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Separation of microcystins by capillary electrochromatography in monolithic columns☆

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Abstract

Contribution on microcystin variant analysis by capillary electrochromatography (CEC) with easily affordable spectrophotometric detection is presented. Two types of reversed-phase capillary columns formed by inorganic or organic polymer monoliths were prepared for this purpose. The analyses were performed isocratically by means of tris(hydroxymethyl) aminomethane (TRIS) buffers of mildly alkaline pH containing 30% (v/v) acetonitrile as the mobile phases. The samples were injected electrokinetically and the analyses were done at the same separation field strength of 500 V/cm. Microcystins were detected at 238 nm. Although both column types differ not only in monolith quality (inorganic versus organic) but also in the length of the aliphatic moiety (C8 versus C12) similar results were achieved. The on-column preconcentration as the encouraging prospect of electrochromatographic technique was also tested. Consequently 5% of column volume was injected in contrast with 0.5% at standard injection scheme resulting in the six times enrichment of the low concentrated cyanobacterial extract at the top of the separation column. From these preliminary results can be seen that the CEC method is fully applicable for rapid microcystin screening.

Keywords: Microcystins; Separation; HPLC; Capillary electrochromatography; Monolithic columns

1. Introduction

Microcystins are a group of hepatotoxic cyclic heptapeptides produced by various genera of cyanobacteria [1]. Hepatotoxins are synthesized and retained in cyanobacteria cells and their majority is released during bloom senescence and cell lysis, although active transport from growing cyanobacterial cells was also recently suggested [2]. As a result microcystins represent important contaminants of freshwater ecosystems and human health risks associated with their occurrence in drinking or recreational waters have been recognized [3,4]. There are more than 70 microcystin variants, and toxic strains usually produce a mixture of different microcystins [3]. The general structure of microcystins is cyclo[-D-Ala-L-R_1-D-MeAsp-L-R_2-Adda-D-Glu-Mdha-] as shown in Fig. 1. Apart from two variable L-amino acids, R_1 and R_2, microcystins consist of three D-amino acids: alanine (D-Ala), methylaspartic acid (D-MeAsp) and glutamic acid (D-Glu), and two unusual amino acids: *N*-methyl dehydroalanine (Mdha) and 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda). The Adda amino acid is responsible for the biological activity of the toxins. The variable L-amino acids give name to the whole molecule. Microcystin-LR (MC-LR) with leucin L and arginin R residues is the most common microcystin variant.

Microcystins are chemically stable [5,6] and can persist in water for several weeks after the bloom [7]. They remain stable and unchanged at irradiation by sunlight; however, the presence of pigments in cyanobacteria cells led to photosensitization and decomposition as a result of the isomerization of a double bond in the Adda chain [8]. Microcystins act as inhibitors of ser-

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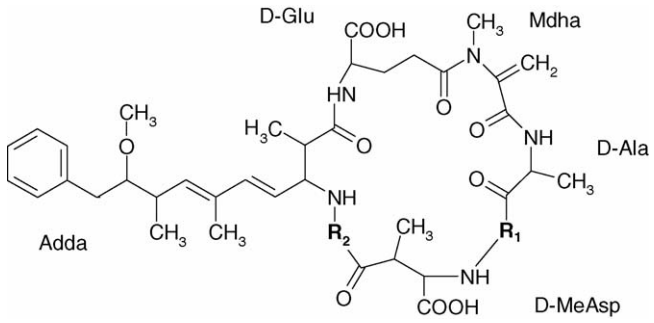

Fig. 1. General structure of microcystin variants; R_1 and R_2 are the variable L-amino acids. For example, in microcystin-LR, R_1 = L-leucine (L), R_2 = L-arginine (R).

ine/threonine protein phosphatase 1 and 2A [9] that can evoke hepatocyte necrosis and hemorrhage [10]. In addition to acute hepatotoxicity, exposure to low concentrations of microcystins can cause chronic effects in mammals such as tumor promotion [11] and evidence on microcystin genotoxicity, nephrotoxicity and neurotoxicity was also reported [4]. Incidences of animal and human fatalities caused by microcystins result in the introduction of guideline values for drinking water by the World Health Organization with a recommended limit of 1 μg of MC-LR per litre [12].

Increased awareness of the hazards presented by microcystins urges the development of diverse methods for their detection and quantification, ranging from biological based screening methods to more sophisticated analytical techniques [13]. Over the past few years, routine sample screening by mouse bioassay has been replaced with more sensitive and reliable assay methods such as enzyme-linked immunosorbent assay [14,15] and the protein phosphatase assay [16]. Although these methods are useful in determining the presence of microcystins in samples, they cannot discriminate microcystin variants.

Analytical separation of microcystins is most commonly carried out by reversed-phase high performance liquid chromatography (HPLC) [17,18], thin layer chromatography [19], and capillary electrophoresis (CE) combined with UV detection [20,21]. The UV detection is based on the absorption of conjugated system of double bounds in Adda, which corresponds with microcystin's characteristic spectral maximum at 238 nm. Besides the above-mentioned separation methods also challenging capillary electrochromatography (CEC) was used for separation of cyanobacterial toxins [22,23]. Further, the mass spectrometry (MS) might be successfully used to identify and quantify microcystins [24–26]. The HPLC–MS combination is capable to determine trace amounts of each microcystin separately at the same time and simultaneously it provides enough structural information to identify them.

CEC is a modern technique that combines high efficiency of CE and selectivity of chromatographic analysis [27], especially in terms of low sample consumption, selectivity and speed of analysis. Another advantage of CEC is the ability to analyze both charged and neutral solutes at the same time. Neutral analytes are separated via differences in their interaction with the stationary phase while charged analytes are further separated according to their diversities in electrophoretic mobilities.

The monolithic columns are a current trend in CEC instrumentation [28–30] and they are applied for the analysis of biological and environmental materials [31–33]. The monolithic column contains a continuous porous structure of organic or inorganic polymer. The monolith is prepared by polymerization of monomers (or precursors) units within the confines of a capillary. The monolith is attached to the capillary walls by covalent bonds or rarely by adhesion. Such structure fills the interior of the column and represents the stationary phase of the separation system. The preparation of monolithic columns is easy and has many advantages in comparison with traditional particle filled columns. Frits are not necessary, so the bubble formation is reduced. For their good flow characteristics, the monoliths have a low backpressure. The created polymeric separation bed has high resistance to chemical attack against various organic solvents, acid or alkaline medium, and it withstands a broad range of temperatures.

CEC has chance to be a very efficient and selective method for separation of peptides [34] and in this paper we demonstrate its suitability for microcystin analysis. In association with UV detection CEC has potential to be an accessible prompt alternative to HPLC.

2. Experimental

2.1. Toxin samples and extraction

Cyanobacterial water bloom (dominated by hepatotoxic *Microcystis aeruginosa*) was collected in Brno reservoir (Czech Republic) during the 1998 and 2001 summer seasons and stored at −20 °C before extraction. After thawing, cyanobacterial biomass was homogenized and microcystins were extracted with 10% (v/v) aqueous methanol using sonification (60 min). Crude extract was obtained after centrifugation (10 min, 3000 × *g*) and filtration of supernatant through the GF/C filter (Whatman International Ltd., Maidstone, Kent, UK). Solid phase extraction was used for microcystin concentration and partial purification. Filtered supernatant was passed through ODS Sep-Pak 35cc 10 g C_{18} cartridge (Waters Associates, Inc., Milford, MA, USA) activated with 100 ml methanol and Milli-Q water. After the sample application, the cartridge was washed with water and 20% (v/v) aqueous methanol (100 ml each) and the peptide fraction (mostly microcystins) was eluted with 100 ml of 60% (v/v) methanol. The aliquots of the eluate were freeze-dried and then redissolved in the appropriate solvent.

2.2. Chemicals

Tris(hydroxymethyl) aminomethane (TRIS), 4-morpholinoethanesulfonic acid monohydrate (MES), trifluoroacetic acid (TFA) and boric acid were purchased from Fluka (Buchs, Switzerland), alkylphenones, *N*,*N*,*N*′,*N*′-tetramethylethylene diamine (TEMED), tetramethoxysilane, tetraethoxysilane and octyldimethylchlorsilane for the sol–gel column preparation were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solutions were prepared in deionised water. For preparation of the organic monolith we used poly(ethylene glycol) M_r 10,000

and *N*-methylformamide obtained from Fluka. Methanol and HPLC grade acetonitrile were purchased from Scharlau Chemie (Barcelona, Spain). Standards of microcystin-LR and -RR were supplied by Alexis Biochemicals (Läufelfingen, Switzerland) and microcystin-YR standard by Sigma–Aldrich (Prague, Czech Republic).

2.3. Methods and columns

The HPLC separation was accomplished on Agilent 1100 chromatographic system with diode array detection (Agilent Technologies, Waldbronn, Germany) and evaluated by HP Chemstation 1100 software. The Supelcosil ABZ + Plus column 150 mm × 4.6 mm, 5 μm particles (Sigma–Aldrich, Prague, Czech Republic) was used for the chromatographic separation. Mobile phases consisted of 0.1% (v/v) TFA in water (solution A) and 0.1% (v/v) TFA in acetonitrile (solution B) with gradient elution programme: 20% B v 0 min, steepness 1.3% B/min; flow rate: 1 ml/min; temperature 30 °C.

For CEC experiments we have used laboratory made capillary columns (of organic and inorganic types). The sol–gel monolithic column was prepared according to the method described by Tanaka et al. [35] and octyldimethylchlorsilane was used for silica surface modification. The acrylamide monolith was synthesized according to the published papers [36,37] with a slight modification. The polymerization mixture contained acrylamide monomer, piperazine diacrylamide as a crosslinker, laurylacrylate for reversed phase character and vinylsufonic acid for electroosmotic flow (EOF) generation. The monomers and the crosslinker were dissolved in *N*-methylformamide/50 mM phosphate buffer (pH 7.8) (95/5, v/v) solution and the mixture was shortly purged by nitrogen. The polymerization was iniciated by the additional of 10 μl of 40% (m/v) ammonium persulfate and 4 μl of TEMED. Then the polymerization mixture was sucked into the capillary and sealed with septum. The reaction was performed at laboratory temperature overnight. All separation columns were tested on their reversed phase character by standard alkylphenone mixture (data are not shown) at the appropriate separation buffer.

The CEC separation was performed at laboratory made apparatus consisting of a high voltage power supply Spellman CZE 1000R (New York, NY, USA) and a UV-970 detector (Jasco, Tokyo, Japan). Data were collected using CSW 32 Software (Data Apex Ltd., Prague, Czech Republic). The proper buffer solutions (see below) with given content of acetonitrile were used as a mobile phase. The stock solution was degassed and filtered each day before filling electrolyte reservoirs. The electrolyte reservoirs were not pressurized during separations and injection was performed electrokinetically. All CEC separations were performed at laboratory temperature and the on-column UV detection at 238 nm was used.

3. Results and discussion

The cyanobacterial extract was analysed for microcystin variant by the classic HPLC method and by CEC. The first method of choice was HPLC as a standard and recommended technique [38]. The HPLC separation of microcystins often involves the use of gradient of mobile phase consisting of acetonitrile in water at acidic pH. The addition of TFA also sustains low pH to suppress ionization of silanolic groups and acts as an ion-pairing agent. In our study the linear gradient of acetonitrile was set from 20 to 46% for 20 min, and the resulting separation of individual microcystins is shown in Fig. 2. The microcystin concentrations presented in Table 1 were calculated from calibration curve of external standards analysed by HPLC under the same conditions.

The mobile phase had to be optimised for the CEC separation. The presence of TFA is not compatible with the requirement

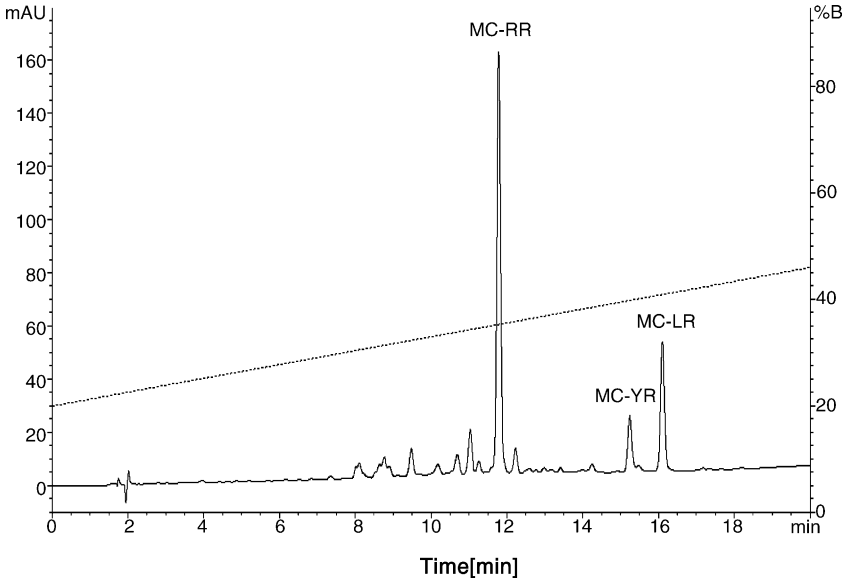

Fig. 2. HPLC chromatogram of the cyanobacterial extract (year 2001). Injected sample volume: 1 μl; column temperature: 30 °C; detection: λ = 238 nm. MC-RR stands for microcystin RR (it contains L-arginine in both variable positions in microcystin structure. Similarly MC-LR marks microcystin-LR (L-leucine in position R_1, L-arginine in position R_2) and MC-YR identically possesses L-tyrosine in R_1 position and L-arginine in position R_2). See Section 2.3 for other HPLC details.

Table 1
Microcystin concentrations in cyanobacterial extracts collected during 1998 and 2001 summer seasons at Brno reservoir as determined with HPLC

Brno reservoir	c (μg/ml)	
	1998	2001
MC-RR	57.8	253.5
MC-YR	3.6	48.5
MC-LR	31.7	82.0

Microcystin abbreviations are elucidated in Fig. 2.

of low conductance of mobile phase and for this reason it was excluded. For the CEC separation the most suitable mobile phase was the mixture of the appropriate buffer with 30% (v/v) of acetonitrile as shown in Figs. 3 and 4. In both cases mildly alkaline pH of mobile phase was used. The possibility of alkaline pH for microcystin separation was supported by Ruangyuttikarn et al. [39]. The detection wavelength was chosen at the absorption

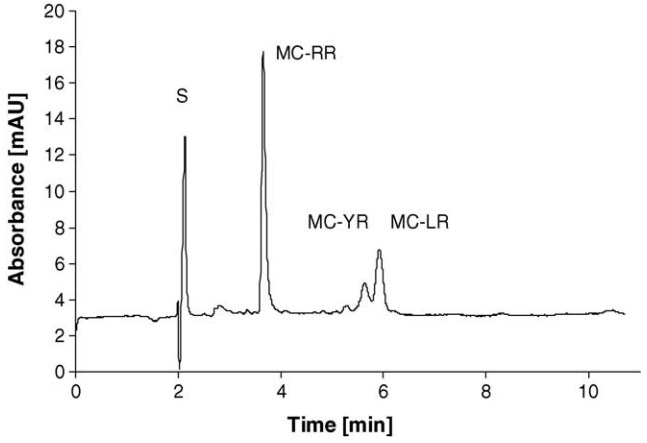

Fig. 3. CEC separation of microcystins on sol–gel C8 monolithic column: 75 μm I.D., 125/95 mm (tot./eff. length); mobile phase: 10 mM TRIS/15 mM borate (pH 8.5)–acetonitrile (30%, v/v), sample: cyanobacterial extract (year 2001); electrokinetic injection: 1.11 kV, 6 s; separation voltage: 6.3 kV (positive polarity); detection: λ = 238 nm. Microcystin abbreviations are elucidated in Fig. 2.

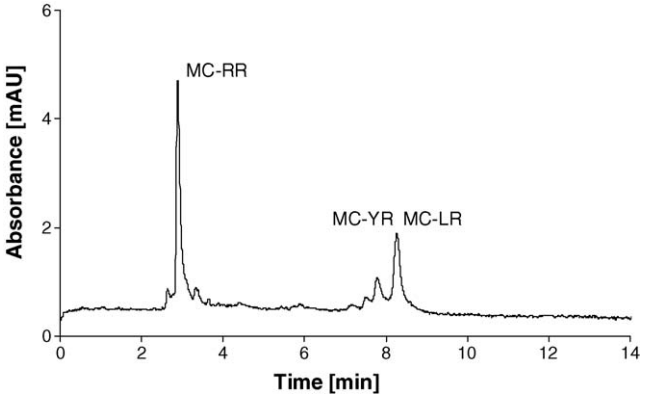

Fig. 4. CEC separation of microcystins on C12 monolithic column: 100 μm I.D., 160/110 cm (tot./eff. length); mobile phase: 10 mM TRIS/10 mM MES (pH 7.7)–acetonitrile (30%, v/v); sample: cyanobacterial extract (year 2001); electrokinetic injection: 0.5 kV, 6 s; separation voltage: 8 kV (positive polarity); detection: λ = 238 nm. Microcystin abbreviations are the same as in Fig. 2.

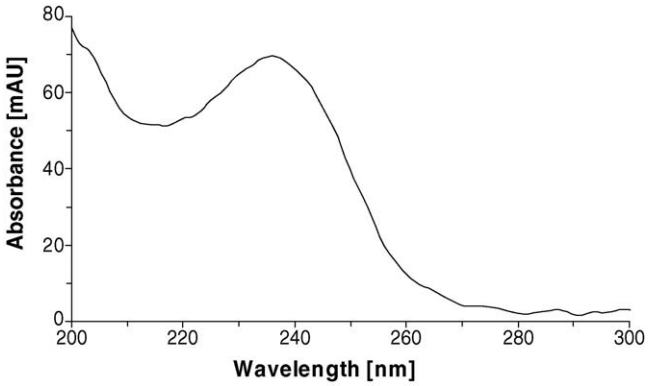

Fig. 5. UV spectrum of MC-LR collected at stop flow during the analysis as presented at Fig. 3 (from the third peak).

maximum of microcystins, which is at 238 nm and allows distinguishing them from other substances. Moreover, the spectral characteristic of microcystins was exploited for their initial identification. Fig. 5 represents the absorption spectrum of MC-LR measured at the stop flow during the CEC analysis with a distinct maximum in the region of 238 nm. The acquired spectrum is in agreement with the published MC-LR spectrum [40].

In this preliminary study we concentrated mainly on qualitative analyses therefore no quantification data from CEC were evaluated. Just for a rough estimate the peak areas from the CEC separations were linked with concentrations obtained from the chromatographic separation (as indicated in Table 1) and their ratios were in accord.

Generally, the usual injected volume of the sample in CEC analyses should not exceed 1% of column volume; nevertheless, for on-column preconcentration we can introduce theoretically the whole column volume. In Fig. 6 the preconcentration of the sample on the column head is documented. The cyanobacterial extract was dissolved in water and the elongated injection was carried out. For on-column preconcentration the 5% of the column volume was injected on the contrary to 0.5% at standard

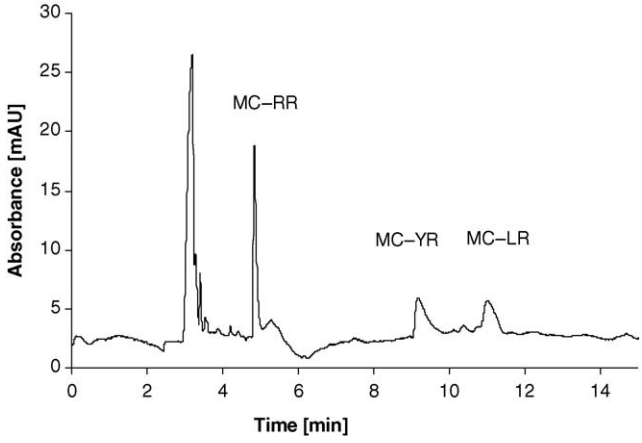

Fig. 6. CEC on-column preconcetration and separation of low concentrated cyanobacterial extract. Sol–gel C8 monolithic column: 100 μm I.D., 167/135 mm (tot./eff. length); mobile phase: 10 mM TRIS/15 mM borate (pH 8.5)–acetonitrile (30%, v/v); sample: cyanobacterial extract (year 1998); electrokinetic injection: 4.5 kV, 20 s; separation voltage: 8.5 kV (positive polarity); detection: λ = 238 nm. Microcystin abbreviations are elucidated in Fig. 2.

injection scheme as presented in Figs. 3 and 4. Although the 10 times higher sample volume was injected, the microcystins were concentrated by a factor of six according to the peak areas. We assume that such result was caused by the absence of acetonitrile in the matrix of low concentrated sample.

The CEC analysis is rapid and instrumentally easy. We used laboratory made reversed phase monolithic columns with that enable to obtain the same elution order of microcystins at given conditions as in the HPLC analysis. The column preparation is not difficult and we can easily handle it with a possibility to adapt its length or restore the capillary ends. Frits are not required for monolithic columns, which facilitates their effortless operating. Moreover, other instrumental equipment is unpretentious. A high-pressure pump is not necessary because the liquid movement is mediated by electroosmotic flow and it is sometimes possible to perform the analysis without need of gradient elution. Anyway, we can use external pressure if desirable. In comparison with the HPLC analysis CEC makes minimal demands on sample amount and it keeps the possibility of on-column preconcentration. In addition the CEC analysis is obtained within short time span at adequate efficiency. To date, there are two publications focused on CEC separation of cyanobacterial alkaloid toxins on commercial packed columns [22,23], but as we have shown here, also microcystins-toxic peptides, can be successfully separated by CEC on laboratory made monolithic columns.

4. Conclusions

Problems of cyanobacterial water bloom occurrence and their toxic secondary metabolites are of serious concern today worldwide. The important part of projects aiming to manage and control water blooms is the monitoring of microcystin concentrations using rapid analytical methods. CEC could be such method that – as a microseparation method – allows the quick separation and determination of microcystin variants.

The standard chromatographic separation uses the gradient elution and so the CEC separation seems to be advantageous. CEC does not need in this particular case gradient elution that makes the analytical system instrumentally easy. Further, no additives are necessary. Another asset is a possibility to concentrate the sample on the top of the column and thus simplify the sample preparation. From these preliminary results can be seen that the CEC method could be fully applicable for rapid microcystin screening in contaminated drinking or recreational waters.

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