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A novel method to detect seven microcystins in hard clam and corbicula fluminea by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A simple and reliable method to detect seven microcystins in hard clam and corbicula fluminea, based on liquid chromatography with electrospray ionization and tandem mass spectrometry (LC–ESI-MS/MS), was developed and validated. The sample preparation procedure includes extraction of tissue by methanol, followed by cleanup on a reversed-phase solid phase extraction (SPE) cartridge. With the optimized method, recoveries were between 43.7% and 92.3% for hard clam, 54.3% and 93.8% for corbicula fluminea, the relative standard deviations (RSD) were less than or equal to 16.2% and 15.7% in hard clam and corbicula fluminea at spiking levels of 1 μg/kg, 2 μg/kg and 5 μg/kg for MC-RR, MC-YR, MC-LR, and MC-LY, and 2 μg/kg, 5 μg/kg and 10 μg/kg for MC-LA, MC-LW and MC-LF, respectively, the limits of quantitation (LOQ) of this method were ranged from 0.7 μg/kg to 2.0 μg/kg.

1. Introduction

Cyanobacterial blooms have occurred in eutrophic waterbodies all around the world as the result of high surface water temperature [1] and a persistent stratification of the water column via sediment deposition or by biological uptake [2]. Some cyanobacteria species produce potent toxins, which are classified according to their mode of action into hepatotoxins, neurotoxins, skins irritants and other toxins [3–5]. The most frequently reported cyanobacterial toxins are cyclic heptapeptide hepatotoxins [6–10], microcystins (MCs) (Fig. 1), named from the species *Microcystis aeruginosa* [3]. They possess a general structure consisting of five amino acids: 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (ADDA), N-methyldehydroalanine (Mdha), D-alanine, β-linked D-erythro-β-methylaspartic acid and γ-linked D-glutamic acid, and two L-amino acids as variants that give the name to the molecule, e.g. two -arg- (RR), -tyr- and -arg- (YR), -leu- and -arg- (LR), -leu- and -arg- (LA), -leu- and -tyr- (LY), -leu- and -tyr- (LW), -leu- and -leu- (LF) [11,12].

Microcystins are usually associated with the poisoning of aquatic organisms, wildlife, domestic animals, and humans through drinking or ingesting cyanobacteria in the water. Especially, aquatic organisms including mollusks, fish and shrimp could accumulate microcystins in their tissues and transfer them to the higher trophic level species along food chain [1,13–15]. And when people consume the contaminated aquatic organisms, serious toxicosis happens [16]. Therefore, World Health Organization (WHO) has recommended a guideline level of MC-LR be 0.1 μg/L in drinking water for a long-time exposure or 1 μg/L for a short-time exposure on the base of toxicity tests on mice and pigs [17]. Consequently, in order to assess the hazards presented by microcystins, a sensitive and reliable analytical method for the detection and quantification of microcystins in aquatic organisms is crucial.

Many analytical methods have been developed to detect microcystins, such as protein phosphatase inhibition assays (PPIA) [18], enzyme-linked immunosorbent assay (ELISA) [19,20], liquid chromatography with Diode Array detection (HPLC-DAD) [21], and more recently, liquid chromatography coupled with mass spectrometry (LC–MS) [22,31] or tandem mass spectrometry (LC–MS/MS) [11,23], various type of mass spectrometers were reported for this analysis, e.g. ion-trap tandem mass spectrometry (LC-IT/MS) [24] and time-of-flight mass spectrometry(LC-TOF/MS) [3]. These methods are more sensitive and reliable than the traditional bioassay method, by measuring different characteristic of the microcystins and providing different information on the micro-

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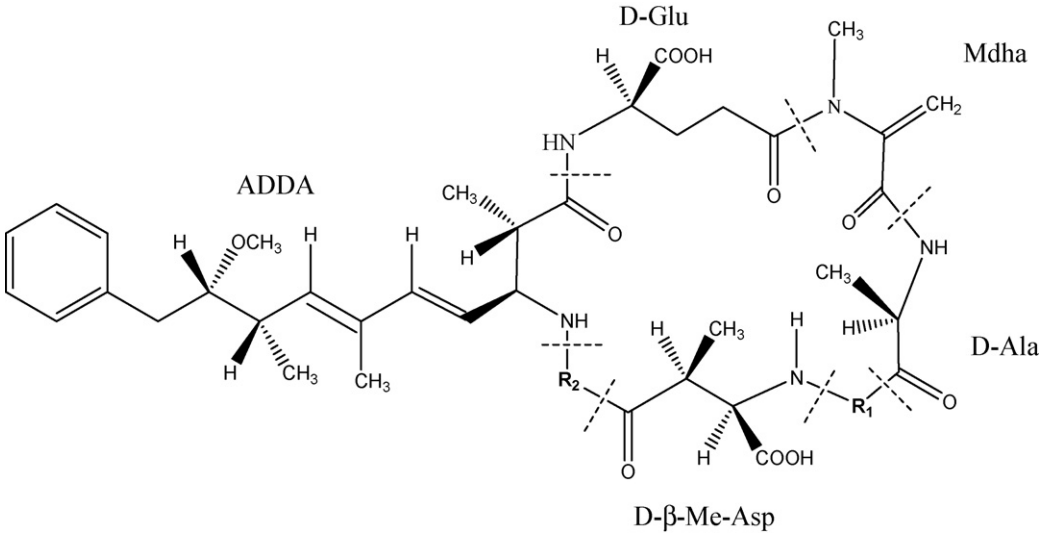

Fig. 1. Chemical structure of MC.

cystins content of unknown samples. Among all these methods, LC–MS/MS has become the most powerful method in determining microcystins in water and aquatic animals. It is capable of identification and quantification of MCs in complex matrixes and offers good specificity and sensitivity.

The feasibility of LC–MS/MS techniques to identify and quantify microcystins in water and fish samples has been well described in many reports [25–27]. Shellfish is an important source of food and global production has increased significantly through the rapid growth of aquaculture during the past decade [28]. Unlike fish, or mussels, hard clam and corbicula fluminea are raised in muddy beaches or silver sands near coastal regions and have more chances of contamination, therefore, hard clam and corbicula fluminea could serve as good samples for risk assessment of aquatic products. However, to our best knowledge, the determination of seven microcystins in some species of shellfish e.g. hard clam and corbicula fluminea by LC–MS/MS has so far not been reported. This study was carried out with the aim to develop a sensitive and reliable method for the determination of MCs in several aquatic species. This was accomplished by simultaneously detecting MC-RR, -LR, -LR, -LA, -LY, -LW, and -LF using HPLC coupled to triple quadrupole tandem mass spectrometry (LC–MS/MS) based on selected reaction monitoring (SRM) scan mode. This method includes sampling procedure, sample handling and extraction methodologies, and was validated in terms of precision, linearity, limits of detection and matrix effect and successfully applied to the analysis of MCs in several aquatic species, trace amount of MC-RR, -LR, -LR, -LA, -LY, -LW, and -LF were detected in hard clam and corbicula fluminea samples.

2. Experimental

2.1. Reagents

The reference standards, including MC-RR, MC-YR, MC-LR, MC-LA, MC-LY, MC-LW, and MC-LF (purity >95.0%) were purchased from Alexis (Lausen, Switzerland). Standard stock solutions were prepared in methanol at 0.5 μg/mL. HPLC-grade acetonitrile, methanol were supplied by Merck KGaA (Darmstadt, Germany). All other solvents and chemicals used in this study were of analytical grade. Water was purified from a Milli-Q deionization unit (Millipore simplicity, Millipore, France).

2.2. Aquatic products

The aquatic product samples were provided from daily testing sample in the laboratory. The hard clam and corbicula fluminea were filleted, the shells were removed, and hard clam and corbicula fluminea meat were homogenized and stored at −20 °C in airtight containers until analysis.

2.3. Sample preparation

5 g of tissue samples was weighed into a 50 mL conical polypropylene centrifuge tube. 10 mL of methanol was added, and the mixture was thoroughly homogenized using an Ultra-Turrax homogenizer for 30 s at its highest speed, and followed by 15 min sonication. The extract was then centrifuged at 8000 rmp for 5 min using a centrifuge (Sigma, Germany). The supernatant was transferred into a glass tube and placed into an N-Evap evaporation system (Hopkinton, MA, USA). The extract was evaporated to a residual volume of approximately 2 mL, under a nitrogen stream, in a 45 °C water bath. The remaining fraction was vortex-mixed with 8 mL of water. The diluted extract was applied to an Oasis HLB reversed-phase cartridge (3 mL, 60 mg), which had been preconditioned with 3 mL of methanol and 3 mL of water at a flow rate of 1 mL/min. Then, the sample solution was passed through the cartridge at a flow rate of 1 mL/min. The cartridge was rinsed with 3 mL of water and 3 mL of 20% methanol, the microcystins were finally eluted with 5 mL of methanol. The eluent was evaporated to dryness under a nitrogen stream at 45 °C and then re-dissolved in 1 mL of 30/70 (v/v) methanol/water solution. The resulting solution was filtered through a 0.45 μm nylon filter and 25 μL of which was injected onto the LC–MS/MS system for analysis.

2.4. LC–MS–MS analysis

A Thermo Finnigan high performance liquid chromatography system combined with a TSQ Quantum Ultra AM triple quadrupole tandem mass spectrometer (Thermo Electron, Waltham, MA, USA) equipped with electrospray ionization probe (Thermo Elctron) was used for analysis. Room temperature was controlled at 20 °C by air conditioner. Chromatographic separation was performed using a Symmetry 300 C18 (150 mm × 2.1 mm i.d. 5 μm) HPLC column (Waters Corporation, Milford, MA, USA), which was preceded inline by a precolumn filter of 3 mm frit (Phenomenex, Torrance, CA,

Table 1
LC–MS/MS acquisition parameters for the targeted compounds.

Name	Molecular weight	Precursor ion	Product ion	Collision energy (eV)
MC-RR	1038.2	519.8 $[M+2H]^{2+}$	135.0[a] 440.4	30 25
MC-YR	1044.0	1045.6 $[M+H]^{+}$	135.0[a] 375.0	60 45
MC-LR	995.2	995.6 $[M+H]^{+}$	135.0[a] 553.0	56 45
MC-LA	910.0	910.6 $[M+H]^{+}$	135.0 375.0[a]	35 31
MC-LY	1002.2	1002.6 $[M+H]^{+}$	135.0 375.0[a]	34 30
MC-LW	1025.2	1025.6 $[M+H]^{+}$	135.0 375.0[a]	60 43
MC-LF	986.2	986.6 $[M+H]^{+}$	135.0 375.0[a]	50 22

[a] Quantificational ion.

Table 2
Microcystin solid phase extraction results (mean ± SD, $n = 3$).

Compound	C18 Mean ± SD (%)	HLB Mean ± SD (%)
MC-RR	30.1 ± 13.5	99.3 ± 4.2
MC-YR	51.5 ± 4.5	80.1 ± 6.1
MC-LR	63.1 ± 9.7	83.7 ± 9.9
MC-LA	92.6 ± 11.8	90.9 ± 4.7
MC-LY	39.9 ± 6.2	92.5 ± 6.9
MC-LW	29.1 ± 11.3	95.6 ± 9.2
MC-LF	50.1 ± 8.2	90.5 ± 5.1

USA). The mobile phases were composed of 0.1% (v/v) formic acid in water (A), 100% acetonitrile (B) and a mobile phase flow rate of 200 μL/min was used. The gradient ran from 20% B to 60% B over 3 min, hold at 60% B for 1 min then the column was washed with 80% B for 2 min, and equilibrated at 20% B for 4 min, the total chromatographic separation time was 10 min.

The mass spectrometer was operated in positive ion mode in this study. The optimal ionization and ion transmission conditions were tuned by an automated tuning procedure to maximize the signal of ions monitored and the optimal values were set as follows: spray voltage was 4500 V; capillary temperature was 350 °C; nitrogen was served as the sheath gas, auxiliary gas, and ion sweep gas and the values of these gases were set to 30, 5, and 1 arbitrary units, respectively; skimmer offset was 10 eV. The mass spectrometer was operated in selected reaction monitoring (SRM) mode with a signal time segment. The scan width for SRM was 0.01 m/z, scan time was 0.02 s, and the peak width settings (FWHM) for both Q1 and Q3 were 0.7. Data acquisition and processing was performed with Thermo Xcalibur software. The relevant collision energy (CE) is shown in Table 1.

2.5. Method validation

The method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision, recovery and matrix effect.

The linearity of the method was assessed at MCs concentration ranging from 1 to 50 ng/mL. Seven calibration curves with six concentration points were constructed. Calibration curves were fitted by least squares linear regression using $1/x$ as the weighting factor of the peak area versus the nominal concentration of MCs. The linearity of the calibration curves was evaluated on the basis of linear regression analysis and correlation coefficients (r^2). A correlation coefficient above 0.99 was desirable for all the calibration curves. LOD and LOQ were defined as concentrations in a sample resulting in signal-to-noise ratios of 3 and 10, respectively. Precision of the assay was expressed by percent relative standard deviation (%RSD).

The recoveries were obtained by analyzing seven MCs in hard clam and corbicula fluminea at three spiked concentration (1 μg/kg, 2 μg/kg, and 5 μg/kg for MC-RR, MC-YR, MC-LR, and MC-LY, and 2 μg/kg, 5 μg/kg, and 10 μg/kg for MC-LA, MC-LW and MC-LF). In all cases, experiments were performed in triplicate ($n = 3$). The limit of detection (LOD) and limit of quantification (LOQ) were estimated sextuplicate.

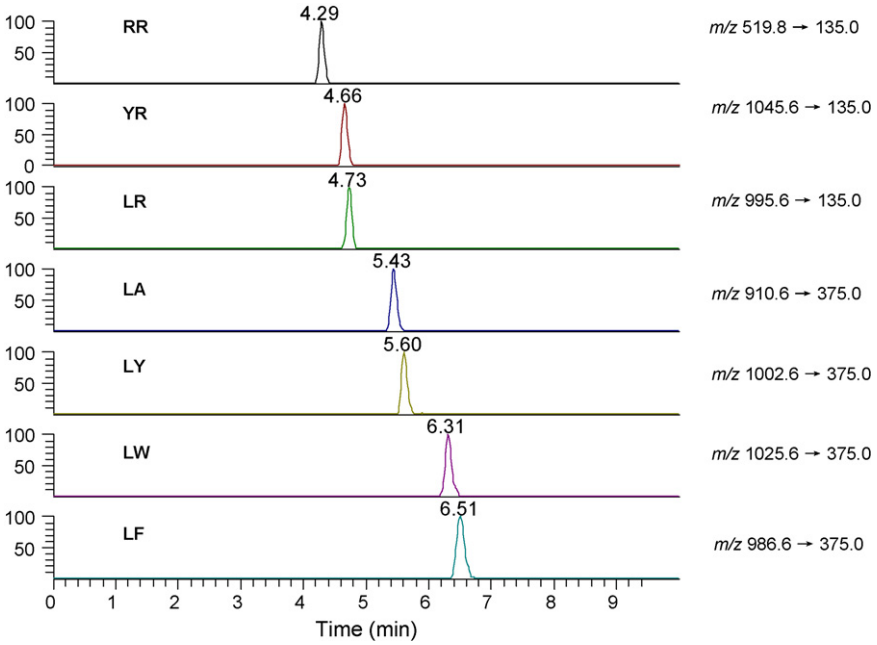

Fig. 2. Extracted chromatograms (XIC) of the selected MCs analyzed by LC–MS/MS using Symmetry 300 C18 Column (150 mm × 2.1 mm i.d., pore size 300 Å) with mobile phases: 0.1% (v/v) formic acid/water and acetonitrile.

Table 3
Linear relationships of microcystin variants.

Compound	Standard curve	r^2	Limit of detection (ng/mL)	Limit of quantification (ng/mL)
MC-RR	$Y = -25304.8 + 409{,}231 \times X$	0.9993	0.2	0.7
MC-YR	$Y = -11983.1 + 32454.2 \times X$	0.9987	0.3	1.0
MC-LR	$Y = -10366.2 + 32903.1 \times X$	0.9989	0.3	0.8
MC-LA	$Y = -6104.83 + 11193.7 \times X$	0.9924	0.6	2.0
MC-LY	$Y = -5899.21 + 32{,}730 \times X$	0.9995	0.3	0.9
MC-LW	$Y = -9771.21 + 23{,}205 \times X$	0.9975	0.4	1.2
MC-LF	$Y = -9138.37 + 14{,}623 \times X$	0.9952	0.4	1.3

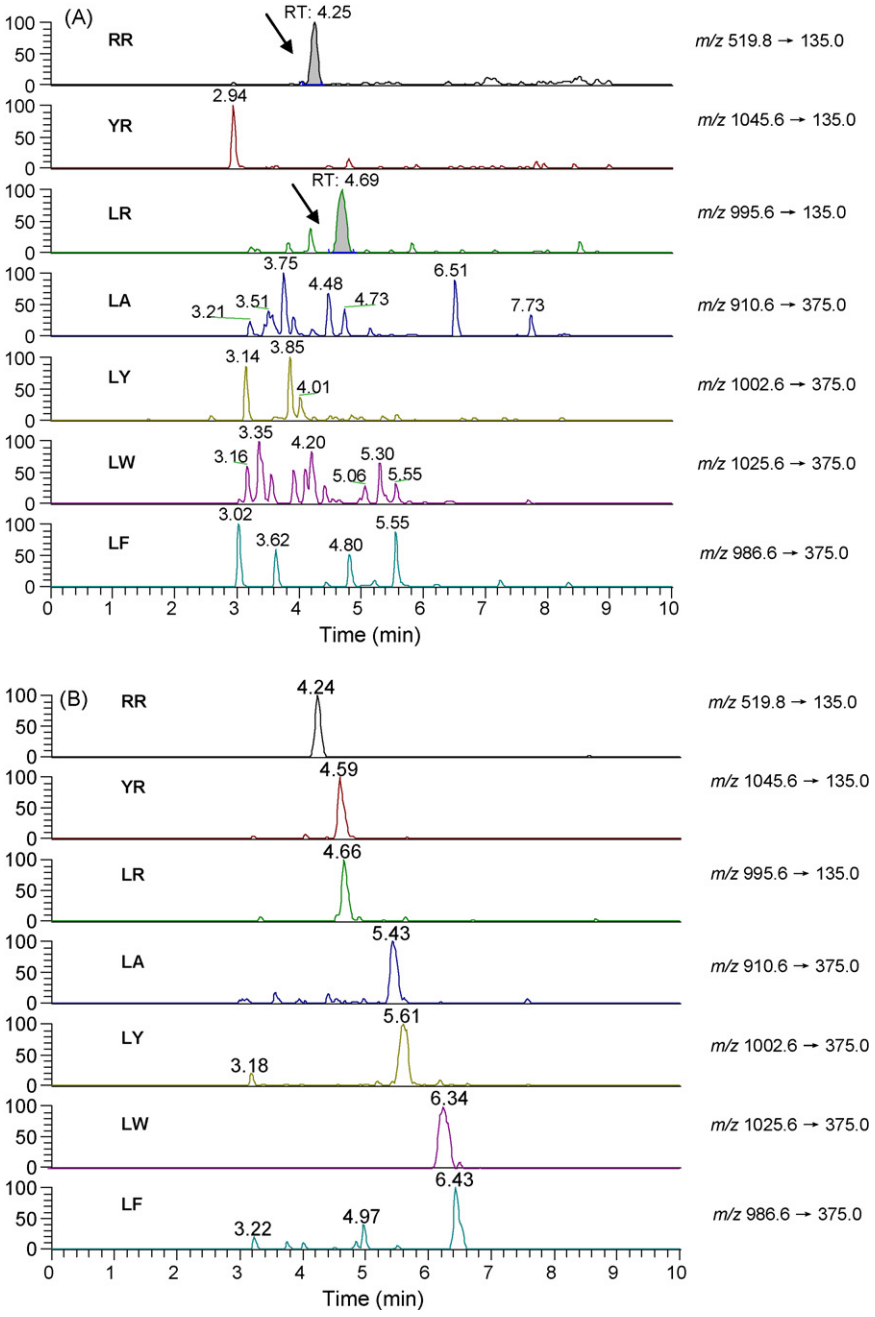

Fig. 3. Extracted chromatograms (XIC) of the selected MCs in (A) hard clam, (B) hard clam with standards at 2 μg/kg for MC-RR, MC-YR, MC-LR, and MC-LY and 5 μg/kg for MC-LA, MC-LW and MC-LF, (C) corbicula fluminea, and (D) corbicula fluminea with standards at 2 μg/kg for MC-RR, MC-YR, MC-LR, and MC-LY and 5 μg/kg for MC-LA, MC-LW and MC-LF.

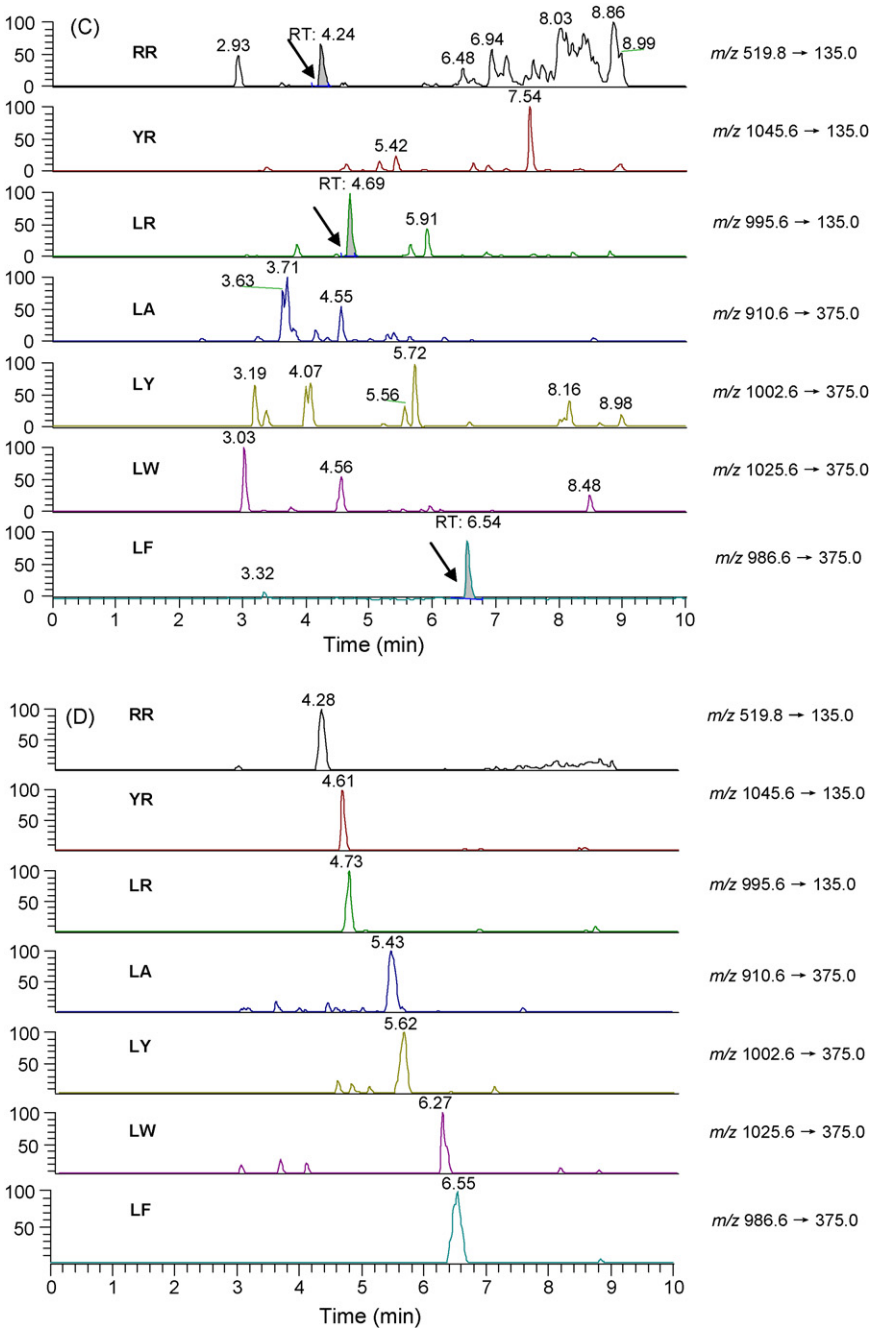

Fig. 3. (*Continued*).

The matrix effect was assessed by comparing the peak areas of the neat analyte standards, standards spiked before and after extraction in hard clam and corbicula fluminea samples.

3. Results and discussion

3.1. Extraction solvent

The extraction solvent and the extraction method are both critical to obtain a satisfactory recovery of microcystins from hard clam and corbicula fluminea tissues. The microcystins have good solubility in water and in organic solvent. Initially a binary extraction solvent system composed of methanol and water with different volume ratios were employed. However, the recoveries were obviously lower than single methanol extraction. Acetonitrile was also tested as extraction solvent. For the complex nature of muscle tissues of hard clam and corbicula fluminea (excessive endogenous interference and high content of protein), acetonitrile has the advantage of better protein precipitation and lower lipids solubility than methanol [29], however, the recoveries of MC-LA and MC-LY were lower by using acetonitrile. The targeted compounds obtained higher recoveries by using methanol as extraction solvent. Therefore, from the efficiency point of view, methanol was chosen as extraction solvent for all investigated shellfish products.

3.2. Solid phase extraction

Several commercially available reverse solid phase extraction (SPE) cartridges including Sep-Pak C18 (Waters, USA) and Oasis HLB (Waters, USA) were evaluated and it was observed that HLB gave

the best sample clean up. The recoveries of the targeted compounds with 5 ng/g spiking level were from 29.1% to 92.6% for Sep-Pak C18 and from 80.1% to 99.3% for HLB. Oasis HLB was the best cartridge for all the tested microcystins as shown in Table 2, and was selected for further experiments in this study. There are many interfering compositions in complex aquatic products, which ideally have to be washed off before elution, 20% methanol in water was found to be able to wash off the interference of the matrix while remain the targeted compounds on the cartridge, pure methanol was chosen for elution because of its highest recovery and conveniently dryness by nitrogen flush. The SPE clean up steps presented in this study are very simple and fast with efficient and adequate purification of MCs extracts.

3.3. LC optimization

LC–MS/MS is considered to be the first choice for the quantitative determination of MCs in aquatic product samples. The LC separation of microcystins was adapted from the method of Cong et al. [30] with minor modification. Two reverse-phase chromatography columns Polaris C18 (150 mm × 2.1 mm i.d., particle size 5 μm, pore size 100 Å) (Varian, Walnut Creek, CA, USA) and Waters Symmetry 300 C18 (150 mm × 2.1 mm i.d., pore size 300 Å) were compared for separation. Waters symmetry 300 showed better separating performance. It was shown to present higher resolution and more reproducible retention time and peak area for the seven microcystins. The reasonable explanation is that the column with larger pore size packing material has lower surface area and this is beneficial for the separation of the microcystins variants with large molecule weight (>1000). Next, mobile phases were tested to further optimize the separation, narrower peak shapes were obtained by using acetonitrile instead of methanol as organic mobile phase, under optimized condition. All MCs were separated with high sensitivity and selectivity, within a run time of 10.0 min as shown in Fig. 2.

3.4. MS optimization

To create the ion transitions of the seven MCs under the SRM mode, the individual MC standard working solution of 0.5 μg/mL was infused into ESI source by using a syringe pump at a flow rate of 5 μL/min and ionized in positive ionization mode. All MCs were successfully ionized and the protonated molecule, $[M+H]^+$, was the base peak for all MCs in the full scan mode, except MC-RR, which formed a doubly charged ion $[M+2H]^{2+}$. The protonated molecule for each analyte was chosen as precursor ion and was subject to fragmentation and product ion scan was performed automatically by instrumental function. Two major ion transitions for each analyte and the optimal collision energy for each ion transition were summarized in Table 2. These results were similar with Refs. [11,30].

3.5. Method validation

3.5.1. Matrix effect

Co-extracted and co-eluted matrix constituents could have an influence on the ionization efficiency of the analyte and adversely affect the reproducibility and accuracy of the method, especially when external calibration curves are used for quantification. For this reason, matrix effects were closely monitored during method development. The recoveries of the spiked analytes in matrices (hard clam and corbicula fluminea) were measured, peak areas before and after spiked at the concentration level of 5 μg/g MCs were compared and they were not significantly affected by the matrix components. Since the signal change was less than 20%, it could be concluded as there was no significant matrix effect.

Table 4
Hard clam and corbicula fluminea recovery tests of current method ($n = 6$).

Compound	Mass concentration (ng/g)	Hard clam		Corbicula fluminea	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
MC-RR	1.0	65.0	5.5	54.3	8.5
	2.0	57.1	11.8	62.1	5.9
	5.0	62.4	9.9	74.8	4.8
MC-YR	1.0	50.7	10.4	73.8	9.7
	2.0	43.7	12.8	70.6	8.5
	5.0	51.8	10.2	81.0	10.1
MC-LR	1.0	56.7	16.2	83.9	12.7
	2.0	75.2	14.3	76.2	11.8
	5.0	81.3	6.1	90.7	7.2
MC-LA	2.0	58.3	13.1	68.2	12.9
	5.0	64.8	13.7	65.9	10.3
	10.0	78.2	13.4	70.3	13.1
MC-LY	1.0	55.9	13.7	58.9	12.5
	2.0	64.9	14.3	60.8	10.3
	5.0	74.1	14.4	67.1	10.8
MC-LW	2.0	75.5	14.8	81.7	15.7
	5.0	66.8	16.2	85.2	13.7
	10.0	84.6	7.1	93.8	10.4
MC-LF	2.0	64.8	14.6	74.8	12.5
	5.0	71.4	13.2	85.7	12.9
	10.0	92.3	8.7	89.4	10.3

3.5.2. Calibration curve, linearity, LOD and LOQ

The calibration curves of the MCs were composed of six concentration levels, 1.0, 2.0, 5.0, 10.0, 20.0, and 50.0 μg/L. The standard curve was constructed by plotting peak areas of the SRM signal of different ion transitions against sample concentration. Good linearity was obtained for all analytes, with the square correlation coefficients (r^2) ranged from 0.9914 to 0.9967. The LODs and LOQs of seven microcystins in this method were ranged from 0.2 μg/kg to 0.6 μg/kg and from 0.7 μg/kg to 2.0 μg/kg, respectively. The results are summarized in Table 3.

3.5.3. Recovery and precision

In-house validation was carried out to evaluate performances of the method. Hard clam and corbicula fluminea samples containing no microcystins were used as blank matrix. Blank samples were pooled and divided into three groups, and each group was spiked with MCs standard solution to a final concentration of 1 μg/kg, 2 μg/kg and 5 μg/kg for MC-RR, MC-YR, MC-LR, and MC-LY, and 2 μg/kg, 5 μg/kg and 10 μg/kg for MC-LA, MC-LW and MC-LF, respectively. The results are shown in Table 4, satisfactory MCs recoveries ranging between 43.7% and 92.3% from hard clam, 54.3% and 93.8% from corbicula fluminea, were obtained by using the proposed method. The precision was satisfactory since the RSD of the mean recovery ranged from 5.5% to 16.2% in hard clam and from 4.8% to 15.7% in corbicula fluminea at spiking levels of 1 μg/kg, 2 μg/kg and 5 μg/kg for MC-RR, MC-YR, MC-LR, and MC-LY, and 2 μg/kg, 5 μg/kg and 10 μg/kg for MC-LA, MC-LW and MC-LF, respectively. The results show that this method has acceptable precision and repeatability at ppb level.

3.6. Application of the method to hard clam and corbicula fluminea samples

Ten hard clam samples and ten corbicula fluminea samples from different region were analyzed using the proposed method. In this batch of samples, only two hard clam samples and three corbicula fluminea samples were found to be polluted. Table 5 shows

Table 5
Results of positive samples (ng/g).

MC	Hard clam		Corbicula fluminea		
	1	2	1	2	3
RR	2.3	1.1	2.6	1.9	1.7
YR	ND	ND	ND	ND	ND
LR	1.5	0.9	1.8	1.4	1.4
LA	ND	ND	ND	ND	ND
LY	ND	ND	ND	ND	ND
LW	ND	ND	ND	ND	ND
LF	ND	ND	ND	3.8	2.3

the results of positive samples. MC-YR, -LA, -LY, and -LW were not observed in all the samples. However, RR, and LR were detected in both the muscle of hard clam and corbicula samples and LF was detected in two samples of corbicula samples. The typical figures are shown in Fig. 3. The concentrations of MC-RR in the selected two species were range from 1.1 ppb to 2.6 ppb. The result indicates that status of pollution of microcystins from the selected samples is not a serious issue.

4. Conclusions

A novel method was developed for simultaneous analysis of the seven microcystins compounds, MC-RR, MC-YR, MC-LR, MC-LA, MC-LY, MC-LW, and MC-LF in hard clam and corbicula fluminea tissues. It includes solvent extraction, cleanup on SPE cartridge and analysis by LC–ESI-MS/MS in SRM scan mode. By using this method, matrix interferences were minimized and the recovery rate of targeted compounds exceeded 43.7%. The limit of quantitation of this method meets the requirement of analyzing trace amounts of MCs when applied to the analysis of hard clam and corbicula fluminea samples. The simple sample pretreatment and fast instrument run time enables this method a practical choice for routine pollution monitoring in shellfish.

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