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An Automatic Solid Phase Extraction and Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry for Determination of Seven Microcystins at Ultra-Trace Levels in Surface Water

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Abstract: A method was developed for the detection of seven microcystins (microcystin-LR, RR, YR, LA, LY, LW and LF) in surface water using automatic solid-phase extraction (A-SPE) coupled with ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The automated solid-phase extraction system was used to extract microcystins (MCs) from water samples. UPLC-MS/MS was used to determine MCs concentrations in just 5 min. Method detection limits were from 0.3 to 0.9 ng/L, microcystin recoveries ranged from 83.8% to 114%, and the relative standard deviation (RSD) varied from 5.6% to 12.5%. This analytical approach was found to be simple, highly sensitive, accurate, which required little manual operation. Additionally, to validate this analytical method, A-SPE+UPLC-MS/MS was applied to characterize the concentration of MCs in Taihu Lake, Wuxi, China.

Key words: microcystins; automatic solid-phase extraction; ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS)

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0 Introduction

Algal contamination (via water eutrophication) has become an important environmental issue across the globe[1]. Microcystins (MCs), toxins produced by cyanobacteria (e.g. blue-green algae), are the most common toxic byproducts of algal blooms in eutrophic water. This is an issue as MCs have posed risks to human health, including hepatic diseases and liver cancer[2]. MCs have been found at detectable concentrations in waters in different countries: China, 0.003 6-7.97 μg/L[3]; Sweden, 0.68-9.1 μg/L[4]; Canada, 0.3-24 000 ng/L[5]; Portugal, 17-344 ng/L[6]. This has resulted in some regulatory and human health issues. For example, in 2014, MC pollution resulted in a shut-down of water utilities in Toledo for three days[7].

Currently, the World Health Organization (WHO) water quality guidelines for microcystin-LR are 1.0 μg/L in drinking water and 20 μg/L in recreational water[8]. Therefore, an analytical method is required to accurately identify and quantify MCs in water so as to determine if the water quality guideline is met.

Several analytical methods have been used for the qualitative and quantitative detection of MCs in water samples, including protein phosphatase inhibition assays (PPIA)[9], enzyme linked immuno-sorbent assay (ELISA)[10], 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB)[5], and high performance liquid chromatography (HPLC)

coupled with ultra-violet or mass spectrometry. Although PPIA, ELISA and MMPB can achieve detection limits as low as 0.5 μg/L[9], 40 ng/L[10] and 0.5 ng/L[5], respectively, these methods cannot characterize the molecular structures of detected MCs or detect false-positive results in real samples. Ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) has extremely high sensitivity and selectivity. Thus in recent years it has been deemed as the best technique for MC detection[11,12]. To lower method detection limits, extraction and pre-concentration of MC from water samples via solid phase extraction (SPE) has been proposed[13]. However, conventional manual SPE is a heavy and time-consuming work, and during MC extraction, precision is often affected by the load flow (as the flow velocity is not precisely controlled by a pump).

On-line SPE is another method that is widely used for MC extraction and concentration. However, the detection limits for MC variants in this method is 2-40 ng/L[11]. The automatic solid phase extraction (A-SPE) system is designed to isolate trace quantities or organics from large aqueous samples (20 mL-20 L). The system is advantageous as it improves analytical precision and saves time, solvent, and labor for it can operate unattended. It has been extensively applied to a range of fields including medical[14, 15], water quality[16] and agricultural pesticides[17].

In this study, A-SPE system[16] paired with UPLC-MS/MS was used to detect ultra-trace levels of MC-LR, MC-YR, MC-RR, MC-LA, MC-LY, MC-LW and MC-LF in surface water samples. This method allowed for automatic and efficient extraction and concentration of microcystin variants from water samples, and represented a new analytical method for detection of MCs in surface water. The use of UPLC-MS/MS in combination with automatic solid-phase extraction was expected to improve the method detection, allowing for the detection of MC in ng/L concentrations.

1 Materials and Methods

1.1 Reagents and Chemicals

MC-LR, MC-YR, MC-RR, MC-LA, MC-LY, MC-LW and MC-LF (95%) were acquired from Alexis Biochemicals (Lausen, Switzerland). Formic acid (98%) was purchased from Sigma-Aldrich Co. (Shanghai, China). C_{18} solid-phase extraction cartridges (500 mg, 6 mL), methanol (pesticide residue grade), and acetonitrile (pesticide residue grade) were obtained from CNW Technologies GmbH (Shanghai, China). High-purity water was obtained from a Milli-Q filtration system from Millipore (Millipore, USA).

1.2 Sample Preparation

Water samples were collected in glass bottles, and analyzed within a 4 h period, then filtered through a 500 mesh stainless steel screen to remove plankton and suspended solids. Water sample (500 mL) was added to 25 mL methanol prior to A-SPE. A laboratory blank was run with each extraction batch, and laboratory fortified sample matrices and a field duplicates were run for every 10 samples.

1.3 Extraction and Analysis

Samples were extracted with an A-SPE (Dionex Autotrace 280, Thermo Scientific, Waltham, Massachusetts) which was carried out as follows: 1) Rinsing cartridge with 20.0 mL methanol containing 0.1% (*V*/*V*) formic acid (10.0 mL/min); 2) Activating cartridge via incubation in 10.0 mL methanol (10.0 mL/min); 3) Rinsing cartridge with 10.0 mL water (10.0 mL/min); 4) Loading 550.0 mL of sample onto cartridge (10.0 mL/min); 5) Rinsing cartridge with 10.0 mL water (10.0 mL/min); 6) Drying cartridge with nitrogen gas for 30.0 minutes (15.0 mL/min); 7) Collecting 8.0 mL eluent into sample tube using methanol containing 0.1% (*V*/*V*) formic acid (2.0 mL/min). The eluent was then reduced to 1.0 mL by nitrogen gas flow, transferred into an auto-sampler vial (2 mL, Waters Corporation, China) and analyzed via UPLC-MS/MS.

1.4 UPLC-MS/MS

UPLC was performed using the AQUITY UPLC™ system (H-Class, Waters Company, USA). The analytical column used was an ACQUITY BEH130 C_{18} column (100 mm×2.1 mm, 1.7 μm particle size, Waters Co. Ltd). The column oven temperature was 40 ℃. The flow rate was 0.4 mL/min, and the injection volume was 10 μL. Acidified H_2O (0.1% formic acid) was used as solvent A, and acidified acetonitrile (0.1% formic acid) was used as solvent B. The gradient elution program was as follows: 20% B (0-1 min), 20%-95% B (1-2.5 min), 95% B (2.5-3.5 min), 95%-20% B (3.5-3.6 min), and 20% B (3.6-5 min)[12].

MS/MS analyses were performed using the API4000$^+$ MS/MS System (AB Sciex, USA) with electrospray ionization (ESI) in positive mode and multiple reaction monitoring (MRM). Source block voltage was 5 500 V and the heated nebulizer temperature was 450 ℃. Curtain, nebulizing, and turbo spray gas pressures were set at 0.138, 0.379 and 0.414 MPa, respectively.

2 Results and Discussion

2.1 Optimization of UPLC-MS/MS Conditions

Although a system using methanol and water can obtain better sensitivity and a higher mass spectrum response for the MC analytes, acetonitrile-water systems have lower system pressures (compared with methanol-water systems at the same flow rate). Thus, the acetonitrile-water system was selected for further use in UPLC-MS/MS. The 0.1% formic acid added in the mobile phase improved the ionization efficiency of target compounds, increased equilibrium concentrations of $[M+2H]^{2+}$ and $[M+H]^{+}$ ions in solution, and enhanced the abundances of these ions detected in MS/MS[18,19]. Chromatograms were shown in Fig. 1.

In the initial experiments, we directly injected high concentrations of individual MC standards using Flow Injection Analysis. The detailed information of MS/MS conditions for each MC analyzed are described in Table 1. The protonated molecular ions of MCs were shown in full scan spectra. The relative abundances of doubly charged ions $[M+2H]^{2+}$ ions from MC-RR, MC-YR and MC-LR were stronger than solo charged ions $[M+H]^{+}$ ions. Therefore, we used $[M+2H]^{2+}$ ions of MC-RR (*m*/*z* 520.0), MC-YR (*m*/*z* 523.4) and MC-LR (*m*/*z* 498.4) as precursor ions in this experiment.

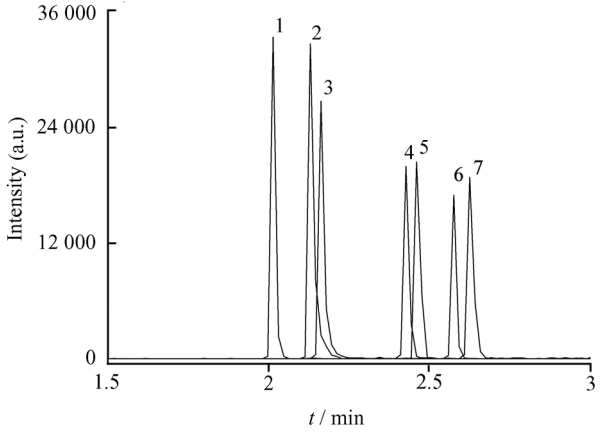

Fig. 1 UPLC-MS/MS chromatogram of MC standard mixture (10 μg/L)

1. MC-RR, 2.04 min; 2. MC-YR, 2.14 min; 3. MC-LR, 2.18 min; 4. MC-LA, 2.45 min; 5. MC-LY, 2.47 min; 6. MC-LW, 2.57 min; 7. MC-LF, 2.62 min

MC-LA, MC-LY, MC-LW and MC-LF gave clear $[M+H]^{+}$ spectra at *m*/*z* 911.5, 1 002.5, 1 025.5 and 986.5 under positive ion mode. These protonated molecules were respectively confirmed as precursor ions. Precursor and product ions are described in Table 1. For the positive ionization mode, the ions (*m*/*z* 135.0) can be considered as quantification ions as they are intense and stable product ions. The other product ions are set as qualitative ions for characteristic analysis.

Table 1 Declustering potential, collision energy, precursor ion and product ion for MCs

Compound	Declustering potential /V	Collision energy /eV	*m*/*z*	
			Precursor ion	Product ion
MC-RR	105	45	520.0	135.1, 620.3
MC-YR	65	19	523.4	134.9, 911.4
MC-LR	55	19	498.4	135.0, 861.6
MC-LA	160	80	911.5	135.0, 213.0
MC-LY	118	85	1 002.5	135.3, 103.1
MC-LW	125	91	1 025.5	135.1, 213.1, 375.0
MC-LF	120	84	986.5	135.1, 163.1, 213.1

2.2 Effects of Sample Loading Flow Rate

An appropriate sample loading flow rate can shorten the time of the preconcentration step and increase sample recovery. In this study, four different flow rates were tested (5, 10, 15 and 20 mL/min) while loading the spiking samples (20 ng/L). As shown in Table 2, the best sample loading flow rate was between 5.0 and 10.0 mL/min, with recovery ranging from 89.1% to 101%, which was deemed satisfactory. Therefore, to improve recovery and reduce extraction time, we chose 10.0 mL/min as the sample loading flow rate.

2.3 Cartridge Drying Method

The A-SPE system loaded samples via positive pressure (i.e. using a mechanical pump). This is different from the traditional manual solid phase extraction, which loads samples via negative pressure (i.e. a vacuum pump). In this study, the A-SPE system left a lot of residual water in cartridges after loading samples, which could affect the efficiency in the elution step. Therefore, we dried the cartridges via nitrogen gas flow and found that residual water in cartridges could be completely removed by the nitrogen gas drying 30 min at 15.0 mL/min.

Table 2 Recovery of MCs at different loading flow rates(n=3)

%

Compound	Loading flow rate /mL·min^{-1}			
	5	10	15	20
MC-LR	90.3	89.1	78.7	65.1
MC-YR	94.6	95.5	81.6	71.6
MC-RR	93.5	92.7	76.9	63.4
MC-LA	101	97.8	84.3	81.7
MC-LY	96.6	97.2	79.5	77.3
MC-LW	95.9	96.0	77.8	79.3
MC-LF	98.1	95.8	82.9	74.1

2.4 Effects of Eluting Volume

Addition of organic acid to methanol significantly improved MC yield, as the hydrophobicity of MCs was improved by decreasing pH[20]. In this study, the organic acid solvent, 0.1% (*V*/*V*) formic acid were selected[21] and completely eluted MCs using 5 mL acidified methanol. The A-SPE system was eluted by mechanical pressure, thus elution flow rate could be controlled in a low flow rate (2 mL/min). As shown in Fig. 2, when the eluent volume was greater than 8 mL, MCs have been completely eluted. Thus, the optimum elution volume was 8 mL.

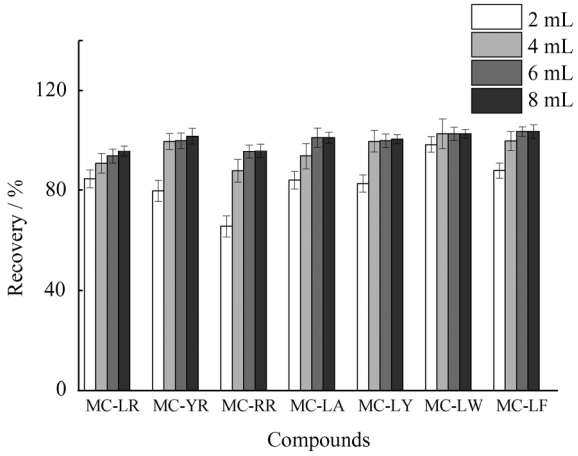

Fig. 2 Recovery of MCs at different elution volumes

Standard errors shown are calculated from biological experiments with triplicate measurements

2.5 Effect of Methanol Addition

In fortified extraction experiment, addition of methanol to the sample increased the solubility of MCs which might initially have been sorbed to particles, vessel and pipes. Without the methanol addition, the recovery of MC-RR in the surface water samples was lower than 43.8%. Low recovery of MC-RR using C_{18} material was also reported in previous research[21]. It is possibly because MC-RR can adhere to particles or glassware via the arginines in their molecular structures[22]. The results (Fig. 3) showed that addition of methanol greatly improved the recovery of MC-RR. Optimal results were obtained when the amount of the added methanol ranged from 25-50 mL (5%-10% of the sample volume).

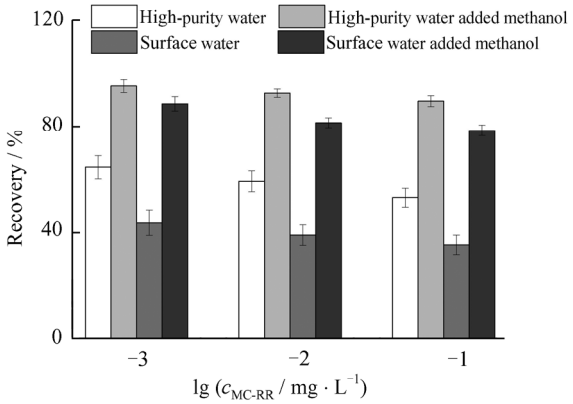

Fig. 3 Recovery of MC-RR at different spiking concentrations in high-purity water, high-purity water+methanol, surface water, and surface water+methanol

Standard errors shown are calculated from biological experiments (n=3) with triplicate measurements

2.6 Standard Curves and Method Detection Limit

Linear calibration curves were obtained in the tested concentration ranges for all of the compounds (Table 3).

The external standard method was used in all quantitative analyses. The method detection limit (MDL) was

Table 3 Linear regression data and method detection limit

Compound	Regression equation	Linear range /ng·L^{-1}	R^2	Method detection limit /ng·L^{-1}
MC-LR	y = 3 770.427x−16.097	1.0-20.0	0.999 4	0.3
MC-YR	y = 2 227.139x+115.579	1.0-20.0	0.999 0	0.3
MC-RR	y = 2 459.535x−166.137	1.0-20.0	0.999 0	0.4
MC-LA	y = 249.094x+24.570	2.0-20.0	0.999 7	0.6
MC-LY	y = 592.248x+97.705	2.0-40.0	0.999 4	0.7
MC-LW	y = 202.504x+33.432	2.0-80.0	0.999 9	0.8
MC-LF	y = 542.394x+188.979	2.0-40.0	0.999 5	0.9

defined as MDL=$S \cdot t_{(n-1,\ 1-\alpha=0.99)}$, where S was the standard deviation of replicate analyses. When seven replicates were used (n=7), the $t_{(n-1,\ 1-\alpha=0.99)}$ was 3.143[23]. The method detection limit was determined using samples spiked with MC internal standards. MC-LR, MC-YR and MC-RR concentrations were 1.5 ng/L in spiked samples, and MC-LA, MC-LY, MC-LW and MC-LF concentrations were 3.0 ng/L in spiked samples. The method detection limits in this method were 0.3-0.9 ng/L, which was 2-40 ng/L in on-line SPE[11].

2.7 Precision and Accuracy

Low, medium, and high concentrations of the standard MC mixture were added to water samples obtained from drinking water source in Taihu Lake. As shown in Table 4, under the optimum conditions, the recoveries using optimal UPLC-MS/MS methods derived here ranged from 83.8% to 114%. Relative standard deviation (RSD) ranged from 5.6% to 12.5%, with a mean precision of 9.5%, which was deemed satisfactory in EPA Method 544[24].

2.8 Application in Real Samples

Water samples were collected from different locations in Taihu Lake (Jiangsu, China), on March, 2017. The analytical method derived in this study was applied to quantifying the concentrations of MCs in the collected samples. As shown in Table 5, the concentration of MCs in Taihu Lake was from 0.4 to 9.0 ng/L, which is lower than the water quality guidelines of WHO[8]. Taihu Lake was not heavily polluted with MCs. This might be due to the fact that the aquatic environment has not yet reached the levels necessary for optimal algal growth. The method can be used for the determination of MCs in surface water.

Table 4 Recovery and repeatability of water samples spiked with three concentrations of MCs (n = 7)

Compound	Spike concentration /ng·L^{-1}	Recovery /%	RSD /%
MC-LR	1.0	90.1	8.2
	10.0	83.8	10.4
	20.0	85.7	11.3
MC-YR	1.0	113.0	8.2
	10.0	91.4	10.2
	20.0	112.0	9.3
MC-RR	1.0	87.2	11.5
	10.0	93.1	5.6
	20.0	92.7	7.3
MC-LA	2.0	114.0	9.1
	20.0	93.1	10.0
	40.0	91.6	10.5
MC-LY	2.0	87.9	9.5
	20.0	91.8	12.5
	40.0	104.0	8.4
MC-LW	1.6	97.6	11.5
	8.0	88.3	10.2
	16.0	98.6	7.5
MC-LF	2.0	89.2	9.7
	20.0	85.7	11.4
	40.0	90.7	8.3

Table 5 MC concentrations in water samples collected from Taihu Lake ng/L

Sample	MC-LR	MC-YR	MC-RR	MC-LA	MC-LY	MC-LW	MC-LF
1# (Meiliang Bay)	0.7	—	—	—	—	—	—
2# (Meiliang Bay)	2.8	0.4	—	—	—	—	—
3# (The center of Taihu Lake)	2.4	—	0.7	—	—	—	—
4# (The center of Taihu Lake)	6.6	—	—	1.5	1.5	2.0	—
5# (Zhushan Bay)	0.8	—	—	—	—	—	—
6# (Zhushan Bay)	2.0	—	—	—	—	—	—
7# (Zhushan Bay)	9.0	—	—	1.3	4.0	1.0	—
8# (Gong Bay)	5.8	0.8	5.0	0.9	—	—	—
9# (Gong Bay)	0.9	—	—	—	—	—	1.2
10# (Gong Bay)	4.4	—	—	—	1.0	—	—

—Not detected

3 Conclusion

This study presented a method based on A-SPE and UPLC-MS/MS that can be used to quantify the concentrations of MCs in water. A-SPE extracted MCs by adding methanol in water sample, drying the cartridge using nitrogen gas, and eluting with acidified methanol (0.1% formic acid, *V*/*V*). A-SPE minimized the sample manipulation and made the pretreatment process faster than the conventional SPE using automation program. The method allowed the simultaneous determination of MCs at ng/L and has been validated in surface waters. Obtained results met the requirements for analyzing ultra-trace levels of MCs in surface water, which helped to further the study of MCs in surface water.

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