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Development and validation of a liquid chromatography–tandem mass spectrometry assay for the simultaneous quantitation of microcystin-RR and its metabolites in fish liver

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ABSTRACT

A novel method for identification and quantification of microcystin-RR (MC-RR) and its metabolites (MC-RR-GSH and MC-RR-Cys) in the fish liver was developed and validated. These analytes were simultaneously extracted from fish liver using water containing EDTA with 5% acetic acid, followed by a mixed-mode cation-exchange SPE (Oasis MCX) and subsequently determined by liquid chromatography–electrospray ionization ion trap mass spectrometry (LC–ESI-ITMS). Extraction parameters including volume and pH of eluting solvents, were optimized. Best recoveries were obtained by using 10 mL of 15% ammonia solution in methanol. The mean recoveries at three concentrations (0.2, 1.0, and 5.0 $\mu g\,g^{-1}$ dry weight [DW]) for MC-RR, MC-RR-GSH and MC-RR-Cys were 93.6–99%, 68.1–73.6% and 90.0–95.2%, respectively. Method detection limit (MDL) were 4, 7 and 5 $ng\,g^{-1}$ DW for MC-RR, MC-RR-GSH and MC-RR-Cys, respectively. Limits of quantification (LOQs) for MC-RR, MC-RR-GSH and MC-RR-Cys were calculated to be 10, 18 and 13 $ng\,g^{-1}$ DW, respectively. Finally, this method was successfully applied to the identification and quantification of MC-RR, MC-RR-GSH and MC-RR-Cys in the liver of bighead carp with acute exposure of MCs.

1. Introduction

Microcystins (MCs) are a family of cyclic hepatotoxic heptapeptides produced by several genera and species of blue-green algae (cyanobacteria) in fresh and brackish water blooms. The general structure is cyclic [-D-Ala-L-X-erythro-β-methyl-D-isoAsp-L-Y-Adda-D-iso-Glu-N-Methyldehydro-Ala-], where X and Z represent two variable L-amino acids, and Adda refers to β-amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid [1]. Among the 80+ variants of MCs that have been reported [2,3], MC-LR, MC-RR and MC-YR are the most common species in cyanobacterial blooms [4,5], and MC-RR is the dominant variant found in eutrophic lakes in China [6–8].

It is well-known that MCs have significant harmful impacts on both wild animals and humans by inhibiting the serine/threonine protein phosphatases1 and 2A (PP1 and PP2A) [9]. It can cause poisoning or death of fish, birds, domestic and wild animals [10,11], as well as illnesses and mortality in humans [12,13]. Recently, microcystins were identified for the first time in the serum (average 0.228 ng MC-LReq mL^{-1}) of a chronically exposed human population (fishermen at Lake Chaohu, China) together with indication of hepatocellular damage [14]. A great deal of studies have revealed that many organisms, especially the liver, develop a complex detoxication metabolism, and that glutathione plays an important role in the metabolic pathway of MCs in both mammals and aquatic organisms [15–19]. Pflugmacher et al. also suggested that the glutathione conjugate of microcystins appears to be the first step of detoxication of MCs in aquatic organisms [18].

Up to now, there have been several studies on the presence and qualitative analyses of conjugates of MC-LR in various organisms [15–22]. The conjugates of MCs to glutathione and cysteine were firstly synthesized and identified by Kondo et al. with Frit-FAB LC/MS [20]. They also identified the presence of MCs glutathione and cysteine conjugates formed in the liver of mouse and rat treated with MCs [15]. Subsequently, Pflugmacher et al. identified MC-LR conjugates formed enzymatically in aquatic macrophyte, invertebrates, fish eggs, and fish by LC–MALDI-TOF-MS [17]. Ito et al. studied the distribution of MC-LR and its glutathione and cysteine conjugates in different tissues in mice by immunostaining method [16]. Recently, LC–ESI-MS in selected reaction monitoring mode (SRM) after SPE (Oasis HLB and silica cartridges) was used to quantify MC-LR and its glutathione conjugates in fish tissues [21]. Based

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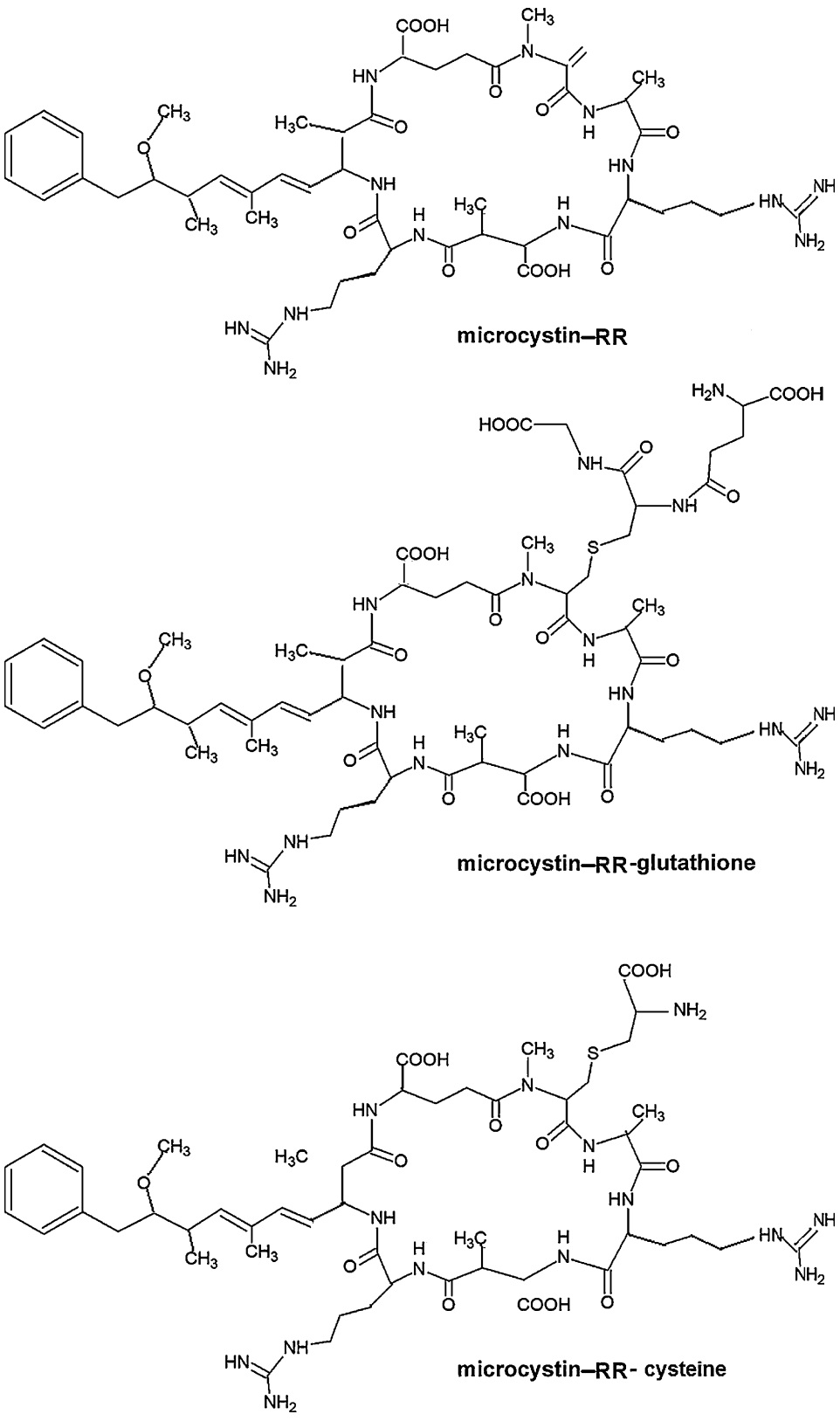

Fig. 1. Molecular structures of MC-RR, MC-RR-GSH and MC-RR-Cys used in this study.

on this method, Zhang et al. described the seasonal changes of MC-LR and its glutathione and cysteine conjugates in three aquatic animals from Lake Taihu [22]. The previous studies have mainly focused on MC-LR and its conjugates. Due to (1) more strong polarity of MC-RR, MC-RR-GSH and MC-RR-Cys, (2) low concentration of target analytes, (3) complex biological matrices, and/or (4) covalent binding of the target compounds with proteins, so far, there have been no published studies to quantitatively determine MC-RR and its metabolites in animal tissues. As a common and abundant hepatotoxin [23], it is an imperative need to develop a method

Table 1
Characteristic fragment ions in the (+)-ESI–MS/MS spectra of MC-RR-GSH and MC-RR-Cys.

MC-RR-GSH		MC-RR-Cys	
Fragment ions	*m/z*	Fragment ions	*m/z*
$[M+H]^+$	1345.73	$[M+H]^+$	1159.56
$[M+2H]^{2+}$	673.61	$[M+2H]^{2+}$	580.56
$[M+3H]^{3+}$	449.49	$[M-CO+H]^+$	1131.34
$[M-Glu+2H]^{2+}$	608.91	$[M-C_9H_{11}O+2H]^{2+}$	513.28
$[Ala-Arg-MeAsp-Arg-Adda-Glu-Mdha-SH+2H]^{2+}$	536.86	$[M-Cys+2H]^{2+}$	519.95
$[M-GSH+2H]^{2+}$	519.87	$[M-Gly+H_2O+2H]^{2+}$	550.23
$[M-GSH-C_9H_{11}O-NH_3+2H]^{2+}$	443.22	$[M-Cys+2H-CO]^{2+}$	504.84
$[MeAsp-Arg+H]^+$	284.72	$[MeAsp-Arg+H]^+$	285.05
$[M-C_9H_{11}O-NH_3+H]^+$	1194.03	$[M-C_9H_{11}O-NH_3+H]^+$	1008.30

for extraction, identification and quantification of MC-RR and its metabolites (MC-RR-GSH and MC-RR-Cys).

This paper firstly reports an analytical method for the simultaneous qualitative and quantitative determination of MC-RR and its metabolites (MC-RR-GSH and MC-RR-Cys) in fish liver. The procedure of this method is based on a mixed-mode strong cation-exchange (Oasis MCX) SPE enrichment, and subsequent quantification by LC–ESI-ITMS in positive ionization mode. Great efforts were taken to obtain superior recoveries by optimization of SPE and MS/MS parameters. The precision, linearity, mean recovery, MDL, LOQ and selectivity of method were assessed. Moreover, the method was applied to examine the concentration of MC-RR and its metabolites in liver samples of the fish injected intraperitoneally with *Microcystis* cell extracts.

2. Experimental

2.1. Chemicals and reagents

MC-RR (Fig. 1) was isolated and purified from *Microcystis aeruginosa* collected from Lake Dianchi according to the method of Ramanan et al. [24]. The purity of MC-RR was determined by HPLC (LC-20A, Shimadze, Kyoto, Japan) (>95%) and its identity was confirmed with LC–MS (Thermo Electron, Waltham, MA, USA). Product ions (*m/z*) of MC-RR include 452.78, 505.21, 887.19 and 904.29, which are identical with that of the standard sample (MC-RR, Wako Pure Chemical Industries, Japan).

MC-RR-GSH and MC-RR-Cys (Fig. 1) were prepared by the method of Kondo et al. [20] and Dai and Xie [21]. Briefly, MC-RR (2 mg) reacted with L-GSH (62 mg) in 4 mL of 5% potassium carbonate aqueous solution while stirring for 2 h at room temperature. The reaction mixture was neutralized with 4 mL of 0.04 M hydrochloric acid and applied to an ODS C_{18} cartridge (2 g, Waters, Milford, MA, USA). The cartridge was washed with 5 mL water and eluted by 10 mL methanol. The synthesis was purified further by a semipreparative reversed-phase liquid chromatography (Waters 600, USA; flow rate 2.8 mL min^{-1}; detection, UV (238 nm)) with an ODS C_{18} reversed-phase semipreparative column (7.8 mm × 300 mm, d_p 10 μm, Waters, Milford, MA, USA) to yield 1.6 mg of MC-RR-GSH. MC-RR-Cys was formed and purified similarly and yielded 1.64 mg. The purity of MC-RR-GSH and MC-RR-Cys were over 95% and checked by HPLC.

In LC–ESI-ITMS analysis the most intense ions of analytes are doubly charged ($[M+H]^{2+}$ at *m/z* 673.61, 580.56 and 520.05), which is typical for MCs containing two Arg residues [25,26], as well as its metabolites formed via addition of GSH and Cys. The $[M+2H]^{2+}$ at *m/z* 673.6 and 580.6 were then used as precursor ion for a product-ion MS/MS scan. Characteristic fragment ions in the (+)-ESI–MS/MS spectra of MC-RR-GSH and MC-RR-Cys were shown in Table 1.

L-Glutathione (L-GSH) and L-cysteine (L-Cys) were purchased from Acros Organics (Geel, Belgium, GR), Aqueous ammonia (25%, AR) and formic acid (GR) were purchased from SCRC and Aldrich. Methanol and acetonitrile were of HPLC grade (TEDIA Company, Inc., Fairfield, OH, USA), and ultra-pure water was obtained from a Milli-Q water purification system from Millipore (Bedford, MA, USA). Other reagents were all analytical reagent grade.

2.2. Equipment parameters and set up

Analyses were performed by the LCQ Advantage MAX ion trap LC/MS in the electrospray ionization (+) mode with a Surveyor HPLC system equipped with autosampler and photoelectric diode array (PDA) detector (Thermo Electron Corporation, San Jose, CA, USA). A Waters XBridge C_{18} column (2.1 mm × 100 mm, d_p 3.5 μm, Waters Corporation, USA) with a C_{18} guard column (2.1 mm × 10 mm, d_p 5 μm, Thermo Electron Corporation, USA) was applied.

2.2.1. Chromatography

The mobile phase consisted of water (A) and acetonitrile (B) (both contained 0.05% formic acid (v/v)). An injection volume of 10 μL was used. The system was programmed to deliver the following linear gradient: 0 min (95% A, 5% B), 1.0 min (65% A, 35% B), 17.0 min (55% A, 45% B), 17.5 min (30% A, 70% B), 18.0 min (5% A, 95% B), 20.0 min (5% A, 95% B), 20.01 min (95% A, 5% B), 25.0 min (95% A, 5% B). The total flow rate was held at 0.2 mL min^{-1} during the analysis stage. After the analysis stage, the percentage of solution B was adjusted to 5% and the flow rate was increased to 0.3 mL min^{-1} for 5 min before the next injection to renew the initial condition rapidly.

2.2.2. MS/MS parameters

Syringe pump was used for tuning the mass spectrometer and optimizing the ESI source. Nitrogen was used as a sheath and auxiliary gas, while helium was used as collision gas in the ion trap. All the analysis were performed by using the following settings: sheath gas flow rate, 20 units; auxiliary gas, 4.5 units; the capillary temperature, 250 °C; spray needle voltage, 4.5 kV; multiplier voltage −852 V; tube lens voltage, 55.5 V for MC-RR, 45.5 V for MC-RR-GSH and MC-RR-Cys; collision energy 40% for MC-RR and MC-RR-GSH, and 34% for MC-RR-Cys; and three microscans per scan were acquired.

The temperatures of vial tray and column oven in the autosampler were set to 10 and 40 °C, respectively. Various parameters were adjusted for the tested sample to optimize signal and to get maximal structural information from the ion of interest.

2.3. Sample preparation

The liver samples were obtained from several healthy bighead carp (*Aristichthys nobilis*, 3540 ± 220 g) and lyophilized immediately by a Christ® Alpha 2-4 freeze dryer (Martin Christ, Osterode, Germany) for analytical method development. No analytes (MC-RR, MC-RR-GSH and MC-RR-Cys) were detected in these samples by using our developed method.

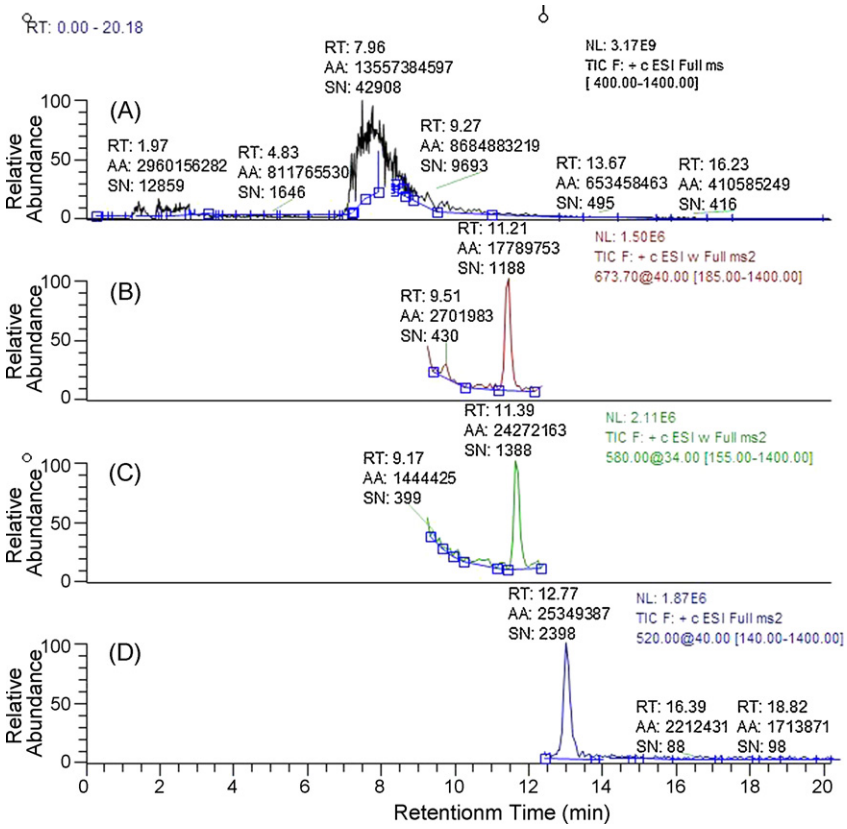

Fig. 2. TIC chromatography for LC–ESI-ITMS analysis of (A) full san LC–MS for spiked sample, and LC–MS/MS for (B) MC-RR-GSH, (C) MC-RR-Cys and (D) MC-RR. The concentrations of analytes spiked in the fish liver were 1 $\mu g\,g^{-1}$.

The lyophilized blank liver samples (50 ± 0.8 mg) were spiked with MC-RR and MC-RR-GSH, MC-RR-Cys at low, medium, and high levels, consisted of 0.01, 0.05, and 0.25 μg of analytes, respectively. Aliquots of the spiked samples were extracted three times with 5 mL of water with EDTA–Na_2 (0.01 M)–5% acetic acid for sonicating 3 min (30% aptitude, 60 W, 20 kHz, Branson Digital Sonifier, Danbury, CT, USA) at 0 °C, and then centrifuged at 15,000 × *g* (BR4, Jouan, Winchester, VA, France).

Stock solutions (50 $\mu g\,mL^{-1}$) were prepared by dissolving MC-RR, MC-RR-GSH and MC-RR-Cys in pure water. Standard solutions (0.1, 0.5, and 2.5 $\mu g\,mL^{-1}$) were prepared by serial dilutions of stock solutions using pure water. Quality control (QC) samples were prepared from the SPE extraction of liver samples spiked with low, medium and high concentrations (0.2, 1.0 and 5 $\mu g\,g^{-1}$ DW). All solutions were stored at −80 °C before use.

2.4. Clean-up procedure

Three solid phase extraction (SPE) cartridges, Oasis MCX (mix-mode strong anion exchanger containing bonded sulfonic acid), MAX (mix-mode strong anion exchanger containing bonded quaternary ammonium), WCX (mix-mode weak exchanger containing bonded carboxyl, 3 cm^3/60 mg, Waters, MA, USA) were used to extract MCs and its metabolites, and the procedures were as follows: the cartridges were conditioned and equilibrated with methanol and ultra-pure water in sequence. Supernatant samples were loaded onto the MCX cartridges, washed with 2% aqueous formic acid and methanol, and then eluted with aqueous ammonia in methanol. For MAX and WCX, 5% aqueous ammonia and methanol were selected as washing solution, and cartridges were then eluted by 2% aqueous formic acid in methanol and directly collected. These eluates were evaporated to dryness by rotary evaporators and the residue was dissolved in 5 mL 100% MeOH. Finally, the eluate was evaporated to dryness and redissolved in 100 μL of the LC mobile phase (95% A, 5% B), 10 μL of above fraction was injected into the LC–MS system.

2.5. Method development

Method was developed and established according to the guidelines for analysis of biological samples by the FDA (available from URL: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107).

External calibration samples were prepared by mixing analytes to blank samples extraction after SPE, and the concentrations of series were 0.02, 0.10, 0.2, 1.0, 2.0 and 5.0 $\mu g\,g^{-1}$ DW. The recovery was calculated by comparing the peak areas of sample spiked prior to extraction to the standard solution. All extractions were performed in triplicate and the values given in tables are average of triplicate runs.

Method detection limit (MDL) was calculated using the following equation proposed by the U.S. EPAsw-864 [27–29]. It is defined as "the minimum concentration that can be determined with 99% confidence that the true concentration is greater than zero", and it takes into account not only matrix effect, but also the variability introduced by all the sample processing steps. The procedure described by the U.S. EPA was carried out. In brief, seven samples were spiked at a concentration of 0.02 $\mu g\,g^{-1}$, each replicate was processed through the entire analytical method and an initial estimate of the MDL was then calculated by multiplying the standard deviation of the results by the appropriate one-sided 99% *t*-statistic. Limit of quantitation (LOQ) is defined as the lowest concentration of an analyte that the bioanalytical procedure can reliably differ-

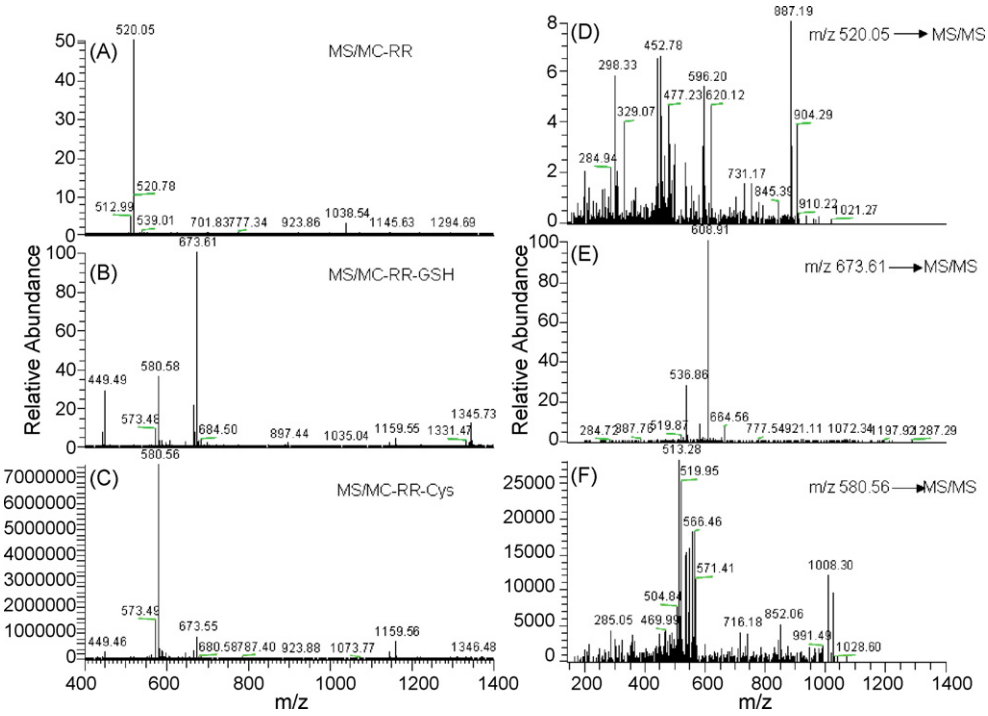

Fig. 3. Product-ion mass spectrum for MC-RR and its two metabolites in the spiked fish liver at the concentrations of 1 μg g^{-1}. Shown are LC–MS spectra (A, B, and C) for MC-RR, MC-RR-GSH and MC-RR-Cys; LC–MS/MS product-ion mass spectra (D, E, and F) for MC-RR, MC-RR-GSH and MC-RR-Cys.

entiate from background noise and was calculated according to Eq. (2):

$$\mathrm{MDL} = t_{(n-1,\ a=0.01)} \times \text{Std. Deviation} \quad (1)$$

$$\mathrm{LOQ} = 2.5 \times \mathrm{MDL} \quad (2)$$

2.6. Application

The LC–MS/MS method was applied to quantify MC-RR, MC-RR-GSH and MC-RR-Cys in the liver of bighead carp. The carp were fasted for 48 h before the experiment. Then a dose of an approximately 1-mL extracted solution of MCs was injected. The content of MC-RR in the extracted microcystin were determined by external calibration curves of the standards available (Wako Pure Chemical Industries, Osaka, Japan). The concentration of MC-RR was 167.7 μg mL^{-1}.

Liver samples were collected at 0, 1, 3, 12, 24 and 48 h postinjection, respectively. Samples were sealed and stored at −80 °C until the analysis. Lyophilized samples were extracted and cleaned up by the methods described in Sections 2.3 and 2.4.

3. Results and discussion

3.1. LC–ESI-MS analysis of MC-RR, MC-RR-GSH and MC-RR-Cys

In this study, MC-RR, MC-RR-GSH and MC-RR-Cys were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) with ion trap in positive mode. Fig. 2 shows the TIC chromatography of MC-RR, MC-RR-GSH and MC-RR-Cys in fish liver. The relative retention times of MC-RR, MC-RR-GSH and MC-RR-Cys were 12.77, 11.22 and 11.39 min, respectively.

The full-scan mass spectra for spiking samples are shown in Fig. 3. The product ions were abundant at *m*/*z* 608.91 and 536.86 of MC-RR-GSH, and they were formed from the precursor ion of 673.61 by loss of fragmentation 129.40 (MeAsp) and 273.50 (-GSH+SH) (Fig. 3E). The ion at *m*/*z* 513.28 and 519.95 were the abundant product ions of MC-RR-Cys (Fig. 3F), and they were correspondent to the ion at *m*/*z* 580.56 by loss of Adda-derived fragment of *m*/*z* 134.56 and -Cys residue of *m*/*z* 121.22 [30]. The resulting product ions were identical with the characteristic fragment ions of MC-RR-GSH and MC-RR-Cys in Table 1. The data obtained from fragment ion sequentially by MS/MS were selected as qualitative ions and also consistent with the structure of MC-RR-GSH and MC-RR-Cys described in Fig. 1.

3.2. Optimization of SPE conditions

Oasis mixed-mode ion-exchange cartridges (MAX, MCX and WCX) were used to extract and clean-up analytes without liver. MCX cartridge was chosen for further development since it had a higher recovery (98.9 ± 3.7%, 83.2 ± 5.6% and 96.0 ± 5.7%), which was remarkably higher than those of MAX (59 ± 3.6%,

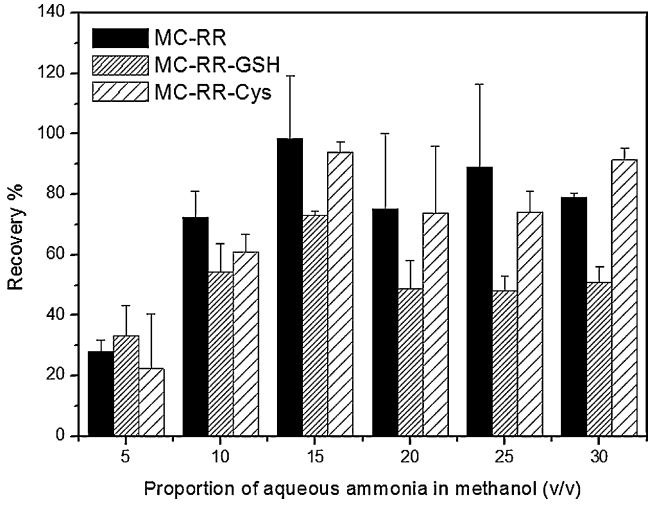

Fig. 4. Proportions of aqueous ammonia in methanol in the elution step.

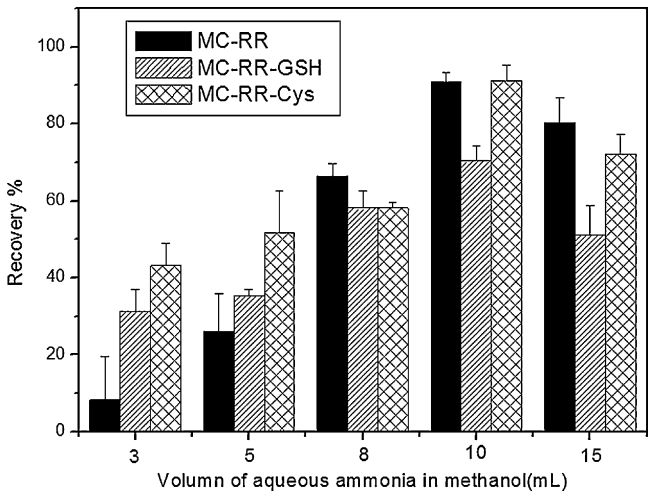

Fig. 5. Effect of eluting volume on the recoveries of MC-RR and its metabolites (the proportions of aqueous ammonia in methanol was 15%).

30.8 ± 6.7% and 41.5 ± 9.2%) and WCX (69.3 ± 3.6%, 25.1 ± 2.1% and 59.1 ± 4.1%).

Methanol was selected as appropriate solvent in this procedure for its volatility and polarity [31,32]. As an ion-exchange SPE, pH of the elution step must be considered and it was adjusted by adding aqueous ammonia into methanol to make its proportions of 5%, 10%, 15%, 20%, 25% and 30%, respectively. According to Fig. 4, 15% aqueous ammonia in methanol was sufficient enough to get best recoveries of all the analytes (98.4%, 73% and 93.9% for MC-RR, MC-RR-GSH and MC-RR-Cys, respectively). To obtain the best eluting volume of the solvent for analytes, 3, 5, 8, 10 and 15 mL of solvent was selected. According to Fig. 5, 10 mL of 15% aqueous ammonia in methanol yielded the best recoveries (90.9%, 70.4% and 91.3%). The recoveries of MC-RR and MC-RR-Cys were higher than that of MC-RR-GSH, and that is because, besides the ion-exchange retention, the reversed-phase retention also contributed to retain the analytes in MCX sorbent. Since MC-RR and MC-RR-Cys are more hydrophobic than MC-RR-GSH, they could be retained tightly on the MCX than MC-RR-GSH in the wash steps.

3.3. Precision and stability

Table 2 summarizes the within day and between days precision of the analytes. Precision was assessed at three different concentrations (0.2, 1.0, and 5.0 μg g^{-1} dry DW) by performing replicated analyses (n = 5) of spiked samples against external calibration curves, using standard solutions in solvent. Precisions are determined as coefficient of variation (CV). The procedure was repeated on the same day and for different days on the same standard series. The precisions of within-assay and between assays ranged between 2.1–11.2% and 2.9–12.8%, respectively, and both were under the acceptable level (<15%).

The stability of the analytes was tested by freeze–thaw day-cycle (−20 °C to room temperature) for three times before analysis. The results showed that MC-RR, MC-RR-GSH and MC-RR-Cys were stable for 72 h. The degradation of the low and high QC levels at 4 °C for 24 h was less than 10.6%. Therefore, analytes could be considered to be stable in liver matrices under frozen storage and assay processing.

3.4. MDL and LOQ

MDL and LOQ were calculated using QC samples with low concentration of MC-RR, MC-RR-GSH and MC-RR-Cys. The MDL values of MC-RR, MC-RR-GSH and MC-RR-Cys were 4, 7 and 5 ng g^{-1} DW, respectively, while their LOQs were 10, 18 and 13 ng g^{-1} DW, respectively.

3.5. Selectivity and linearity

LC–MS analysis of the extracts from fish liver (Fig. 2) and blank samples (data not shown) showed that analytes were clearly detected by the TIC chromatogram for LC–MS/MS at 12.77, 11.22 and 11.39 min, and few interfering peaks were present at the known retention time.

Calibration curves were created for MC-RR, MC-RR-GSH and MC-RR-Cys in liver matrices. A linear calibration curve for analytes quantitated from 0.02 to 5.0 μg g^{-1} DW was achieved. The correlation coefficient (r^2) values (0.9924, 0.9942 and 0.9996 for MC-RR, MC-RR-GSH and MC-RR-Cys, respectively) suggested that LC–MS/MS method was suitable for the quantitative detection of these analytes in the liver of fish at this concentration range.

3.6. Mean recovery and ion suppression

The mean recoveries were evaluated to test the efficiency and trueness of the procedure. The determination of the recovery was made at low, medium and high concentrations for three replicates and was conducted as described in Sections 2.3 and 2.4. The

Table 2
Precisions of the method for different analytes.

Analyte	Within-assay CV (%, n = 5)			Between assays CV (%, 3 days)		
	Low[a]	Medium[b]	High[c]	Low	Medium	High
MC-RR	11.2	7.5	2.5	11.4	12.8	4.8
MC-RR-GSH	2.3	8.8	8.8	12.8	8.6	8.3
MC-RR-Cys	4.5	6.9	2.1	7.1	4.9	2.9

[a] Low fortification: 0.2 μg g^{-1} for MC-RR, MC-RR-GSH and MC-RR-Cys.
[b] Medium fortification: 1.0 μg g^{-1} for MC-RR, MC-RR-GSH and MC-RR-Cys.
[c] High fortification: 5.0 μg g^{-1} for MC-RR, MC-RR-GSH and MC-RR-Cys.

Table 3
Mean recoveries of MC-RR, MC-RR-GSH and MC-RR-Cys in fish liver (mean ± SD, n = 3).

Spiking levels (μg g^{-1})	MC-RR		MC-RR-GSH		MC-RR-Cys	
	Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)
0.2	93.6 ± 3.7	4.0	68.8 ± 3.4	4.9	92.6 ± 7.4	8.0
1	98.8 ± 3.3	3.3	68.1 ± 5.7	8.4	90.0 ± 5.5	6.1
5	99.0 ± 6.2	6.3	73.6 ± 3.5	4.8	95.2 ± 6.6	6.9

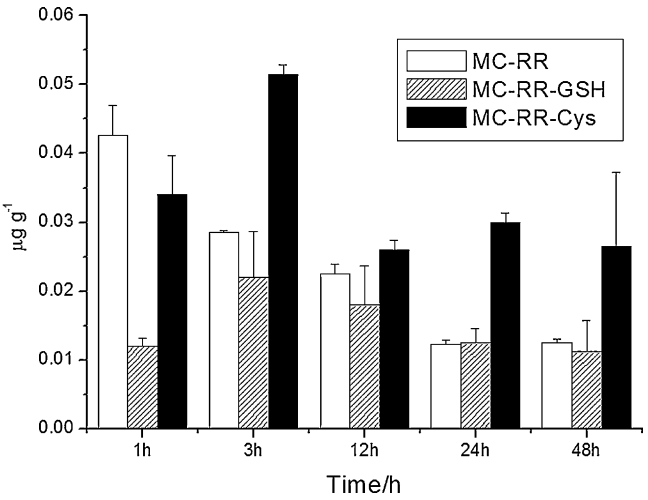

Fig. 6. The mean (n = 3) liver concentration–time profiles for MC-RR, MC-RR-GSH and MC-RR-Cys after the intraperitoneal injection of MCs to bighead carp.

mean recoveries were 93.6–99.0% (MC-RR), 68.1–73.6% (MC-RR-GSH) and 90.0–95.2% (MC-RR-Cys), respectively (Table 3).

Matrix effects occurred in the analysis of microcystin by using ESI-MS [21,33,34]. Endogenous compounds (i.e., from matrices) or exogenous contaminants (i.e., from plastics or solvents) may co-elute with analytes during analysis and it causes an increase (enhancement) or decrease (suppression) in the analyte's signal. In present study, the ion suppression was determined as the relative signal decrease of the liver sample spiked in the SPE eluate, compared to the standard solution. Results indicated that MCX cartridge decreased the amount of matrix injected onto the column and the ion suppression effect was less than 19.8% (n = 6, detailed data not shown).

3.7. Method application

Extracted MCs was injected intraperitoneally to bighead carp and the developed method was applied to detect the concentration of analytes in liver samples. The mean (n = 3) liver concentration–time profiles for MC-RR, MC-RR-GSH and MC-RR-Cys were shown in Fig. 6.

In the liver samples, concentrations of MC-RR, MC-RR-GSH and MC-RR-Cys ranged from 0.013 to 0.043 $\mu g\,g^{-1}$ DW, from 0.011 to 0.022 $\mu g\,g^{-1}$ DW and from 0.03 to 0.051 $\mu g\,g^{-1}$ DW, respectively. MC-RR shows the highest concentration at 1 h postinjection, while MC-RR-GSH and MC-RR-Cys peaked at 3 h postinjection.

4. Conclusion

In this study, a method for simultaneous determination of MC-RR, MC-RR-GSH and MC-RR-Cys in fish liver was developed and validated for the first time. In this method, a cation-exchange cartridge was used and shown to be efficient to extract and concentrate the MC-RR, MC-RR-GSH and MC-RR-Cys from fish liver. Mean recoveries of analytes at different concentrations ranged from 93.6% to 99.0%, from 68.1% to 73.6% and from 90.0% to 95.2% for MC-RR, MC-RR-GSH and MC-RR-Cys, respectively. Validation results showed a low MDL (4, 7 and 5 $ng\,g^{-1}$ DW) and LOQ (10, 18 and 13 $ng\,g^{-1}$ DW). Finally, this method was applied for detecting low concentrations of MC-RR, MC-RR-GSH and MC-RR-Cys in liver samples of fish injected intraperitoneally.

Metabolites of toxins provide essential information on the toxicological and pharmacological studies [35,36]. It is expected that the quantitative method developed in this study could be used to document the patterns of the absorption, distribution and excretion of MC-RR and its metabolites, and to evaluate the kinetics of MC-RR and its metabolites in blood, body fluids and various organs, so as to provide possible explanation for the great difference in MC induced toxicity among different animals, especially between aquatic vertebrates (e.g., fish) and mammals (e.g., mice). Moreover, a method for the simultaneous determination of microcystin-LR, MC-RR and their glutathione and cysteine conjugates in animal tissues are also needed in our future studies.

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