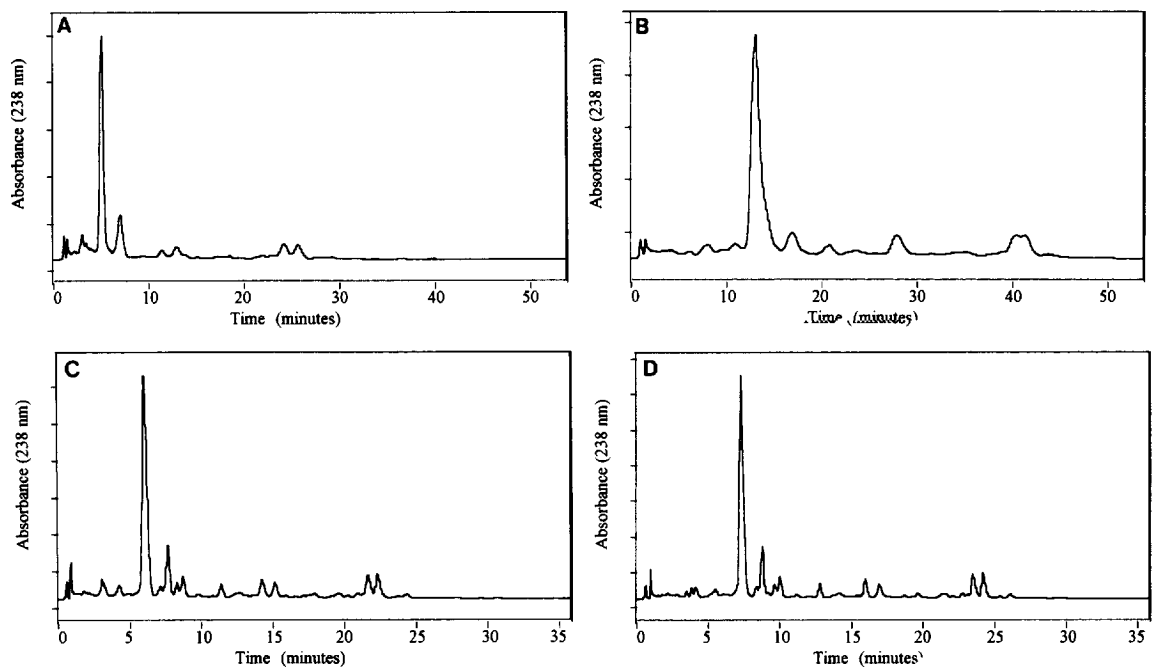

Fig. 2. Separation of microcystin extract (0.1 mg) on (A) Vydac C_{18} (15–20 μm, 300 Å), (B) YMC ODS (15 μm, 120 Å), (C) Hyperprep HS BDS C_{18} (12 μm, 100 Å) and (D) Hyperprep HS BDS C_{18} (8 μm, 100 Å).

3. Results

3.1. Extraction and clean-up

Although it took 7 h to load 80 l of aqueous extract, this was far less time consuming than the several days required to remove the methanol by rotary evaporation. There was no breakthrough of microcystins during sample loading.

3.2. Identification of microcystins

Seven major microcystins were identified in the extract after concentration and clean-up using reversed-phase flash chromatography. These included MC-LR (**4**), MC-LY (**5**), MC-LW (**6**) and MC-LF (**7**) along with three uncharacterized components which had UV spectra indicative of microcystins (Fig. 1). The objective was to purify as many of these compounds in a single gradient separation.

Table 1
Reproducibility of retention in four preparative gradient separations

Peak No.[a]	Mean retention ±S.D. (variation)
1[a]	752 ± 6.0 (0.8%)
2	861 ± 7.6 (0.9%)
3	924 ± 4.6 (0.5%)
4	993 ± 2.5 (0.3%)
5	1669 ± 12.12 (0.7%)
6	1928 ± 12.10 (0.6%)
7	2025 ± 14.20 (0.7%)

[a] Numbers refer to peaks in Fig. 1.

Table 2
Recovery of microcystin-LR (**4**) from preparative gradient separations

Run No.	Total load (g)	Recovery (%)	Purity[a] (%)
1	0.3	84	98
2	0.5	83	98
3	0.5	82	96
4	0.5	85	97

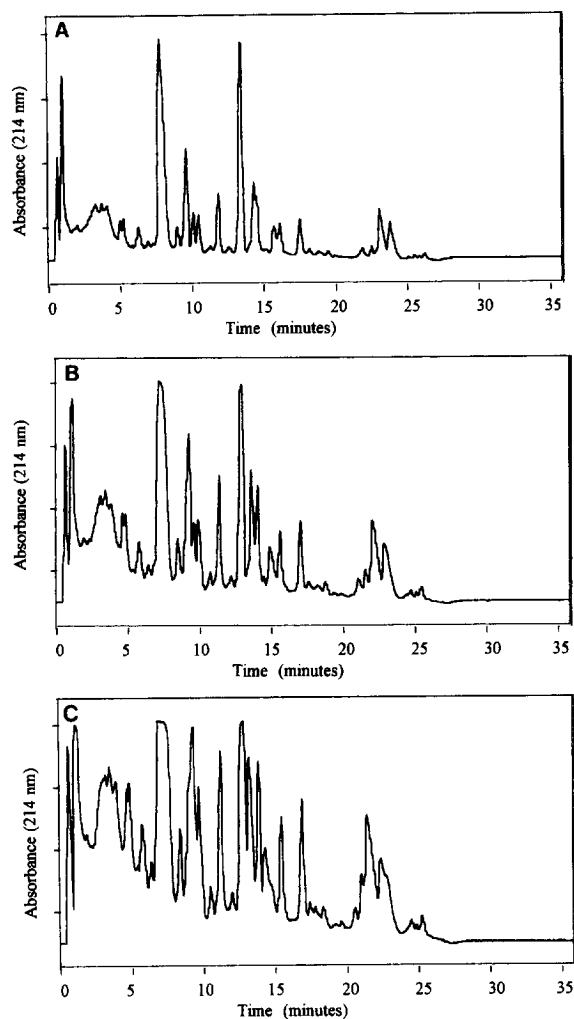[a] As determined by analytical HPLC with diode-array detection.

Fig. 3. Optimization of load on Hyperprep C_{18} (15 cm × 0.46 cm I.D., 8 μm particle size). Total loads of (A) 0.3, (B) 0.75 and (C) 1.5 mg/g packing material were examined.
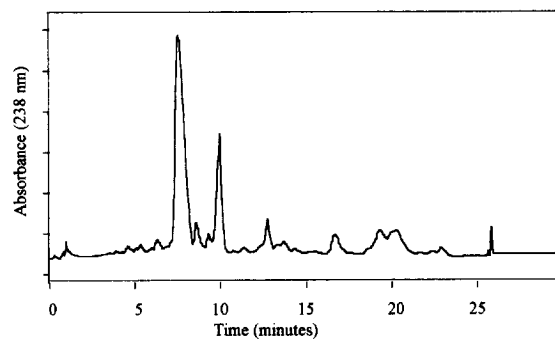

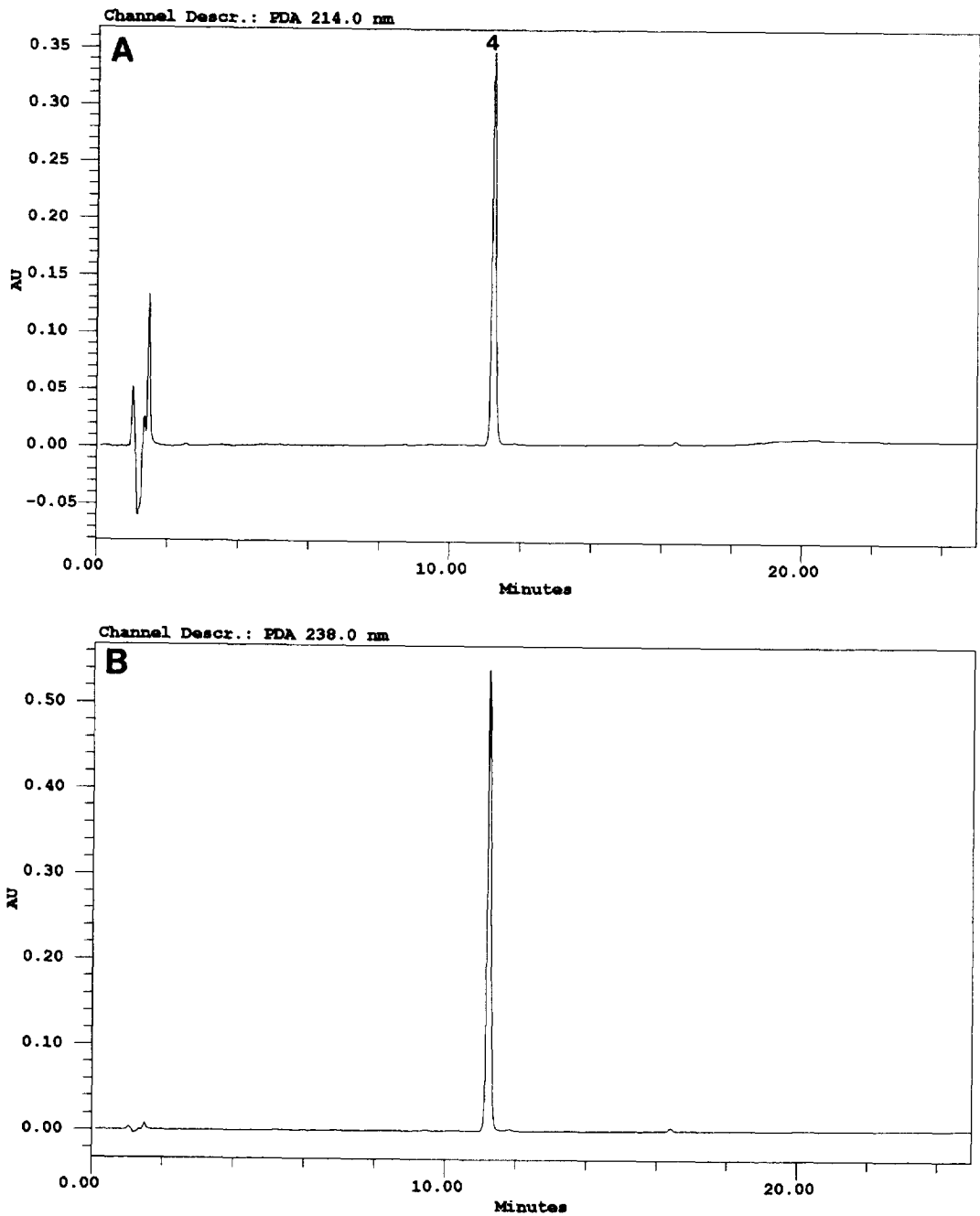Fig. 4. Preparative separation of 0.5 g of extract on the 15 cm × 7.5 cm I.D. column.

Channel Descr.: PDA 214.0 nm

Channel Descr.: PDA 238.0 nm

Fig. 5. Analysis of fraction containing MC-LR (**4**) from preparative run by HPLC using the method described in the caption of Fig. 1.

3.3. Stationary phase selection

Using the gradient described, the best resolution was obtained on columns packed with smaller particle size material, i.e. the Hyperprep C_{18} (8 and 12 μm particle size) (Fig. 2). Although there was very little difference in the resolution achieved on the Hyperprep columns, the C_{18} column (8 μm particle size) was selected for the study since MC-LR (**4**) had a greater

retention and MC-LW (**6**) and MC-LF (**7**) were slightly more resolved.

3.4. Load optimization

As the load was increased to 1.5 mg of extract per gram of packing material, the separation of MC-LW (**6**) and MC-LF (**7**) deteriorated, and therefore a load of 1 mg/g was selected for scaling-up (Fig. 3). This was equivalent to a load of 0.5 g on the 15cm × 7.5 cm I.D. column which contained 485 g of Hyperprep HS BDS C_{18}.

3.5. Preparative HPLC

Separation of the extract was scaled up to the 15 cm × 7.5 cm I.D. column as shown in Fig. 4. The retention and resolution for most components were similar to those obtained on the scouting column, although peak 1 eluted after peaks 2 and 3 and the resolution of MC-LW (**6**) and MC-LF (**7**) was poor. This was possibly the result of loading the sample via the sample pump in a large volume (100 ml). Gradient separation of the microcystins was highly reproducible from run to run, as indicated by retention times from four separations (Table 1).

Microcystin-LR (**4**) was successfully purified using gradient elution as shown by the analytical HPLC of pooled fractions (Fig. 5). Yields of MC-LR (**4**), where the purity was greater than 95%, were in excess of 80% (Table 2). Unfortunately, the purity of the other microcystins ranged from 60 to 80% and therefore further purification was necessary. MC-LW (**6**) and MC-LF (**7**) were purified using closed-loop recycling as described previously [17].

4. Conclusions

Microcystins (approximately 1 g in 80 l of aqueous methanol) were successfully concentrated and cleaned up using reversed-phase flash chromatography.

Separation of the extract was examined on several stationary phases frequently used for the purification of peptides. Gradient separation and loading were optimized on the analytical scale

using a scouting column packed with Hyperprep C_{18}. The method was successfully scaled up to a 15 cm × 7.5 cm I.D. column where sample introduction, gradient control and fraction collection were automated. Good yields of pure MC-LR (**4**) were obtained using automated gradient elution. However, owing to the complexity of the sample, it was not possible with a short gradient to obtain other microcystins with acceptable purity, i.e. >95%. Further work on gradient development is necessary to improve the yield of these other microcystins. However, this method would be suitable for the less complex cyanobacterial extracts obtained from cultured cells.

Desalting on the smaller flash cartridge was highly successful; components were eluted in smaller volumes without risk of contamination from components retained on the cartridge from previous separations.

Acknowledgements

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