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Journal of Chromatography A, 848 (1999) 515–522

JOURNAL OF CHROMATOGRAPHY A

Short communication

Purification of closely eluting hydrophobic microcystins (peptide cyanotoxins) by normal-phase and reversed-phase flash chromatography

Linda A. Lawton[a,*], Jacqui McElhiney[a], Christine Edwards[b]

[a]*School of Applied Sciences, The Robert Gordon University, St. Andrew Street, Aberdeen AB25 1HG, UK*

[b]*Biotage (UK) Ltd., a Division of Dyax Corporation, 15, Harforde Court, Foxholes Business Park, Hertford SG13 7NW, UK*

Received 1 December 1998; received in revised form 30 March 1999; accepted 31 March 1999

Abstract

Two closely eluting hydrophobic peptides, microcystin-LW and -LF had proved in the past to be particularly difficult to purify by reversed-phase HPLC. Initial extraction by reversed-phase flash chromatography provided a good method of concentrating microcystins and also partially purifying them, although the separation of closely eluting variants was not possible. The use of normal-phase flash chromatography after initial reversed-phase extraction was found to be a suitable method for achieving high purity and gave a good yield of both microcystins. However, a final reversed-phase flash step was necessary to eliminate trace contaminants. The method described provides a simple three step flash chromatography extraction and purification eliminating the need to use preparative HPLC.

Keywords: Flash chromatography; Microcystins; Peptides; Toxins

1. Introduction

Microcystins are a group of toxic, cyclic peptides which are produced by a number of genera of cyanobacteria (blue–green algae) including *Microcystis*, *Planktothrix* (*Oscillatoria*) and *Anabaena* [1]. These toxins have been implicated in frequent animal poisonings [2–4], and more recently in the fatality of a significant number of dialysis patients in Brazil [5,6].

Microcystins are cyclic heptapeptides named according to the single letter abbreviation for the variable amino acids which they contain hence those under investigation here, microcystin-LW and -LF both contain leucine (L), with tryptophan (W) and phenylalanine (F), respectively. Different amino acid substitutions and a number of minor chemical modifications yield a large family of related homologs. The microcystins studied here are relatively hydrophobic compared to most other microcystins which is due to the presence of the more hydrophobic amino acids, tryptophan and phenylalanine [4].

Microcystin-LW and -LF have been reported in a number of naturally occurring cyanobacteria and are produced in laboratory culture by several strains of *Microcystis aeruginosa* [4,7]. Many of the commonly used extraction methods utilise aqueous solvents which would not be suitable for recovering more

*Corresponding author. Tel.: +44-1224-262-823; fax: +44-1224-262-828.

E-mail address: l.lawton@rgu.ac.uk (L.A. Lawton)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00462-8

hydrophobic microcystins [8,9]. Furthermore, the commonly used analytical high-performance liquid chromatography (HPLC) protocols fail to elute over a broad enough range of polarity, resulting in an under reporting of the occurrence of hydrophobic microcystins [10]. It is therefore important that purified hydrophobic microcystins are readily available to enable their detection and subsequent reporting.

Previously, we reported the use of reversed-phase flash chromatography as a suitable method for concentration and preliminary purification [11,12]. This facilitated simple isocratic preparative HPLC of several microcystins. However, successful purification of microcystin-LW and -LF was only achieved using closed-loop recycling. Although this method provided good purity and yield, it relies on the availability of specialised equipment and expertise. This paper describes the development of a normal-phase flash chromatography method for the purification of microcystin-LW and -LF after preliminary extraction and separation by reversed-phase flash chromatography. The method eliminates need for the relatively expensive, preparative HPLC and exploits the use of a simple pre-packed flash cartridge system.

2. Experimental

2.1. Chemicals

Chemicals were of analytical-reagent grade unless stated and obtained from Merck, Darmstadt, Germany. HPLC-grade methanol, acetonitrile and dichloromethane (DCM) were obtained from Rathburn (Walkerburn, UK). Pure water was obtained from a Milli-Q system (Millipore, Watford, UK). Microcystin standards were purified from cultured cyanobacterial cells as previously described [11].

2.2. Cyanobacterial material

Batch cultures of *Microcystis aeruginosa* PCC7820 (Pasteur Culture Collection, Paris, France) were grown in BG-11 plus nitrate (8.8 m*M*) under continuous illumination and sparging with sterile air. Cells were harvested after approximately five weeks growth by tangential flow filtration (Pellicon-2; fitted with three 0.22 μm, type GVPP-V filters, Millipore) and stored as a wet pellet at −20°C until required.

2.3. Extraction of cyanobacterial cells

Wet pellet (equivalent to 30 g dry mass) was thawed and extracted as previously described. The aqueous extract was applied to a pre-conditioned KP-C_{18}-HS flash cartridge (15×4 cm I.D., 35–70 μm particle size, 60 Å pore size) using a Biotage Flash 40 system (Biotage, a Division of Dyax Corporation, Charlottesville, VA, USA) at a flow-rate of 40 ml/min. The microcystins were eluted using a step gradient from 0 to 100% methanol in 10% increments (1 l per step) with each step fraction collected separately and analysed by analytical HPLC. After use, reversed-phase flash cartridge was cleaned with DCM (1 l) then flushed (1 l) and stored in methanol for future use. It has been found that reversed-phase flash cartridge can be used a number of times depending on the sample and thoroughness of the cartridge cleaning.

2.4. Analytical HPLC

All fractions were monitored as described previously [9] with the following modifications. Quantification, identification and purity were determined by HPLC with high-resolution diode array detection using a Waters 996 detector. Samples were separated on a Symmetry C_{18} column (250×4.6 mm I.D.; 5 μm particle size; Waters). Detector resolution was set at 1.2 nm and data acquired from 200 to 300 nm.

2.5. Method development for normal-phase separation of microcystin-LW and -LF

Solvent optimisation was carried out initially using normal-phase thin-layer chromatography (TLC) (10×5 cm, silica gel 60; Merck). A range of solvents with different selectivities were evaluated and components were visualised by developing TLC plates in iodine. R_F values were calculated and converted into column volumes ($CV=1/R_F$) which represented the approximate number of column volumes of solvent required to elute each component. In order to determine suitable separation conditions, the difference

between predicted column volumes for each microcystin was determined (ΔCV).

Using the solvent selected by TLC, final optimisation of separation conditions was carried out using a pre-packed flash KP-Sil silica cartridge (15×1.2 cm I.D., 32–63 μm particle size, 60 Å pore size; Biotage) which was conditioned by washing with methanol (200 ml) then DCM (100 ml). Mobile phase A was DCM and B contained methanol–acetic acid (10:2). The cartridge was equilibrated with initial mobile phase, A–B (95:5) (100 ml). For method optimisation, a partially purified sample containing 1.3 mg microcystin-LF and 1.5 mg microcystin-LW was resuspended in a small volume of methanol then diluted in DCM prior to injection. A step gradient was performed as follows: three CVs (30 ml) A–B (95:5); 10 CVs A–B (92:8); 12 CVs A–B (90:10). Fractions (approximately 5 ml) were analysed by TLC (DCM–methanol–acetic acid, 88:10:2) and those containing microcystins were dried, resuspended in methanol, and analysed by HPLC.

2.6. Scale-up of normal-phase flash chromatography method

The fraction (60% aqueous methanol) eluted from the reversed-phase flash extraction and found to contain predominantly the two microcystins of interest (Table 1), was dried by rotary evaporation and the sample resuspended in a small volume of methanol. The KP-Sil silica cartridge (15×4 cm I.D., 32–63 μm, 60 Å pore size; Biotage) was prepared by flushing with methanol (2400 ml), DCM (1200 ml) and initial mobile phase (1200 ml) as described in Section 2.5. The sample (9.3 mg microcystin-LW and 12.2 mg microcystin-LF) was diluted in DCM prior to injection onto the prepared cartridge. Elution of the microcystins was carried out as before (Section 2.5) maintaining the number of CVs used to separate the toxins. Fractions (50 ml) were dried, resuspended in methanol and analysed by HPLC. Fractions containing a single microcystin with purity (by HPLC) greater than 90% were pooled, diluted to 20% (v/v) methanol and passed through a KP-C_{18}-HS flash cartridge (7.5×4 cm I.D., 35–70 μm particle size, 60 Å pore size; Biotage) and the cartridge washed with water (500 ml) to ensure the of removal any residual acetic acid. Each purified microcystin was eluted from the cartridge in methanol (500 ml) then rotary evaporated to dryness prior to quantification by gravimetric analysis.

Gravimetric analysis and visual appearance suggested the presence of contaminants not revealed by HPLC. Individual purified microcystins were therefore resuspended in methanol and the sample added to water (1 l). This was passed through a preconditioned C_{18} flash cartridge (7.5×4 cm I.D., 35–70 μm particle size, 60 Å pore size; Biotage) and the sample eluted by step gradient from 0 to 100% methanol in 10% increments (500 ml per step). Each increment was collected separately and analysed by HPLC. The fraction containing purified microcystin

Table 1
Recovery of microcystins, determined by HPLC–photodiode array detection, from *Microcystis aeruginosa* PCC7820 following reversed-phase flash chromatography using an aqueous methanol step elution

Methanol fraction (%)[a]	Microcystin variant	Amount of microcystin (mg)	Recovery (%)[b]
40	LR	18	22
	LY	1	9
50	LR	52	64
	LY	9	91
	LW	3	19
60	LW	12	67
	LF	15	93

[a] Percentage of aqueous methanol used to elute that fraction.
[b] Percentage of microcystin recovered in that fraction relative to total amount applied to the cartridge.

was rotary evaporated to dryness, resuspended in a small volume of methanol, dried and quantified gravimetrically.

3. Results

3.1. Solvent and reversed-phase extraction of microcystins

HPLC analysis of the cell extract prior to reversed-phase extraction revealed four main microcystins (Fig. 1) which were identified and quantified as microcystin-LR (82 mg), microcystin-LY (10 mg), microcystin-LW (17 mg) and microcystin-LF (16 mg). Analysis of the flash fractions eluted using a methanolic step gradient revealed that the microcystins were eluted in the 40 to 60% fractions (Table 1) with the fraction eluted in 60% methanol found to contain primarily microcystin-LW and -LF.

3.2. Method development of normal-phase separation of microcystin-LW and -LF

The most suitable solvent system indicated by TLC was DCM–methanol–acetic acid (88:10:2). The addition of acetic acid minimised tailing. R_F values were 0.08 and 0.18 for microcystin-LW and -LF, respectively. This gave a theoretical ΔCV of 7.3 which would indicate a potentially good separation when chromatographed on a column. This mobile phase was evaluated using small (15×1.2 cm I.D.) pre-packed flash cartridges. It was found that pre-washing of the cartridge with methanol followed by the mobile phase was necessary to prevent recovery problems.

Direct transfer of the TLC method to the 12 mm column did not result in the simple separation as predicted, thus it was necessary to use a step gradient. The conditions described (Section 2.5) provided the optimum separation and although no

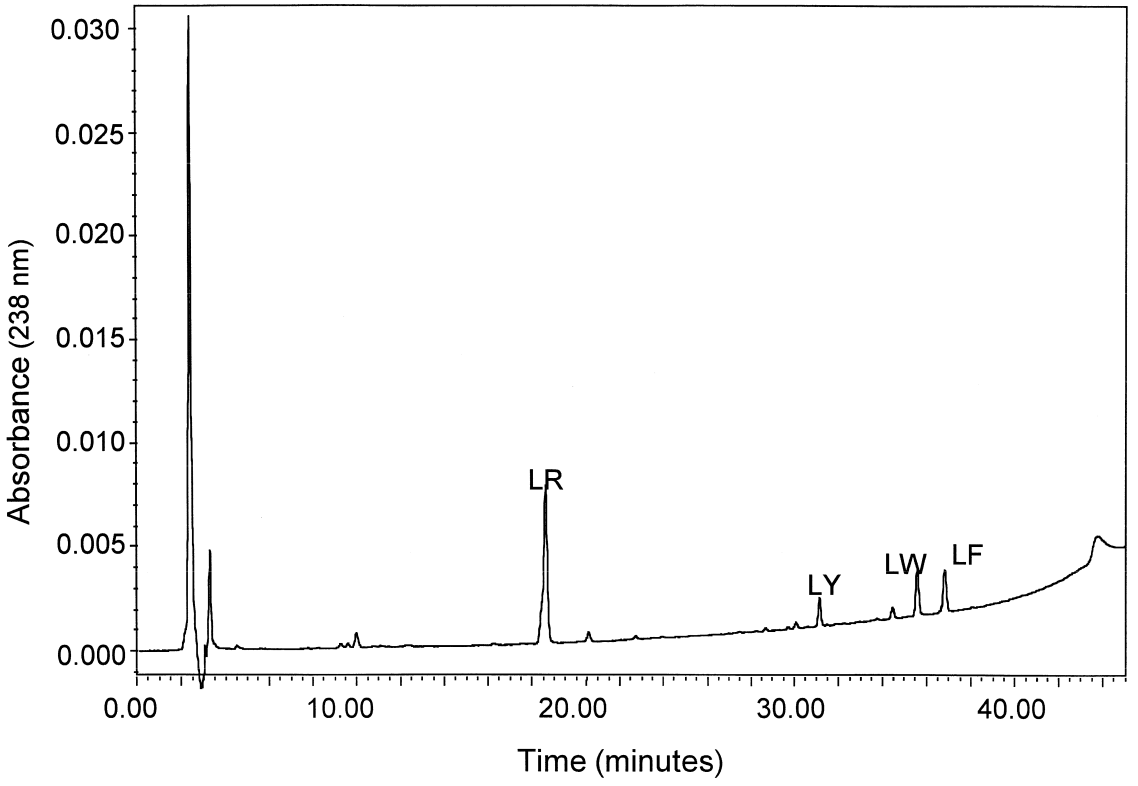

Fig. 1. Reversed-phase HPLC chromatogram of aqueous methanolic extract of *Microcystis aeruginosa* PCC7820 prior to application to C_{18} flash cartridge. Four main microcystins present in the extract: microcystin-LR; microcystin-LY; microcystin-LW; and microcystin-LF.

microcystins were eluted in the first step (A–B, 95:5) it was found to be essential to the initial retention and subsequent satisfactory separation of the microcystins.

3.3. Scale-up of normal-phase purification

The optimised method was used to purify microcystin-LW and -LF from the initial reversed-phase flash (Section 2.3). Direct scale-up was achieved using a 4 cm I.D. cartridge where volumes of solvent in the step gradient, volume of sample injected, flow-rate and fraction size were increased proportionally. Due to limited amount of sample, the load injected onto the 4 cm column was equivalent to 0.23 mg per gram of packing material compared to 0.35 mg injected onto the 1.2 cm I.D. column. A similar separation was achieved (Fig. 2a) although all toxin-containing fractions were eluted during the final step of the gradient (A–B, 90:10). It can be seen (Fig 2b) that a significant number of fractions contained a single microcystin at a purity greater that 90%.

3.4. Final reversed-phase purification

Reversed-phase flash was successfully used to ensure the removal of acid from the sample. Further analysis, however, revealed the sample still contained contaminants although these were successfully removed by re-applying the sample to a C_{18} flash cartridge and eluted stepwise, successfully removing the contaminants. Final purity of both microcystins by HPLC was determined to be 95% (Fig. 3), with the yield at this purity for microcystin-LW and -LF of 5.5 mg and 7.2 mg, respectively.

4. Conclusions

As previously reported [11], reversed-phase flash chromatography enabled sample concentration, and when combined with step elution, it provided an easy method for partial purification allowing simplification of subsequent chromatography. It was successfully used to separate the two microcystins of interest from both the other microcystins present in the sample and from a large proportion of the co-extracted contaminants.

The purification of microcystin-LW and -LF has previously presented difficulties for a number of reasons. Firstly, although analytical HPLC provides good separation [9], it utilises trifluoroacetic acid (TFA) which when applied to preparative separations, has been found to greatly reduce recovery due to effects on the stability of the microcystins. In particular, microcystin-LW has been observed to be particularly unstable in solutions containing TFA. Secondly, when alternative mobile phases have been investigated, separation deteriorated rapidly with increased sample loading. This was successfully overcome using closed-loop recycling where the toxins were passed through the column two additional times which enhanced resolution [11]. However, this requires specialised equipment and expertise, hence prompting the search for an alternative method.

Normal-phase flash chromatography was found to provide a suitable alternative. TLC provided a guide to a suitable solvent system, but did not result in the predicted separation when scaled-up to the 12 mm I.D. column. This was not unexpected since modes of elution in TLC compared to LC are very different especially in the presence of polar modifiers such as methanol and acetic acid. The 12 mm I.D. cartridges provided an ideal tool for rapid method optimisation with minimum consumption of sample and solvents. The optimised method was readily applied to a scaled-up purification. Although the mobile phase used was acidified (acetic acid), stability of the microcystins was not affected (unpublished data).

The combination of normal and reversed-phase flash chromatography yielded 5.5 mg (59%) of microcystin-LW and 7.2 mg (59%) of microcystin-LF at a purity of 95% determined by analytical HPLC. Less pure material was retained for reprocessing. Although a four stage process has been described in this paper, this could be simplified to three stages by replacing the 100% methanol elution stage with a aqueous methanol step gradient during the acid removal step.

In summary, two closely related, hydrophobic microcystins, which represent less than 0.1% of the cyanobacterial biomass, were concentrated and partially purified from an aqueous extract using re-

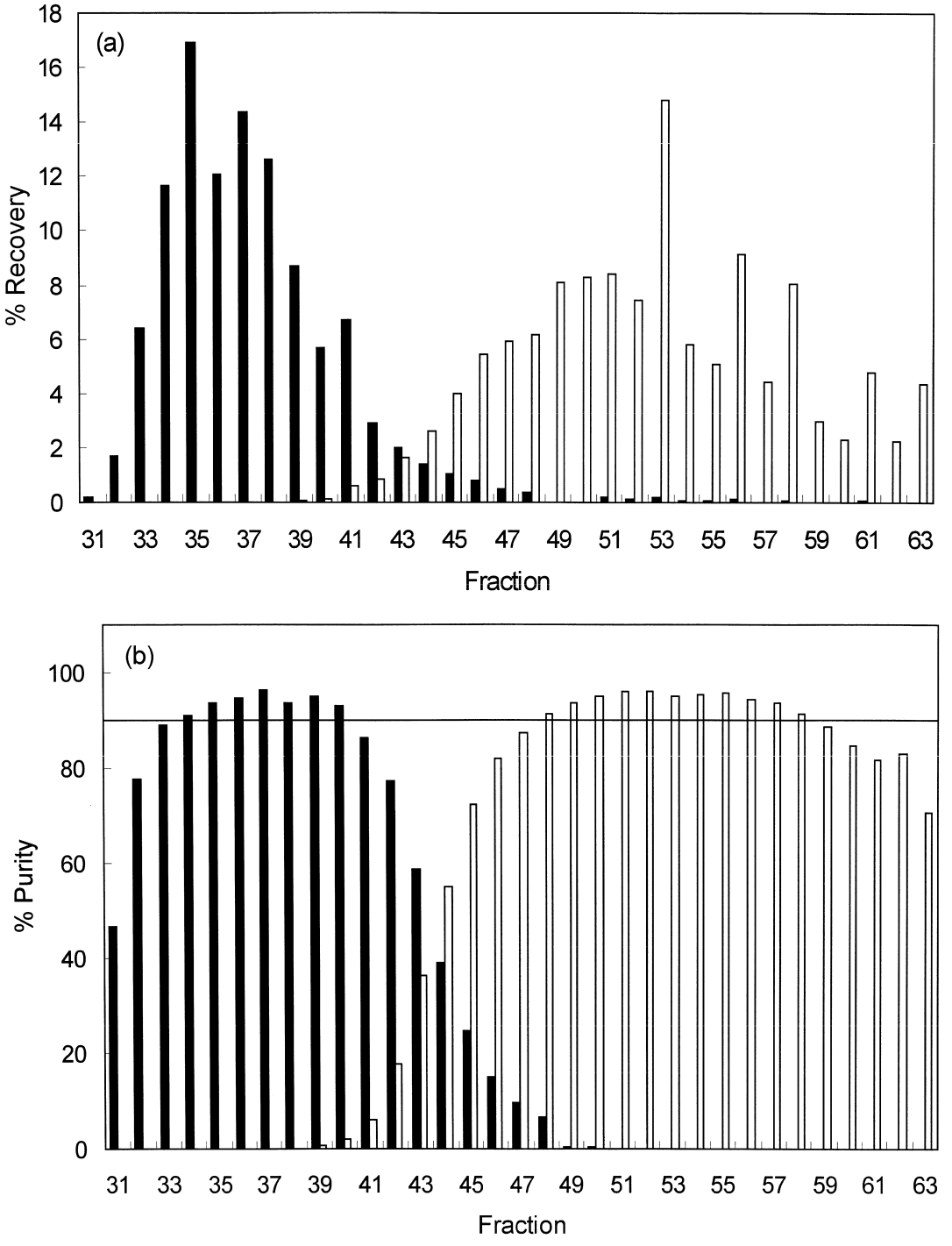

Fig. 2. Results of HPLC analysis of fractions collected from normal-phase flash separation indicating, (a) recovery and (b) purity of microcystin-LF (black) and microcystin-LW (white) in each fraction. The line across (b) indicates samples above 90% purity.

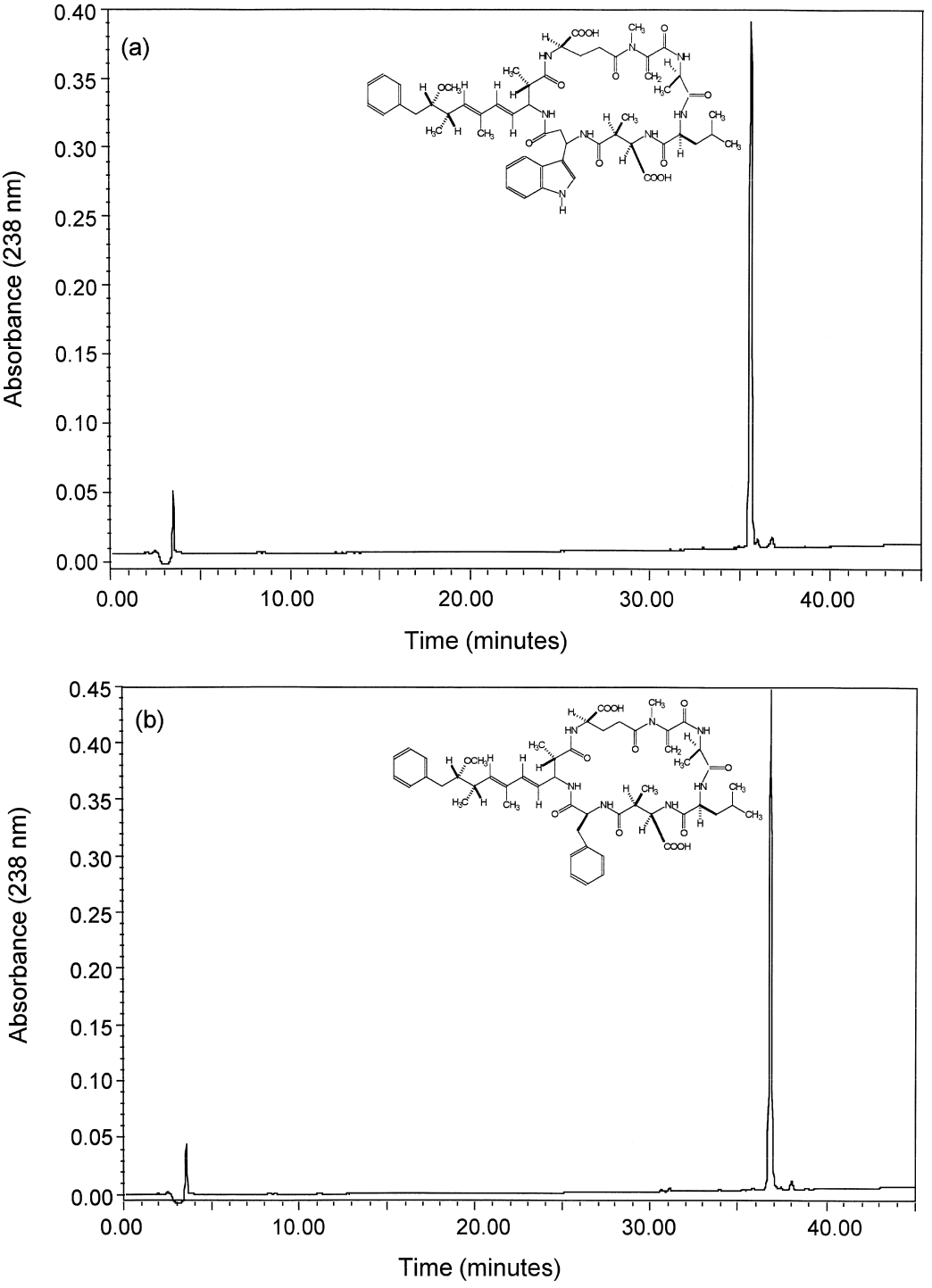

Fig. 3. Reversed-phase HPLC chromatograms of purified (a) microcystin-LW (1.93 μg injected) and (b) microcystin-LF (2.29 μg injected).

versed-phase flash chromatography. They were subsequently separated by normal-phase flash chromatography followed by acid removal and final polishing on reversed-phase flash to provide high purity microcystins.

Acknowledgements

The authors wish to thank Nathan Hammond for his assistance during the early development of this method.

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