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Highly Sensitive Liquid Chromatography-Mass Spectrometry Detection of Microcystins with Molecularly Imprinted Polymer Extraction from Complicated Aqueous Ecosystems

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Abstract

In the present study, a liquid chromatography-mass spectrometry (LC-MS) method has been developed and validated to monitor traces of microcystins (MCs) in lake and marine waters. The molecularly imprinted polymer (MIP) formulated with itaconic acid as the functional monomer and ethylene glycol dimethacrylate as the cross-linking monomer has been used to selectively enrich MCs from the aqueous solutions. The extraction capacity and selectivity of MIP was higher when comparison with conventionally used resin XAD and powdered activated carbon (PAC). The MIP showed an outstanding selectivity for microcystin-LR (MC-LR) in a mixture of MCs from aqueous solutions in the pH range 6-9. The LC-MS analysis of MCs after MIP extraction showed an excellent linearity in the working range (R^2=0.998) with high repeatability (RSD%, <6.3) and recoveries above 90%. Interference of dissolved ions and solution pH on MCs trace quantification in the lake and marine water samples were quantified. The limits of quantification (LOQ) and lower limit of detection (LOD) for the MC-LR were 10 and 1 ng L^{-1}, respectively, which satisfies the strictest World Health Organization standard for MC-LR in drinking water (1 ng mL^{-1}). The proposed analytical approach is simple, efficient and comparable with the detection limit of the traditional and expensive ELISA method of MCs analysis.

Keywords: Microcystins; Molecularly imprinted polymer; Solid-phase extraction; Liquid chromatography; Environmental trace analysis

Introduction

The production and release of cyanotoxins by cyanobacteria in freshwaters around the world has been well documented [1,2]. Microcystins (MCs) are the most frequently occurring class of cyanobacterial toxins, of which microcystin-LR (MC-LR) is the most toxic and primarily detected congener [3]. The general structure of MCs is cyclo-(-D-Ala-L-R1-D-erythro-β-methyllisoAsp-L-R2-Adda-D-iso-Glu-N-methylde hydroAla), where R1 and R2 represent two variable L-amino acids and Adda stands for 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. There are about 60 MC variants known, and MC-LR, MC-RR and MC-YR are the three most abundant vairants reported in natural algal blooms across the world [4]. Contamination of drinking water by MCs has been linked to cases of primary liver cancer in China and the deaths of 76 patients undergoing dialysis in Brazil [5,6]. Recently, blooms of microcystis have resulted in health alerts in Nebraska and other parts of the Midwestern United States [7]. Due to adverse health effects, the World Health Organization (WHO) established a provisional concentration limit of 1 μg L^{-1} for MC-LR in drinking water [8] and the United States Environmental Protection Agency (USEPA) has placed MCs on the Drinking Water Contaminants List [9]. Reduction of threats to human health and aquatic life involves toxic cyanobacteria blooms and/or MCs to be monitored and removed from water columns, in particular, public water supplies. Because of this, there is a growing demand for the development of more reliable, rapid, cost-effective and sensitive analytical methods for monitoring of MCs in water.

MCs are commonly measured by HPLC with photodiode array (PDA) in full UV spectrum acquisition, over the range 200-300 nm [10]. They developed a SPME-microbore-LC/Q-TOF-MS method for measuring MCs in water [11]. This technique requires small volumes (2 mL) and provides sensitive and information-rich analysis of unknown toxins. Another sensitive method reported for MCs and nodularins analysis in tap water by [12]. Using the LC/ESI-MS/MS method the quantification limits of 0.25-0.90 μg L^{-1} were achieved for MCs, with a short run time of 10 min. [13,14] reported the analysis of polar dimethyl microcystin variants that are common in nature but for which there exist no commercial standards. [15] developed a capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) to the simultaneous separation of cyanobacterial toxins (anatoxin-a, MC-LR, cylindrospermopsin) [14]. Long et al. [16] developed a fast and sensitive detection method for MC-LR with a portable trace organic pollutant analyzer (TOPA) based on the principle of immunoassay and total internal reflection fluorescence [15]. The limit of detection of 0.03 μg L^{-1} and the quantitative detection range of 0.1–10.1 μg L^{-1} was obtained while the cross-reactivity against a few compounds structurally similar to MC-LR was little. The recovery of MC-LR added to water samples at different concentrations ranged from 80 to 110% with RSD values less than 5%. [17] reported eight reversed-phase columns intended for rapid HPLC were assessed for the separation of thirteen MCs and nodularins [16]. Cong et al. [18] presented a novel chromatography electron spray ionization (ESI-MS) tandem triple quadruple mass spectrometry method to determine the trace amounts of major MCs in water. Solid phase extraction consuming 10 mL of water samples was used for sample clean-up and analyte enrichment. Limits of quantification (LOQ) and lower limit of detection were 0.05 and 1.0 ug/L, respectively [19].

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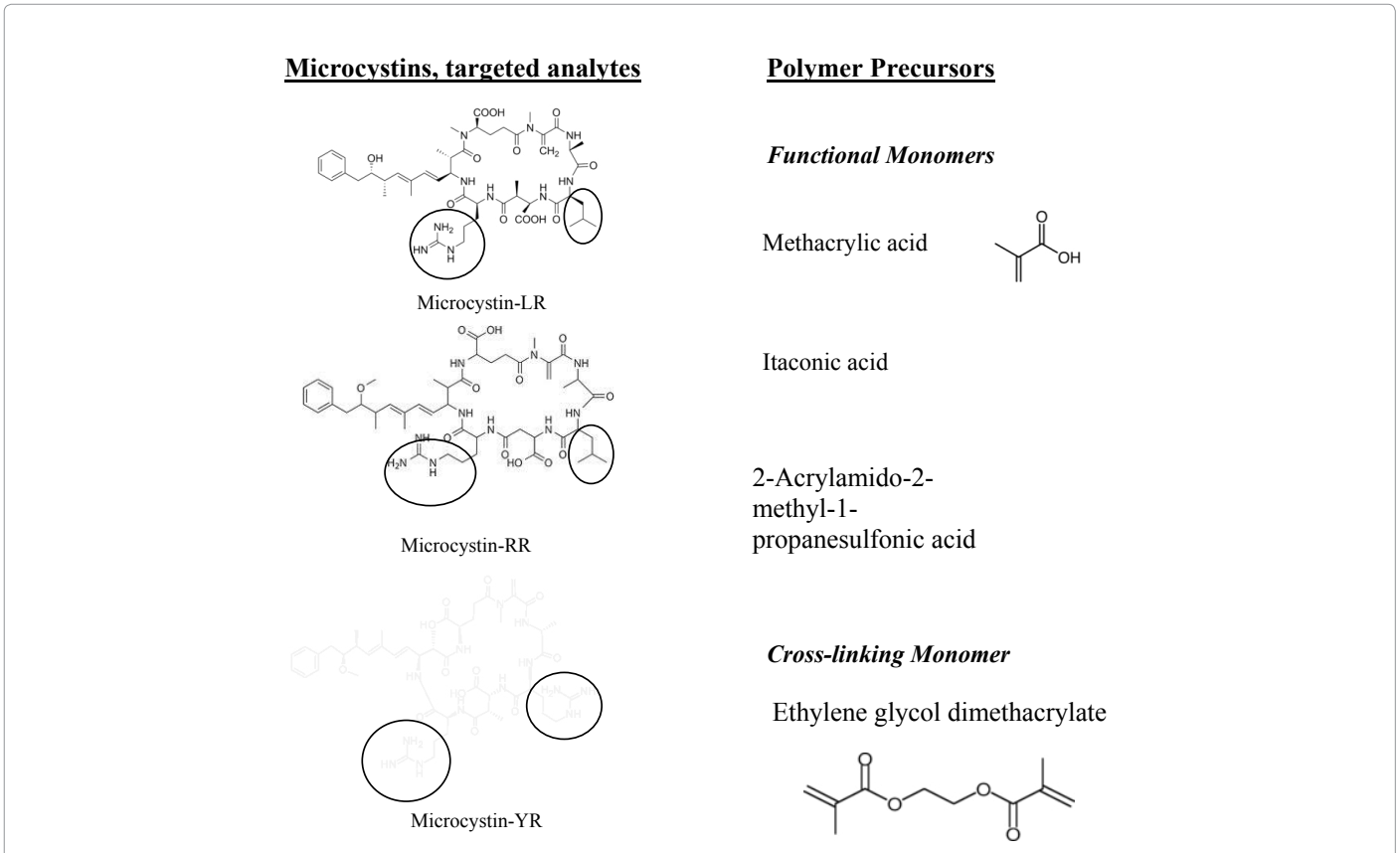
Successful extraction of target compounds from complex environmental samples using molecularly imprinted polyerms (MIPs) have been demonstrated for triazine herbicides [20], carcinogenic air pollutants polycyclic aromatic hydrocarbons [21,22], nicotine [23] and other environmentally relevant analytes [24]. Various experimental designs have been used including MIP packed into columns or cartridges, batch-mode where MIP is incubated with samples, and on-line SPE in combination with HPLC or HPLC-MS [25]. Very limited reports are available in literature on MC-LR imprinted polymers for both the purposes - solid phase extraction and sensory material. Using the combination of SPE followed by detection with piezoelectric sensor the minimum detectable amount of MC-LR was 0.35 nM [26]. The use of MIP-SPE provided up to 1000 fold pre-concentration, which was more than sufficient for achieving the required detection limit for MC-LR in drinking water (1 μg L^{-1}) [27]. In the present study, three MIPs specific for MC-LR were prepared with different formulations and then optimized the SPE conditions for MC-LR analysis in lake and marine waters by LC/MS detection. The merit of MIPs as specific SPE material for extraction of MC-LR with regard to sample pH and concentration of dissolved ions was described. The analytical data was treated for the quantitative aspects of the analysis, and accuracy, precision, and limits of detection.

Experimental

Chemical and Reagents

The analyte, MC-LR and other structural analogues of MCs namely MC-RR and MC-YR were purchased from Alexis Biochemicals (San Diego, USA). The functional monomers itaconic acid (IA), methacrylic acid (MAA), and 2-Acrylamido-2-methyl-1-propanesulfonic acid (AMPSA) and cross-linking monomer ethylene glycol dimethacrylate (EGDMA) were purchased from Sigma-Aldrich (Bachs, Switzerland); while the solvents acetonitrile, methanol and trifluoroacetic acid were procured from Merck (Darmstadt, Germany). 2,2'-azobisisobutyronitrile (AIBN) was brought from Acros Organics (Geel, Belgium). The molecular structures of chemicals used in the study are shown in (Figure 1). The monomers were purified prior to use via standard procedures in order to remove stabilizers. The polymerization initiator, AIBN was re-crystallised from acetone and the acetonitrile was purified by passing over over molecular sieves. All reagents used



MC variant	Molecular Weight	X	Z
MC-LR	994	Leucine	Arginine
MC-RR	1037	Arginine	Arginine
MC-YR	1044	Tyrosine	Arginine

Figure 1: Molecular structures of MCs and other polymer precursors used in the preparation of molecularly imprinted polymers.

Polymer SPE material	Template	Functional monomers (µmol)			Cross-linking Monomer (mmol)	ACN mL	*S* m^2 g^{-1}	V_p cm^3 g^{-1}	d_p (Å)
	MC-LR (µmol)	APMSA	MAA	IA	EGDMA				
MIP-1	**1**	**-**	**10**	**-**	**1.5**	**5**	**380 ± 5**	**0.632**	**125**
MIP-2	1	10	-	-	1.5	5	393 ± 4	0.645	118
MIP-3	1	-	-	10	1.5	5	416 ± 3	0.653	97

SPE, Solid Phase Extraction; MIP-1, molecularly imprinted polymer formulated with MAA and EGDMA; MIP-2, molecularly imprinted polymer formulated with APMSA and EGDMA and MIP-3, molecularly imprinted polymer formulated with IA and EGDMA.
MC-LR, microcystin-LR; APMSA, 2-Acrylamido-2-methyl-1-propanesulfonic acid; MAA, methacrylic acid; IA, itaconic acid; EGDMA, ethylene glycol dimethacrylate; ACN, acetinitrile.
S, BET surface area; V_p, specific pore volume; and d_p, average pore diameter were calculated from nitrogen absorption measurements.

Table 1: Composition and surface properties of molecularly imprinted polymers used in this study.

were either HPLC grade or analytical grade. Deionized water was obtained by passing tap water through a Milli-Q system and the treated water showed conductivity <15 MΩ cm^{-1} and TOC <5 µg L^{-1}. The stock solution of MCs were prepared at the concentration of 10 µg mL^{-1} in deionized water containing 0.1% trifluoroacetic acid. The reference SPE materials powdered activated carbon (PAC) and polystyrene divinylbenze resin (XAD) were purchased from Merck (Darmstadt, Germany). The lake water sample was collected from Ambazari lake (Nagpur, India) while the marine water sample was collected from Bay of Bengal near East Coast of Kakinada where enriched mangrove vegetation was found; and the salinity of the water was 17%.

Preparation of MIPs

Molecularly imprinted polymer specific to MC-LR was prepared by dissolving (9.93 mg, 1.0 mmol) in 10 mL acetonitrile in a 10 mL glass vial. To this, the functional monomer itaconic acid (210 mg, 2 mmol) was added and then the glass vial was placed in a refrigerator at 0°C for 30 min. Later, 5 mmol (991 mg) cross-linking monomer EGDMA and 10 mg AIBN were added to the solution. The sealed glass vial containing reaction mixture was freeze- thaw-degassed by submerging the tube in liquid nitrogen and holding the frozen tube under a vacuum of 100 mTorr for a period of 15 min. The polymer reaction mixture in the tube was sonicated for 5 min and kept in water bath at 40°C for 16 h. Upon completion of polymerization, the tube was taken out of the water bath and polymer monolith was ground in a ball mill to polymer particles of size 75 µm or less (200 mesh). The template MC-LR was extracted from the polymer matrix washing with methanol. The washing procedure was repeated (10 times) until no MC-LR found in the extraction solvent. Finally, the particles were dried under vacuum for further use. Apart from this MIP, two different MIPs were prepared with using methacrylic acid (MAA) and 2-Acrylamido-2-methyl-1-propanesulfonic acid (AMPSA) as the functional monomers. The composition of polymers was given in Table 1. The corresponding non-imprinted polymers (NIPs) was prepared in parallel in the absence of MC-LR and treated in the same manner.

MC-LR extraction experiments

The extraction capacity of MIP and other SPE materials was determined in batch mode. The dry SPE materials (MIPs formulated with 3 different compositions, XAD, or PAC; 10 mg) were weighed into 5mL glass vessels, and to this were added 0.1, 0.2, 0.3, 0.4, and 0.5 mL of a standard MC-LR solution (10 µg L^{-1}) mixed with a water in separate glass vessels. The samples were then shaken in a water bath at 25°C for 1 h. After sedimentation of the SPE materials, the residual concentration of MC-LR in aqueous phase was measured using LC/MS. The amount of MC-LR adsorbed was calculated by subtraction, using a calibrating curve obtained from the same experiment leaving out the SPE material. The reference SPE materials powdered activated carbon (PAC) and resin XAD were used for comparison with MIP for their adsorption capacity and selectivity. For each SPE material, these experiments were repeated at least three times.

SPE cartridges and pre-concentration experiments

The SPE protocol was developed in an off-line mode using a SPE manifold supplied by SUPELCO (Bellefonte, PA) connected to vacuum pump. Two hundred milligrams of MIP was transferred into a 10 ml screw-cap scintillation vial and incubated with 3 ml of methanol. The sealed vial was allowed to stand 24 h at ambient temperature with occasional shaking. Then, the slurry was transferred into an 1 ml SPE cartridge equipped with a polyethylene frit. The polymer was allowed to settle for 5 min. The MIP bed was stabilized by careful insertion of a second frit, avoiding any compression of the polymer filling. The pre-conditioned MIP cartridges were sealed with pluggers and stored at 4 °C to prevent drying out by solvent evaporation. Prior to any extraction the polymer was washed with an eluting mixture of methanol/water (4:1) containing 1% trifluoroacetic acid until no more residual analyte (MC-LR) was eluted from the polymer. For SPE experiments, the MIP was conditioned with 10 mL of methanol and 10 mL of acidified milli-Q (pH. 5.4). The required sample volume was applied to the conditioned cartridges and the polymer then washed with 10 mL of methanol.

For investigation of the reusability of the MIP , a single MIP cartridge was employed for 10 consecutive SPE clean-up cycles for a given MC-LR spiked deionized water (10 µg L^{-1}). In between the cycles, the MIP packed SPE column was reconditioned by washing with methanol/water (4:1) containing 1% trifluoroacetic acid (10 mL). The same procedure was followed for PAC and XAD filling, conditioning and pre-concentration of MC-LR.

The standard solution (10 µg L^{-1}) of MC-LR was prepared in methanol:water (4:1) solution, diluted with water obtained from the Milli-Q system, and then filtered through an empore disc to prepare a calibration curve ranging from 0.1-100 µg L^{-1}. Environmental water samples (lake water and marine waters) were filtered through a 0.45 um filter prior to any experiments. A standard spiked lake and marine water samples were used. The lake and marine water samples was preliminarily applied to total dissolved ions, and pH measurements to clarify its matrix effect, then filtered through 0.45 µm glass fiber filter prior to use without pH adjustment. The dissolved ions measurement was performed using conductivity meter. To verify the accomplishment of the molecular imprinting, the performance of the MIP was compared to that of non-imprinted polymer. Samples of 1 mL of lake water or marine water were analyzed using identical dimensions of SPE cartridge packed with MIP, XAD and PAC cartridges, separately, and the operational conditions of SPE were optimized. The selectivity of the MIP was determined by comparing the binding capacity of MC-LR on to the NIP and MIP packed SPE cartridges by analyzing 1 mL of samples of lake and marine waters with the optimized operational conditions.

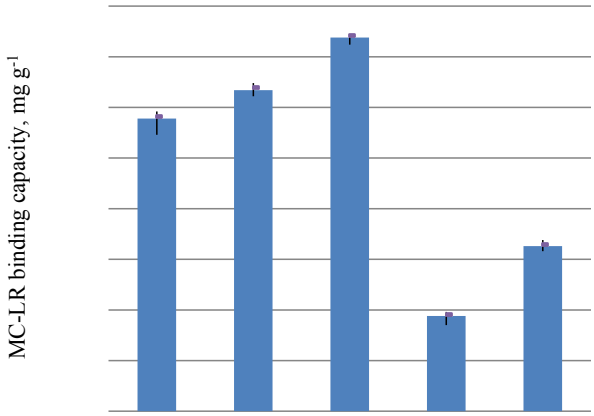


Figure 2: Binding capacity of MC-LR onto molecularly imprinted polymers (MIPs) and other SPE materials used in the present study.

SPE materials	Extraction capacity at different solution pH, mg g^{-1}					
	5.0	6.0	7.0	8.0	9.0	10.0
MIP-1	2.76 ± 0.13	2.83 ± 0.11	2.89 ± 0.13	2.82 ± 0.16	2.85 ± 0.16	2.72 ± 0.11
MIP-2	3.11 ± 0.15	3.15 ± 0.13	3.17 ± 0.16	3.15 ± 0.11	3.17 ± 0.11	2.96 ± 0.13
MIP-3	3.63 ± 0.11	3.65 ± 0.12	3.69 ± 0.19	3.65 ± 0.12	3.68 ± 0.13	3.12 ± 0.09
XAD	0.62 ± 0.08	0.85 ± 0.06	0.94 ± 0.11	0.96 ± 0.11	0.82 ± 0.15	0.59 ± 0.13
PAC	1.47 ± 0.11	1.51 ± 0.13	1.63 ± 0.12	1.68 ± 0.11	1.55 ± 0.09	1.32 ± 0.15
The values reported in the table are mean of three experimental determinations (n=3) and the initial concentration of MC-LR was 10 µg L^{-1}.						

Table 2: Effect of solution pH on MC-LR extraction by SPE materials.

Instrumentation

The microcystins were qauntified using LC/MS with a PE series 200 Quaternary Pump and a PE series 200 auto sampler (PerkinElmer, Shelton CT, USA). The chromatographic separation was carried out on a reversed-phase column C18 with dimensions-3.9 mm×150 mm id 5µm (Aqua, Torrance CA, USA). Apart from using C18, the columns packed with different SPE materials such as MIPs, PAC and XAD were also used for chromatographic separation of MCs. The injection volume was 1 mL min^{-1}. The mobile phase was a mixture of deionized water, ammonium acetate (10 mMl pH, 7.0), methanol, and acetonitrile. Mass spectrometry (MS) measurements were performed using an API 165 MS with an atmospheric pressure ionization (API) source operating in turbo-ion spray (TIS) mode (Applied Biosystems, Foster city CA, USA). The elute from the LC column was transferred to the MS device using split ratio of 5:1 (volume to waste/volume transferred) and nitrogen (heated to 450°C; 7 L min^{-1}) was applied to dry ion spray aerosol. Nitrogen was also used as nebulizer gas with a glow of 0.7 L^{-1}. The ionization voltage of the TIS interface was set to 5.5 kV. The MS system was operated in positive multiple ion detection (MID) mode to give highest sensitivity and selectivity $[M+H]^+$ ions cantered at: *m/z,* 520.5 (MC-RR); 995.7 (MC-YR); and 1045.8 (MC-LR) were monitored. For determination of unknown MCs the following mass ranges were scanned in additional runs: 500-600 Da (doubly charged MCs) and 800-1200 Da (singly charged MCs).

Analytical Performance

MIP- SPE coupled to LC/MS was developed to determine MCs in lake and marine water samples. The linearity of the analytical method was evaluated by a calibration curve in the range of 0.1–100 ng L^{-1} of MC-LR (n = 5). The Milli-Q water was spiked with MC-LR to achieve final concentrations of 0.1, 0.5. 1. 5, 10, 20, 50, and 100 ng L^{-1}. The limit of detection (LOD) was defined as three times ratio of signal to noise. The loading volume of aqueous MC-LR standard solution was 100 mL. Finally, quantitative figures of merit for the optimized SPE-LC/MS method were determined through analytical curves estimated using water samples of the MCs in the concentration range from 0.1 to 100 ng L^{-1}. The environmental samples were collected in glass bottles from lake and marine waters; then filtered through 0.45 um glass fibre filter before storing in dark at 4°C. The SPE procedure was accomplished within 24 h to avoid any microbial degradation of MCs.

Results and Discussion

Extraction of MCs

The MCs extraction capacity of MIPs (formulated with different polymer composition) and other SPE materials were determined using batch equilibrium experiments. The extraction capacity of MIP, XAD and PAC were 3.69, 0.94, and 1.63 mg g^{-1} respectively, and also extraction capacity of MIP was 6-folds higher than the corresponding non-imprinted polymer (NIP). The MIP formulated with IA and EGDMA showed significantly higher capacity than the other MIPs and SPE materials (Figure 2). Such high MCs extraction capacity is expected because of existence of specific binding sites in the polymer. The binding capacity of MIPs with the formulation of MAA - EGDMA and AFMSA -EGDMA were less pronounced than the MIP prepared with IA-EGDMA formulation; this could be explained based on formation of the lower affinity binding sites during molecular imprinting of former cases. The extraction capacities of the MIP prepared with IA-EGDMA formulation showed superior MCs binding capacity than those of most MIPs reported in the literature [25,26]. The highest extraction capacity of MIP for MC-LR reported in the literature was 0.98 mg g^{-1} which was about one-third of the MIP prepared in this study. The reason may be formation of clear and high populous binding sites during molecular imprinting process, thereby increase in the surface area of the MIP (formulated with IA-*co*-EDGMA) compared with NIP suitably justify

high MCs extraction capacity. The parameters contributing to the molecular imprinting was formation of the porous structure with an excellent specific surface areas for binding and the increasing number of effective binding sites. The microscopic characteristics of the imprinted polymer, and porous surface could be clearly observed. The specific surface area, pore volume, and pore size distribution obtained from nitrogen adsorption experiments were given in Table 2. The nitrogen porosimeter data showed that the pore size of IA formulated MIP was quite smaller than the MIPs formulated with AFMSA and MAA functional monomers. Earlier studies related to MC-LR were reported primarily in organic media, where higher binding capacity and selectivity were reported [27,28]. In this study, the high affinity of MIP for MC-LR in water was demonstrated, where the MIP bind kinetically faster to MC-LR (more than 90% of MC-LR within 60 min) and the binding equilibrium was attained within 10 min between MIP and MC-LR (Figure 3). However, the conventional SPE materials, PAC and XAD extracted lower than 25% of the MC-LR when compared with MIP in the same period. The binding equilibrium attained for other SPE materials XAD and PAC was about 2 h. The short contact time needed to reach the binding equilibrium as well as the high binding capacity of the MIP are very much relevant for practical application of sample pretreatment.

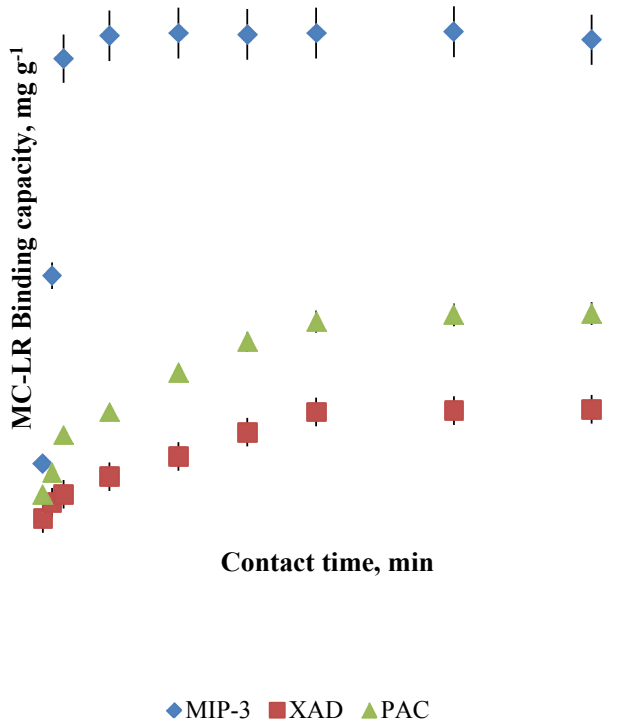


Figure 3: MC-LR binding kinetics of MIP and other SPE materials.

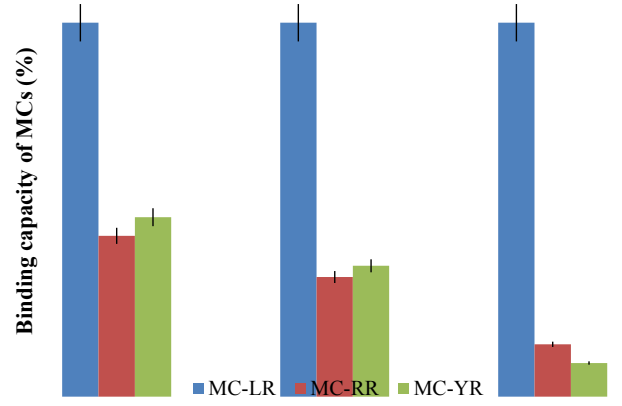


Figure 4: Cross-selectivity of MC-LR imprinted polymer and other SPE materials for MCs.

The selective extraction of MC-LR among other MCs (MC-YR, and -RR) was studied onto MIP and other SPE materials. From the (Figure 4), it would be clear that only MC-LR was selectively adsorbed onto the MIP from aqueous solution, however, 14% and 19% of MC-RR and -YR variants were adsorbed, respectively. Based on the adsorption equilibrium experimental data MIP formulated with IA-*co*-EGDMA was used as SPE material for packing column, while the non-MIPs packed SPE columns showed almost equal adsorption capacity for all the MCs and the nature of adsorption would be non-specific.

Chromatographic evaluation of the SPE materials

The extraction capacity and selectivity data showed that the MIP possesses higher selective extraction capacity than the other SPE materials studied for MC-LR extraction from water. The hydrogen bonding between MC-LR and the functional groups of IA, which often occurs in a polar solvents cause specific hydrogen bonds responsible for suppressing the nonspecific hydrophobic interactions. Nevertheless, nonspecific hydrophobic interactions always exist in the adsorption process of MIP in the water samples. To avoid interference of water soluble compounds in the analysis of the target molecule, a washing step is normally included in the analytical protocol.

The washing step was the most crucial procedure to maximize the specific interactions between the MCs and binding sites of MIP, and to simultaneously decrease non-specific interactions to discard matrix components in the polymer [29]. However, the comparative analysis between NIP and MIP was carried out denote useful information about MIP's selective extraction of MC-LR from water samples. Acetonitrile was proved the most effective washing solvent, though MC-LR could not be eluted from NIP completely. Thus, acetonitrile was chosen as the washing solution. When acetonitrile was used at a volume of 1 mL, about 40% of MC-LR loaded on NIP cartridge was washed off while MC-LR bound on MIP was still retained. With an increase of the acetonitrile volume to 2 mL, the amount of MC-LR eluted from the NIP cartridge increased to 70%. Therefore, acetonitrile was mixed with small quantity of water (4:1, v/v) to improve the polarity showed an excellent washing step. 2 mL of MC-LR standard solution (10 ng L^{-1}) in water was applied to the MIP and NIP cartridges. After loading MC-LR solution, both the MIP and NIP cartridges were submitted to washing step, then the cartridges were eluted with 2 mL of acetonitrile/water (4:1, v/v). Both the washing and elution fractions of the solvent were collected and analyzed. Table 3 shows the recoveries of MC-LR in the washing and elution fractions after pre-concentration on the MIP and NIP cartridges by using 2 mL of each of the washing solvents. For the elution, five aliquots of methanol: trifluoroacetic acid (4:1, v/v), each of 1 mL in volume, were used to elute MC-LR from the MIP cartridge after washing step. The recovery for every 1 mL aliquot of methanol:trofluoroacetic acid (4:1, v/v) was calculated separately. The results showed that 2 mL of the eluting solution was sufficient to elute MC-LR from MIP cartridge completely.

The pre-concentration capacity of a SPE column was evaluated using breakthrough volume experiments. The breakthrough volume

Compound	Linearity			DL[a] (µg L^{-1})	RSD (%); n=5
	Range (ug/L)	Equation	r		
Microcystin-LR	0.1-10	Y=264+4272X	0.996	0.25	4.2
Microcystin-RR	0.5-10	Y=186+2164X	0.991	0.39	5.8
Microcystin-YR	0.3-10	Y=490+1550X	0.993	0.43	4.7
[a]Detection limits were estimated on the basis of 3:1 signal to noise ratios. RSD, relative standard deviation (concentration range tested: 01 - 10 µg MC-LR on MIP packed column.					

Table 3: The linear range, detection limit (DL) and RSD of MIP-SPE method coupled with HPLC for the determination of microcystins (microcystin-LR, RR, YR).

	Recovery parameter	MIP			XAD			PAC		
		MC-LR	MC-RR	MC-YR	MC-LR	MC-RR	MC-YR	MC-LR	MC-RR	MC-YR
Lake water	Recovery (%)	96	91	93	82	86	77	78	75	77
	RSD (%)	3.2	3.7	4.1	5.8	4.9	6.2	6.5	7.2	6.2
Marine water	Recovery (%)	89	76	82	73	61	77	68	65	61
	RSD (%)	3.5	8.9	5.3	6.4	8.9	7.3	8.3	7.9	8.1
Recovery is based on comparison of peak areas obtained by MC-LR and mixture of MCs by injecting the same amount of cyanotoxin at a concentration of 10 µg L^{-1}.										

Table 4: Recoveries of microcytins from the spiked lake and marine waters (n=5).

of the MIP packed SPE column was thrice as much as PAC and XAD columns; which indicates that MIP used in the present study could be used as SPE material for extracting MC-LR from aqueous samples, where high volumes of water are usually loaded. As shown in Table 4, there is no MC-LR loss even if 100 mL of MC-LR standard solution was loaded. Moreover, the MIP packed SPE column had two advantages than the reported MC-LR imprinted polymers; one was the MIP prepared in this study was high extraction capacity for the MC-LR extraction from aqueous solutions than the earlier reported. The other one was that the MIP packed SPE could selectively retain the MC-LR from lake and marine samples and avoided the bleeding of traces of retained MC-LR from the MIP packed SPE column.

Matrix effect on MC-LR extraction

The level of total dissolved solids (TDS) in water and wastewater detrimentally interfere in extraction of MC-LR onto the SPE materials. The interference of TDS was investigated using different strengths of sodium chloride solution. In deionized water, the TDS solutions of concentration from 100 to 5000 mg/L were prepared. The extraction of MC-LR from different strengths of TDS was investigated in batch mode and the data obtained is shown in Figure 5. As the increase in TDS strength, it was found that, MC-LR extraction also increased exponentially. The possible reason would be the addition of NaCl enhanced the mass transfer in the extraction process, and also water molecules solvate around ions leads to poor solubility [30]. This enhanced the extraction of MC-LR on MIP. Since, the diffusion of MC-LR onto the MIP from water is the rate-limiting step, it suggested that the addition of salt speeds up diffusion of MC-LR. Another important matrix parameter is the solution pH. The effect of pH on MC-LR binding onto MIP was investigated by varying the pH of the MC-LR solution from pH 2 to pH 9. Interestingly, as the pH of MC-LR solution increases, MIP displayed much higher retention on MIP than the corresponding NIP. This binding could be due to the formation of ionic interactions between MC-LR and the MIP. The optimum pH for MC-LR binding was 7.3. It would be possible that at the optimum pH (7.3), the MIP lose its charge and the non-specific binding caused by the ionic bonds decreases. This statement would be supported by the dominance of H-bonding arising from functional monomer, IA during molecular recognition of MIP.

LC/MS analysis of MCs

20 mL of standard aqueous MC-LR solution containing 10 µg was passed through the SPE column packed with MIP was passing with the sample flow rate 2 ml min^{-1}. The columns packed with NIP and other SPE materials (XAD and PAC) were also used for extraction of MC-LR from standard solution. The chromatogram obtained using the MIP packed SPE column showed clear and distinct peaks for MCs; while the chromatograms of cartridges packed with XAD and PAC were quite small and almost similar to the chromatogram of non-imprinted polymer. The sample preparation using MIP packed columns is very effective for MC-LR at trace quantities. These results indicated that the MC-LR was selectively extracted onto the MIP column, and the selectivity of MIPs was quite high for MC-LR, and the recognition sites formed in the MIP originated from the molecular imprinting.

The analytical figures of merit for the proposed sample preparation with MIP packed SPE extraction followed by LC/MS analysis for trace quantification of MC-LR was evaluated under optimal experimental conditions. The mass spectra of $[M+H]^+$ ions of MCs revealed that the site of protonation for MCs was, the methoxy group in the Adda. While the differences in mass spectra between MC-LR and MC-YR was explained based on structural variations. Besides, detecting typical fragment ions of MCs in the mass spectra of MC-YR (*m/z*, 135 and 213), the other fragment ions, such as *m/z* 141, 271, 602 and 952, were characteristic for MC-YR. The fragmented ions observed for the three variants of MCs (MC-LR, -RR, and -YR) arise predominantly from the loss of water and then consecutive cleavages of the amide bonds that provide information concerning the amino acid sequences. With a sample loading flow rate of 2 ml min^{-1} for a 10 min extraction, the enrichment factor obtained by the slopes of the linear portion in comparison with the direct injection of 10 µL standard sample solution was 1.045. The detection limit (S/N=3) of 1.0 ng L^{-1} was based on three times of the signal-to-noise (S/N) ratio of baseline near the analyte peak obtained from the LC/MS analysis. The chromatograms of standard mixture of MCs are depicted in Figure 6a. Reproducibility was evaluated by pre-concentrating nine replicate runs of water samples spiked at 10 µg L^{-1}, and the results were satisfactory with relative standard deviation (RSD) of <7%. Calibration curve was obtained for MC-LR in this range by five-point calibration with concentration coefficients for the linear regression curve of 0.998, and the linear range of the calibration graph was 0.05-10 µg L^{-1}. The reliability of an analytical method is proven when it is applied to the real samples. For this purpose, two different type of samples were analyzed with the methodology developed in this work. One sample was from the Lake Ambazari (Nagpur, India) and the another sample was collected from Godavari estuary (Bay of Bengal Sea, India). The chromatograms obtained after pre-concentration of

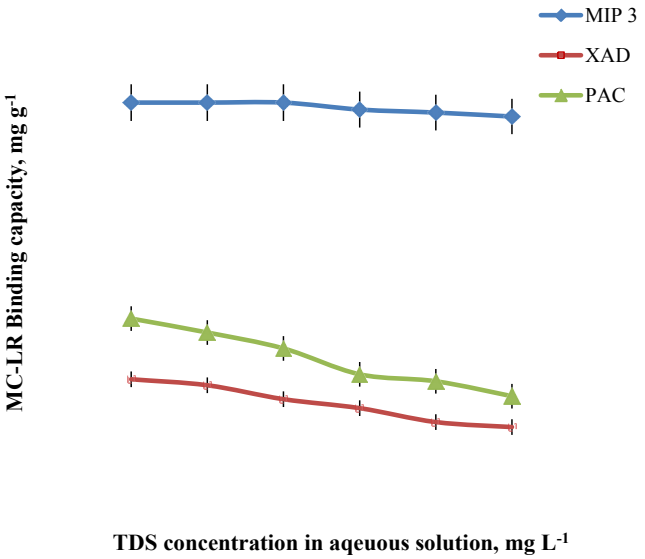


Figure 5: Interference of solution TDS on MC-LR extraction onto MIP and other SPE materials.

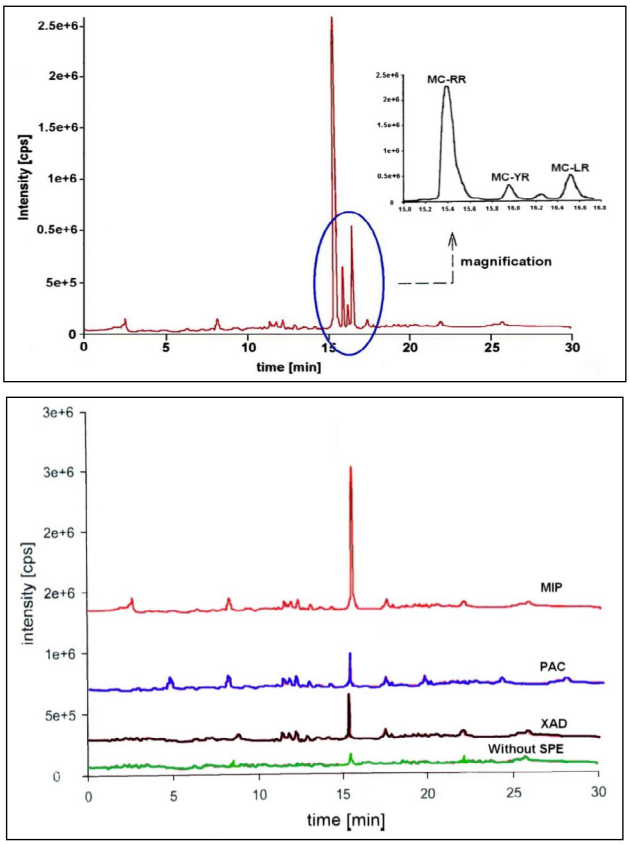


Figure 6: (a): Chromatographic separation of MCs and (b): Chromatograms of MC-LR after extraction with different SPE materials.

aqueous samples through the cartridges of (i) the MIP (ii) the XAD and (iii) the PAC, the MIP chromatogram presents a clearer baseline, and the MCs were identified and detected (Figure 6b). The concentration of MC-LR in aqueous samples were given in Table 4. Recovery of MCs from spiked lake water and marine water was determined by the comparison of peak areas obtained by standard MC-LR (10 µg L^{-1}) and the mixture of MCs (each MC concentration was 10µg L^{-1}) injecting into HPLC of sample volume 2 mL. The reproducibility of this method was observed because of the high recoveries of MIP. To our surprise, the observed reproducibility of the method was satisfactory in three sequential cycles of extraction. Certainly, the extractions were repeated on a single MIP packed SPE column, and thus results also demonstrated the regeneration of the column. MIP imprinted with MC-LR showed highest recovery (96%) for MC-LR demonstrates that the imprinting is based on the synergistic effect of shape and size complementarily and the interaction of functional groups of the analyte, MC-LR with those of binding cavities. The lower recoveries for MC-RR and MC-YR are due to mismatch of size and shape of MIP's binding cavity. The other SPE materials XAD and PAC showed lower recoveries for all MCs, however, the recoveries are non-selective in nature. The amount of MC-LR in the lake water and marine water were 0.87 and 1.13 mg L^{-1} respectively, and these values are higher than the WHO standard for MC-LR. The concentration of MC-LR was sufficiently high and could be easily assayed using SPE coupled with LC/MS detection. Normally, an additional clean-up step before LC/MS detection is very much essential to remove matrix interfering parameters (dissolved solids and organic matter). However, since selective interactions between the MC-LR and the binding sites of MIP are achieved using imprinted polymers allows lower coefficients of variation in the measurements, where there is no need of sample clean-up step.

Conclusions

The new analytical method proposed using LC/MS analysis with MIP extraction is highly efficient method for trace quantification of MC-LR in lake and marine waters. The experimental variables were optimized for MIP packed column for selective separation of MCs from aqueous samples. The MC-LR recovery data suggested that the MIP packed column provided a reliable and effective recovery of MC-LR (i.e., about 95%) in the concentration range 0.1-10 µg L^{-1} from aqueous samples. The results obtained for calibration linearity, precision, accuracy and mass ratio stability of MC-LR represents that the proposed method is quite efficient for the trace quantification of MC-LR in environmental samples. With a 100-fold selective pre-concentration step, the LOD is 1.0 µg L^{-1} which represent one of the most sensitive detection methods of MC-LR in environmental analysis.

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References

1. Carmichael WW (1992) Cyanobacteria secondary metabolites--the cyanotoxins. J Appl Bacteriol 72: 445-459.
2. Carmichael WW, Azevedo SM, An JS, Molica RJ, Jochimsen EM, et al. (2001) Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environ Health Perspect 109: 663-668.
3. Kaebernick M, Neilan BA (2001) Ecological and molecular investigations of cyanotoxin production. FEMS Microbiol Ecol 35: 1-9.
4. Welker M, Fastner J, Erhard M, von Döhren H (2002) Applications of MALDI-TOF MS analysis in cyanotoxin research. Environ Toxicol 17: 367-374.
5. Lindon M, Heiskary S (2009) Blue-green algal toxin (microcystin) levels in Minnesota lakes. Lake and Reservior Manag 25: 240-252.
6. Dodds WK, Bouska WW, Eitzmann JL, Pilger TJ, Pitts KL, et al. (2009) Eutrophication of U.S. freshwaters: analysis of potential economic damages. Environ Sci Technol 43: 12-19.

7. Pouria S, de Andrade A, Barbosa J, Cavalcanti RL, Barreto VT, et al. (1998) Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. Lancet 352: 21-26.

8. Martínez Hernández J, López-Rodas V, Costas E (2009) Microcystins from tap water could be a risk factor for liver and colorectal cancer: a risk intensified by global change. Med Hypotheses 72: 539-540.

9. Chen J, Xie P (2005) Seasonal dynamics of the hepatotoxic microcystins in various organs of four freshwater bivalves from the large eutrophic lake Taihu of subtropical China and the risk to human consumption. Environ Toxicol 20: 572-584.

10. Fastner J, Codd GA, Metcalf JS, Woitke P, Wiedner C, et al. (2002) An international intercomparison exercise for the determination of purified microcystin-LR and microcystins in cyanobacterial field material. Anal Bioanal Chem 374: 437-444.

11. Hyenstrand P, Metcalf JS, Beattie KA, Codd GA (2001) Effects of adsorption to plastics and solvent conditions in the analysis of the cyanobacterial toxin microcystin-LR by high performance liquid chromatography. Water Res 35: 3508-3511.

12. Allis O, Dauphard J, Hamilton B, Shuilleabhain AN, Lehane M, et al. (2007) Liquid chromatography-tandem mass spectrometry application, for the determination of extracellular hepatotoxins in Irish lake and drinking waters. Anal Chem 79: 3436-3447.

13. Wang J, Pang X, Ge F, Ma Z (2007) An ultra-performance liquid chromatography-tandem mass spectrometry method for determination of microcystins occurrence in surface water in Zhejiang Province, China. Toxicon 49: 1120-1128.

14. Rapala J, Erkomaa K, Kukkonen J, Sivonen K, Lahti K (2002) Detection of microcystins with protein phosphatase inhibition assay, high performance liquid-chromatography-UV detection with enzyme linked immunosorbent assay: Comparison of methods. Anal Chim Acta 466: 213-231.

15. Vasas G, Szydlowska D, Gáspár A, Welker M, Trojanowicz M, et al. (2006) Determination of microcystins in environmental samples using capillary electrophoresis. J Biochem Biophys Methods 66: 87-97.

16. Long F, He M, Zhu AN, Shi HC (2009) Portable optical immunosensor for highly sensitive detection of microcystin-LR in water samples. Biosens Bioelectron 24: 2346-2351.

17. Neffling MR, Spoof L, Meriluoto J (2009) Rapid LC-MS detection of cyanobacterial hepatotoxins microcystins and nodularins--comparison of columns. Anal Chim Acta 653: 234-241.

18. Cong L, Haung B, Chen Q, Lu B, Zhang J, et al. (2006) Determination of trace amount of microcystins in water samples using liquid chromatography coupled with triple quadruple mass spectrometry. Anal Chim Acta 569: 157-168.

19. Fontanals N, Marce RM, Borrull F (2005) New hydrophilic materials for solid-phase extraction. Trends Anal Chem 24: 394-406.

20. Krupadam RJ, Khan MS, Wate SR (2010) Removal of probable human carcinogenic polycyclic aromatic hydrocarbons from contaminated water using molecularly imprinted polymer. Water Res 44: 681-688.

21. Krupadam RJ, Bhagat B, Wate SR, Bodhe GL, Sellergren B, et al. (2009) Fluorescence spectrophotometer analysis of polycyclic aromatic hydrocarbons in environmental samples based on solid phase extraction using molecularly imprinted polymer. Environ Sci Technol 43: 2871-2877.

22. Krupadam RJ, Venkatesh A, Piletsky SA (2013) Molecularly imprinted polymer receptors for nicotine recognition in biological systems. Mol Imprint 1: 27-34.

23. Pichon V, Chapuis-Hugon F (2008) Role of molecularly imprinted polymers for selective determination of environmental pollutants--a review. Anal Chim Acta 622: 48-61.

24. He C, Long Y, Pan J, Li K, Liu F (2007) Application of molecularly imprinted polymers to solid-phase extraction of analytes from real samples. J Biochem Biophys Methods 70: 133-150.

25. Oehrle SA, Southwell B, Westrick J (2010) Detection of various freshwater cyanobacterial toxins using ultra-performance liquid chromatography tandem mass spectrometry. Toxicon 55: 965-972.

26. Mekebri A, Blondina GJ, Crane DB (2009) Method validation of microcystins in water and tissue by enhanced liquid chromatography tandem mass spectrometry. J Chromatogr A 1216: 3147-3155.

27. Chianella I, Lotierzo M, Piletsky SA, Tothill IE, Chen B, et al. (2002) Rational design of a polymer specific for microcystin-LR using a computational approach. Anal Chem 74: 1288-1293.

28. Chianella I, Piletsky SA, Tothill IE, Chen B, Turner AP (2003) MIP-based solid phase extraction cartridges combined with MIP-based sensors for the detection of microcystin-LR. Biosens Bioelectron 18: 119-127.

29. Pichon V (2007) Selective sample treatment using molecularly imprinted polymers. J Chromatogr A 1152: 41-53.

30. Turner NW, Piletska EV, Karim K, Whitcombe M, Malecha M, et al. (2004) Effect of the solvent on recognition properties of molecularly imprinted polymer specific for ochratoxin A. Biosens Bioelectron 20: 1060-1067.