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Analysis of microcystins by capillary zone electrophoresis coupling with electrospray ionization mass spectrometry

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ABSTRACT

In this paper, a rapid and effective method based on capillary zone electrophoresis (CZE) coupled with electrospray ionization mass spectrometry (ESI-MS) was established for the trace analysis of microcystin (MC) isomers in crude algae sample. The experimental conditions including the composition, acidity and concentration of buffer, separation voltage, injection time, and MS detection parameters were investigated in detail. A capillary separation system was as follows: a uncoated fused-silica capillary tube (50 μm i.d. × 90 cm), 40 mmol L^{-1} ammonium acetate solution (pH 9.86) as running buffer, 25 kV as separation voltage, 20 kV × 3 s water first and 20 kV × 20 s for sample injection. Mass analysis was performed in ESI source, with sheath gas temperature 150 °C, sheath gas pressure 10 psi, and sheath gas flow 6 L min^{-1}. And sheath liquid was 7.5 mmol L^{-1} acetic acid in 50% isopropanol–water (3 μL min^{-1}). Protonation and ammonium adduct molecular ions *m*/*z* 506.9 (MC-LR) and 532.0 (MC-YR) were used for the quantification of MCs. Under these conditions, two MCs were baseline separated within 9 min, the calibration curves were obtained in the range of 0.11–10.0 μg mL^{-1} and 0.16–10.5 μg mL^{-1} for MC-LR and MC-YR, respectively. Meanwhile, limits of detection were 0.05 and 0.08 μg mL^{-1} for MC-LR and MC-YR, respectively. The recoveries for the two MCs were in the range of 95.8–108%. The developed approach had been successfully applied to the analysis of MCs in crude algae samples.

1. Introduction

A multitude of occurrences of toxic *cyanobacteria* have been reported in freshwater lakes, drinking water reservoirs and brackish seawaters all over the world [1,2]. Waterblooms of *cyanobacteria* (also named blue-green algae) are recognized as an important problem for the aquatic environment due to the potential toxins that they can produce. *Cyanobacteria* have been responsible for the death of wild and domestic animals and would also be a health hazard for humans [3,4]. Microcystins (MCs) are the best known and most widespread of the cyanotoxins. MCs have a common structure (see Fig. 1) containing three D-amino acids (alanine, β-linked erythro-β-methylaspartic acid and α-lined glutamic acid), two unusual amino acids, N-methyldehydroalanine (Mdha) and 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda), and two variable L-amino acids, R_1 and R_2 [5]. More than 60 MCs have been identified so far from bloom sample and isolated strains of *cyanobacteria* [2], but the majority of the analytical studies only focus on the three well known toxic heptapeptides, including MC-LR, MC-RR and MC-YR, especially MC-LR [2]. Several cases of animal and human intoxication have been reported, showing that these toxins can lead to liver cancer and even death [3,6,7]. World Health Organization (WHO) has recommended a maximum level of 1 μg L^{-1} for MC-LR in the guidelines for the drinking water quality [8]. The human fatalities have highlighted the need to develop fast, sensitive and reliable methods to detect these toxins.

Several analytical methods have been developed for MCs analysis, including mouse bioassay (MBA) [9], protein phosphatase inhibition assay (PPA) [10], enzyme-linked imunosorbent assay (ELISA) [11,12], and HPLC coupled with various detectors [13–16]. As a conventional screening method, the three former is simple. However, poor sensitivity, low specificity and the potential false positives for MCs limited the further application. Unlike the

Abbreviations: MC, microcystin; Mdha, methyldehydroalanine; Adda, 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid; CZE, capillary zone electrophoresis; ESI-MS, electrospray ionization mass spectrometry; WHO, World Health Organization; MBA, mouse bioassay; PPA, phosphatase inhibition assay; ELISA, enzyme-linked imunosorbent assay; HPLC, high performance liquid chromatography; UV, ultraviolet; MS, mass spectrometry; SIM, selective ion monitoring; SPE, solid phase extraction; Rs, resolution; MEKC, micellar electrokinetic capillary chromatography; EOF, electroosmotic flow; TIC, total ion current; *m*/*z*, molecular mass–charge ratios; MSD, Mass Spectrometry Detection.

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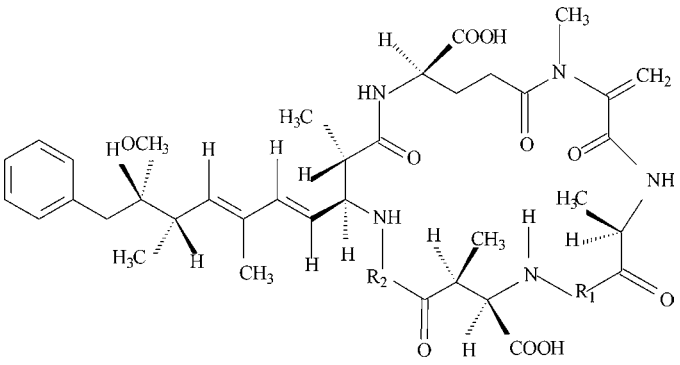

Fig. 1. Structures of MC. MC-LR ($C_{49}H_{74}N_{10}O_{12}$): R_1-Leu, R_2-Arg; MC-YR ($C_{52}H_{72}N_{10}O_{13}$): R_1-Tyr, R_2-Arg.

former, HPLC–UV and HPLC–MS can afford high separation selectivity and precisely quantitative analysis. Recently, capillary zone electrophoresis (CZE) [17–20] and micellar electrokinetic capillary chromatography (MEKC) [21–24] have been also purposed for the determination of MCs analysis due to the merits of the excellent resolving power, short analysis time, low cost and little sample consumption compared with HPLC. At the same time, since MS can provide the capability to determine the molecular weight as to obtain structure related fragmentation information on the analytes, it has been an attractive detection method for CE analysis [25–28]. Few works were reported in the application of CE–MS on the analysis of MCs. Bateman et al. had identified several MCs including MC-LR in extracted from *M. aeruginosa* cell by LC–ESI-MS and CE–ESI-MS, and just made a quantification on MC-LR [19]. Siren et al. have discussed the separation conditions of MCs with CZE and MEKC, and used off-line ESI-MS to identify and quantify of the isolated MCs [29]. So far, still no research work focusing on the simultaneous quantification of MCs by on-line CE–MS was reported.

In this paper, CZE coupled with ESI-MS was developed for the quantification of the MCs, as well as the identification of MC in crude algae sample. The quantification was carried out in selective ion monitoring (SIM) mode with characteristic ions *m/z* 506.9 (MC-LR) and 532.0 (MC-YR). The detection limit of MC-LR was 0.05 μg mL^{-1}, which was better than those of 0.2 and 1.5 μg mL^{-1} presented by Bateman et al. [19] and Birungi and Li [20], respectively. In addition, the proposed method was further applied to the analysis of MCs in crude algae sample. The result implies that the CZE–ESI-MS method has a potentiality in MCs analysis of crude algae sample, which will be an alternative approach for the water quality control in the field of aquatic environment.

2. Materials and methods

2.1. Apparatus

All CZE experiments were carried out on an Agilent ^{3D}CE model (Agilent Technologies Inc., Waldbronn, Germany) coupled to an Agilent G1956B series single quadrupole mass spectrometry (Agilent Technologies Inc., Waldbronn, Germany). The mass spectrometry was equipped with Agilent coaxial sheath liquid interface and ESI source. The sheath flow was supplied by an LC-pump (1100 series LC; Agilent Technologies Inc., Waldbronn, Germany) with a 1:100 splitter. Agilent CE ChemStation was used for the instrument control, data acquisition, and data analysis. A 90 cm length of 50 μm i.d., 360 μm o.d. uncoated fused-silica capillary was obtained from Polymicro Technologies Inc. (Phoenix, AZ, USA).

2.2. Chemicals

MC-LR (95%) and MC-YR (95%) were purchased from Alexis Corporation (Lausen, Switzerland). Acetonitrile, methanol and isopropanol (HPLC grade) were obtained from Sinopharm Chemical reagents (Shanghai, China). Acetic acid glacial, ammonium acetate, formic acid, ammonium formate, and ammonium hydroxide were analytical reagent grade, which were purchased from Sinopharm Chemical reagents (Shanghai, China). Deionized water supplied by a Milli-Q purification system (Bedford, UK) was used to prepare buffer, standard solution and sample solution.

2.3. Preparation of solution

MCs stock samples with the concentration of 100 μg mL^{-1} in 20% methanol were used in this experiment. A mixture consisting of 5.0 μg mL^{-1} MC-LR and MC-YR was used to investigate the optimum conditions for the separation of the two MCs. All the solutions were stocked at −20 °C. A buffer solution of 0.1 mol L^{-1} ammonium acetate was adjusted to the desired pH in the range of 9.70–10.0 with 0.1 mol L^{-1} ammonium hydroxide. All the solutions were filtered through 0.22 μm membrane filters.

2.4. Conditions of CZE–ESI-MS analysis

Fresh capillaries were pretreated by flushing with 0.1 M NaOH for 30 min, and then rinsed with 0.1 mol L^{-1} HCl and deionized water for 10 min, respectively. Between runs, the capillary was rinsed with deionized water and running buffer for 5 min, respectively.

In this experiment, CZE method was developed for the separation of MC-LR and MC-YR, the CZE separation conditions were as follows: 40 mmol L^{-1} ammonium acetate–ammonia solution with pH 9.86 was as the running buffer; 20 kV was as separation voltage; water injection 20 kV for 3 s and then sample injection 20 kV for 20 s was as the injection mode.

The samples were identified by ESI-MS. Both scan and SIM mode were adopted. For scan mode, the mass range was set in the range of 400–1100 Da. The characteristic ions in MS detection were 506.9 and 532.0 corresponding to $[M+NH_4+H]^{2+}$ both for MC-LR and for MC-YR, respectively. The capillary voltage of MS was maintained at +5000 V. The fragment, step size, and gain were set at 70, 0.2 and 2.0, respectively. Nitrogen was used as the nebulizer and the drying gas pressure was set at 10 psi with the flow-rate of 6 L min^{-1} at the temperature of 150 °C. The sheath liquid composing of isopropanol–water (1:1, v/v) containing 7.5 mmol L^{-1} acetic acid with the flow-rate of 3 μL min^{-1} was introduced to the interface by a 1100 series Agilent Iso Pump.

2.5. Sampling and isolation of microcystins

A 25 # plankton net (mesh size 64 μm, Beijing Purity Instrument Co., Ltd., China) was used to collect the crude algae sample. Centrifugation was required at 4000 r min^{-1} for 20 min in order to remove the extra water, the obtained algae paste was stored at −20 °C. Algae paste (1.0 g) was freeze–thaw for 3 cycles, and then dissolved in 50.0 mL acetic acid: water (5:95, v/v) solution. The mixture was ultrasonic extracted for 10 min and centrifuged at 4000 r min^{-1} for 10 min, respectively. The supernatant was collected and the residue was mixed again with 50.0 mL acetic acid:water (5:95, v/v) solution, repeated the above extraction steps for twice, all the supernatant was collected finally. Prior to loading the sample, the C_{18} solid phase extraction (SPE) cartridge was conditioned with 10.0 mL methanol following 15.0 mL deionized water. The supernatant was loaded into the cartridge, and rinsed with 15.0 mL methanol:water (20:80, v/v). The sample was eluted with 10.0 mL of methanol. The efflu-

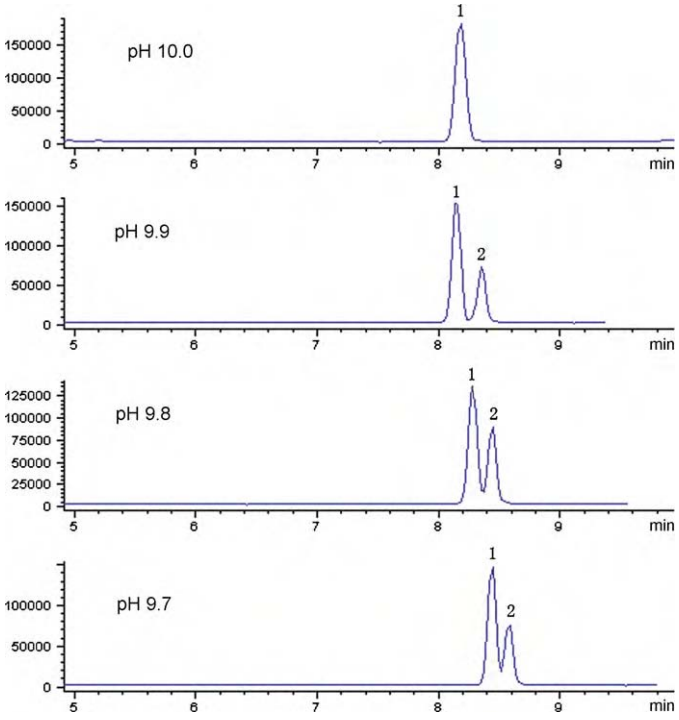

Fig. 2. Effect of buffer pH on the separation of the MCs uncoated fused-silica capillary: 50 μm i.d. × 90 cm; running buffer: 40 mmol L^{-1} ammonium acetate; separation voltage: 25 kV; injections: water 20 kV × 3 s, sample 20 kV × 20 s; nebulizing gas pressure: 10 psi; the drying gas flow-rate: 6 L min^{-1}; drying gas temperature: 150 °C; sheath liquid: 3 μL min^{-1}, 7.5 mmol L^{-1} acetic acid in isopropanol–water (1:1, v/v). 1. MC-LR, 2. MC-YR.

ent was collected in 10.0 mL round bottom flask and evaporated to dryness with nitrogen gas, and residue was redissolved in 1.0 mL methanol and restored at −20 °C.

3. Results and discussion

3.1. CZE separation conditions

The CZE separation conditions including ionic strength and pH of buffer, and viscosity have a direct influence on the intensity of the electroosmotic flow (EOF), which can affect the fluxes of solution reaching the ESI source as well as the resolution (Rs) of two MCs. The optimum of the buffer solution and other conditions was based on the signal stability and Rs of the MCs.

In order to transfer an established CZE separation to CZE–MS compatible conditions, it is advantageous to select volatile components as the buffer composition to avoid any contamination of ESI source. In this experiment, volatile buffer solutions including ammonium formate buffer and ammonium acetate buffer were studied, excellent peak shape and good resolution were obtained with ammonium acetate buffer. The pH value of running buffer would greatly affect the separation because the separation mechanism of CZE is based on the differences in molecular mass–charge ratios (m/z) of the analyte molecules [30]. Experiments showed that the migration of MC-LR and MC-YR were very similar in a wide pH range, except the narrow range of 9.7–10.0, the two MCs showed a trend toward separation as shown in Fig. 2. The pH of 9.78–9.90 effect on Rs was investigated in detail, it was found that the Rs of two MCs achieved to 1.5 in ammonium acetate buffer with pH 9.86. In addition, the concentration of ammonium acetate buffer was investigated in the range of 30–60 mmol L^{-1}. Experimental results showed that higher Rs but lower signal was exhibited when using a high concentration buffer. Considering the Rs and response signal, the 40 mmol L^{-1} ammonium acetate solution with pH 9.86 was selected.

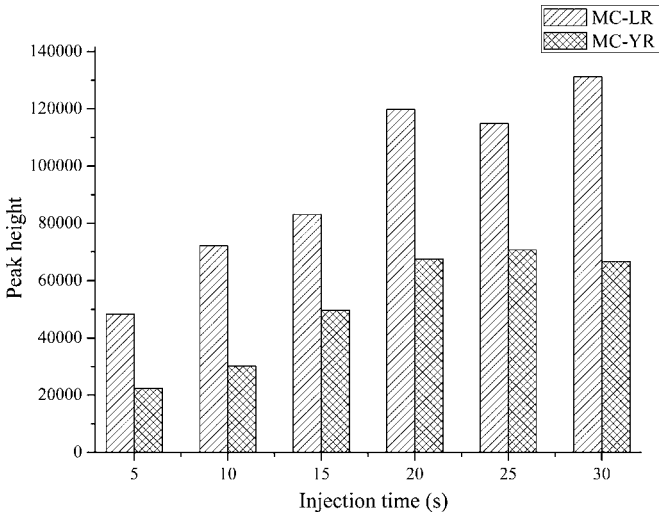

Fig. 3. Effect of injection time on peak height of the MCs pH = 9.86, other conditions were as Fig. 2.

Plotting the voltage in the range of 18–27 kV against current, a straight line was obtained. With the increase of the voltage from 18 to 27 kV, the migration time of the MCs was shortened and the Rs was lower. Therefore, 25 kV was selected as separation voltage in this experiment.

3.2. Sample injection condition

To improve the sensitivity and stability, certain water plug was need before sample injection, 20 kV × 3 s of water plug was applied in this experiment. Furthermore, under the condition of 20 kV as injection voltage, the effects of sample injection time ranging from 5 to 30 s on the peak height were studied. It can be seen from Fig. 3 that the signals increase obviously with the injection time from 5 to 20 s, However, due to the peak broadening, it was level off when the injection time exceeded 20 s. Thus, 20 kV × 20 s was the optimum condition for the sample injection.

3.3. Selection of the MS detection conditions

As for CZE–MS, the addition of a sheath liquid to compensate the low flow-rate of the CZE system (nL min^{-1}) is applied in order to improve the stability and the production of the spray, a nebulization gas is used for the purpose of stabilizing the spray formation and the adjustment of the position between the CE capillary and the stainless steel. Several parameters, such as composition and flow-rate of sheath liquid, flow-rate of sheath gas and so on, had been optimized.

The choice of the sheath liquid parameters is also very important in developing a method employing CZE–ESI-MS. In this work, 7.5 mmol L^{-1} acetic acid in isopropanol–water (1:1, v/v) was used because of its excellent sensitivity and long-term stability in this experiment. The addition of a sheath liquid was expected to reduce the MS signal intensity due to solute zone dilution. Low flow-rate affects negatively spray stability and peak shape that result in low signal intensity, whereas too high flow-rate causes the analytes dilution that also generates low signal intensity. Therefore, under the conditions of keeping stable spray and electrical contact, the flow-rate should be as low as possible to reduce dilution. The flow-rates of 2.0–6.0 μL min^{-1} were investigated, and 3.0 μL min^{-1} was chosen.

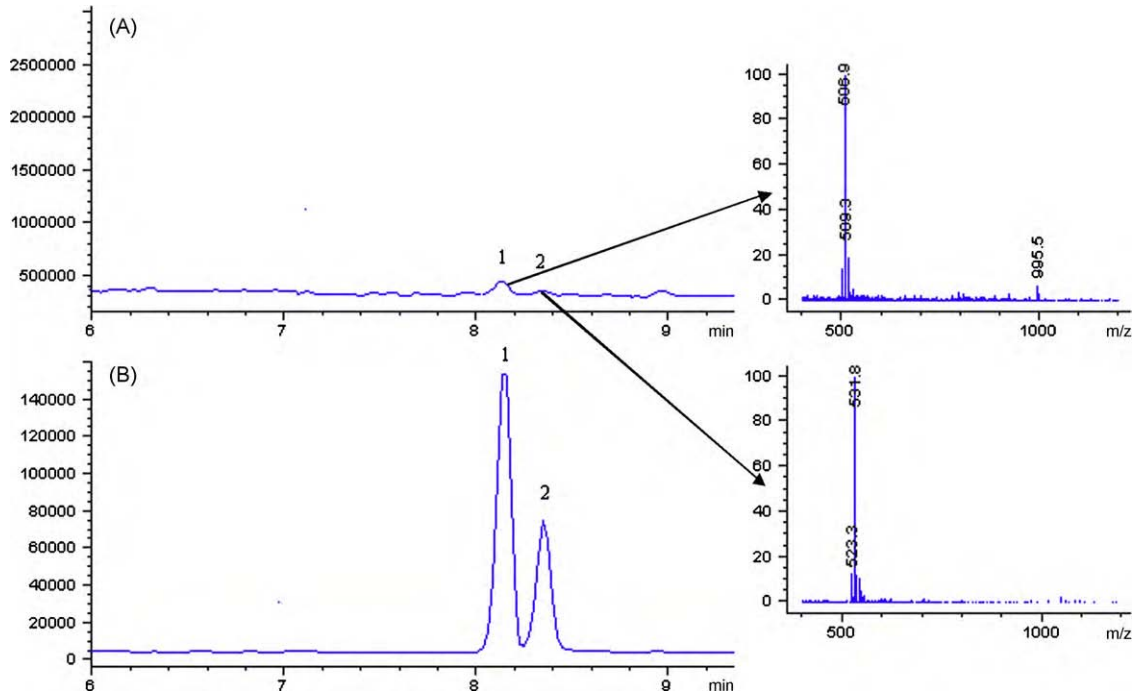

Fig. 4. The scan (A) and SIM (B) TIC electropherograms and mass spectrums of two MCs standard mixture. The conditions were as Fig. 3. 1. MC-LR (10 μg mL^{-1}), 2. MC-YR (10 μg mL^{-1}).

Table 1
Retention time of Mass Spectrometry Detection (MSD) and characteristic ions in the ESI for two MCs.

Compound	$t_{R(MS)}$ (min)	Characteristic ions of ESI	
		$[M+2H]^{2+}$	$[M+NH_4+H]^{2+}$
MC-LR	8.300	498.5	506.9
MC-YR	8.453	523.0	532.0

The sheath gas (nitrogen) helps to nebulize sample solution into a fine mist as the sample solution exits the ESI needle. The sheath gas may increase the speed of the solution in the capillary by suction effect [31]. A low flow-rate of sheath gas should be used for the electrospray since a high flow-rate of sheath gas influences sensitivity. In addition, a too high sheath gas flow-rate could result in a driving force making the electrolytes move faster than the EOF [32]. In this experiment, stable spray and good sensitivity could be achieved at the drying gas flow-rate of 10 psi, 6 L min^{-1} with the temperature of 150 °C.

Mass spectra of cation were acquired in positive ion mode scanning from *m/z* 400 to 1100. Mass spectra for each MC were shown in Fig. 4. Under the examined conditions, doubly charged ions $[M+NH_4+H]^{2+}$ were observed as the most abundant ions. This result can be attributed to the existence of $[NH_4]^+$ in the electrolytes [33]. Corresponding to $[M+NH_4+H]^{2+}$, *m/z* 506.9 for MC-LR and *m/z* 532.0 for MC-YR are shown in Table 1. Consequently, cations were determined with protonation and ammonium adduct molecules in SIM mode.

3.4. Methodological study

Fig. 4 shows scan and SIM total ion current (TIC) electropherograms and mass spectrums of two MCs standard mixture obtained by CZE–ESI-MS under the optimum conditions. The linearity for the various MCs was determined by plotting the peak height of the analyte against the concentration of each analyte in the SIM mode. The regression equations, correlation coefficient, linear ranges, and detection limits are summarized in Table 2. The detection limits were evaluated on the basis of a signal–noise ratio of 3. The calibration curves exhibited excellent linear behavior in the range of 0.11–10.5 μg mL^{-1} with the correlation coefficient higher than 0.998, and the detection limits were 0.05 and 0.08 μg mL^{-1} for MC-LR and MC-YR, respectively. These results clearly demonstrated that the CZE–ESI-MS method studied here was a robust and reliable approach for the analysis of MCs.

The reproducibility was evaluated by analyzing five replicated standard mixture solution containing 1.25 μg mL^{-1} MC-LR and MC-YR. The relative standard deviations (RSDs) were lower than 2.7% for migration time and 4.0% for peak height, respectively.

3.5. Recovery

To verify the reliability of the developed CZE–ESI-MS method, the recovery experiment was carried out by adding two levels of two MCs into the crude samples and calculating the concentration from the calibration curves. From the ratio between found amount

Table 2
Regression equation, linear range and detection limits of MC-LR and MC-YR.[a]

Compound	Regression equation[b]	Correlation coefficient	Linear range (μg mL^{-1})	Detection limit[c] (μg mL^{-1})
MC-LR	$y = 7595.4x + 4938.4$	0.9980	0.11–10.0	0.05
MC-YR	$y = 6148.6x + 2150.5$	0.9992	0.16–10.5	0.08

[a] CZE–ESI-MS conditions are as Fig. 3.
[b] Where *y* and *x* are the peak height of MS detection and concentration of analytes (in μg mL^{-1}), respectively.
[c] (S/N = 3) the detection limits are estimated on the basis of a signal-to-noise ratio of 3.

Table 3
Recoveries of two MCs in the spike sample ($n = 3$).[a]

Compound	Background ($\mu g\,mL^{-1}$)	Added ($\mu g\,mL^{-1}$)	Found ($\mu g\,mL^{-1}$)	Recovery (%)	RSD (%)
MC-LR	0.4	1.20	1.15	95.8	2.3
		3.60	3.90	108	3.5
MC-YR	0.6	1.80	1.82	101	1.4
		5.40	5.26	97.4	3.0

[a] CZE–ESI-MS conditions are as Fig. 3.

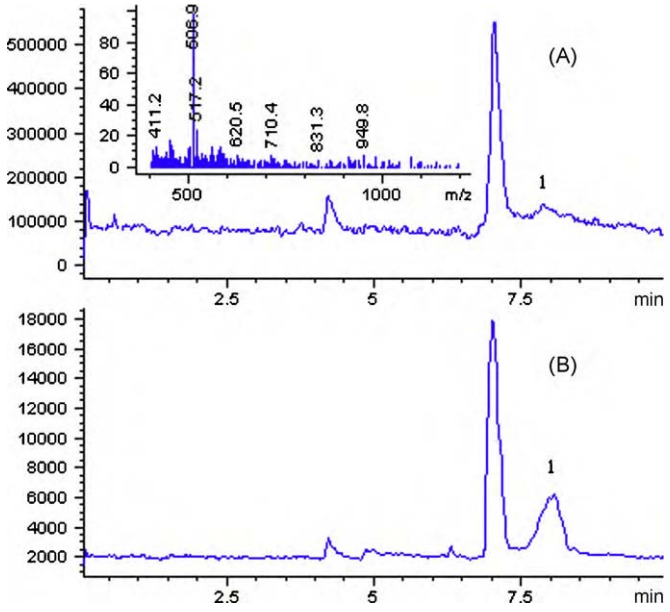

Fig. 5. The scan (A) and SIM (B) TIC electropherograms and mass spectrums of the MCs contained in crude algae sample. The conditions were as Fig. 3. 1. MC-LR.

and added amount, the recovery values were obtained. As shown in Table 3, the recoveries are in the range of 95.8–108% and 97.4–101% for MC-LR and MC-YR, respectively (RSD ≤ 3.5%).

3.6. Sample detection

The developed CZE–ESI-MS method was applied to the direct analysis of crude algae sample collected from lake. After extraction as described in Section 2.5, the sample was determined by CZE–ESI-MS. The scan and SIM TIC electropherograms and mass spectrums of the MCs contained in crude algae sample are presented in Fig. 5. It can be seen from Figs. 4 and 5, both the mass spectrum (m/z 506.9) and the migration time of MC-LR are fully compatible with the standard sample. On the basis of scan mass spectrum, the existence of MC-LR in crude algae sample was confirmed. As a result, the proposed CZE–ESI-MS method appears to be a simple and effective method for MCs assay.

4. Conclusion

This paper describes the method of CZE coupled with ESI-MS for the determination of two MCs (MC-LR and MC-YR). The SIM mode provides the possibility to the quantification of MCs, while the scan mode exhibits the capability to the identification of the target MC in crude algae sample. However, it is regretful that the sensitivity exhibited in this proposed method is still not enough for the trace analysis of the MC-LR in real drinking water which is recommended by WHO. Possible reason is due to the less sample volume of CZE and dilution affected from sheath liquid of MS. From this point, the effective on-line enrichment method of CZE may be a good proposal. Therefore, the on-line enrichment method for the detection of MCs by CZE–ESI-MS is proceeding in our lab.

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