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Rapid separation of microcystins and nodularin using a monolithic silica C_{18} column

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Abstract

A monolithic C_{18}-bonded silica rod column (Merck Chromolith) was compared to particle-based C_{18} and amide C_{16} sorbents in the HPLC separation of eight microcystins and nodularin-R. Two gradient mobile phases of aqueous trifluoroacetic acid modified with acetonitrile or methanol, different flow-rates and different gradient lengths were tested. The performance of the Chromolith column measured as the resolution of some microcystin pairs, the selectivity, efficiency (peak width) and peak asymmetry equalled, or exceeded, the performance of traditional particle-based columns. The Chromolith column allowed a shortening of the total analysis time to 4.3 min with a flow-rate 4 ml min^{-1}.

Keywords: Stationary phases, LC; Monolithic columns; Microcystins; Nodularin; Peptides; Toxins

1. Introduction

Microcystins and nodularins are hepatotoxic cyclic peptides produced by cyanobacteria (blue-green algae). Microcystins are produced by the freshwater cyanobacterial genera *Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*) and *Nostoc*, whereas nodularins are produced by the brackish water cyanobacterium *Nodularia*. Over 70 different analogues of microcystins and less than 10 analogues of nodularins have been isolated from natural blooms and laboratory cultures of cyanobacteria (for a recent review, see Ref. [1]). The general structure of microcystins (Fig. 1) is cyclo(D-Ala-L-**X**-D-*erythro*-methylAsp(iso-linkage)-L-**Z**-Adda-D-Glu(iso-linkage)-*N*-methyldehydro-Ala), where Adda is the unique β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid [2]. The main structural variation in microcystins is observed in the L-amino acid residues 2 (**X**) and 4 (**Z**), which are indicated by a two-letter suffix; for example, the common microcystin-LR contains leucine (L) in position 2 and arginine (R) in position 4. Nodularins are cyclic pentapeptides with the general structure cyclo(D-*erythro*-methylAsp(iso-linkage)-L-**Z**-Adda-D-Glu(iso-linkage)-2-(methylamino)-2(*Z*)-dehydrobutyric acid) (Fig. 1). Microcystins and nodularins are inhibitors of protein phosphatase 1 and 2A. The toxins are widely recognised as a public health problem due to their potent acute liver toxicity [LD_{50} (mouse, i.p.) is 50–500 μg kg^{-1} for most toxins] and their long-term effects as tumour promoters [1,3].

Complex microcystin mixtures have been found in cyanobacterial blooms. For example, 15 and 19

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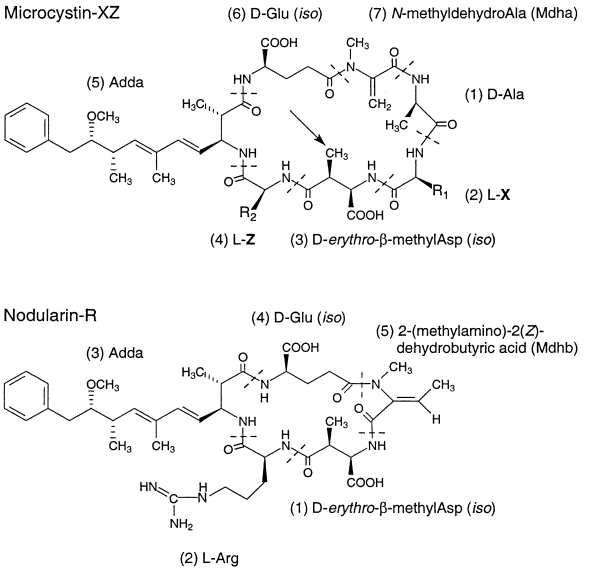

Fig. 1. Structure of microcystins-XZ (the desmethylation site in residue 3 is marked with an arrow) and nodularin-R.

microcystin congeners were reported in *Microcystis* blooms in the UK and the USA, respectively [4,5]. In order to elucidate the toxin profile and thus indirectly estimate the total toxicity of a complex sample, the chemical methods used for toxin analyses should be able to separate and quantify individual microcystins which have different toxicities. This calls for advanced separation methods of high resolution and good selectivity. On the other hand, microcystins and nodularins comprise a useful peptide family for studies of selectivity and other chromatographic properties in different stationary and mobile phase systems.

The most widespread analytical techniques for cyanobacterial peptide toxins are based on commercial enzyme-linked immunosorbent assays (ELISA) and HPLC coupled with photodiode-array UV detection (DAD) [6–8]. Most modern HPLC–DAD protocols for microcystins and nodularins rely on the use of high-resolution reversed-phase columns (typically 25 cm length with 5 μm particles or 15 cm length with 3–4 μm particles) in order to achieve sufficient resolution of the toxin analogues. The mobile phase usually consists of 0.05–0.1% trifluoroacetic acid in water and a gradient of acetonitrile which typically lasts from 25 to 45 min followed by the necessary re-equilibration [8–10]. Although this combination gives excellent performance, the throughput is only one to two samples per hour. The slow throughput is partly due to the high resistance to flow of particulate silica necessitating low flow-rates and partly due to the re-equilibration time required when using solvent gradients. As laboratories dealing with microcystin and nodularin samples can receive up to several hundreds or thousands of samples during the bloom period of cyanobacteria there is an apparent need to increase the analytical throughput without compromising the analytical performance.

Monoliths are special types of stationary phases. They can be made of synthetic organic material (such as acrylate and methacrylate), natural polymers (cellulose) or inorganic material (silica) [11]. The material can be casted in the form of disks, rods or tubes. Based on work carried out by Nakanishi and Soga [12], monolithic silica material has been manufactured using a sol–gel process which includes the hydrolysis and polycondensation of alkoxysilanes in the presence of water-soluble polymers. After ageing, the phase dries to form a rod with a bimodal pore structure consisting of large macropores (diameter 2 μm, comparable to the interstitial voids of a particle-packed column) and mesopores (13 nm in diameter, on the silica skeleton) [13]. The macropores allow high flow-rates due to low flow resistance enabling the analytes to be transported under low pressure to the active surface. The large internal surface of approximately 300 $m^2\ g^{-1}$ comprised by the mesopores facilitates rapid adsorption and desorption kinetics [13]. Monolithic HPLC sorbents have a significantly higher total porosity after octadecylsilylation than conventional particulate columns, over 80% vs. ca 65%, respectively [14]. The commercially available Chromolith monolith silica columns from Merck, which come with a C_{18}-bonded phase and endcapping, are comparable in selectivity to conventional reversed-phase columns [13]. It has been demonstrated that Chromolith columns are equivalent to 3.5–5 μm conventional columns with the additional advantage that flow-rates up to 9 ml min^{-1} can be used due to the flat plate height vs. mobile phase velocity curve [13,15,16].

Monoliths in various forms have been successfully used for the fast separation of biological macro-

molecules such as proteins and polynucleotides [11] and for smaller biomolecules such as the mycotoxin ochratoxin A [17]. Pharmaceutical applications include metabolites of the drug debrisoquine [18], β-blocking drugs [13], preparative isolation of cyclosporin A [19] and separation of drug intermediate diastereomers [20]. In this study we investigated whether a monolithic C_{18}-bonded silica rod (Merck Chromolith) can be applied to the separation of the cyanobacterial peptide toxins microcystins and nodularins. The performance of the monolith column was compared to those of conventional particle-based reversed-phase columns in terms of resolution, selectivity, efficiency (peak width), asymmetry, total analysis time and solvent consumption.

2. Experimental

2.1. Chemicals

HPLC-grade methanol and HPLC S-grade acetonitrile were from Rathburn (Walkerburn, UK). Trifluoroacetic acid (TFA) of protein sequencing grade was from Fluka (Buchs, Switzerland). Water was purified to 18.2 MΩ cm on a Milli-Q plus PF system (Millipore, Molsheim, France)

2.2. HPLC apparatus

The HPLC system consisted of a Degasys DG-2410 degasser from Uniflows (Tokyo, Japan), and an FCV-10AL gradient mixer, an LC-10AT pump and a SIL-9A autosampler from Shimadzu (Kyoto, Japan). Column temperature was regulated with a laboratory-constructed heater (based on water circulation around a dry column compartment made of aluminium). UV detection was performed with a Merck–Hitachi (Darmstadt, Germany) L-7450A photodiode-array detector at 200–300 nm. Spectral bandwidth was 2 nm and spectral interval 100 ms. The absorbance data were analysed with Hitachi D-7000 HPLC System Manager (HSM) software, version 3.1.1, and absorbance data at 238 nm were exported as ASCII files into Microsoft Excel software in order to make publication quality figures.

2.3. Stationary and mobile phases

The Chromolith Performance RP-18e (100 mm×4.6 mm I.D.) and LiChrospher 100 RP-18e LiChroCART (5 μm particles, 125 mm×4 mm I.D.) columns were from Merck (Darmstadt, Germany). A 4 mm×3 mm I.D. Phenomenex (Torrance, CA, USA) C_{18} guard column was used for both columns. For reference purposes, a 150 mm×2.1 mm I.D. RP-Amide C_{16} column from Supelco (Bellefonte, PA, USA) with a 4 mm×2 mm I.D. C_{18} guard column (Phenomenex) was employed [10].

To enable a direct comparison of the performance of the Chromolith and LiChrospher columns, the same mobile phases (with different flow-rates) were used for both. Two mobile phase systems were tested: TFA–MeCN eluent: eluent A: 0.05% aqueous trifluoroacetic acid (TFA), eluent B: 0.05% TFA in acetonitrile, and TFA–MeOH eluent: eluent A: 0.05% aqueous TFA, eluent B: 0.05% TFA in methanol.

The TFA–MeCN gradients were run at 25–70% MeCN and the TFA–MeOH gradients at 45–90% MeOH with the Chromolith and LiChrospher columns. All gradients were linear. The used mobile and stationary phase combinations are listed in Table 1. The highest percentage of the organic component was held for 1 ml (0.25–1 min depending on flow-rate) after the gradient rise. The columns were re-equilibrated with 6 ml (1.5–6 min) of the starting eluent between runs. Column temperature was 25 °C for the Merck columns in order to avoid temperature gradients inside the columns possibly arising from a high flow of mobile phase at room temperature. The reference column, amide C_{16}, was run with the TFA–MeCN mobile phase with the following gradient programme: 0 min 20% B, 25 min 65% B, 27 min 65% B, 28 min 20% B; run interval 45 min; flow-rate 0.3 ml min^{-1}; temperature 40 °C [10].

2.4. Sample preparation

Microcystins of varying hydrophobicity and nodularin-R were chosen as analytes. Microcystin-RR variants with their two guanidino groups are among the most hydrophilic microcystins, whereas microcystin-LW and -LF with their aromatic tryptophan and phenylalanine residues, respectively,

Table 1
Chromatographic data of microcystins in different mobile and stationary phase combinations. Negative resolution values indicate reversed elution order. The values in the three rightmost columns were determined with a sample consisting of microcystin-LR only (159 ng/10 μl injection). All separations were performed at 25 °C except for the separation with the amide C_{16} column, which was run at 40 °C

Stationary phase	Mobile phase	Gradient (% organic modifier)	Length of gradient rise (min)	Total run length (min)	Flow rate (ml min $^{-1}$)	Resolution				Selectivity k(LF)/ k(3-dm-RR)	Retention time of LR (min)	Capacity factor of LR	Width of LR at half height (s)	Asymmetry of LR	Relative area of LR
						3-dm-RR vs. RR	YR vs. LR	3-dm-LR vs. LR	LW vs. LF						
Chromolith	TFA–MeCN	25–70	1.25	4.8	2.0	0.74	0.68	0.00	0.81	1.6	2.10	2.1	1.5	1.27	2.00
Chromolith	TFA–MeCN	25–70	2.5	6.0	2.0	1.20	1.02	0.00	1.31	1.9	2.52	2.6	1.7	1.14	1.99
Chromolith	TFA–MeCN	25–70	5.0	8.5	2.0	1.89	1.66	0.00	1.62	2.3	3.17	3.6	2.1	1.05	2.02
Chromolith	TFA–MeCN	25–70	1.25	3.0	4.0	1.22	1.02	0.00	1.17	1.9	1.24	2.8	0.9	1.14	1.06
Chromolith	TFA–MeCN	25–70	2.5	4.3	4.0	1.77	1.56	0.00	1.53	2.3	1.56	3.7	1.1	0.99	1.02
Chromolith	TFA–MeCN	25–70	5.0	6.8	4.0	2.24	1.97	0.00	1.78	2.9	2.03	5.2	1.6	1.00	1.00
Chromolith	TFA–MeOH	45–90	1.25	4.8	2.0	0.00	1.04	0.00	0.77	1.4	2.33	2.2	1.7	1.33	2.13
Chromolith	TFA–MeOH	45–90	2.5	6.0	2.0	0.77	1.88	0.00	1.35	1.6	2.89	3.0	2.0	1.18	2.14
Chromolith	TFA–MeOH	45–90	5.0	8.5	2.0	1.09	3.26	−0.87	2.04	1.9	3.79	4.3	2.7	1.10	2.10
Chromolith	TFA–MeOH	45–90	1.25	3.0	4.0	0.73	1.86	0.00	0.85	1.6	1.46	3.2	1.1	1.20	1.06
Chromolith	TFA–MeOH	45–90	2.5	4.3	4.0	1.00	2.91	0.00	1.92	1.9	1.86	4.3	1.5	1.08	1.08
Chromolith	TFA–MeOH	45–90	5.0	6.8	4.0	1.08	3.86	−1.35	2.25	2.4	2.54	6.3	2.3	1.03	1.08
LiChrospher	TFA–MeCN	25–70	7.5	14.5	1.0	1.38	1.76	0.81	1.26	1.7	6.57	6.2	5.2	1.21	4.52
LiChrospher	TFA–MeCN	25–70	5.0	9.7	1.5	1.32	1.67	0.75	1.24	1.7	4.34	6.2	3.6	1.18	2.85
LiChrospher	TFA–MeOH	45–90	7.5	14.5	1.0	0.66	1.64	0.00	1.34	1.5	7.56	6.8	6.7	1.17	4.69
LiChrospher	TFA–MeOH	45–90	5.0	9.7	1.5	0.00	1.52	0.00	1.27	1.5	5.04	7.0	4.9	1.14	2.80
Amide C_{16}	TFA–MeCN	20–65	25	45	0.3	2.00	2.42	1.02	0.74	2.4	14.5	7.5	10.2	1.22	7.60

represent more hydrophobic toxin analogues. Methanolic extracts were prepared from samples of *Microcystis* PCC7820 (deposited at Institut Pasteur, Paris, France) and *Anabaena* sp. 90 (culture collection of Professor Kaarina Sivonen, University of Helsinki, Finland). *Microcystis* PCC7820 has been shown to produce microcystin-LR, -LY, -LW and -LF [9,10] and *Anabaena* sp. 90 microcystin-LR and -RR and 3-desmethyl variants of -LR and -RR [10,21]. The pooled extracts were diluted with water to 20% methanol, after which they were concentrated on a BondElut C_{18} solid-phase extraction cartridge (Varian, Harbour City, CA, USA). The toxins were eluted with methanol and spiked with a commercial standard of microcystin-YR (Calbiochem, La Jolla, CA, USA) and methanolic extracts of Baltic Sea *Nodularia* containing nodularin-R. The toxins were then diluted with water to 75% methanol and centrifuged (10 min, 10 000 *g*). The product, a mixture of microcystins and nodularin-R (MC-MIX1), was stored at −20 °C until use. Runs of MC-MIX1 were complemented by runs with fewer toxins or with microcystin-LR only. The following abbreviations are used in the table and figures: RR, microcystin-RR; YR, microcystin-YR; LR, microcystin-LR; LY, microcystin-LY; LW, microcystin-LW; LF, microcystin-LF; 3-dm, 3-desmethyl; Nodln-R, nodularin-R.

The calibration curves for microcystin-LR were constructed from injections of dilutions of purified microcystin-LR. The concentration of the stock solution was determined spectrophotometrically at 238 nm according to method E in Ref. [22]. Dilutions were made in 75% aqueous methanol.

2.5. Calculations of chromatographic parameters

The retention time for an unretained peak was determined by injecting 1 μl of 0.1 *M* $NaNO_3$. Resolution calculations were usually made by the HSM program from runs of MC-MIX1. The calculations were complemented by (manual) calculations based on runs with fewer toxin analogues. Asymmetry was assessed at 5% of peak height.

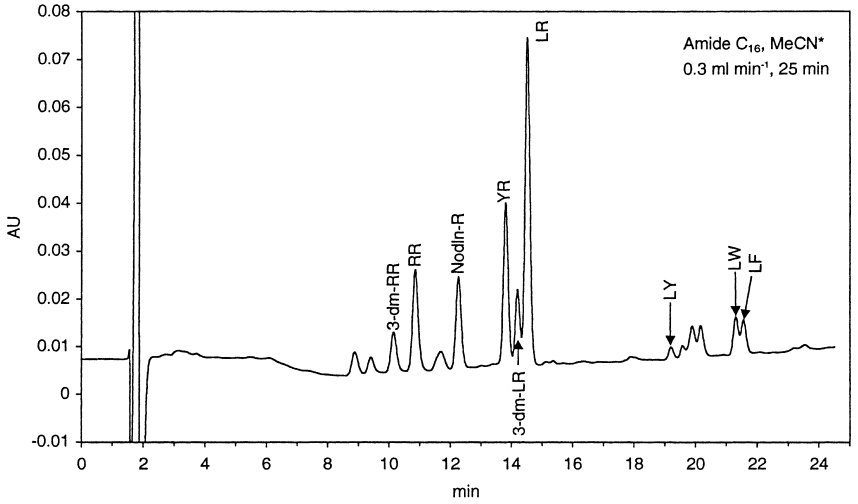

Fig. 2. Chromatogram of MC-MIX1 (121 ng microcystin-LR per 10 μl injection) on the amide C_{16}, 150 mm×2.1 mm I.D., column. Mobile phase TFA–MeCN; A: 0.05% aqueous trifluoroacetic acid (TFA), B: 0.05% TFA in acetonitrile. Flow-rate: 0.3 ml min^{-1}. Linear gradient programme: 0 min 20% B, 25 min 65% B, 27 min 65% B, 28 min 20% B. Detection, absorbance at 238 nm; temperature, 40 °C.

3. Results

A reference chromatogram with the amide C_{16} column is shown in Fig. 2 and some calculated chromatographic parameters are shown in Table 1. The amount of microcystin-LR in MC-MIX1 was 121 ng per 10 μl injection. The amide C_{16} column resolved all the toxins in the mixture, although the resolution of microcystin-LW vs. -LF was low. The ratio of the capacity factors of microcystin-LF and 3-desmethylmicrocystin-RR, a measure of the selectivity of the column, was 2.4.

The high-speed separations with the Chromolith column were generally characterised by good resolution and efficiency, and low backpressures. The following backpressures were registered at 0 min of the gradients (total backpressure including the HPLC system, at 25 °C): Chromolith/TFA–MeCN 4 ml min^{-1} 92 bar, 2 ml min^{-1} 48 bar; Chromolith/TFA–MeOH 4 ml min^{-1} 140 bar, 2 ml min^{-1} 75 bar; LiChrospher/TFA–MeCN 1.5 ml min^{-1} 103 bar, 1 ml min^{-1} 68 bar; LiChrospher/TFA–MeOH 1.5 ml min^{-1} 166 bar, 1 ml min^{-1} 110 bar. The total backpressure of the amide C_{16} column was 70 bar at 40 °C and 0.3 ml min^{-1}.

Chromatographic performance of the Merck columns is described in Table 1 and Figs. 3–5. Chromolith: longer gradient lengths (2.5 and 5.0 min) gave clearly better resolutions for the studied microcystin pairs as compared to the short 1.25 min gradient (Table 1 and Figs. 3 and 5) and visual examination of the chromatograms verified the overall superiority of the longer gradients (Fig. 3). Resolution was also clearly enhanced by the higher flow-rate (4 ml min^{-1}) (Table 1). According to our judgement, the best combination of overall performance and rapid throughput was achieved with the TFA–MeCN mobile phase, 2.5 min gradient length and 4.0 ml min^{-1} flow-rate (Fig. 3, lower left chromatogram). This combination gave good resolution for all other analytes but for the pair 3-desmethylmicrocystin-LR and microcystin-LR. The range of capacity factors in this case was from 2.7 (3-desmethylmicrocystin-RR) to 6.2 (microcystin-LF). Selectivity expressed as the ratio of the capacity factors of microcystin-LF and 3-desmethylmicrocystin-RR was 2.3. The total analysis time including re-equilibration was 4.3 min (14 samples per hour). If a lower consumption of eluents is desired, a flow-rate of 2.0 ml min^{-1} can be used. LiChrospher: the performance of the LiChrospher column and the TFA–MeCN mobile phase is adequate (Table 1 and Fig. 4) in many instances, but, generally speaking, lower than that of Chromolith. For example, the

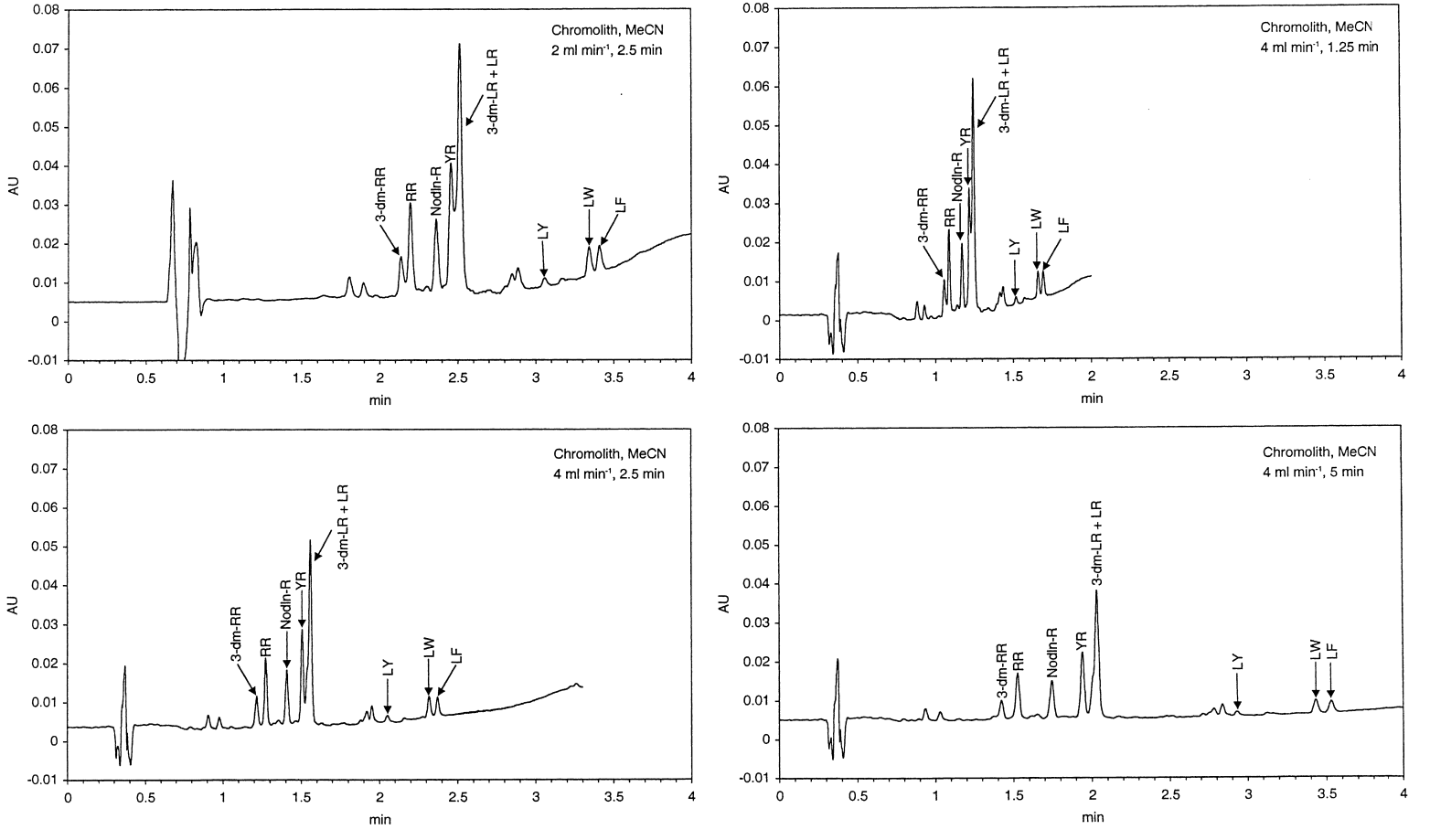

Fig. 3. Chromatograms of MC-MIX1 (121 ng microcystin-LR per 10 μl injection) with the TFA–MeCN mobile phase (25–70% MeCN) and Chromolith column. Flow-rate and length of gradient rise are indicated in the chromatogram panels. Detection, absorbance at 238 nm; temperature, 25 °C.

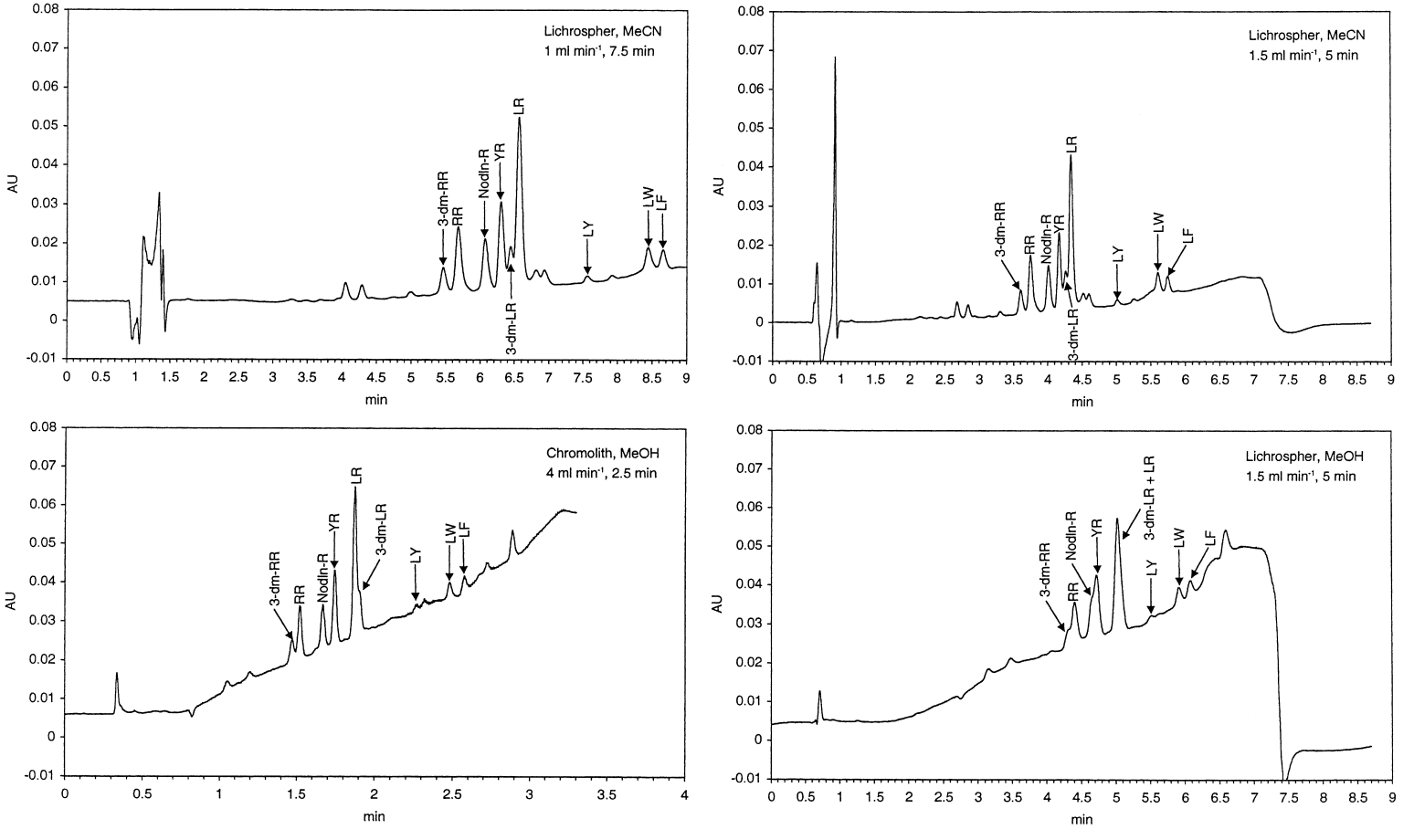

Fig. 4. Chromatograms of MC-MIX1 (121 ng microcystin-LR per 10 μl injection) with the TFA–MeCN mobile phase (25–70% MeCN) and LiChrospher column, and with the TFA–MeOH mobile phase (45–90% MeOH) and LiChrospher and Chromolith columns. Flow-rate and length of gradient rise are indicated in the chromatogram panels. Detection, absorbance at 238 nm; temperature, 25 °C.

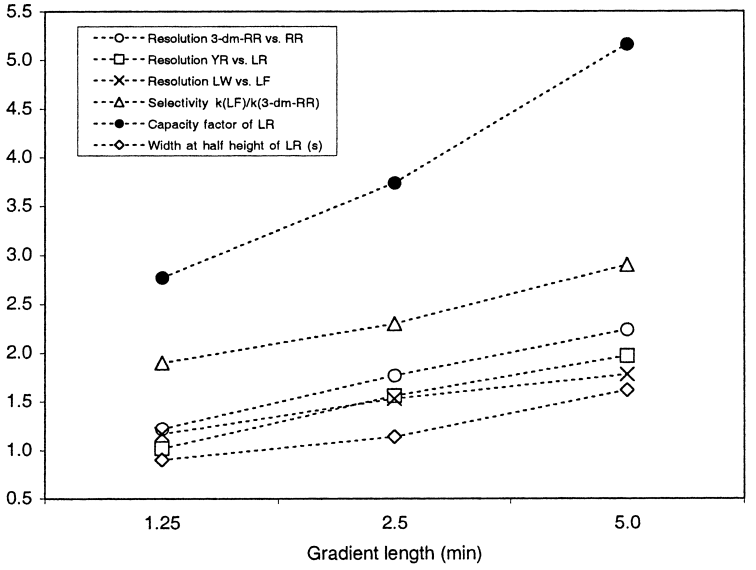

Fig. 5. Effect of the length of gradient rise on some chromatographic parameters. Conditions: TFA–MeCN mobile phase (25–70% MeCN), Chromolith column, flow-rate 4 ml min^{-1}, temperature 25 °C.

selectivity of microcystin-LF vs. 3-desmethyl-microcystin-RR was only 1.7 with the TFA–MeCN eluent (Table 1). Also, the total analysis times were longer than those with the Chromolith column.

The chosen percentages of organic modifiers, 25–70% for acetonitrile and 45–90% for methanol, gave comparable capacity factors for microcystin-LR (Table 1), showing that the higher methanol percentages were necessary. The mobile phase consisting of aqueous trifluoroacetic acid and methanol (TFA–MeOH) could not be regarded as a primary choice for microcystin separations with either LiChrospher or Chromolith columns. This was due to high backpressures, lower efficiencies (broader width at half height of microcystin-LR), coelution of some toxins and high UV background at higher percentages of methanol (Table 1 and Fig. 4). However, some microcystin pairs, for example microcystin-YR vs. -LR, were better resolved with TFA–MeOH on the Chromolith column (Table 1). A peak reversal of 3-desmethylmicrocystin-LR and -LR was observed with the Chromolith column and TFA–MeOH mobile phase.

The limit of detection (LOD, $S/N = 3$) and the limit of quantitation (LOQ, $S/N = 10$) for microcystin-LR were determined for some separations. The approximate LODs and LOQs, respectively, were 0.7 and 2 ng per 10 μl injection (amide C_{16}), 1 and 3 ng per 10 μl injection (LiChrospher, TFA–MeCN mobile phase, flow-rate 1.5 ml min^{-1}), and 3 and 10 ng per 10 μl injection (Chromolith, TFA–MeCN mobile phase, flow-rate 4 ml min^{-1}, length of gradient rise 2.5 min).

4. Discussion

We regard the Chromolith column as a useful tool for rapid separations of microcystins and nodularin. The short total analysis time, 4.3 min in the preferred chromatographic system, allows a high throughput and rapid analytical answers. In comparison, the total analysis time in commonly used chromatographic systems for microcystins is usually 45–60 min [9,10].

The chromatographic performance of the Chromolith column was comparable to that of the amide C_{16} reference column and good separation of most toxin analogues was achieved. The elution order of most microcystins was as predicted by the retention values of the variable amino acid residues [23]. However, a reversal of microcystin-LW and -LF was observed. This is in accordance with earlier runs on other C_{18} columns [10]. The total solvent

consumption was 17.2 ml in the 4.3 min/4 ml min^{-1} Chromolith run and 13.5 ml in the amide C_{16} run. The high flow-rates of the Chromolith runs require splitting in LC–MS work and this consequently raises the detection limit, which could be critical in some analyses. The generally higher limit of detection with the Chromolith column is partly due to the higher background noise resulting from the imperfect mixing of the mobile phase components at high flow-rates. This behaviour is typical for the used low-pressure gradient instrumentation where the opening and closing times of solenoid valves proportioning solvents are controlled for every four cycles of pump operation at flow-rates equal to or higher than 2 ml min^{-1}. However, there were no problems with the reproducibility of the retention times.

The lack of resolution between 3-desmethyl-microcystin-LR and -LR (with the Chromolith column and to a lesser extent with the LiChrospher column) is a common problem in many C_{18} separations run in TFA–acetonitrile. In a previous paper we suggested the use of a 25-cm amide C_{16} column to overcome this separation problem [10].

We expect to see many new applications for the Chromolith and other monolith columns in the near future. A miniaturisation of the column dimensions [24,25] to reduce solvent consumption would render the monolith columns even more useful as general-purpose columns for rapid separations.

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