A sensitive method for the determination of total microcystins in water and sediment samples by liquid chromatography with fluorescence detection†

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Microcystins (MCs) are potent hepatotoxins that comprise a family of more than 90 different variants. MCs can bind to matrices, after which they cannot be completely extracted by the solvent. Currently, the MMPPB (2-methyl-3-methoxy-4-phenylbutyric acid) method is the only effective way of determining total MCs without solvent extraction. In this study, a sensitive method to determine the total MCs in water and sediment was developed based on the MMPPB method. Specifically, MCs were oxidized to MMPPB with an improved oxidation reagent (20 mg mL⁻¹ NaIO₄, 4000 mg L⁻¹ KMnO₄, pH ~ 9) and a stable MMPPB yield of about 35% was obtained for both water and sediment samples. The minimum volume of the oxidation reagent could be determined by the organic content in the matrix. After being concentrated, the MMPPB was derivatized with 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-p-toluenesulfonate (BDETS) and detected by liquid chromatography using a fluorescence detector. Quantification limits of MCs in water and sediment samples were 125 ng L⁻¹ and 100 ng g⁻¹ respectively. The method was successfully applied to Lake Dianchi and the soluble MC concentrations determined by this method were correlated well with those obtained by the enzyme-linked immunosorbent assay (ELISA).

Methods

Different techniques have been applied to quantify MCs in the water column, mainly liquid chromatography with a diode array detector or mass spectrometry (HPLC-PDA, LC-MS), protein phosphatase inhibition assay (PPIA) or enzyme-linked immuno-sorbent assay (ELISA). There are more than 90 different MC variants in natural samples; therefore, measuring the total MC content is the most economic and feasible method. HPLC-PDA is the most widely used approach for quantification of MCs, while LC-MS can provide more sensitive detection and accurate identification. However, both chromatography and mass-spectrometric methods neglect unknown MC variants that are not available as standards. The PPIA and ELISA can detect MCs present at levels below the WHO guidelines without the need for sample pre-concentration. However, false positives may be obtained in PPIA from other phosphatase inhibitors that may occur in environmental samples including okadaic acid, tautomycin and calyculin-A. Moreover, it is doubtful that the PPIA and ELISA are equally sensitive to different MC variants.

Although these methods are generally effective for measuring the dissolved toxins in water, their application to analysis of MCs in other matrices (algae, sediment and tissue) requires prior solvent extraction, which may significantly underestimate the total toxin content. Meissner et al. found that a considerable portion of MCs binds to protein in algae and is neglected by current analysis techniques based on solvent extraction. Bieczynski et al. found that protein-bound MC-LR (L = leucine, R = arginine) represented 66–100% of total MC-LR in the intestine and liver. Our previous studies also revealed that MCs can be strongly adsorbed by sediment, but only be partly recovered by solvent extraction.

The MMPPB (2-methyl-3-methoxy-4-phenylbutyric acid) method has been successfully employed for determination of the total MC content in various matrices. This method is especially well-suited to the analysis of animal tissues and sediment, where toxins are bound to matrix components. This technique involves cleavage of the chemically unique C20...
amino acid, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), to form MMPB, which is then quantified. A stable oxidation yield of MMPB is essential for the precise quantification. However, the MMPB yields were only mentioned in four studies and the reported values were inconsistent. On the other hand, the direct quantification of MMPB using HPLC with a UV detector was not sensitive enough. GC-MS21,22 and LC-MS24 have also been used to quantify MMPB, but the instrument and operation cost of mass spectrometry is much higher than HPLC and GC.

Derivatization followed by HPLC with a fluorescence detector (FLD) has been commonly used to measure organic acids. As a small molecular organic acid, derivatization may also be feasible for sensitive quantification of MMPB. 1,2-Benzo-3,4-dihydrocarbazole-9-ethyl-p-toluenesulfonate (BDETS) was synthesized by You et al. and used in the determination of bile acids and fatty acids. In the present study, we investigated the feasibility of using this derivatization reagent to improve the sensitivity of MMPB.

This study was conducted to develop a comprehensive method that sensitively and accurately analyzes the total MCs in water and sediment. The specific goals were to (i) optimize the oxidation procedures for a stable MMPB yield, (ii) improve the detection sensitivity of MMPB by fluorescence derivatization, and (iii) apply the optimized method for the detection of total MCs in spiked and natural samples.

Experimental

Reagents and chemicals

The MC-LR used in the experiment was isolated and purified in the laboratory as previously described. The concentrations were determined according to the MC standards purchased from Sigma-Aldrich (St. Louis, MO, USA). MMPB sodium salt standards were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). BDETS was kindly provided by the Northwest Plateau Institute of Biology (Chinese Academy of Sciences, Xining, China). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Reagents other than HPLC grade methanol and acetonitrile were of analytical grade.

Sample collection and preparation

Sediment and water samples for toxin analysis were obtained from 18 sites in Lake Dianchi on April 25, 2014 (Fig. S1 of the ESIF). Sediment and water samples for spike experiments were obtained from Lake Donghu in Wuhan. Sediments from Lake Caohai and Lake Taihu were also used.

All sediment samples were transported to the laboratory as soon as possible. After homogenizing, the samples were stored at −20 °C and then freeze-dried at 0.05 mbar in an Alpha 1–2/5D Freeze Dryer (Martin Christ, Germany). Next, the dried samples were crushed, passed through an 80 mesh sieve and stored at −20 °C until analysis.

For water samples, 400 mL of water were filtered through Whatman 0.45 μm nitrocellulose membranes, after which the MCs were concentrated using a Sep-Pak C18 cartridge (500 mg, 6 cm³, Waters, Milford, MA, USA). The cartridge was pre-conditioned with 10 mL methanol followed by 10 mL water. After loading the filtered water, the cartridge was washed with 10 mL water and 10 mL 20% methanol, respectively, and then eluted with 10 mL methanol. The eluate was evaporated to dryness by rotary vacuum evaporation at 40 °C and reconstituted with 0.5 mL 50% v/v methanol. Aliquots of the concentrated MC solutions were used for the ELISA or the following oxidation procedure.

Oxidation of MCs in aqueous solutions and sediments

Sediment and water samples containing natural MCs or spiked with MCs were oxidized by the Lemieux reagent according to Wu et al. Each test was conducted in triplicate.

For sediment samples, 1 g of freeze-dried sediment was oxidized with 10 mL oxidation reagent containing 20 mg mL⁻¹ sodium periodate (NaIO₄) and 4000 mg L⁻¹ potassium permanganate (KMnO₄) for 1 h under weakly alkaline conditions (pH ~ 9) unless otherwise specified. After centrifugation, the supernatants were quenched with 40% sodium bisulphite (NaHSO₃) solution (50 μL NaHSO₃) for 1 mL oxidation reagent. The oxidation yields of MCs in sediments from Lake Tai, Lake Caohai and Lake Dianchi were then compared. The organic contents of the three sediments were 12.78, 76.75 and 438.21 g kg⁻¹, respectively. For water samples, aliquots of the concentrated MC solutions (0.1 mL) were added to 1 mL oxidation reagent with the same composition. One hour later, 50 μL of 40% NaHSO₃ were added to terminate the reaction.

The pretreatment of MMPB in reaction solution

The MMPB in the reaction solutions was concentrated and purified with three commercial SPE disposable cartridges, Sep-Pak cartridge (500 mg, 6 cm³), Oasis MAX cartridge (150 mg, 6 cm³), and Oasis HLB cartridge (200 mg, 6 cm³) (Waters, MA, USA), and with three extraction solutions, n-hexane, diethyl ether and dichloromethane, respectively. Each test was conducted in triplicate.

The cartridge was pre-conditioned with 10 mL methanol followed by 10 mL water. After loading aliquots of reaction solution, the Sep-Pak and Oasis HLB cartridges were washed with 10 mL water and 10 mL 20% methanol, respectively, and then eluted with 10 mL methanol. The Oasis MAX cartridge was subsequently washed with 10 mL 5% ammonia water and 10 mL methanol and eluted with 10 mL 5% formic acid in methanol. In all cases, the eluent was evaporated to dryness and resolved in 0.5 mL acetonitrile.

Derivatization of MMPB

The derivatization of MMPB was conducted according to Wang et al. All reactions were conducted in a 2 mL vial containing 5 mg K₂CO₃, 200 μL dimethyl formamide (DMF), 100 μL BDETS (5 × 10⁻³ mol L⁻¹) and 100 μL of MMPB acetonitrile solution unless otherwise specified. The vial was then sealed and allowed to react in a microfiter thermostat at 100 °C for 60 min unless otherwise specified. Each test was conducted in triplicate. The derivatization procedure is shown in Fig. 1.
Analysis methods
MCs and MMPB were analyzed by HPLC-PDA as described.\textsuperscript{16} MCs in water samples were also detected by the ELISA based on anti-MCLR monoclonal antibodies.\textsuperscript{23,24}

The MMPB derivative (BDETS–MMPB) was analyzed on a Synergi Hydro-RP C18 column (4 \textmu m, 250 mm \times 4.6 mm) using HPLC with a FLD at excitation and emission wavelengths of 273 and 365 nm, respectively. The mobile phase consisted of acetonitrile and 0.05% aqueous trifluoroacetic acid with the following gradient applied at a constant flow of 1.0 mL min\textsuperscript{-1}: initial concentration of acetonitrile at 70%, which was increased gradually to 100% over 20 min, then decreased back to 70% over the next minute, where it was held until the end of the run at 25 min. The retention time of the MMPB derivative was determined by comparing the chromatograms of standard MMPB solutions with that of a blank control.

The structure of the MMPB derivative was confirmed using an Agilent 6460 Triple Quadrupole LC-MS System (Agilent Technologies, Waldbronn, Germany) in positive-ion ESI mode. The ESI source parameters were as follows: gas temperature 300 °C, gas flow 6 L min\textsuperscript{-1}, nebulizer gas pressure 40 psig, sheath gas temperature 300 °C, sheath gas flow 11 L min\textsuperscript{-1}, and capillary voltage 3500 V. The fragmentor voltage and collision energy were 166 V and 45 eV, respectively.

Statistical analysis
All statistical analyses were performed by the software SPSS 13 for Windows (SPSS, Chicago, USA). Graphs were generated with Origin 8.0 software (OriginLab, Northampton, USA).

Results and discussion
Identification of the MMPB derivative
The BDETS–MMPB derivative produced an intense molecular ion peak at \( m/z \left[ M + H \right]^{+} 454.2 \) (Fig. 2). With MS/MS analysis of the derivative, the collision induced dissociation spectra of \( m/z \) 454.2 produced specific fragment ions of \( m/z \) 436.4, 244.0 and 209.3. The fragment ion at \( m/z \) 436.4 corresponded to the dehydration product of the derivative. The fragment ions of \( m/z \) 209.3 and 244.0 originated from the cleavage of BDETS–MMPB as shown in Fig. 1.

Optimization of the derivatization procedure of MMPB with BDETS
The effects of the reaction temperature and time on the derivatization yield were investigated as shown in Fig. 3a. The rate of
Derivatization was positively correlated with temperature. Maximum fluorescence responses were observed at 90 °C for 60 min or 100 °C for 40 min. The latter was adopted for further studies and deemed optimum. You et al. derived the carboxyl groups of bile acids with BDETS in 30 min at 95 °C.22 Wang et al. derived the carboxyl groups of free fatty acids with BDETS in 30 min at 90 °C.21 We adopted a slightly longer time and higher temperature to derive MMPB with BDETS.

Measurement of the derivatization yields of the three different concentrations of MMPB with varying concentrations of BDETS revealed that the yields increased with the concentration of BDETS and became stable at 7.5 × 10^{-3} mol L^{-1} (Fig. 3b); therefore, the concentration of BDETS was set at 7.5 × 10^{-3} mol L^{-1}. You et al. found that the derivative yield reached about 100% when a 10- to 15-fold molar excess of BDETS was used.22 Our results showed that the derivative yields relied on the concentration of BDETS rather than the molar ratio between BDETS and MMPB.

The effects of different amounts of potassium carbonate (K_{2}CO_{3}) and water content on the derivation yield were also compared (Fig. S2 of the ESI†). Changes in the water content had a remarkable effect on the derivatization reaction, while no significant differences were observed in response to variations in the K_{2}CO_{3} concentration (P = 0.865, LSD test). Indeed, as little as 2.5 mL of water could significantly reduce the fluorescence intensity and increasing the water content resulted in a decreased peak area. Thus, an anhydrous condition is essential for a stable derivation yield.

Analysis of serial dilutions of standard MMPB solutions ranging from 1 μg L^{-1} to 1 mg L^{-1} showed that the response was a linear function of the amount of MMPB over a 1000-fold concentration range that could be described by the linear function \( Y = 107.364X - 8796 \), where \( Y \) is the peak area and \( X \) (μg mL^{-1}) is the amount of MMPB (correlation coefficient > 0.9999). The corresponding derivatives were stable and enabled further HPLC analysis for at least 2 weeks with normalized peak areas <0.32%.

### Oxidation of MCs

During Lemieux oxidation, KMnO_{4} is renewable as the product, manganite (K_{2}MnO_{4}), can be oxidized to KMnO_{4} by NaIO_{4}. Thus, sufficient NaIO_{4} and small amounts of KMnO_{4} are needed in the Lemieux oxidation. As shown in Table 1, saturated NaIO_{4} solution (~20 mg mL^{-1}) has been used for oxidation in most previous studies. In all but one study, the MC oxidation was conducted in a weak base (pH ≈ 9). In contrast, the KMnO_{4} concentrations varied significantly among studies. Thus, we investigated the effects of different KMnO_{4} concentrations on the MMPB yield with consistent NaIO_{4} (20 mg mL^{-1}) concentrations and pH (9).

KMnO_{4} concentrations of 50 and 8000 mg L^{-1} were too low or high for the oxidation of MCs (Fig. 4). The oxidation yields in KMnO_{4} solutions with concentrations ranging from 500 to 4000 mg L^{-1} were 22.4–40% (Fig. 4). The oxidation yields in KMnO_{4} solutions with concentrations ranging from 500 to 4000 mg L^{-1} were 22.4–40% (Fig. 4).

![Fig. 3](image-url) Effects of reaction temperature (a), time (a) and BDETS concentrations (b) on the derivatization of MMPB.

![Fig. 4](image-url-1) Effects of the KMnO_{4} concentration on the yield of MMPB.

| Table 1: Summarized oxidation conditions of MCs to produce MMPB |
|---|---|---|---|---|
| Matrix | NaIO_{4} (mg mL^{-1}) | KMnO_{4} (mg L^{-1}) | pH | Yield | Origin |
| Water | 20 | 50 | 1.6 | 1.6 | 16 |
| Water | 12.5 | 1896 | 9 | 96.5% | 19 |
| Water | 20 | 4000 | 9 | 75% | 17 |
| Algae | 20 | 8000 | 9 | 33–45% | 11 |
| Sediment | 20 | 8000 | 9 | 22.4–40% | 3,26 |
| Tissue | 4.3 | 3160 | 9 | 14 |
| Tissue | 20.6 | 562 | 9 | 8 |

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mg L$^{-1}$ showed no significant differences ($P = 0.124$, SNK test). In this range of KMnO$_4$ concentrations, the effects of the oxidation time were compared and no significant differences were observed ($P = 0.124$, SNK test).

Since KMnO$_4$ is not a selective oxidant, other organics in matrices can affect the formation of MMPB. This was verified by Wu et al., who found that the yield of MMPB decreased as the organic material in sediments increased.$^{11}$ Thus, we tested the effects of sediments on the yields of MMPB.

There were no significant differences in the oxidation yields of MCs in water solution and 1 g sediments from Lake Taihu and Lake Dianchi (Fig. 5a). However, significant reductions were found in 0.2 and 0.4 g sediments from Lake Caohai (Fig. 5a). By increasing the volume of the oxidation reagent or repeating the oxidation procedure with more oxidation reagent, the loss of oxidation yield in sediment from Lake Caohai could be recovered (Fig. 5b).

The relationship between the volume of the oxidation reagent and the organic content of sediment was tested by oxidizing 0.5 g spiked sediment from Lake Caohai with different volumes of the oxidation reagent (Fig. 6). Stable yields were obtained when more than 45 mL of the oxidation reagent was used. Since the organic content was 438.21 g kg$^{-1}$, 205 mL of the oxidation reagent would be needed for sediment with 1 g organic matter.

A stable MMPB yield of 35% could be obtained when the MCs were oxidized by an appropriate volume of the oxidation reagent with fixed concentrations. The MMPB yields were reported to be 29–40% for animal tissues spiked with MCs$^3$ and $33–45%$ for sediment samples.$^{11}$ Our results were in accordance with these studies. The maximum MMPB yield from the oxidation of algae powder was found to be higher than 85% by Wu et al.$^{17}$ However, the MMPB yield that was overestimated for the MC concentrations in the algae powder was underestimated in the study.$^{11}$ Xu et al. oxidized MC solutions under similar oxidation conditions to this study$^{19}$ and the MMPB yield, 96.5%, was inconsistent with this study and other research studies.

### Extraction, concentration and clean-up of MMPB in environmental samples

To decrease the adverse impact of impurities of environmental samples on the determination of MMPB derivatives, the extraction capabilities of liquid–liquid extraction or solid-phase extraction were compared. As shown in Table 2, the extraction efficiencies of the SPE cartridges were significantly higher than those of the solvents, and there were no significant differences in the extraction efficiencies of the three SPE cartridges for MMPB. The extraction efficiencies of diethyl ether and dichloromethane did not differ significantly, but the extraction efficiency obtained by $n$-hexane was significantly lower. Consequently, solid-phase extraction with SPE cartridges is more

#### Table 2 Recoveries of MMPB by liquid–liquid extraction or solid-phase extraction

<table>
<thead>
<tr>
<th>Material</th>
<th>Recovery (%)</th>
<th>Material</th>
<th>Recovery (%)</th>
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<tbody>
<tr>
<td>$n$-Hexane</td>
<td>9 ± 0</td>
<td>Sep-Pak cartridge</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>77 ± 2</td>
<td>HLB cartridge</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>70 ± 2</td>
<td>MAX cartridge</td>
<td>96 ± 2</td>
</tr>
</tbody>
</table>
efficient for MMPB and Sep-Pak cartridges were chosen for the subsequent procedures owing to their high cost-effectiveness.

Analysis of spiked samples

A standard curve between the MC amount (10 ng to 2.5 μg) and peak area determined by HPLC-FLD was generated (Fig. 7) and the formula describing the curve was found to be \( Y = \left( 15.298X + 0.265 \right) \times 10^6 \), where \( X \) and \( Y \) are the amount of MCs (μg) and peak area, respectively. The \( R^2 \) was 0.9995.

Water and sediment samples spiked with different amounts of MCs were also detected by optimized procedures and the recoveries were determined by the standard curve. The detection limits for water and sediment samples were respectively 10 ng (80 mL water sample) and 100 ng (1 g sediment), which were equivalent to 125 ng L\(^{-1}\) and 100 ng g\(^{-1}\) (Table 3). The recoveries were 98–109% and most of the relative deviations were below 10%.

Derivatization followed by GC-MS detection was adopted by Xu et al. and the detection limit was 0.56 μg L\(^{-1}\) when 100 mL water sample was used for detection. Roy-Lachapelle et al. detected MMPB with LDTD-APCI-MS/MS and the quantification limit was 0.2 μg L\(^{-1}\). Thus, the sensitivity of the proposed method was comparable with published methods based on mass spectrometry. Due to the low test cost and high sensitivity, this method may be a good option for the routine detection of MCs.

Application to field samples

The total MC concentrations in water samples of Lake Dianchi determined by HPLC-FLD ranged from 0.21 μg L\(^{-1}\) at point 6 to 1.01 μg L\(^{-1}\) at point 18 (Fig. 8). The MC concentration was only higher than 1 μg L\(^{-1}\) at point 18. The results of the two methods were significantly correlated, with a relationship defined by \( Y = 0.666X + 0.052 \), where \( X \) and \( Y \) are the MC concentrations determined by HPLC-FLD and the ELISA, respectively. The correlation coefficient was 0.743. The average concentrations of MCs determined by HPLC-FLD and the ELISA were 0.541 and 0.455 μg L\(^{-1}\), respectively. The results of HPLC-FLD were higher than those obtained by the ELISA (paired sample \( T \) test, \( p = 0.044 \)). Wu et al. found that the average soluble MC content measured by the ELISA was 0.5 μg L\(^{-1}\) in 2009, which was consistent with our results.

Since anti-MCLR monoclonal antibody was used in the ELISA, other variants may not have the same cross-reactivity. It was reported that the antibody had good cross-reactivities with MC–LR, MC–RR, and MC–YR but lower reactivities with MC–LY and MC–LA (Y = tyrosine, A = alanine). Thus, the total MC content may be underestimated by the ELISA.

The World Health Organization has proposed a provisional upper limit for microcystin LR of 1 mg L\(^{-1}\) in drinking water. Our result revealed the low potential risk of MCs for human health in Lake Dianchi.

No MCs were detected in sediment samples from any sampling points. After adsorption by sediment, MCs may be rapidly degraded by microorganisms in sediment as reported by

<table>
<thead>
<tr>
<th>Spiked MCs (μg)</th>
<th>Recovery from lake water (%)</th>
<th>Recovery from sediment (%)</th>
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<tbody>
<tr>
<td>2.5</td>
<td>106 ± 4</td>
<td>107 ± 1</td>
</tr>
<tr>
<td>1</td>
<td>99 ± 2</td>
<td>102 ± 5</td>
</tr>
<tr>
<td>0.5</td>
<td>109 ± 3</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>0.25</td>
<td>101 ± 2</td>
<td>98 ± 12</td>
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<tr>
<td>0.1</td>
<td>107 ± 5</td>
<td>104 ± 15</td>
</tr>
<tr>
<td>0.05</td>
<td>109 ± 9</td>
<td>Not detected</td>
</tr>
<tr>
<td>0.01</td>
<td>103 ± 3</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

Fig. 7  Plot of peak area versus MC content in deionized water.

Fig. 8  The MC contents at the 18 sampling points in Lake Dianchi determined by HPLC-FLD and the ELISA.
Chen et al., who found that MC-LR could be degraded anoxically from 5 mg L\(^{-1}\) to below the detection limit within 2 days.\(^{19}\) It seems that MCs are quickly degraded in sediments.

**Conclusion**

A sensitive method for the determination of total microcystins in water and sediments of a eutrophic lake was developed. MCs were oxidized to MMPB with consistent yield (35%). After being concentrated with a Sep-Pak SPE cartridge, the MMPB was derivatized with BDETS and detected by HPLC with FLD. The detection limits for water and sediment samples were 125 ng L\(^{-1}\) and 100 ng g\(^{-1}\) respectively. The results of this method were correlated well with those obtained by the ELISA. The developed methods were then successfully applied to field samples in Lake Dianchi.

**Acknowledgements**

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**References**