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Rapid and sensitive analysis of microcystins using ionic liquid-based *in situ* dispersive liquid–liquid microextraction

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ABSTRACT

Three structurally different ionic liquids (ILs), namely 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]), 1-(6-hydroxyethyl)-3-methylimidazolium chloride ([HeOHMIM][Cl]) and 1-benzyl-3-(2-hydroxyethyl)imidazolium bromide ([BeEOHIM][Br]), were applied as extraction solvents using *in situ* dispersive liquid–liquid microextraction (*in situ* DLLME) for the preconcentration of two microcystin variants, microcystin-RR (MC-RR) and microcystin-LR (MC-LR) from aqueous samples. Extraction parameters including sample solution pH, ratio of IL to metathesis reagent, sample volume, IL quantity, and salt concentration were optimized to achieve the best extraction efficiency. The [BeEOHIM][Br] IL, which contains both an aromatic moiety and a hydroxyl group within its chemical structure, exhibited superior extraction efficiency compared to the other two ILs. The analytical performance of the [BeEOHIM][Br] IL as an extraction solvent for *in situ* DLLME of microcystins was investigated using HPLC-UV and HPLC-MS. The limits of detection (LODs) for MC-RR and MC-LR were 0.7 $\mu g\,L^{-1}$ using UV detection with a linear range from 1 to 50 $\mu g\,L^{-1}$. The separation method was successfully adapted for ESI-MS/SIM detection, wherein the LODs for MC-RR and MC-LR were greatly improved to 0.005 and 0.003 $\mu g\,L^{-1}$, respectively. The accuracy of the method was demonstrated by examining the relative recovery using tap water and river water and produced recoveries ranging from 45.0 to 109.7% and from 46.3 to 103.2%, respectively.
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1. Introduction

Microcystins are a class of monocyclic heptapeptide hepatotoxins produced by certain species of freshwater cyanobacteria, mainly *Microcystis aeruginosa*. There are more than 90 microcystin variants that have been identified [1]. These variants typically contain three conserved D-amino acids, two varied L-amino acids, and two uncommon amino acids, namely *N*-methyldehydroalanine and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA) [2]. The ADDA amino acid found in microcystin variants contributes to microcystin toxicity by inhibiting two major protein phosphatases in eukaryotic cells, protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A) [3,4]. Interest in the analysis of microcystins has grown rapidly in recent years due to water contamination caused by cyanobacterial blooms [5]. The microcystin variants possess median lethal doses (LD_{50}) ranging from 50 to 1200 $\mu g\,kg^{-1}$ in mice [6]. Microcystin-LR (MC-LR) is one of the variants possessing acute toxicity ($LD_{50} = 50\ \mu g\,kg^{-1}$) [6], prompting the World Health Organization to establish a health-based standard concentration of 1 $\mu g\,L^{-1}$ for MC-LR in drinking water [7].

Solid-phase extraction (SPE) employing a reversed phase sorbent is among the most common sample preparation methods for microcystin analysis [2,8]. Due to the lack of selectivity, the extraction procedure usually requires repeated sample work-up in order to achieve satisfactory extraction efficiency. To address this limitation, immunoaffinity sorbent phases containing microcystin antibodies have been applied in order to improve the extraction selectivity toward microcystins [9–11]. Additionally, bioassay methods such as enzyme-linked immunosorbent assays (ELISAs) and protein phosphatase bioassays have been applied subsequent to SPE pre-treatment [12–15] or utilized as off-line detection methods in conjunction with high-performance liquid chromatography (HPLC) [16]. Unfortunately, these immunological and enzymatic bioassay approaches often require expensive substrates. Therefore, alternative extraction techniques that are rapid, selective, sensitive, and present a lower cost barrier should be explored in order to further improve the analysis of microcystins.

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Dispersive liquid–liquid microextraction (DLLME) was first described by Rezaee and co-workers in 2006 [17]. This sample preconcentration technique can often achieve good extraction efficiency by using a water-miscible organic solvent to disperse a water-immiscible extraction solvent within the aqueous sample. Fine droplets of the extraction solvent generate high contact surface area for the analytes to partition to the immiscible phase. Phase separation of the hydrophobic extraction solvent from the aqueous phase is usually accomplished by centrifugation [18–23] or by decreasing the solution temperature [24–26]. Subsequently, the extraction solvent containing the analyte(s) of interest can be withdrawn and subjected to chromatographic analysis. Ionic liquids (ILs) were first applied as extraction solvents for DLLME in 2008 [24,27]. ILs possess high thermal stabilities and negligible vapor pressure at ambient temperatures. Additionally, their tunable chemical structures and unique solvation properties enable ILs to be attractive extraction solvents in DLLME. IL-based *in situ* DLLME was described by Baghdadi and our group in 2009 [28,29]. In this method, a hydrophilic IL is dissolved in an aqueous sample solution. An anion exchange reagent, such as lithium bis[(trifluoromethyl)sulfonyl)]imide ($LiNTf_2$), is added to the solution resulting in the formation of fine droplets of the hydrophobic IL phase that can easily be separated from the aqueous solution. A significant advantage of IL-based *in situ* DLLME as a preconcentration method is the ability to customize the structure of ILs to achieve high extraction efficiency and selectivity [30]. A number of analytes have been previously studied by this approach, including emerging contaminants, medicinal products, and biological molecules [19,30–36].

In this study, IL-based *in situ* DLLME was adopted for the selective extraction of microcystins. Three structurally different ILs, 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]), 1-(6-hydroxyethyl)-3-methylimidazolium chloride ([HeOHMIM][Cl]) and 1-benzyl-3-(2-hydroxyethyl)imidazolium bromide ([BeEOHIM][Br]), were synthesized and applied for the preconcentration of two selected microcystin variants, namely MC-RR and MC-LR. Extraction parameters including sample solution pH, ratio of IL to metathesis reagent, sample volume, IL quantity, and salt concentration were optimized in this study. The sedimented IL phase was subjected to HPLC for further analysis. A comparison of UV and MS detection was performed by evaluating the sensitivity, linearity of calibration curve, and limits of detection (LODs) of the established method. The accuracy of the analytical method was also investigated by recovery studies in real water samples, including tap water and river water.

2. Experimental

2.1. Reagents

The reagents 1-methylimidazole, 1-benzylimidazole, 1-chlorobutane, 2-bromoethanol, 6-chloro-1-hexanol, and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium chloride, isopropanol, methanol, and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). $LiNTf_2$ was purchased from SynQuest Labs, Inc. (Alachua, FL, USA). The two microcystin variants, namely MC-RR and MC-LR, and a microcystin analog, nodularin (NOD), were purchased from Enzo Life Sciences (Farmingdale, NY, USA). The structures of MC-RR, MC-LR and NOD are shown in Fig. 1.

Stock solutions of MC-RR and MC-LR were individually prepared for *in situ* DLLME coupled to HPLC-UV. The stock solution of MC-RR was prepared by dissolving 250 μg of MC-RR in 250 μL of 80% methanolic aqueous solution. The stock solution of MC-LR was prepared by dissolving 1000 μg of MC-LR in 1000 μL of methanol. The working solutions at concentrations of 10 $\mu g\,mL^{-1}$ and 100 $\mu g\,mL^{-1}$ were obtained by serial dilution of the stock solutions with methanol. All of the stock solutions and working solutions were stored at −20 °C. The aqueous samples were prepared by spiking an aliquot of the microcystin working solution into deionized water (18.2 MΩ cm) produced by a Milli-Q filtration water system (Bedford, MA, USA). Similarly, after salt optimization, the aqueous samples were prepared by spiking an aliquot of the microcystin working solution into the 30% NaCl (w/v) aqueous solution.

Preparation of sample solutions for *in situ* DLLME coupled to HPLC-MS was followed using the aforementioned procedures. The microcystin working solutions at concentrations of 0.1 $\mu g\,mL^{-1}$, 1 $\mu g\,mL^{-1}$ and 10 $\mu g\,mL^{-1}$ were diluted serially from the stock solutions with methanol. Additionally, NOD, a cyclic pentapeptide hepatotoxin produced by the planktonic cyanobacteria *Nodularia spumigena*, was used as an internal standard for all HPLC-MS experiments. The stock solution of NOD was prepared by dissolving 100 μg of NOD in 100 μL of 50% methanolic aqueous solution. The working solution of NOD with a concentration of 1 $\mu g\,mL^{-1}$ was prepared by diluting the NOD stock solution with methanol. The aqueous sample solution was consistently spiked with NOD at a concentration of 0.1 $\mu g\,L^{-1}$.

2.2. Synthesis of ILs

Chemical structures of the three applied ILs are shown in Fig. 2. The [BMIM][Cl] IL was synthesized according to a previous study [29]. Synthesis of the [HeOHMIM][Cl] IL was performed by mixing 60.9 mmol of 1-methylimidazole and 73.1 mmol of 6-chloro-1-hexanol in 10 mL isopropanol and heating at 60 °C for 3 days. After the removal of solvent under reduced pressure, the crude product was dissolved in 8 mL of water. The aqueous solution was then washed with ethyl acetate and chloroform, respectively, in order to remove unreacted starting materials (6 × 4 mL). Finally, the product was dried under vacuum at 70 °C for 24 h. Synthesis of the [BeEOHIM][Br] IL was carried out by mixing 25.3 mmol of 1-benzylimidazole and 30.4 mmol of 2-bromoethanol in 10 mL isopropanol and heating at 60 °C for 3 days. The [BeEOHIM][Br] IL was purified by following the same procedure as the [HeOHMIM][Cl] IL, but with a recrystallization step being adopted to yield the final product. A 2 g aliquot of dried [BeEOHIM][Br] IL, a viscous yellow liquid, was dissolved in 1 mL of isopropanol and stored in a scintillation vial at 4 °C for 2 days. Following this storage process, clear crystals were formed on the bottom of the vial. The crystal layer was washed with 2 mL of cold isopropanol and dried overnight under vacuum at 70 °C. The final product appeared as a viscous liquid with a faint yellow color. All final products were subsequently characterized by proton nuclear magnetic resonance spectroscopy (^1H NMR) and electrospray ionization MS (ESI-MS). Spectral data for the characterization of [HeOHMIM][Cl] and [BeEOHIM][Br] are provided in the supplemental information.

2.3. Instrumentation

Sample solutions were prepared in 15 mL polypropylene conical tubes purchased from Becton Dickinson Labware (Franklin Lakes, NJ, USA). Agitation was accomplished with a vortex mixer from Barnstead/Thermolyne (Dubuque, IA, USA). Post-extraction centrifugation was performed using a model 228 centrifuge from Fisher Scientific at a rate of 3400 rpm (1380 × *g*). Characterization of all synthesized ILs was performed on a Varian Unity Inova 600 MHz NMR spectrometer and Bruker Esquire multipole ion trap mass spectrometer equipped with an ESI source.

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