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Received January 15, 2009
Revised March 22, 2009
Accepted March 23, 2009

Research Article

Determination of cyanobacterial cyclic peptide hepatotoxins in drinking water using CE

Four cyanobacteria hepatotoxins microcystin LR, microcystin RR, microcystin YR, and nodularin were simultaneously determined in drinking water using CZE and MEKC coupled with UV detection. The toxins were satisfactorily separated in both CZE and MEKC modes. Detection limits were in the range of 0.82–4.81 μg/mL, with R^2 values of 0.994–0.999. The linearity range tested for the standards was 5–100 μg/mL and RSD percentages were in the range of 1.0–2.5% for retention time and 3.0–10.2% for peak area. When a known amount of standard was spiked into a known volume of water and extracted, recoveries were 90.3% (RR), 101.5% (nodularin), 90.6% (YR), and 88.2% (LR). The use of SPE enabled cleanup and pre-concentration of a real sample to achieve a 100-fold concentration factor. Detection limits after SPE of the real sample spiked with microcystins were 0.090 μg/L (RR), 0.076 μg/L (YR), and 0.110 μg/L (LR), with RSD percentage values of 9.9–11.7% for peak area and 2.2–3.3% for retention time. The technique developed provides an alternative method for determining microcystins at levels of concentration that will be able to meet WHO drinking water guidelines for microcystins.

Keywords:
CE / Microcystin(s) / Nodularin DOI 10.1002/elps.200900030

1 Introduction

The presence of cyanobacteria (blue-green algae) in water bodies results in unpleasant taste and odor. It is also capable of producing a wide range of potent toxins usually referred to as cyanotoxins [1, 2]. There are about 40 genera of organisms that produce cyanobacteria toxins but the main ones are *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Microcystis, Nostoc,* and *Oscillatoria* (*Planktothrix*) [1]. These organisms thrive in the presence of sunlight and high temperature, especially in polluted waters that are rich in nutrients and have a slow flow rate or are stagnant [1].

Cyanotoxins can be grouped into cyclic peptides (hepatotoxic microcystins and nodularins), alkaloids (hepatotoxic cylindrospermopsin and neurotoxins such as anatoxin-a, anatoxin-a(s) and saxitoxins), and lipopolysaccharides.

Microcystins are cyclic heptapeptides with relative molecular masses (RMM) in the range 500–4000 Da. They are seven peptide-linked amino acids with two terminal amino acids of the linear peptide condensed to form a cyclic compound. Five of the amino acids are non-protein and mostly constant in microcystins. The other two, which distinguish microcystins from each other, are protein amino acids [1]. The general structure is cyclo-(D-alanine-X-D-erythro-β-methyl aspartic acid-Z-2S, 3S, 8S, 9S-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4,6-dienoic acid-D-glutamate-*N*-methyldehydroalanine), short form: [cyclo-(D-alanine-X–D-MeAsp-Z-Adda-D-glutamate-Mdha], in which X and Z represent the variable (protein) amino acids. Nodularins lack the two amino acids that distinguish microcystins and are therefore monocyclic pentapeptides. The general structure is cyclo-(D-MeAsp-L-Arginine-Adda-D-glutamate-2-(methylamino)-2-dehydrobutyric acid [Mdhd]). The Adda amino acid is a typical structure in all cyclic peptide toxins (Fig. 1).

The need to determine the presence of cyanobacteria toxins is mainly due to increased awareness of the harmful effects from exposure. Skin irritations and death of dogs and cattle have been linked to microcystin exposure [3] and human fatalities were reported after exposure to cyanotoxins during dialysis in Brazil [4, 5]. Moreover, exposure to low levels of hepatotoxins has been linked to the development of tumors and cancers by an epidemiological study in China [6]. This increasing evidence shows that low levels of exposure may have chronic effects in humans [7]. Therefore, it is important to determine microcystins in drinking and other use waters even at low concentrations because in addition to immediate effects attributed to exposure at high

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Abbreviation: RMM, relative molecular masses

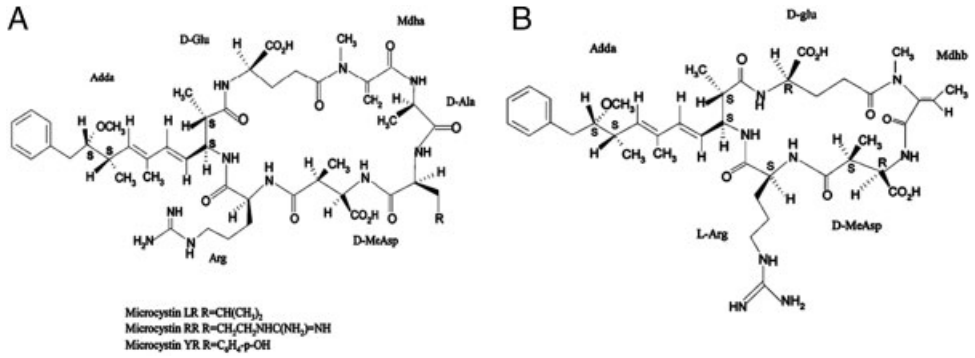

Figure 1. (A) General structure of microcystin and (B) structure of nodularin.

concentrations, long-term exposure to low amounts of the toxins can also cause detrimental effects.

Microcystins are relatively polar molecules (due to carboxylic acids and amino and amido groups) and the Adda residue gives them a partially hydrophobic character [1, 8]. Therefore, they remain in the aqueous phase rather than being adsorbed on sediments or on suspended particulate matter [8]. Nodularins have similar chemical properties, so they can be determined with the same method as for microcystins. Determination of microcystins and nodularin in the same run by HPLC has been reported [9, 10] and CE can be used for their simultaneous determination. Other techniques have been used for the determination of cyanobacterial hepatotoxins, some of which are briefly mentioned below.

Mouse bioassay is a widely used method, but it lacks sensitivity and is not suitable for quantitation [7] considering the number of mice that would be involved and usually a license would be required to use them. Protein phosphatase bioassay and ELISA techniques are used to provide rapid screening for microcystins even though they have poor specific identification ability. So they are usually used as a first screen [7, 11].

The most established and commonly used methods are based on HPLC [2, 12, 13], which is an efficient technique. However, it can involve relatively lengthy analysis time and high solvent and chemical reagent consumption. Compared to HPLC, CE offers short analysis time, high separation efficiency, small sample volume, low solvent cost, and little hazardous waste. Therefore, there are significant advantages to use CE as an alternative in the determination of microcystins.

Vasas *et al.* [14, 15] and Onyewuenyi and Hawkins [16] demonstrated that CE can be used to separate microcystins and the successful mode of CE employed was MEKC using SDS. The use of CEC has also been reported [17]. However, the sensitivity reported in these studies was relatively low (in the mg/L range). Gu *et al.* [18] successfully separated microcystins LR, RR, and YR using pressurized CEC and was able to achieve limits of detection in μg/L range with the aid of SPE. However, the method required home-made monolithic columns and highly specialized instrumentation, which are not readily available for routine analysis of environmental and water samples.

In our study, simple CZE and MEKC methods for the determination of four cyananobacterial hepatotoxins (microcystin RR, microcystin YR, microcystin LR, and nodularin) in drinking water were investigated. Satisfactory separation was obtained and the method was validated for the determination of hepatotoxins in drinking water. The method demonstrated the potential to be applied to a wider range of microcystins because the resolutions between adjacent peaks were good. Furthermore, by using formic acid as a BGE, this method can be extended for use with mass spectroscopy since the BGE is volatile.

2 Materials and methods

2.1 Experimental

CE was performed on a portable CE-P2 system (CE Resources, Singapore). A UV–vis detector (Shimadzu SPD 10A) was used at 238 nm (λ_{max} for microcystin). Fused silica capillaries (Polymicro technologies) of 65 cm total length (55 cm effective length) were used and were conditioned by flushing with 1 M aqueous NaOH for 30 min, followed by deionized water for 30 min, and finally BGE for 30 min. In subsequent use, the capillary was flushed with NaOH (1 M), followed by water and BGE for 20 min each before commencing experiments each morning.

The sample was injected hydrodynamically at 0.3 psi for 15 s and voltage applied was 20–25 or −20 kV for CZE and MEKC modes, respectively. Data were acquired and analyzed using clarity chromatography software from Data Apex Clarity (Prague, The Czech Republic).

2.2 Chemicals

Microcystins LR, RR, and YR and nodularin were obtained from Alexis Biochemicals (Switzerland). Formic acid and sodium hydroxide were from Fluka (Singapore). CTAB and ethylene glycol were from Sigma Aldrich (Singapore). Phosphoric acid was supplied by Riedel-de Haen (Germany) and methanol was obtained from Tedia (Tritech Scientific Pte, Singapore). C_{18} ODS SPE column, 500 mg (Whatman), and SPE manifold from Supelco (Sigma-Aldrich) were used for sample preparation.

2.3 Sample preparation

Standard solutions of 100 μg/mL (100 ppm) concentration were prepared by dissolving 100 μg of each individual standard in deionized water (1 mL). For the test samples, known amounts of the standard solution were spiked in tap water. This was followed by extraction using a C_{18} (octadecyl) silica SPE column aided by an SPE manifold.

Real water samples (1 L each) made up of surface water (collected at 4 m deep in the water reservoir) were collected from Kranji water reservoir (Singapore). An aliquot of the real sample (50–500 mL) was spiked with microcystin standards and extracted. Another aliquot of equivalent volume was extracted using the same procedure as the spiked sample and the two were compared.

2.4 Extraction

Sometimes the water sample under test may contain other impurities. Furthermore, the provisional guideline for microcystin in drinking water is 1 μg/L [11, 13, 19]. SPE can be used to remove some interference and to concentrate the sample to an amount that can be detected by UV using the methods investigated here.

The SPE column used was activated by 10 mL of methanol (100 %, HPLC grade) followed by deionized water (10 mL). The sample solution was prepared by spiking 5–50 μL each of the 100 ppm standard solution of microcystins LR, RR, and nodularin into tap water or reservoir water sample (50–500 mL). This was extracted using C_{18} SPE column. Finally, the test sample was eluted with methanol (5 mL) and concentrated by heating the sample in a water bath. The test solution was made by reconstituting the sample with deionized water (100–500 μL).

3 Results and discussion

3.1 CZE separation conditions

Formic acid (1 M) was used as a BGE in the CZE mode. The microcystins were very well resolved. No special pretreatment or conditioning of the capillary was necessary and the hepatotoxins could be detected at low μg/L concentration levels (refer to Fig. 2A and Table 1). The elution time was slightly long (~17 min). This is in agreement with other research works [14–16, 20],] which show that the microcystins migrate slowly in acidic pH. The use of formic acid as a BGE was first reported by Bateman *et al.* [20] in the analysis of microcystin LR. In this study, we investigated simultaneous determination and quantification of three related microcystins LR (RMM 995.17), RR (RMM 1038.2), and YR (RMM 1045.19) and a related hepatotoxin nodularin (RMM 825.0). Phosphoric acid used as a BGE behaved similarly as shown in the separation of the microcystins LR, RR, and YR (refer to Fig. 3).

At acidic pH, RR has two positive charges (from arginine) and therefore will migrate toward the cathode faster. Moreover, arginine is very hydrophilic and so solubility in polar solvents is high. Microcystins LR and YR have a single positive charge, but the amino acid leucine is more hydrophobic than tyrosine. Therefore, YR migrates slightly faster and, hence, they can be separated. Nodularin has a single positive charge as well and migrates faster than microcystins LR and YR since it is smaller.

3.2 MEKC

For the MEKC separation, the BGE used was made up of formic acid (1 M), CTAB (0.1%), and ethylene glycol (0.1%). Figure 2B shows the results obtained. The migration order

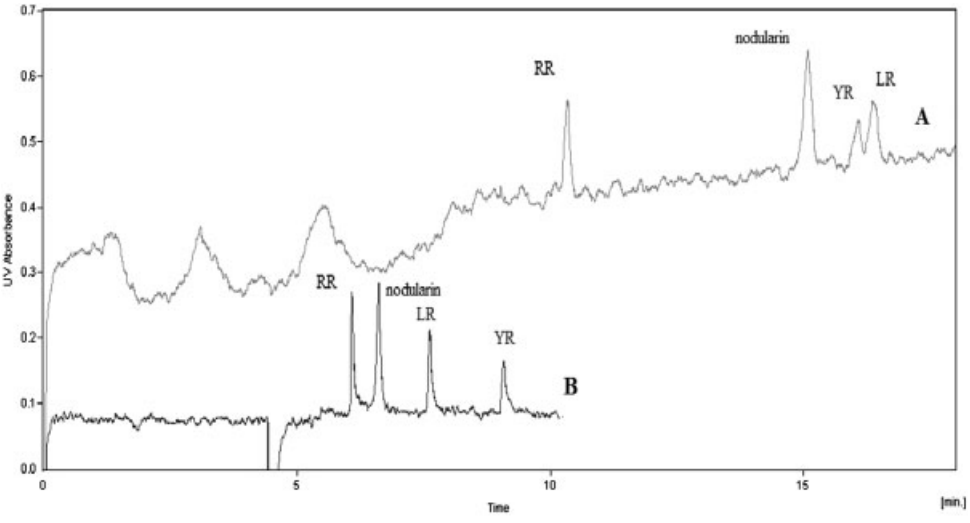

Figure 2. (A) CZE electropherogram of microcystin RR, nodularin, and microcystins YR and LR. BGE- formic acid (1M), Voltage + 25 KV. The concentration of each of the hepatotoxins was 5μ g/mL; (B) MEKC Electropherogram for Microcystins RR, LR and YR and nodularin Voltage −20 KV ; UV detection was at 238 nm; the concentration of each of the hepatotoxins was 5 mg/mL, BGE was made up of formic acid (1 M), CTAB (0.1 %) and Ethylene glycol (0.1%).

Table 1. Analytical performance of the CZE and MEKC methods

Part A: CZE

Analyte	LOD (before stacking) (μg/mL)	LOD (after stacking) (μg/mL)	RSD% peak area (intra-day) $n = 6$	RSD% peak area (inter-day)	RSD% retention time (min) (intra-day) $n = 6$	RSD% retention time (min) (inter-day)	R^2 before stacking	R^2 after stacking
Microcystin RR	4.075	0.824	3.648	4.666	1.000	1.485	0.994	0.990
Nodularin	4.813	0.826	3.349	3.354	1.674	2.119	0.988	0.999
Microcystin YR	4.075	1.770	2.981	3.313	1.696	2.165	0.996	0.995
Microcystin LR	4.730	1.500	7.454	10.164	1.736	2.144	0.997	0.998

Part B: MEKC no stacking

Analyte	Range (μg/mL)	LOD (μg/mL)	RSD% peak area	RSD% retention time	R^2
Microcystin RR	5.00–100	1.400	6.441	0.621	0.995
Nodularin	5.00–100	1.026	5.846	0.626	0.999
Microcystin LR	5.00–100	2.700	2.257	0.751	0.988
Microcystin YR	5.00–100	3.200	2.702	0.850	0.997

Part C: CZE of a real sample spiked with RR, YR, and LR

	Migration time (min)	%RSD in migration time	LOD 3σ (μg/L)	%RSD in peak area
Microcystin RR	13.430	2.060	0.092	9.910
Microcystin YR	21.160	3.230	0.076	11.700
Microcystin LR	21.610	3.310	0.110	9.960

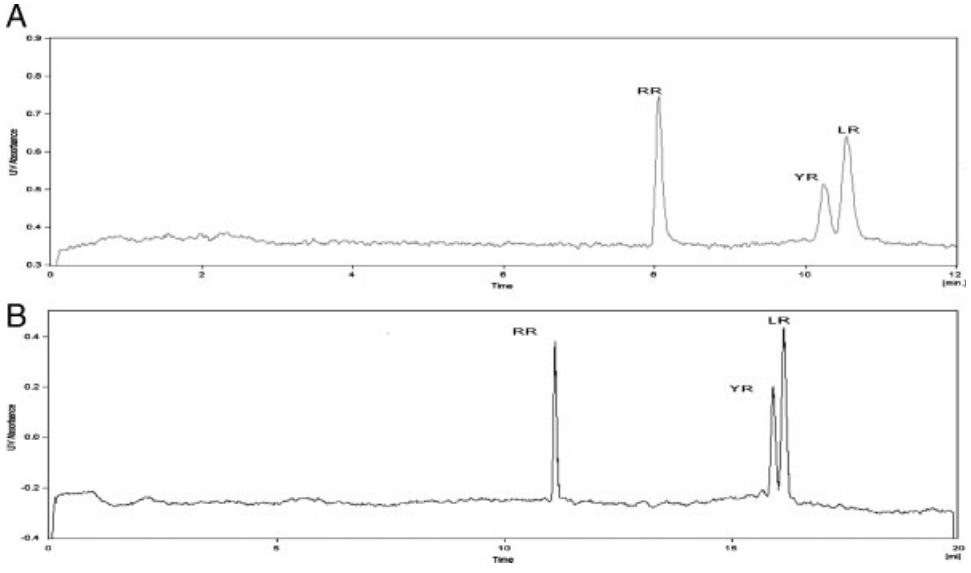

Figure 3. Electropherograms obtained using (A) 50 μg/mL each of microcystins RR, YR, and LR with phosphoric acid (0.1 M) and (B) 30 μg/mL each of the microcystins with formic acid (1 M) as BGE.

between microcystins LR and YR reversed and MEKC gave a very good resolution between the analytes (4.3 between RR and nodularin, 7.1 between nodularin and LR, and 10.2 between LR and YR).

The use of both CZE and MEKC provides a way of confirming the results and can be useful especially if the peak of the analyte overlaps with that from matrix component [15] in cases where no sample pretreatment is done or where sample pre-concentration does not include removal of interfering substances. The CZE method and the MEKC method were evaluated in their analytical performance and the data is shown in Table 1.

3.3 Sample stacking

The small sample volumes injected in CE are partly responsible for low sensitivity. Therefore, a larger volume was injected and the sample was stacked with the aid of an organic solvent to improve on the sensitivity. Water, methanol, and acetone were studied for use as the solvent in the plug to optimize the stacking efficiency. Injection times (volume) for the sample and for solvent were also investigated. Optimum results were obtained by injecting methanol in the solvent plug for 5 s followed by the sample at 0.3 psi for 40 s. The peak heights for the analytes increased RR (3.9 times), YR (4.8 times), and LR (2 times) in CZE and at least twice in MEKC. Table 2 and the electropherograms in Fig. 4 demonstrate that there was a slight sensitivity enhancement.

3.4 Comparison of the CZE and MEKC methods

The comparison can be summarized into the electropherograms in Fig. 2. Both techniques offer a good separation. Good resolution (4.3 between RR and nodularin, 7.1 between nodularin and LR, and 10.2 between LR and YR) and faster migration times were obtained using the MEKC technique. However, in both cases the resolution obtained is acceptable (18.7 between RR and nodularin, 3.5 between nodularin and YR; 0.95 between YR and LR) and the detection limits were in the same range.

3.5 Tap water analysis

Tap water was spiked with microcystins RR, LR, and nodularin (5 μg/mL each), and the sample was analyzed by CZE with stacking at 20 kV. The results are shown in the electropherogram in Fig. 5A. The use of both CZE and MEKC is particularly useful for cases where no sample cleanup is carried out. This is because the different separation mechanism involved can allow for identification of peaks in case of the matrix peak overlapping with the microcystin peaks. When sample cleanup is done, either CZE or MEKC can be used as a stand-alone technique in the determination of the microcystins.

3.6 Reservoir water sample analysis

A reservoir water sample (50 mL) was extracted as described in the Section 2.1 and eluted with methanol (5 mL). The sample was evaporated in a water bath and the final test solution was reconstituted with deionized water (500 μL). To another aliquot of the reservoir water sample (50 mL), microcystin standards RR, LR, and YR (50 μL each of the 100 μg/mL) were added. This sample was also extracted, eluted, and reconstituted as the first sample. Microcystins LR, RR, and YR were not detected in the reservoir water sample. Good reproducibility was obtained for the spiked sample as shown in Table 1 part C. Detection limits for microcystins RR, YR, and LR were all below 0.11 μg/L. The sensitivity will be able to meet WHO drinking water guidelines for microcystins (*i.e.* 1 μg/L for microcystin LR). Figure 5B shows the electropherograms obtained.

Table 2. Stacking with the aid of methanol in CZE

Condition	Microcystin RR peak height (mV)	Microcystin YR peak height (mV)	Microcystin LR peak height (mV)
Injection of sample for 10 s	0.154	0.031	0.163
Injection of sample for 40 s	0.411	0.131	0.292
Injection of methanol for 5 s followed by sample for 40 s	0.601	0.149	0.330
Enhancement	3.903	4.806	2.035

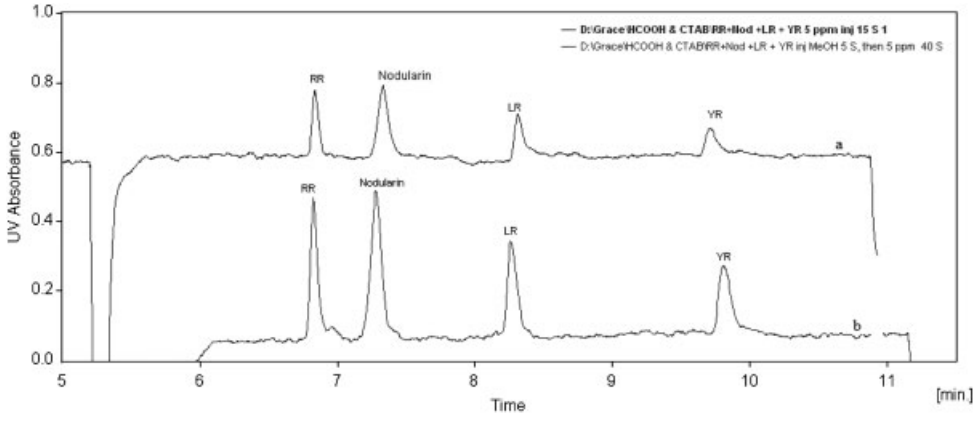

Figure 4. MEKC separation of the microcystins. (a) Without stacking and (b) after stacking with the aid of methanol. The test solution was 5 μg/mL of the hepatotoxins.

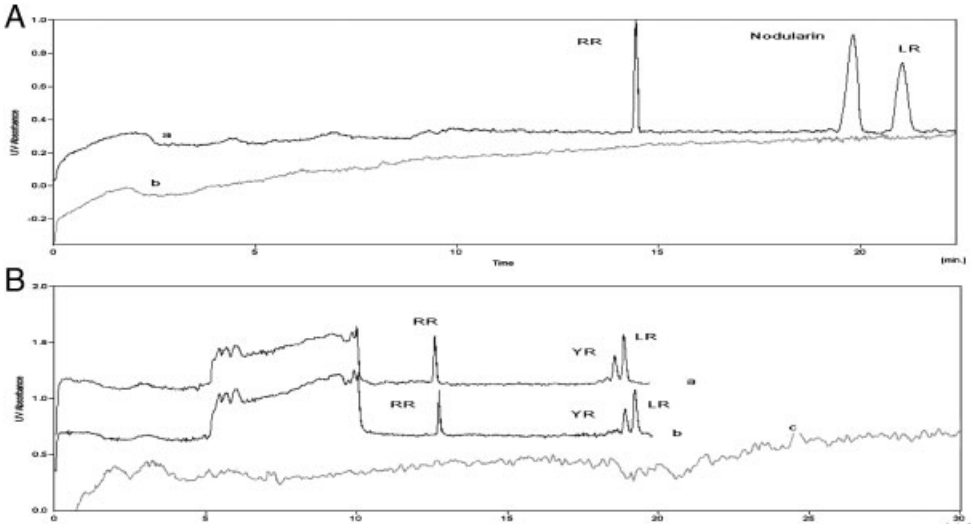

Figure 5. Electropherograms of real samples: (A) – tap water: (a) spiked with 5 μg/mL each of microcystins LR, RR, and nodularin after stacking with the aid of methanol; (b) tap water that was not spiked, also stacked with the aid of methanol. (B) – reservoir water sample: (a) spiked with microcystins LR, RR, and YR (10 μg/mL each); (b) a repeat run of the spiked sample; and (c) reservoir water sample that was not spiked.

4 Concluding remarks

The use of CE was demonstrated to be applicable in the determination of cyclic hepatotoxins in tap and reservoir water. The technique is very simple and can be readily extended to a wide range of microcystins. By using both CZE and MEKC, the microcystins can be determined even for cases where no sample cleanup is carried out. By performing sample cleanup and pre-concentration using SPE, the method enables detection of the microcystins at concentration levels (below 1 μg/L), which will meet the WHO drinking water guidelines. With the inherent advantages of CE (*e.g.* rapid, low consumption of reagents and samples, high sensitivity, and automated), this method will be useful in routine monitoring of presence of microcystins in drinking water. In addition, based on the volatile buffer used in this study, we are pursuing further work on the application of CE-MS for the analysis of samples collected in contaminated areas.

Financial supports from The Third World Organization for Women in Science (TWOWS), National University of Singapore (NUS), and EWI (MEWR C651/06/144) are acknowledged. We would also like to thank Dr. Yuk Chun Chiam-Tai and Mr. Seng Chen Tan of Public Utilities Board (PUB) of Singapore's national water agency, for their assistance in the collection of the water samples.

The authors have declared no conflict of interest.

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