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Separation, purity testing and identification of cyanobacterial hepatotoxins with capillary electrophoresis and electrospray mass spectrometry

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Abstract

Capillary zone electrophoretic and micellar electrokinetic chromatographic methods with UV detection were investigated for separation and identification of some microcystins isolated from cyanobacterial *Anabaena 90* strains in Finland. The low-sensitivity purity tests with full scan UV–Vis spectra were done with a CZE method. All the test compounds, [D-Asp^3,Dha^7]MCYST-LR, MCYST-LR, MCYST-YR, [Dha^7]MCYST-LR, MCYST-RR, [Dha^7]MCYST-RR, [D-Asp^3,Dha^7]MCYST-RR, [D-Asp^3]MCYST-LR and [D-Asp^3]MCYST-RR (where MCYST stands for microcystin) isolated with a preparative HPLC method, could be separated from each other in a MECC method. The detection limits of the microcystins were below ppm level. The repeatability of the MECC technique was tested by comparing the absolute migration times with the indices calculated with ''in-laboratory'' designed programs operating in MATLAB Mathworks Inc. The identification and the high-sensitivity purity tests of the isolated microcystins were made with an off-line electrospray mass spectrometer. Identification could be done for MCYST-YR, MCYST-LR, MCYST-RR, [Dha^7]MCYST-RR, [Dha^7]MCYST-RR, [D-Asp^3]MCYST-LR, [D-Asp^3]MCYST-RR, [D-Asp^3, Dha^7]MCYST-LR and [D-Asp^3, Dha^7]MCYST-RR on the basis of their $[M+H]^+$ or $[M+2H]^{2+}$ ions in the MS mode and by using the protonated molecule as precursor in the MS–MS mode.

Keywords: Hepatotoxins; Toxins; Microcystins

1. Introduction

Cyanobacteria produce neurotoxins and peptide hepatotoxins, of which microcystins (MCYSTs) (Fig. 1) have the general structure cyclo(–D–Ala^1–X^2–D-MeAsp^3–Z^4–Adda^5–D-Glu^6–Mdha^7–), where X

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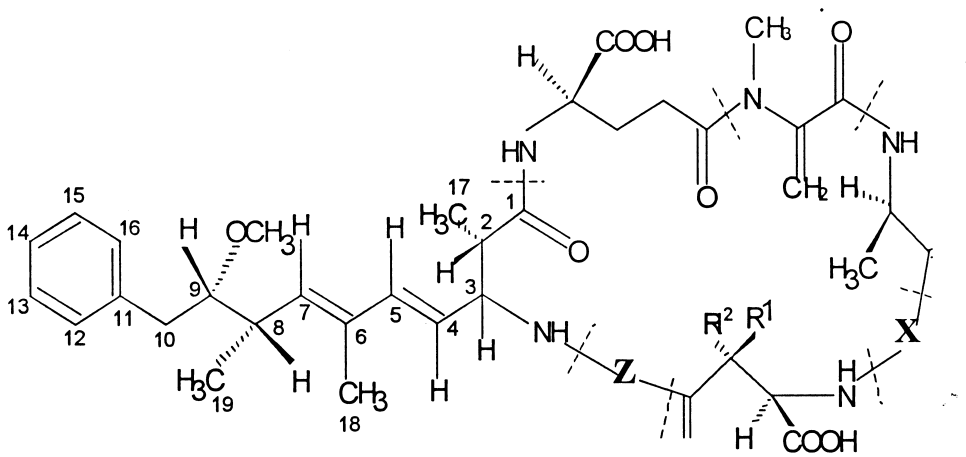

Microcystins	X	Z	R^1	R^2	Formula
[Dha^7] MCYST-LR	Leucine	Arginine	CH_3	H	$C_{48}H_{72}N_{10}O_{12}$
[Dha^7] MCYST-RR	Arginine	Arginine	CH_3	H	$C_{48}H_{73}N_{13}O_{12}$
[D-Asp^3,Dha^7]-MCYST-LR	Leucine	Arginine	H	H	$C_{47}H_{70}N_{10}O_{12}$
[D-Asp^3,Dha^7]-MCYST-RR	Arginine	Arginine	H	H	$C_{47}H_{71}N_{13}O_{12}$
MCYST-YR	Tyrocine	Arginine	CH_3	CH_3	$C_{52}H_{72}N_{10}O_{13}$
MCYST-LR	Leucine	Arginine	CH_3	CH_3	$C_{49}H_{74}N_{10}O_{12}$
[D-Asp^3]MCYST-RR	Arginine	Arginine	H	CH_3	$C_{48}H_{73}N_{13}O_{12}$
[D-Asp^3]MCYST-LR	Leucine	Arginine	H	CH_3	$C_{48}H_{72}N_{10}O_{12}$
MCYST-RR	Arginine	Arginine	CH_3	CH_3	$C_{49}H_{75}N_{13}O_{12}$

Fig. 1. Microcystins studied.

and Z are L-amino acids, D-MeAsp is D-*erythro-b*-methylaspartic acid, Mdha is *N*-methylaldehyroalanine and Adda is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyl-deca-4,6-dienonic acid [1–3].

In several European surveys the toxic cyano bacterial blooms containing microcystins are shown to occur the most frequently [4]. Toxic cyano bacteria have been detected in freshwater lakes, in drinking water reservoirs and in brackish seawaters. One of the most toxic microcystins is microcystin-LR (LD_{50} 50 mg/kg) [3]. Recently there have been evidences of microcystins, activity as tumour promotors [5,6].

Several chemical differences in microcystin structures have made increasing demands for chromatographic techniques ending separations of the individual compounds. Methods like thin-layer chromatography (TLC) [7], micro high-performance liquid chromatography (HPLC) [8,9] and capillary electrophoresis (CE) [10–13] have been used for the analysis. Especially, the chromatographic methods have been successfully applied to isolate and identify several microcystins from the extracts of water, bloom and strain samples. The disadvantage of these analytical methods has been the time required for sample processing, which unfortunately cannot be avoided. CE has proven to be a powerful micro separation technique for peptide analysis [14–17] and its combination to mass spectrometry provides a powerful analytical tool for analyte identification [12,18]. Advantages in operation modes available, high separation efficiency, good resolution and small amounts of samples needed, have made CE a reference technique with resolution of multicomponent mixtures for chromatographic methods.

Due to the chemical nature of microcystins, CE separation can be impeded by adsorption of the analyte onto the inner wall of the capillary. Therefore, in the studies of microcystins by CE it is preferable to select separation conditions enabling the analysis of anions or zwitterions, under basic conditions with micelles e.g., sodium dodecyl sulphate (SDS). Successful separations have been performed with additives or ligands used in order to prevent the interactions between the analyte molecules and the silanol groups of the capillary wall surface.

This study focussed on the development of one selective analytical method for separation of microcystins and to obtain reliable identification of these hepatotoxic peptides by electrospray ionisation mass spectrometry (ESI-MS). It was necessary to optimise and to validate a method for the separation of microcystins and to verify compound identity by multi-wavelength and diode array detectors together with index values obtained by mathematical matrices.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical grade. Tris(hydroxymethyl)aminomethane, 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) and SDS were from Sigma (St. Louis, MO, USA). Sodium acetate, sodium tetraborate, sodium salts of dihydrogenphosphate and hydrogenphosphate and acetic acid (100%) were from Merck (Darmstadt, Germany). Water was purified (distilled water treated through anion, cation and carbon black sorbents) with a Water-I System (Gelman Sciences, Ann Arbor, MI, USA), then filtered through a 0.45-μm membrane (Waters, Molsheim, France).

2.2. Sample preparation

Microcystins were isolated from cyanobacterial strain *Anabaena 90* using HPLC in isocratic elution with 10 m*M* ammonium acetate (GR grade, Merck) containing 0.01% trifluoroacetic acid (TFA) (GR, Fluka, Buchs, Switzerland)–acetonitrile (HPLC grade) (75:25, v/v) at a flow-rate of 1 ml/min. A Waters Delta Prep 3000 system, with a μBondapak C_{18} semipreparative column (190×50 mm, 125 Å, 10 μm) and a Varian 4070 integrator, was used for the further purification of these toxins [18,19]. UV detection was set at 238 nm and fractions were collected manually (retention time for MCYST-LR was about 60 min). The collected fraction was then dried using a gentle flow of nitrogen. Finally, solid-phase extraction (SPE) was applied to remove the residual of ammonium acetate from these samples. A C_{18} cartridge (Varian, Palo Alto, CA, USA) was

activated with methanol (20 ml), then flushed with water (20 ml) before a sample was loaded by using a syringe at a rate of 8 ml/min. After that, the loaded sorbent was washed with 20 ml of water, and the microcystins were eluted by methanol (10 ml). After evaporation of the solvent, the dried samples were dissolved in methanol–water (5:95, v/v) for capillary zone electrophoresis (CZE).

2.3. Standards

The purified microcystins were dissolved separately and mixed together at different concentrations in methanol–water (5:95, v/v) solution for the analyses by CZE. For micellar electrokinetic chromatography (MECC) [D-Asp^3,Dha^7]MCYST-LR, MCYST-LR, MCYST-YR, [Dha^7]MCYST-LR, MCYST-RR, [Dha^7]MCYST-RR, [D-Asp^3,Dha^7]-MCYST-RR, [D-Asp^3]MCYST-LR and [D-Asp^3]-MCYST-RR were mixed at concentrations of 1 μg/ml. The mixtures were further diluted with methanol–water (1:5, v/v) mixture to get a better resolution.

2.4. CE instruments and separation conditions

A BioFocus 3000 capillary electrophoresis (Bio-Rad Labs, Hercules, CA, USA) with a multi-wavelength detector and a HP^{3D} CE system (Hewlett-Packard, Waldbronn, Germany) with a diode array detection (DAD) system were used. The wavelength used for screening of the microcystins was 238 nm. The total lengths of uncoated fused-silica capillaries (Composite Metal Services, Hallow, UK) were 49.6 cm (45 cm to detector) and 58.5 cm (50 cm to detector) for Bio-Rad and HP systems in CZE analyses, respectively. The MECC separations were performed in a 78.5 cm capillary (70 cm to detector) with the HP instrument. The sample injection was performed hydrodynamically by pressure at 3–20 p.s.i. in the Bio-Rad instrument and at 50 mbar for 5 s in the HP^{3D} CE system (1 p.s.i.=6894.76 Pa and 1 bar=10^5 Pa). The capillaries were flushed with 0.1 *M* NaOH, washed with purified water and rinsed with the running buffer then balanced in the running buffer for 1.0 min before each injection, in order to improve experimental repeatability. The applied voltage used during the analyses was from +13 to +28 kV at capillary temperature of 25°C. UV spectra (190–350 nm) were collected for every peak identified with the HP system.

2.5. Electrolyte solutions

2.5.1. CZE

To find the most effective electrolyte solution in our study, inorganic and organic buffer systems were prepared from sodium acetate, disodium hydrogenphosphate–sodium dihydrogenphosphate or tris-(hydroxymethyl)aminomethane at concentrations varying from 10 to 200 m*M* at various pH from 2.0 to 6.0. In basic pH region, disodium tetraborate and CAPS solutions at concentrations of 10 to 80 m*M* were used to obtain pH of 8.11 to11.2. Finally, the electrolyte solution for purity testing prepared was a 40 m*M* solution, which was made from sodium acetate to keep the pH at 4.0.

2.5.2. MECC

To evaluate the suitable MECC method different concentrations of CAPS and SDS were used in electrolyte solutions at pH 10.6, which pH was chosen according to the preliminary CZE studies. The standard procedure to prepare the 40 m*M* solution is as follows: CAPS buffer was prepared from 0.2 M stock solution of CAPS to get a 40 m*M* by mixing 40 ml of 0.1 M NaOH solution (Titrisol), 40 ml of Water-I water and 20 ml of 0.2 M CAPS. The pH of the electrolyte solution was 10.6 without pH adjustment. To that solution, 0.4326 g of SDS and 60 ml of acetic acid (100%) were added to get the micellar system and to keep the pH at 10.6.

2.6. Mass spectrometric conditions

The samples were identified by electrospray mass spectrometry (Bruker Instruments, Bremen, Germany) with positive ESI. Samples dissolved in methanol–acetonitrile–acetic acid mixture were introduced to the interface with flow of 65 ml/min. Nitrogen was used as the reagent gas with the flow-rate of 200 ml/min at the temperature of 170–180°C. The voltages in producing the ion spray were −4800 V, −4300 V and −3270–3700 V for the capillary, the end plate and the cylinder, respectively. The

voltages of the multiplier, dynodes, capillary skimmer and exit lens were −1400 V, −8.0 V, 70–110 V, 11–30 V and −160–230 V, respectively. The d.c. offset was changed from 8.5 to 20.5 depending on the background noise. The low-molecular-mass cut-off was 210 u and summation was 20–200 s. The width of isolation in fragmentation was done with cut-off set 270–280 by using voltage of 1.2–1.3 V. During all the analyses the delay time was 5 ms and the radio frequency (RF) amplitude was 350 V. Sheath gas or liquid was not used.

2.7. Samples in mass spectrometric studies

The stock solutions of the isolated microcystins were diluted with water–methanol–acetic acid (50:49.5:0.5, v/v/v) mixture. The dilution factor was 1:50.

2.8. Calculations

The migration indices were calculated in MATLAB Mathworks Inc. with ''in-laboratory'' designed programs which are based on the algorithms described in Ref. [20]. The equations used for the migration index values (Ind_x) for the analyte x were

$$Ind2 = Ind1\ (t_{eo}/t_2 - 1)/(t_{eo}/t_1 - 1) \quad (1)$$

$$Ind_x = [t_1 t_2\ (Ind1 - Ind2) - t_x(Ind1 t_1 - Ind2 t_2)]/t_x(t_2 - t_1) \quad (2)$$

where Ind1 and Ind2 are the indices of the marker compounds xanthene-9-carboxylic acid and *o*-phtalic acid, respectively, and t_1 and t_2 are their respective absolute migration times and t_{eo} is the electroosmotic migration time. The value of Ind1 was fixed to be 1000 leading to the Ind2 value of 1814.

3. Results and discussion

3.1. Electrophoresis in CZE

By using the acetate buffer of pH 4.0, the separation mechanism is based on the differences in molecular mass-charge ratios (m/z) of the analyte molecules [10]. The results obtained by the CZE method were not satisfactory (Table 1), since in a mixture the microcystins were not separated from each other. However, the CZE method was very suitable at low-sensitivity UV detection in peak purity tests of the individual compound when it was separated from its by-products after preparative HPLC (Figs. 2 and 3).

As heptapeptides, the ionization of these microcystins was reduced at lower pH values, resulting in an increase of their electrophoretic mobilities (μ_e). Positively charged MCYST-RR and negatively charged MCYST-LR resulted in a wide migration window, which, of course, is favourable for simultaneous separation of different microcystins. Here the

Table 1
Studies on repeatability of the separation of microcystins in (a) CZE and (b) MECC method estimation by absolute migration times and indices ($n=9$)

Compound	t_R (min)	RSD (%)
[D-Asp^3, Dha^7]MCYST-LR	11.21 (b)	1.18
MCYST-LR	7.89 (a)	0.22
	11.42 (b)	0.77
[Dha^7]MCYST-LR	12.05 (b)	0.80
MCYST-RR	7.89 (a)	0.25
	13.43 (b)	0.88
[D-Asp^3, Dha^7]MCYST-RR	13.96 (b)	0.93
[D-Asp^3]MCYST-LR	8.08 (a)	0.22
	15.85 (b)	0.95
[D-Asp^3]MCYST-RR	16.18 (b)	1.02

Table 2
Repeatability of the MECC method (indices with 9 inter-day replicates. Equations described in Refs. [21,22])

Compound[a]	Index	RSD [%]
[D-Asp^3, Dha^7]MCYST-LR	1046	1.06
MCYST-LR	1346	0.21
[Dha^7]MCYST-LR	1392	0.15
MCYST-RR	1487	0.15
[Dha^7]MCYST-RR	1519	0.16
[D-Asp^3]MCYST-RR	1577	0.18

[a] Marker compounds: xanthene-9-carboxylic acid (index 1000) and *o*-phtalic acid (index 1814).

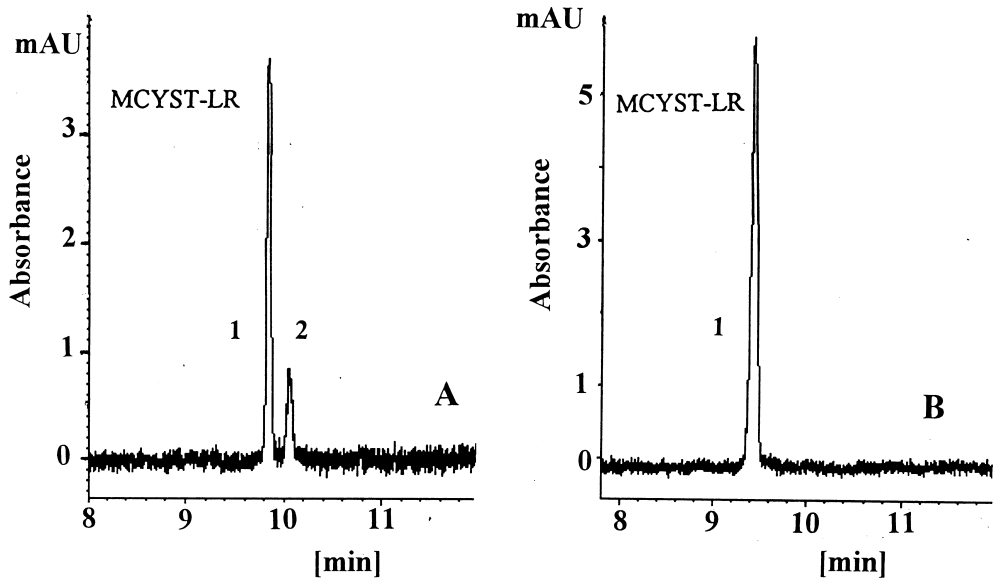

Fig. 2. CZE electropherograms of a MCYST-LR sample (A) before and (B) after further purification with semipreparative HPLC and SPE. See Section 2.4 for CZE conditions.

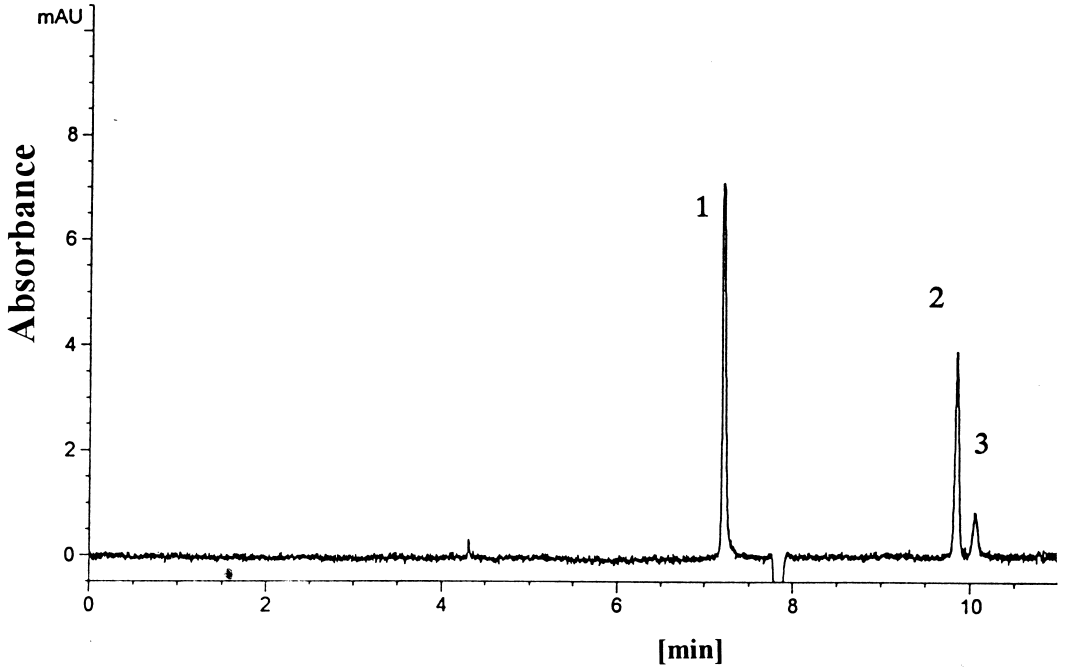

Fig. 3. CZE electropherogram of three microcystins in 40 m*M* acetate buffer at pH 4.0. Sample containing (1) MCYST-RR (30 μg/ml), (2) MCYST-LR (20 μg/ml) and (3) [D-Asp^3]MCYST-LR (5 μg/ml).

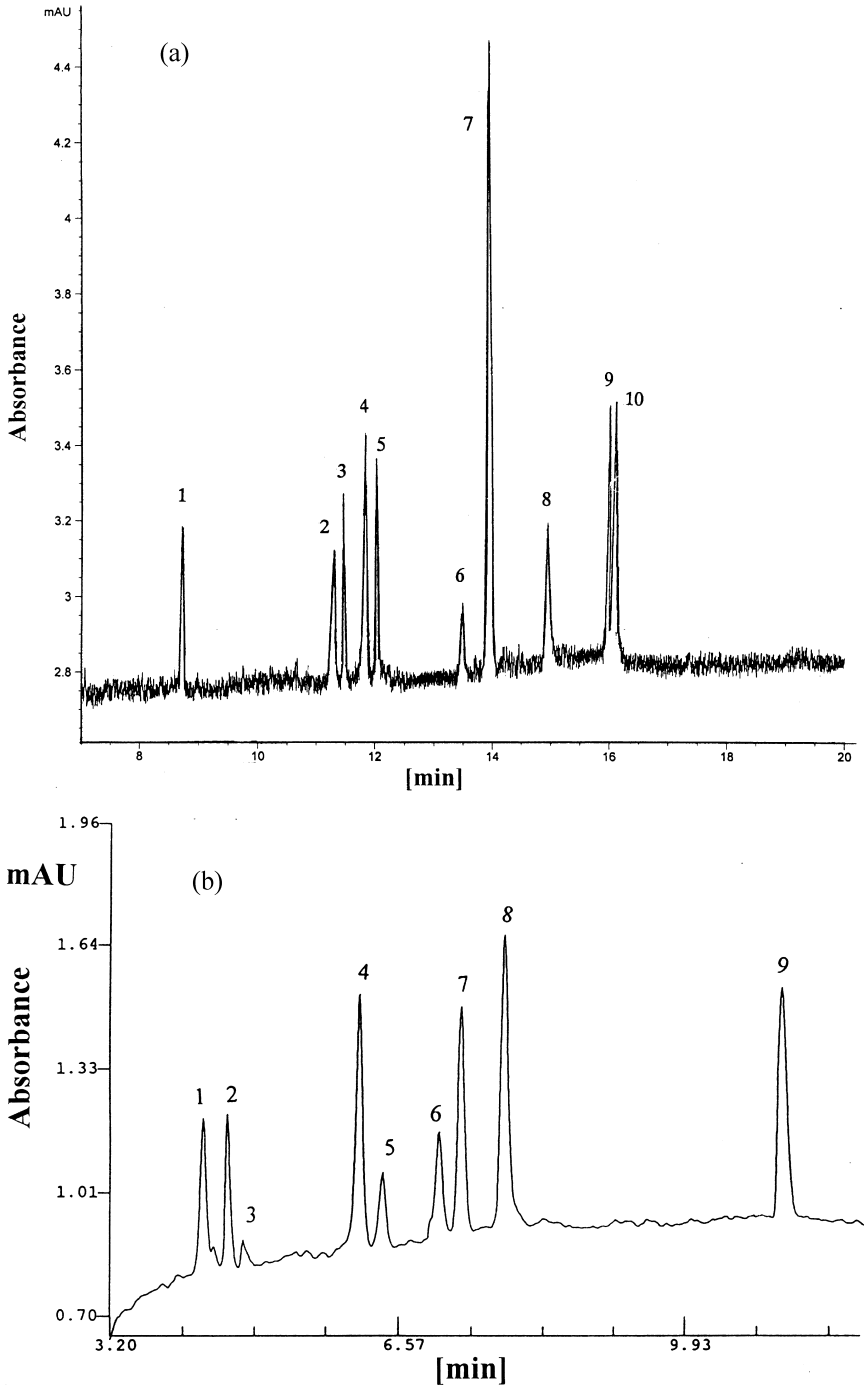

Fig. 4. MECC separation of (a) microcystins in the study and (b) microcystins isolated from some blue–green algae mixed with compounds used in calculation of indices for the microcystins. Peaks in (A): 1=[D-Asp^3, Dha^7]MCYST-LR, 2=MCYST-LR, 3=MCYST-YR, 4=[Dha^7]MCYST-LR, 5=MCYST-RR, 6=[Dha^7]MCYST-RR, 7=[D-Asp^3, Dha^7]MCYST-RR, 8=[D-Asp^3]MCYST-LR, 9=[D-Asp^3]MCYST-RR and 10=MCYST-YA. Peaks in (B): 1=*o*-cresol, 2=xanthene-9-carboxylic acid, 3=[D-Asp^3, Dha^7]MCYST-LR, 4=MCYST-LR, 5=[Dha^7]MCYST-LR, 6=MCYST-RR, 7=[Dha^7]MCYST-RR, 8=[D-Asp^3]MCYST-LR and 9=*o*-phtalic acid.

analysis time is reasonable short and can be used for calculations of their isoelectric points, which are between 3 and 5, and for checking their purity. The result was calculated from laboratory data obtained with capillary electrophoresis by changing pH of acetic acid buffer step by step by value of 0.2 at pH range of 4.00–5.80 and marker for electroosmosis. According to our studies, electroosmosis and the effective mobilities ($\mu_e = \mu_a - \mu_{eo}$) for the analytes were not changed when the ionic strength of the electrolyte solution was over 0.08 *M* in the MECC method. The electrophoretic mobilities of MCYST-RR, MCYST-LR and [D-Asp^3]MCYST-LR were $3.80 \cdot 10^{-4}$, $3.36 \cdot 10^{-4}$ and $3.32 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}, respectively.

The CZE method developed in this study was used to monitor the purity of MCYST-LR and the results indicated that this sample contained certain amount of [D-Asp^3]MCYST-LR, as shown in Fig. 2a. After further purification with semi-preparative HPLC and SPE, no impurity peak was detected (Fig. 2b). Thus the CZE method has potential for quality control testing of microcystins. However, it should be noted that MCYST-RR, [D-Asp^3]MCYST-RR and [Dha^7]MCYST-RR cannot be separated by the CZE method developed.

3.2. Electrophoresis in MECC

Microcystins could be separated and their peaks in electropherograms could be reliably identified using *o*-cresol and xanthene-9-carboxylic acid as mobility markers (Table 2) [20,21] in basic micellar electrokinetic technique (pH 10.6, 40 m*M* CAPS, 15 m*M* SDS, Fig. 4) [9]. The electrophoretic mobilities of

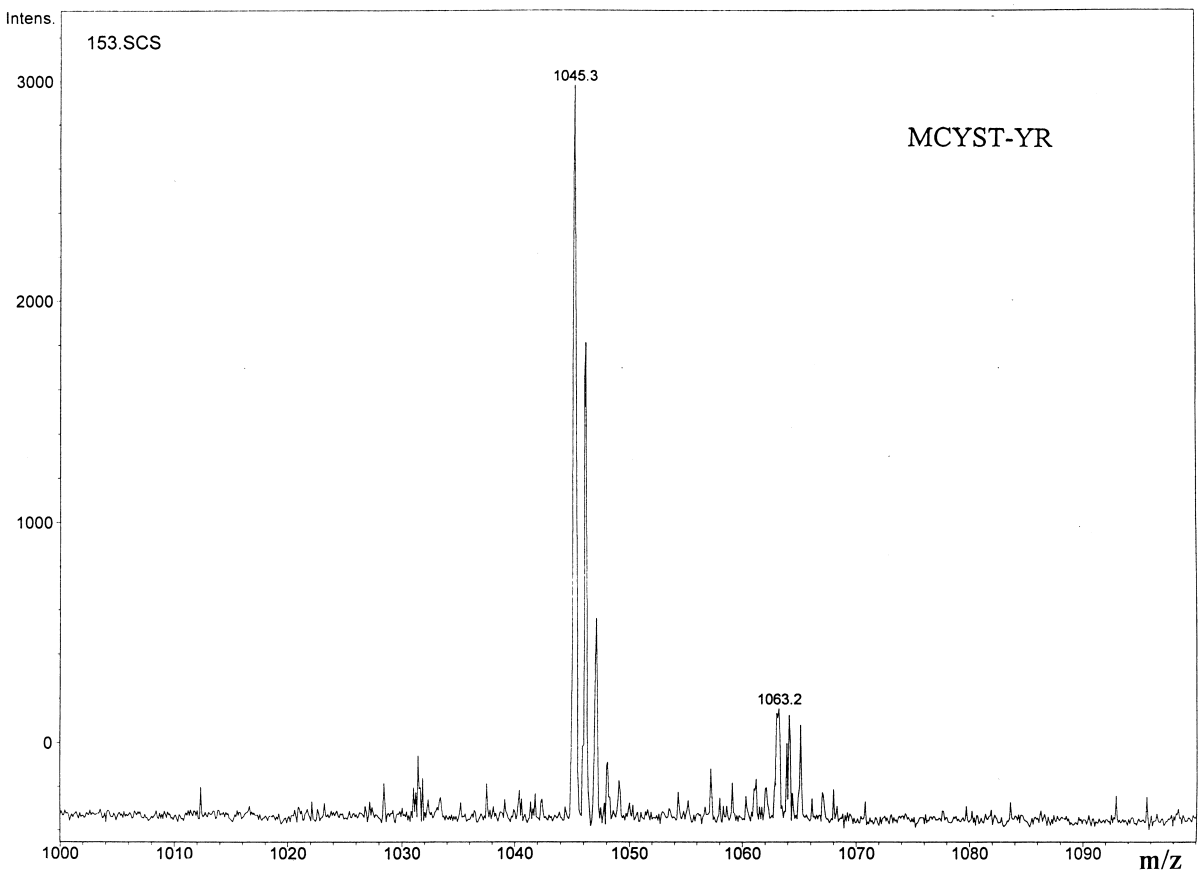

Fig. 5. ESI-MS from microcystins MCYST-YR.

[D-Asp^3,Dha^7]MCYST-LR, MCYST-LR, [Dha^7]-MCYST-LR, MCYST-RR, [Dha^7]MCYST-RR and [D-Asp^3]MCYST-RR were $-2.49\cdot10^{-8}$, $-3.20\cdot10^{-8}$, $-3.31\cdot10^{-8}$, $-3.54\cdot10^{-8}$, $-3.61\cdot10^{-8}$ and $-3.75\cdot10^{-8}$ $m^2\ V^{-1}\ s^{-1}$, respectively. The new MECC method could also be used to monitor the purity of the microcystin, when their concentrations were high enough for UV response (signal-to-noise 2). When marker compounds were used, the repeatability of the analyses was very good (RSD below 0.1%) giving excellent possibility to quantitative identification using Q_{id} index. [Its value should be more than 2 when calculated by equation $Q_{id}=(x_2-x_1)/(s_1-s_2)$ where x_1 and x_2 are the responses of interest and s_1 and s_2 are their standard deviations] [21].

3.3. Identification and Purity Testing with ESI-MS and ESI-MS–MS

The micellar technique was not suitable for on-line studies of the microcystins. On the contrary, although the different compounds could not be separated, the method used for CZE separations was good for mass detection. But, when performed on-line, the concentrations of the microcystins introduced into the capillary were too low (signal-to-noise ratio below 2) for reliable mass detection (total spectrum) with electrospray in scan mode. Therefore, only off-line identification is presented here in Figs. 5–7 and in Table 3.

Identification could be done for MCYST-YR, MCYST-LR, [Dha^7]MCYST-LR, [D-Asp^3, Dha^7]MCYST-LR and [D-Asp^3, Dha^7]MCYST-LR on the basis of their protonated molecule ion $(M+H)^+$. MCYST-RR, [Dha^7]MCYST-RR and [D-Asp^3]MCYST-RR on the basis of its doubly charged ion $M+2H)^{2+}$. The results in Table 3 were compared with the values available in the literature, which showed that all our results were one m/z unit higher than reported in Refs. [8,9]. Figs. 5–7 show the typical fragmentation pattern for MCYST-YR

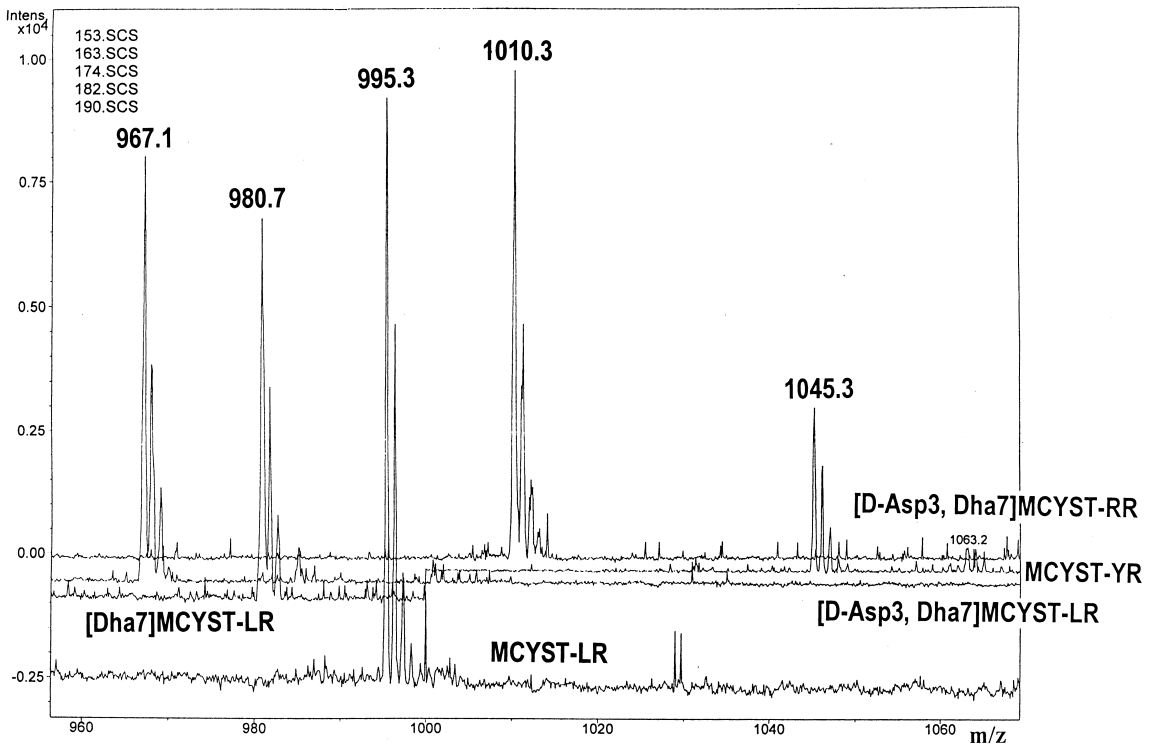

Fig. 6. ESI-MS from microcystins [D-Asp^3,Dha^7]MCYST-RR (ion 1010.3), MCYST-YR (ion 1045.3), [D-Asp^3, Dha^7]MCYST-LR (ion 967.1), [Dha^7]MCYST-LR (ion 980.7) and MCYST-LR (ion 995.3).

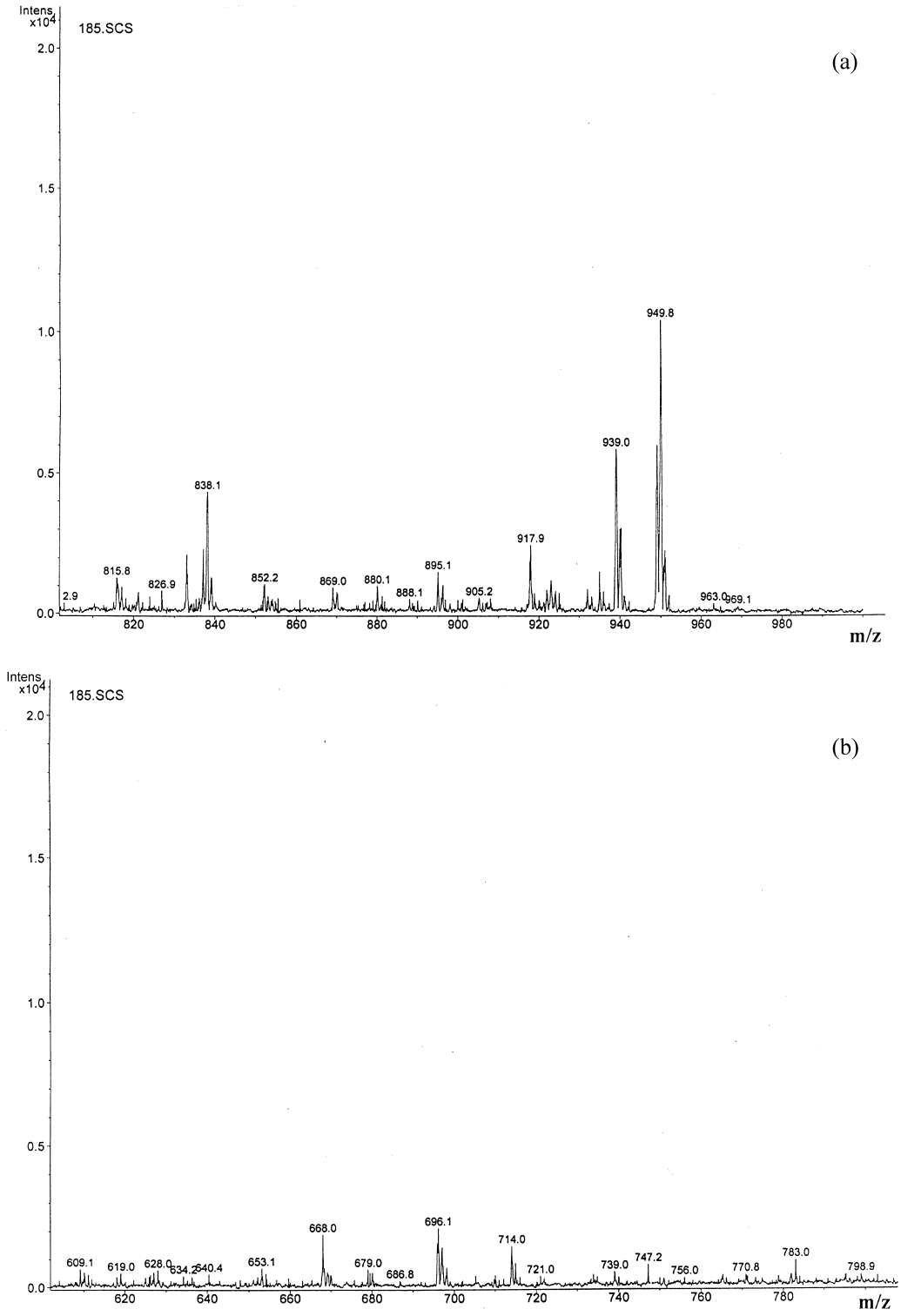

Fig. 7. ESI-MS–MS from [D-Asp^3, Dha^7]MCYST-LR (from ion m/z 967).

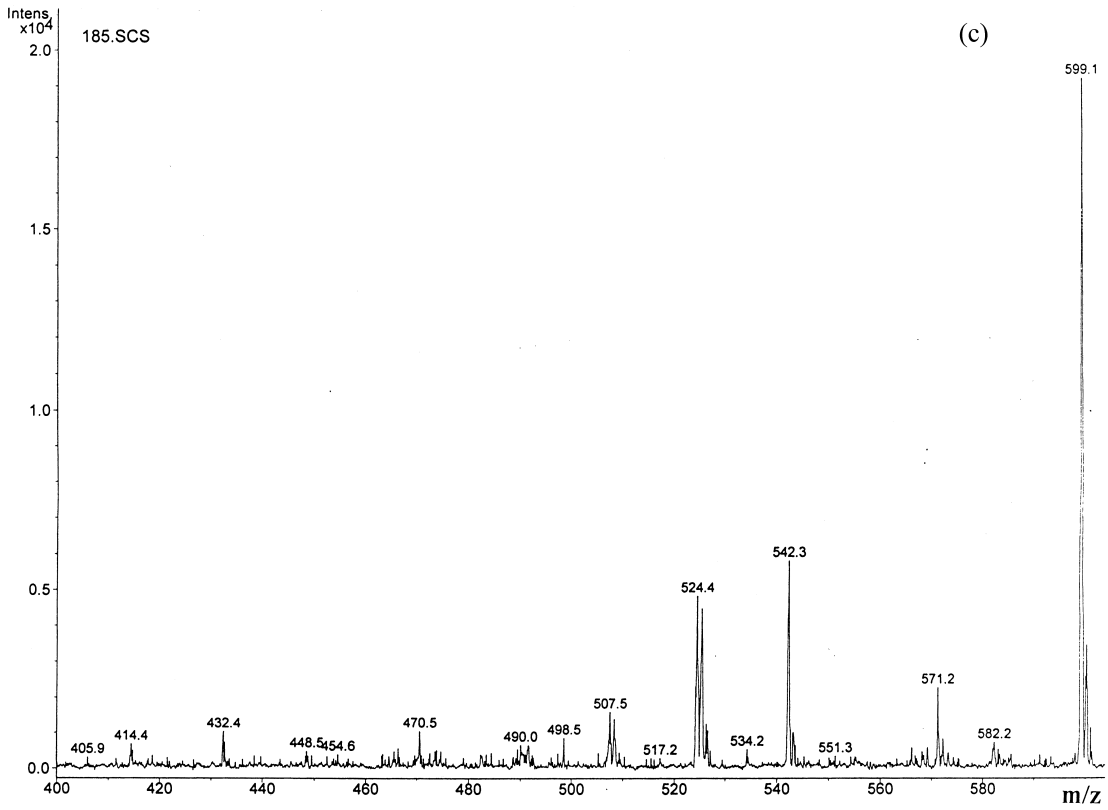

Fig. 7. (*continued*)

and a displayed report of the fragmentation of [D-Asp^3]MCYST-RR, MCYST-YR, MCYST-LR, [D-Asp^3]MCYST-LR and [Dha^7]MCYST-RR.

3.4. Quantification

As for the repeatability of this method, the relative standard deviation (RSD) was better than 0.2% for absolute migration time and 2% for corrected peak areas. The relationship between peak parameters (peak height, peak area and corrected peak area) versus the concentrations of MCYST-LR was investigated at the range of 0.5 to 100 μg/ml giving a good linearity of $R^2 > 0.997$. In MECC the detection limits were at approximately 100 ng/ml (S/N 2) at wavelength 238 nm, whereas the detection limit for concentration of 1.0 μg/ml was observed for CZE–UV.

4. Conclusions

The CZE method is useful only in separation of microcystin-LR structures from -RR forms, where pH seemed not to play any role, if separation was done in acidic medium. When the MECC method was used, it was noticed that the most effective factor influencing the resolution was the total ionic strength of the electrolyte solution and the concentration of SDS. On the contrary, small differences in the pH of the electrolyte solution at the range 9.6–11.0 had only a very minor effect on separation pattern.

The identification and the purity of the isolated microcystins were made with off-line ESI-MS due to the very low concentrations of the analytes. The results showed that the concentrations of the microcystins are so low that the identification for whole mass range was needed. Also, due to the impurities

Table 3. Mass fragmentation of the microcystins in electrospray mass spectrometry[a]

M_r (+H)	-YR	-R	-RR	Dha^7-LR	Dha^7-RR	D-Asp^3-LR	D-Asp^3-RR	D-Asp^3-Dha^7-LR	D-Asp^3-Dha^7-RR
1045	M+								
1038			M++						
1027	+								
1024					M++		M++		
1020			++						
1017	+								
1010									M+
1006									+
1001	+								
995		M+							
981				M+		M+			
977		+							
975						+			+
967		+						M+	+?
963				+		+			
957									+
953						+			
950								+	+
939								+	
932									+
916	+								
904			+						
895								+	+
890					+		+		
887			+						
879									+
873					+		+		
869								+	
866		+				+			+?
852								+	
851				+		+?			
838								+	
783		+							
731									+
728		+							
717					+		+		
714								+	+
710	+	+		+?					
696								+	+
682						+			
668								+	
667						+			
620	+						+		
599	+	+		+	No data obtained	+	+	+	+
582					No data obtained		+	+	
571	+				No data obtained			+	
570		+			No data obtained				
556				+	No data obtained				
553		+			No data obtained				
550					No data obtained				+
542					No data obtained			+	
538				+?	No data obtained	+			
535		+			No data obtained				
533					No data obtained				+
524					No data obtained			+	
Literature	1044	994	1037	980	1023	980	1023	966	1009

[a] Notation: + = Singly charged ion, ++ = original double charged fragment ion (converted to single charged), M = molecular ion, ? = uncertain peak.

in the samples the whole fragmentation was needed. Most of the microcystins could be identified on the basis of their $[M+H]^+$ and $[M+2H]^{2+}$ ions in the MS mode or by using the MS–MS technique.

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