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High-performance liquid chromatographic separation of microcystins and nodularin, cyanobacterial peptide toxins, on C_{18} and amide C_{16} sorbents

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Abstract

Four C_{18} columns and a novel amide C_{16} column were assessed in the HPLC separation of eight microcystins and nodularin-R. Gradient mobile phases of acetonitrile combined with trifluoroacetic acid, formic acid or ammonium acetate were compared. Special attention was paid to the resolution of four possible coeluting microcystin pairs. Generally speaking, the acidic mobile phases were superior to the ammonium acetate-based mobile phase in terms of resolution and selectivity. The amide C_{16} column had the best overall performance and unique selectivity properties.

Keywords: Stationary phases, LC; Mobile phase composition; Microcystins; Toxins; Nodularin

1. Introduction

Microcystins and nodularins are potent liver toxins and tumour promoters from cyanobacteria also known as blue–green algae. Microcystins are produced by several common freshwater cyanobacterial genera including *Microcystis*, *Anabaena* and *Oscillatoria* (*Planktothrix*) while nodularin is produced by the brackish water cyanobacterium *Nodularia*. More than 60 different analogues of microcystins and about 10 analogues of nodularins have been described from natural blooms or laboratory cultures of cyanobacteria (for reviews, see Refs. [1–3]). The general structure of microcystins is cyclo(-D-Ala–L–*X* – D - *erythro* - methylAsp – L - *Z* - Adda–D - Glu – *N* - methyldehydro-Ala) where Adda stands for the unique β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid [4] (Fig. 1). The main structural variation in microcystin analogues is seen in the L-amino acid residues 2 (*X*) and 4 (*Z*) which are indicated by a two-letter suffix, e.g., the common microcystin-LR contains leucine (L) in position 2 and arginine (R) in position 4. Altered residues other than 2 and 4 are often described by a prefix: thus [D-Asp^3]microcystin-LR contains a desmethylated residue, D-aspartic acid, in position 3. In this paper, a common alternative notation is used for the desmethylated toxins, e.g., 3-desmethylmicrocystin-LR for [D-Asp^3]microcystin-LR. Microcystins are relatively polar molecules (due to the carboxylic acids in positions 3 and 6 and the frequent presence of arginine in positions 2 and 4)

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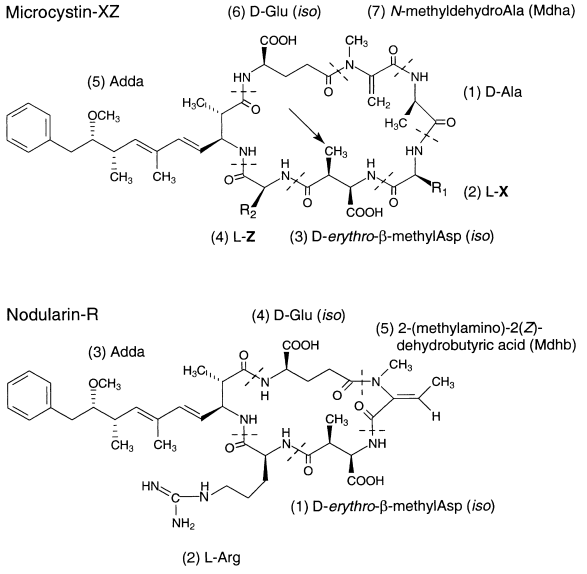

Fig. 1. Structures of microcystin-XZ (the 3-desmethylation site is marked with an arrow) and nodularin-R.

with some more hydrophobic parts like the Adda residue or hydrophobic substituents as the variable amino acids. Nodularins are cyclic pentapeptides with the general structure cyclo(-D-*erythro*-methylAsp-L-*Z*-Adda-D-Glu-2-(methylamino)-2(*Z*)-dehydrobutyric acid). The second residue (designated as *Z* above) is L-Arg in the common nodularin-R (Fig. 1). The lethal dose giving 50% deaths (LD_{50}) values (mouse, i.p.) for the different microcystins and nodularins vary from about 50 μg kg^{-1} to over 1 mg kg^{-1}. Minor differences in structure may lead to pronounced differences in toxicity. For instance, the reported LD_{50} values (mouse, i.p.) are 180–250 μg kg^{-1} for desmethylmicrocystin-RRs but 600 μg kg^{-1} for microcystin-RR [3]. In contrast, desmethylmicrocystin-LRs are weaker toxins (LD_{50} 90–300 μg kg^{-1}) than microcystin-LR (50 μg kg^{-1}) [3].

Cyanobacterial blooms can produce very complex microcystin profiles. Fifteen and 19 microcystins were reported in *Microcystis* blooms in the UK and USA, respectively [5,6]. Complex microcystin patterns together with the different potencies of the toxins call for sophisticated separation methods. On the other hand, microcystins and nodularins constitute a unique family of closely related cyclic peptides for chromatographic studies. They comprise a useful peptide material for studies of selectivity and other chromatographic properties in different stationary and mobile phase systems. In addition, they are a good target for studies of derivatisation techniques and detection methods based on, e.g., fluorimetry [7] or chemiluminometry [8].

Reversed-phase high-performance liquid chromatography (HPLC) on C_{18} or C_8 phases is a common choice for separating smaller peptides, e.g., those occurring naturally or present in enzymic digests of proteins [9]. The mobile phases for peptides often consist of acetonitrile gradients in the presence of perfluorinated alkyl carboxylic acids, usually trifluoroacetic acid (TFA). Addition of TFA improves the chromatographic efficiency by maintaining a low pH in order to protonate carboxylic acid groups in peptides. It also acts as an ion-pairing agent that increases retention of poorly retained peptides and minimises the interaction between basic groups and silanol groups on the silica surface. In theory, it is possible to resolve mixtures of tens of peptides in these chromatographic systems but, in reality, analytes with minor differences tend to overlap.

Reversed-phase separations of microcystins and nodularins follow the general trends in peptide chromatography [10]. The mobile phases used in microcystin separations fall into three categories: neutral eluents with ammonium acetate and acetonitrile, methanol-containing eluents with different buffers and pH values, and acidic eluents with TFA and acetonitrile. Neutral ammonium acetate-based eluents are commonly used in preparative toxin separations, e.g., Ref. [5], but also in analytical chromatography [11]. In some cases, acidic conditions have to be avoided due to the instability of tryptophan-containing microcystins.

Acidic mobile phase systems are generally considered to be capable of separating more microcystin variants than neutral mobile phases [10]. Guo and co-workers developed retention coefficients for amino acid residues and they could predict the retention times of small linear peptides (not microcystins) on reversed-phase HPLC with water–acetonitrile–0.1% TFA gradients [12,13]. These coefficients should give an indication of the elution order of microcystins in TFA–acetonitrile eluents. Lawton et al. [14] as well as Wicks and Thiel [15] developed chromatographic procedures that permit

the determination of several microcystin variants on C_{18} columns with water–acetonitrile–TFA-based eluents. The observed elution orders match the predicted ones [12] within the microcystin-XR and microcystin-LZ series with one exception: the elution order of microcystin-LW and -LF is reversed. Internal surface reversed-phase HPLC [16,17], which has both reversed-phase and cation-exchange characteristics, and anion-exchange HPLC [18] have been advocated as alternative techniques in microcystin separations. However, the potential of stationary phases other than C_{18} has not been fully evaluated in the separation of microcystins.

There are some microcystin pairs, which are difficult to separate in certain chromatographic systems. For example, microcystin-LR and -YR co-eluted in a C_{18} system using 26% acetonitrile in 10 m*M* ammonium acetate but they were easily separated by anion-exchange HPLC [18]. Another often-encountered problem is the separation of desmethylated toxins from their non-desmethylated analogues (see later in this paper). Microcystin-LW and -LF constitute a further example of a microcystin pair which is difficult to separate, especially in preparative runs of lower resolution [5].

The aim of this work was to compare the chromatography of eight different microcystins and nodularin-R in different gradient mobile phases and on different reversed-phase sorbents. Four C_{18} sorbents and an amide C_{16} sorbent [with the bonded-phase ligand $Si–(CH_2)_3NHCO(CH_2)_{14}CH_3$] were tested as stationary phases. Among the chromatographic parameters studied special attention was paid to the resolution of the above described microcystin pairs (desmethyl microcystins vs. non-desmethylated ones, -YR vs. -LR, and -LW vs. -LF).

2. Experimental

2.1. Chemicals

HPLC-grade methanol was purchased from Baker (Deventer, The Netherlands) and HPLC S-grade acetonitrile from Rathburn (Walkerburn, UK). TFA of protein sequencing grade was from Fluka (Buchs, Switzerland). The following reagents were of analytical-reagent grade: formic acid from Riedel-de Haën (Seelze, Germany) and ammonium acetate from Sigma (St. Louis, MO, USA). Amino acid standards and *o*-phthaldialdehyde reagent solution (P-0532) were from Sigma. 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), an FCV-10AL gradient mixer, an LC-10AT pump and an SIL-9A autosampler from Shimadzu (Kyoto, Japan). The HPLC columns were thermostated to 40°C 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 800 ms. The absorbance data was 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 phases

Column and run parameters are presented in Table 1. The Nucleosil column was from Phenomenex (Torrance, CA, USA). The Discovery columns were from Supelco (Bellefonte, PA, USA). The Nucleosil and Discovery columns were protected by a C_{18} guard column, 4 mm×3 mm I.D., from Phenomenex. The Purospher and LiChrospher LiChroCART columns were from Merck (Darmstadt, Germany) and protected by a 4 mm×4 mm I.D. Purospher RP-18e guard column (Merck).

2.4. Mobile phases

Four different mobile phase combinations were tested.

(I) TFA1; eluent A: 0.05% TFA, eluent B: 100% acetonitrile (no TFA in acetonitrile).

(II) TFA2; eluent A: 0.05% TFA, eluent B: 0.05% TFA in acetonitrile.

Table 1
Column and run characteristics

Column	Particle size (μm)	Poresize (Å)	Pore volume (ml g^{-1})	Surface area (m^2 g^{-1})	Carbon load (%)	Endcapped	Length (mm)	I.D. (mm)	Flow-rate (ml min^{-1})	Time for $NaNO_3$ (min)
Nucleosil C_{18} (specifications[a])	5	100	1.0	350	14	Yes	250	4.6	1.00	2.33
Discovery C_{18} (lot analysis)	4.11 (mean)	185	0.97	199	12.54	Yes	250	4.6	1.00	2.91
Discovery RP-AmideC_{16} (lot analysis)	4.09 (mean)	181	0.98	199	11.54	Yes	250	4.6	1.00	3.02
Purospher RP-18e (specifications[b])	5	120	1.0	350	18.0	Yes	250	4.0	0.75	2.47
LiChrospher RP-18e (specifications[c])	5	100	1.25	350	21.6	Yes	250	4.0	0.75	2.33

[a] From Ref. [23].
[b] From Merck Technical Service.
[c] From Ref. [24].

(III) Formic acid; eluent A: 0.5% formic acid, eluent B: 0.2% formic acid in acetonitrile.

(IV) Ammonium acetate; eluent A: 0.0105 *M* ammonium acetate–acetonitrile (95:5), eluent B: 0.05 *M* ammonium acetate–acetonitrile (20:80).

The acidic mobile phases were used for a maximum period of 6 days without renewal (no stability problems encountered in test runs) while the ammonium acetate-based eluents were renewed daily. Gradient conditions were chosen according to the following criteria: (1) gradient steepness should not exceed 1.3% units of the organic modifier per min. (2) Microcystin-LR should elute at about 15–20 min and the hydrophobic microcystins LW and LF at about 30 min. The retention factors (*k*) of the toxins should be in the range of 2–12. (3) The maximum allowed total run time was determined to be 1 h. The linear gradient programme for the acidic mobile phases was: 0 min 25% B, 35 min 70% B, 37 min 70% B, 38 min 25% B, 60 min 25% B. The linear gradient programme for the ammonium acetate-based mobile phase was: 0 min 15% B, 35 min 60% B, 37 min 60% B, 38 min 15% B, 60 min 15% B. Samples were run at 1-h intervals and sample volume was 10 μl. Flow-rate was 1 ml min^{-1} for 4.6 mm I.D. columns and 0.75 ml min^{-1} for 4 mm I.D. columns.

2.5. Sample preparation

Methanolic extracts were prepared from samples of *Microcystis aeruginosa* PCC7820 (deposited at Institut Pasteur, Paris, France) and *Anabaena* sp. 90 (culture collection of Dr. Kaarina Sivonen, University of Helsinki, Finland). *Microcystis aeruginosa* PCC7820 has been previously shown to produce microcystin-LR, -LY, -LW and -LF [14] and *Anabaena* sp. 90 microcystin-LR and -RR and 3-desmethyl variants of -LR and -RR [19]. The extracts were diluted with water to 20% methanol after which they were concentrated on BondElut C_{18} solid-phase extraction cartridges (Varian, Harbour City, CA, USA). The toxins were eluted in methanol, combined, spiked with a commercial standard of microcystin-YR (Calbiochem, La Jolla, CA, USA) and methanolic extracts of Baltic Sea *Nodularia* containing nodularin-R, and centrifuged (10 min, 10 000 *g*). The product, a methanolic mixture of microcystins and nodularin-R (MC-MIX), was stored at −20°C until use. The following abbreviations are used in the tables 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.

2.6. Toxin identification

The identities of the microcystins and nodularin-R in MC-MIX were confirmed by amino acid analysis (*o*-phthaldialdehyde label) of purified toxins isolated from the same sources [20] and by liquid chromatography–mass spectrometry (LC–MS) of MC-MIX. Amino acid analysis was done primarily in order to clarify the desmethylation site of the desmethylated

Table 2
LC–MS data of microcystins and nodularin-R[a]

Microcystin/nodularin	Observed *m/z* (partial data)	Structure of fragment	Calculated mass of M (u)
3-dm-RR	1024.7	$[M+H]^+$	1023
	513.2	$[M+2H]^{2+}$	
RR	1038.8	$[M+H]^+$	1037
	520.1	$[M+2H]^{2+}$	
Nodln-R	825.5	$[M+H]^+$	824
YR	1045.7	$[M+H]^+$	1044
3-dm-LR	981.8	$[M+H]^+$	980
LR	995.9	$[M+H]^+$	994
LY	1002.5	$[M+H]^+$	1001
LW	1025.9	$[M+H]^+$	1024
LF	986.6	$[M+H]^+$	985

[a] Chromatographic conditions as described in the Experimental section.

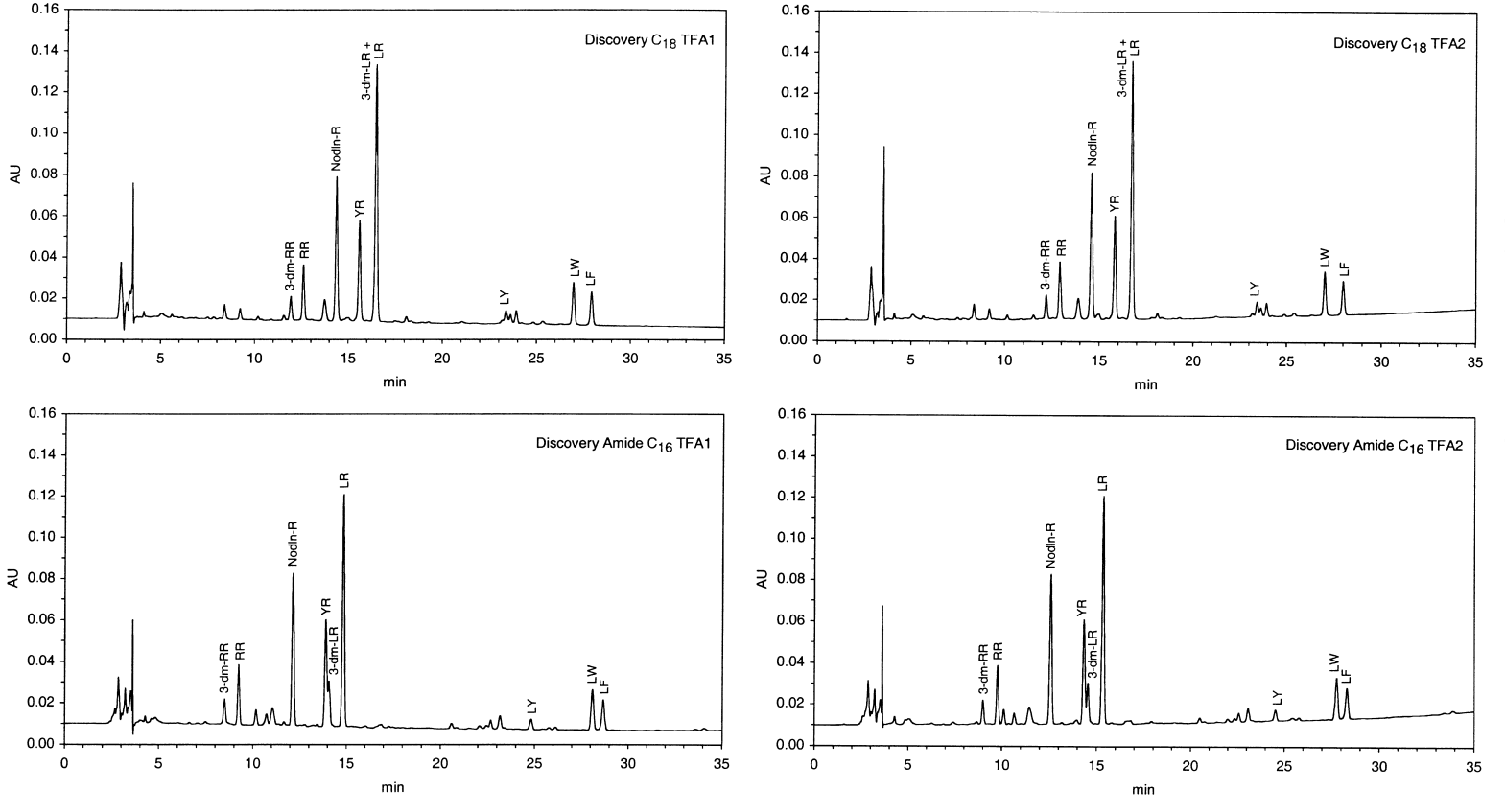

Fig. 2. Chromatograms of MC-MIX in the TFA1 and TFA2 mobile phases with the Discovery columns. Chromatographic conditions were as described in the Experimental section. Chromatograms shown at 238 nm. The amount of microcystin-LR was ca. 0.5 μg/10 μl injection. AU=Absorbance units.

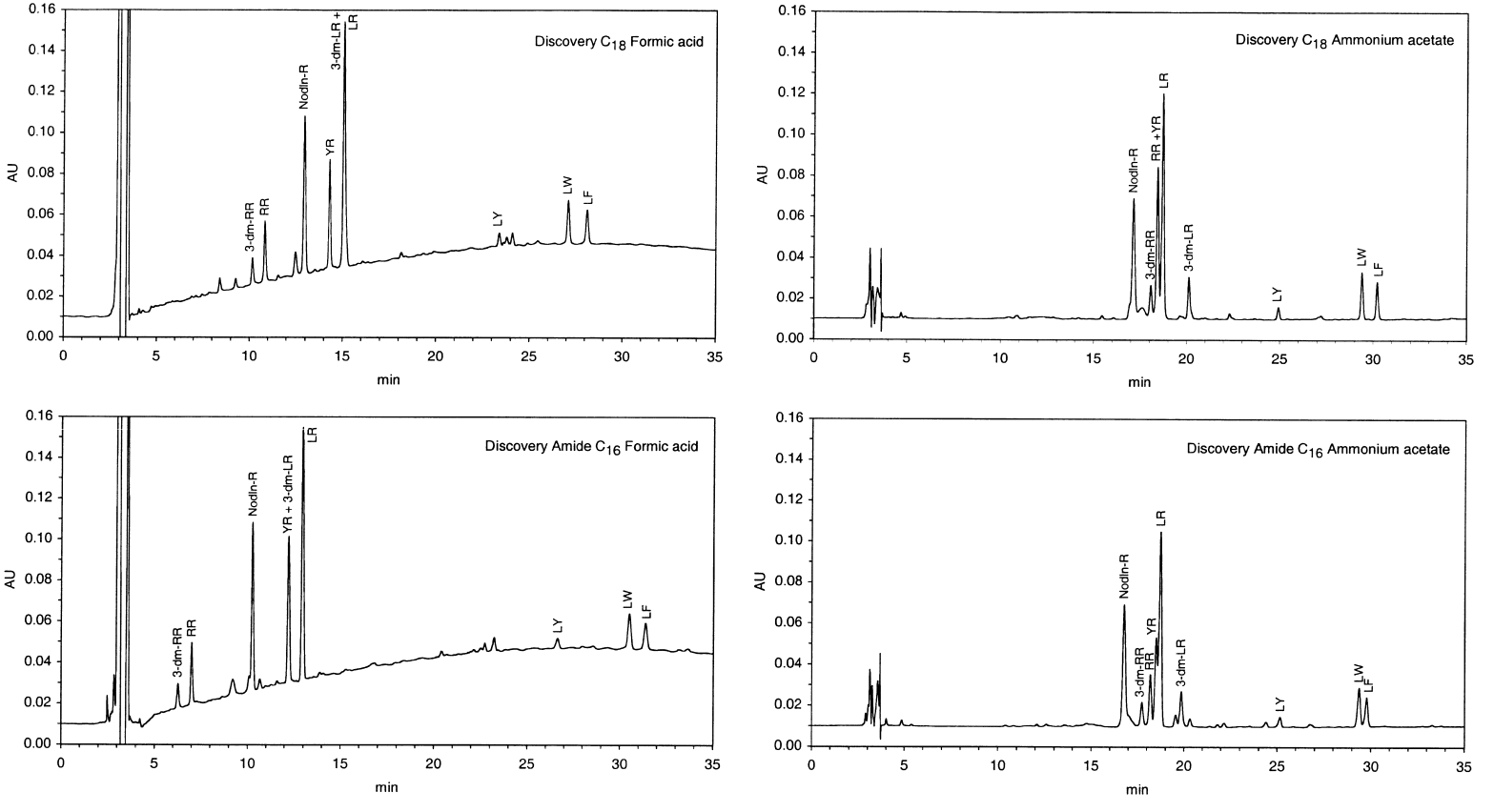

Fig. 3. Chromatograms of MC-MIX in the formic acid and ammonium acetate mobile phases with the Discovery columns. Chromatographic conditions were as described in the Experimental section. Chromatograms shown at 238 nm. The amount of microcystin-LR was ca. 0.5 μg/10 μl injection. AU=Absorbance units.

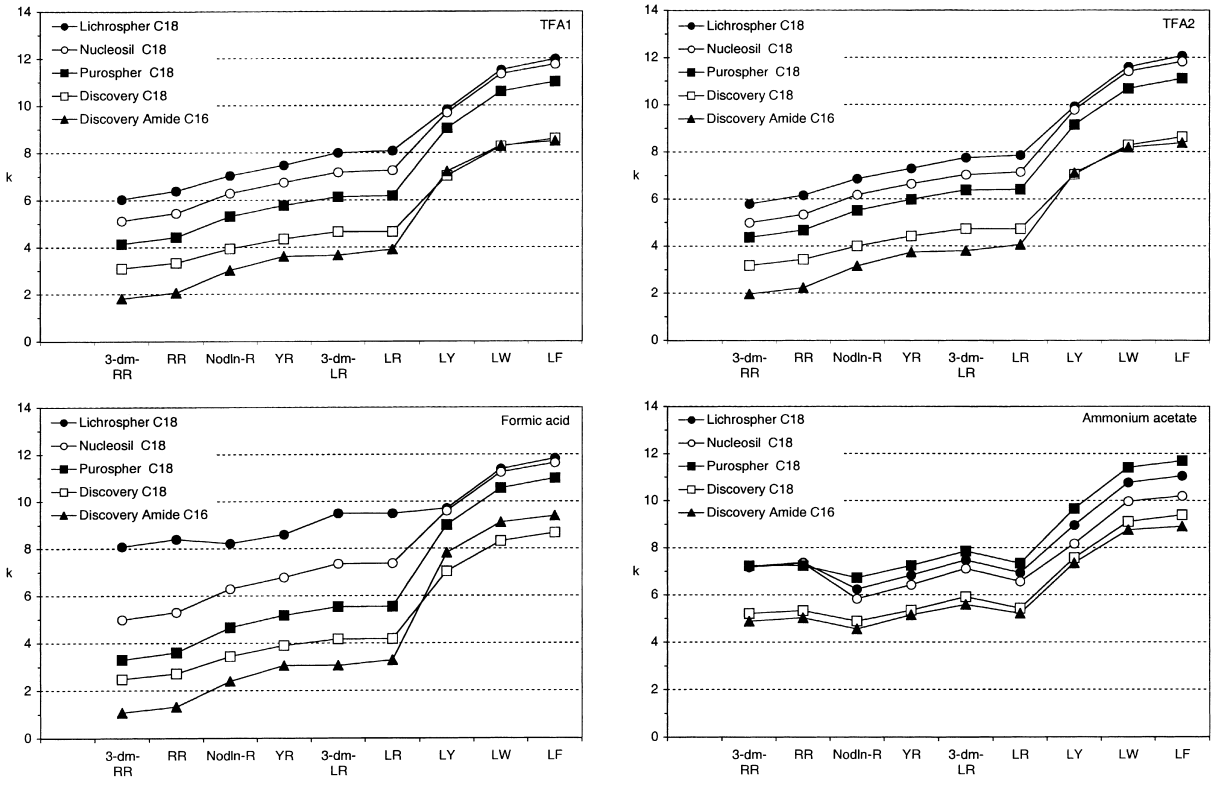

Fig. 4. Retention factors (*k*) of the toxins in different chromatographic systems. Chromatographic conditions were as described in the Experimental section.

microcystin-LR and -RR. The LC–MS experiment was conducted on a Perkin-Elmer (Norwalk, CT, USA) HPLC system with a Series 200 micropump and a 785A UV–Vis detector coupled to a PE Sciex (Foster City, CA, USA) Triple Quadrupole Model API 365 LC–MS–MS system operated in electrospray mode (TurboIonSpray). The HPLC mobile phase TFA1 and the amide C_{16} column were used in the LC–MS run. A total ion chromatogram (TIC) was collected from 110 to 1500 u and the CID (collision-induced dissociation) energy was set to 30 eV. Runs of MC-MIX in the diode-array detection–HPLC system were complemented by runs of individual pure toxins and extracts with fewer microcystins in order to securely identify the toxins and to give peak information.

2.7. Calculations of chromatographic parameters

The retention times for an unretained peak in each column were determined in the ammonium acetate-based mobile phase by injecting 1 μl of 0.1 *M* $NaNO_3$ suspended in the corresponding eluent A (0.0105 *M* ammonium acetate–acetonitrile, 95:5). Resolution calculations were usually made by the HPLC System Manager program from runs of MC-MIX. In cases of severe coelution of the involved peaks these calculations were complemented by (manual) calculations based on runs with fewer toxin analogues. Asymmetry was assessed at 5% of peak height.

3. Results

3.1. Toxin identification

The results of the LC–MS experiment are listed in Table 2. The TIC of the microcystins containing two arginine residues showed an m/z corresponding to a

double charged ion besides the molecular ion $[M+H]^+$. Additional information about the peak identities was obtained from the on-line UV spectra in TFA and ammonium acetate mobile phases. For example, microcystin-LW had an additional UV maximum at ca. 222 nm in addition to the typical maximum at 238–240 nm found in other microcystins and nodularin-R. The desmethylation sites of the 3-desmethylmicrocystin-LR and 3-desmethylmicrocystin-RR were confirmed by the presence of a peak coeluting with *o*-phthaldialdehyde-labelled D-Asp standard. This peak was not present in the non-desmethylated microcystins.

3.2. Chromatograms

Chromatograms of the Discovery columns are shown in Figs. 2 and 3. Retention factors presented in Fig. 4 reflect the chromatogram appearance of the Nucleosil, LiChrospher and Purospher columns. The LiChrospher column produced a rather densely packed chromatogram in the formic acid mobile phase and the retention factors of seven analytes were in the range of 8–10. The retention patterns of the Purospher column were much like those of the Nucleosil column but the retention factors of the Purospher column were lower.

Table 3
Resolution data for some microcystin pairs[a]

Mobile and stationary phases	Resolution				Retention time of LR (min)	Width at half height of LR (min)	Asymmetry of LR
	3-dm-RR vs. RR	YR vs. LR	3-dm-LR vs. LR	LW vs. LF			
TFA1							
LiChrospher RP-18e	2.95	5.40	**0.69**	3.91	21.18	0.16	1.07
Nucleosil C_{18}	2.59	4.40	**0.78**	3.24	19.26	0.16	**0.89**
Purospher RP-18e	2.60	3.55	**0.37**	3.43	17.74	0.16	1.06
Discovery C_{18}	3.25	4.01	**0**	3.94	16.48	0.13	1.05
Discovery RP-AmideC_{16}	3.7	4.41	3.63	2.15	14.83	0.12	0.95
TFA2							
LiChrospher RP-18e	3.28	5.22	**0.97**	3.90	20.65	0.15	1.01
Nucleosil C_{18}	2.92	4.55	**1.07**	3.26	19.00	0.15	**0.88**
Purospher RP-18e	2.74	3.76	**0.37**	3.43	18.32	0.16	1.06
Discovery C_{18}	3.53	4.18	**0**	4.05	16.72	0.12	0.97
Discovery RP-AmideC_{16}	3.73	4.52	3.57	2.09	15.31	0.13	0.95
Formic acid							
LiChrospher RP-18e	**0.94**	5.71	**0**	3.98	24.44	**0.23**	**1.30**
Nucleosil C_{18}	2.49	4.37	**0**	3.30	19.52	0.19	1.10
Purospher RP-18e	2.68	3.40	**0**	3.49	16.18	0.16	**1.17**
Discovery C_{18}	3.35	3.68	**0**	4.24	15.06	0.12	1.10
Discovery RP-AmideC_{16}	3.97	3.62	3.62	2.63	12.97	0.12	1.04
Ammonium acetate							
LiChrospher RP-18e	1.50	**0.99**	−5.29	2.22	18.51	0.14	**1.42**
Nucleosil C_{18}	1.55	**1.29**	−4.30	2.19	17.63	0.15	0.97
Purospher RP-18e	**0**	**0.68**	−4.02	2.23	20.59	0.17	1.05
Discovery C_{18}	**1.48**	**1.22**	−6.45	3.63	18.72	0.13	1.01
Discovery RP-AmideC_{16}	1.81	**0.83**	−4.40	**1.45**	18.70	0.15	0.95

[a] Retention times, widths at half height and asymmetries of microcystin-LR are also presented. Chromatographic conditions as described in the Experimental section. Resolution values less than 1.5, widths at half height wider than 0.2 min and asymmetry values outside 0.95–1.1 (regarded as not acceptable) are boldfaced. Negative resolution values indicate reversed elution order.

Generally speaking, the acidic mobile phases produced well spaced chromatograms with large differences in analyte retention (Fig. 2 and Fig. 3, left panel). The elution order in the acidic mobile phases was 3-desmethylmicrocystin-RR, microcystin-RR, nodularin-R, microcystin-YR, 3-desmethylmicrocystin-LR, microcystin-LR, -LY, -LW and -LF. However, coelution of microcystin-YR and 3-desmethylmicrocystin-LR, or 3-desmethylmicrocystin-LR and microcystin-LR occurred. The strong UV absorbance of formic acid (Fig. 3, left panel) limited the usefulness of the formic acid mobile phase because poor UV spectra were obtained.

Separations with ammonium acetate resulted in inadequate resolution between the arginine containing microcystins. The more hydrophobic microcystin analogues with aromatic residues in position 4 eluted later than the arginine containing toxins also in this eluent. The elution order of the arginine containing toxins varied to some extent. For example, 3-desmethylmicrocystin-RR and microcystin-RR eluted after microcystin-LR on the Nucleosil column but before -LR on the Discovery columns (Fig. 3, right panel and Fig. 4).

Nodularin-R was separated from microcystins in all systems except with LiChrospher/formic acid (Figs. 2–4).

3.3. Chromatographic parameters

Fig. 4 shows the retention factors, *k*, for the analytes in different chromatographic systems. Steep curves (obtained with the acidic mobile phases) indicate good separation of the analytes. Carbon loads of the columns (Table 1) did not correlate completely with the retention strengths of the columns. Purospher had lower retentive strength than Nucleosil in the acidic mobile phases despite its higher carbon load. The high carbon load of the LiChrospher column was reflected by high retention factors in all eluents. The Discovery columns with a lighter carbon load had rather low retention factors. The amide C_{16} (see also Fig. 3) and the Purospher C_{18} columns in the formic acid mobile phase had the widest ranges of retention factors.

Table 3 lists the resolutions between four microcystin pairs. The resolution between 3-desmethylmicrocystin-RR and microcystin-RR was rather poor in the ammonium acetate mobile phase, and the same applied to the pair microcystin-YR and -LR. The C_{18} columns failed to resolve 3-desmethylmicrocystin-LR and microcystin-LR in the acidic mobile phases whereas the same separation could be easily accomplished on the amide C_{16} column. In the ammonium acetate mobile phase the elution order was reversed, i.e., 3-desmethylmicrocystin-LR elutes after microcystin-LR. The resolution of microcystin-LW and -LF was adequate in practically all systems including the amide C_{16} column but the resolution was clearly better on the C_{18} columns.

Widths at half height for microcystin-LR were below 0.2 min in all systems with the exception of LiChrospher in the formic acid eluent (0.23 min). The Discovery columns stood out with their sharp and high peaks (Figs. 2 and 3). This could be due to the particle size smaller than the nominal 5 μm (Table 1). The Nucleosil column had some tendency to fronting in the TFA eluents (asymmetry$<$1, Table 3). Tailing was encountered with the LiChrospher column (formic acid, ammonium acetate, Table 3) and the Purospher column (formic acid, Table 3).

4. Discussion

Microcystins and nodularins are not only a demanding challenge for the analyst, but also very useful compounds for comparisons of mobile and stationary phases. This study found considerable differences in the chromatographic performance of five reversed-phase columns and four mobile phase systems. While it is practically impossible to test the separation characteristics of all known microcystins (and nodularins) the present results give a good indication of efficient and convenient chromatographic systems.

The ammonium acetate-based mobile phase had only limited separation capabilities of the arginine-containing toxins. Therefore, it cannot be considered a primary choice for analytical microcystin separations as arginine is a key component in microcystins. Sivonen and Jones listed 60 microcystins in their review [3]. In 44 (73%) of the toxins arginine or homoarginine was present. In preparative separations, ammonium acetate is useful because of

its mild nature. Ammonium acetate-based eluents are prone to changes in selectivity as the buffering capacity (e.g., against carbon dioxide from air) is poor. We have found these selectivity changes also in microcystin separations if the eluent had been used for a period of two days. Because ammonium acetate supports microbial growth inclusion of acetonitrile in both eluents A and B was regarded as essential. This in turn caused gas bubble formation in the eluents (especially A).

The three tested acidic mobile phase systems all functioned well from a chromatographic point of view but the high absorbance of formic acid distorted the baseline and made acquisition of UV spectra impossible. Of course, these concerns are not important in mass spectrometric detection. Of the two TFA mobile phases we prefer TFA1 which has no TFA in acetonitrile although the TFA2 mobile phase gave marginally better resolution in some cases (Fig. 2, Table 3). The reason for this preference is purely of practical nature.

In our laboratory, we have now chosen the Discovery RP-AmideC_{16} column as a routine tool for microcystin and nodularin separations. We perform the separations with a 150 mm×2.1 mm I.D. RP-AmideC_{16} column using the TFA1 mobile phase (see Experimental) with the following linear gradient programme: 0 min 20% B, 25 min 65% B, 27 min 65% B, 28 min 20% B (45 min run interval, column temperature 40°C, flow-rate 0.3 ml min^{-1}, sample volume 5 μl). The main reasons for our choice were the following: (1) the amide C_{16} column separates the desmethylated toxins, which are often encountered in field samples, well from the non-desmethylated toxins. (2) Adequate differences in the retention of the toxins are achieved. This might suggest successful chromatography of toxin variants not tested in this paper. (3) The runs can be performed with a slightly lower percentage of organic modifier in the eluent (as compared to the C_{18} columns). Furthermore, scaling down to 2.1 mm I.D. gives a higher signal-to-noise ratio and less mobile phase consumption. A column length of 150 mm gives a reasonable separation and allows higher sample throughput by making the total analysis time shorter. In LC–MS applications with more specific detection the use of even shorter columns should be possible.

A further application where the unique selectivity of the amide C_{16} phase could be useful is the separation of certain stereoisomers. Microcystins have been found to contain a small amount of geometrical isomers of Adda [6(*Z*)-Adda] [21,22] but the chromatography of these microcystin variants has received little attention. However, it would be beneficial in both analytical and preparative work to be able to resolve the toxic microcystins (with the correct stereochemistry) from the non-toxic ones (having the inactive Adda stereochemistry). Harada and co-workers were able to separate geometrical isomers of microcystin-LR and -RR using methanol-based eluents on a C_{18} phase [21,22]. The amide C_{16} bonded phase contains an amide linkage, which has a partial double bond character and therefore very limited rotation. This property could possibly be used in separations involving stereoisomers.

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