Determination of Microcystins in Water Using Integrated Solid-Phase Microextraction with Microbore High-Performance Liquid Chromatography–Electrospray Quadruple Time-of-Flight Mass Spectrometry

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Abstract

The development of a technique combining solid-phase microextraction (SPME) with microbore high-performance liquid chromatography (micro-HPLC)–tandem quadrupole time-of-flight (QTOF) mass spectrometry (MS) for determination of dissolved microcystins in water is reported. Several important parameters affecting the efficiency of SPME extraction of microcystins are investigated. A microbore C18 column HPLC coupled with tandem QTOF-MS with information-dependent acquisition (IDA) is developed to effectively analyze microcystins in microliter volumes of SPME extracts. The micro-HPLC–QTOF-MS with IDA technique provides comprehensive information, including a survey chromatogram (total ion chromatogram), full scan mass spectrum, and product ion scan mass spectra at different collision energies for individual analytes, which allows for both identification and quantitation in the same run. Linear calibration curves of microcystin standard [microcystin (MC)-arginine (R)R] 1–100 µg/L and of microcystin standard [MC-leucine (L)R] 1–250 µg/L are obtained with a correlation coefficient of 0.996. The combination of SPME with HPLC–QTOF-MS and IDA offers limits of detection of 0.6 pg for MC-RR and 1.6 pg for MC-LR. Analysis of spiked lake-water samples shows a recovery of > 86% for MC-RR and > 70% for MC-LR. This technique requires small sample volumes, minimizes the use of organic solvents, and provides sensitive and information-rich analysis of unknown samples.

Introduction

Microcystins (Figure 1) are a group of cyclic heptapeptide hepatotoxins that cause serious damage to the liver architecture, cellular organelles, and reorganization of microfilaments (1–4). They inhibit the activity of protein phosphatases 1 and 2A (5–7) and act as tumor promoters (8). Microcystins are released into lake water from some species of freshwater cyanobacteria (blue-green algae), *Microcystis*, *Oscillatoria* (also known as *Planktothrix*), *Anabanena*, and *Nostoc*, when the blue-green algae die naturally or because of algaecide treatment. More than 70 microcystins have been isolated and characterized. Microcystins are a serious global health concern regarding safe water supplies for both humans and livestock because of their toxicity and the frequent occurrence of blue-green algae blooms in lakes and rivers around the world. The World Health Organization has recommended a guideline limit in drinking water of 1 µg/L microcystin-leucine (L) arginine (R) for short-term exposure and 0.1 µg/L for long-term exposure (9). Reliable determination of microcystins is critical for protection of public health because a false-negative result can cause serious health problems; on the other hand, a false-positive result can have unnecessarily high cost, waste resources, and scare the population. The need for routine surveillance of microcystins in lakes led to the development of an integrated analytical technique that can provide multiplex information for both reliable identifi-

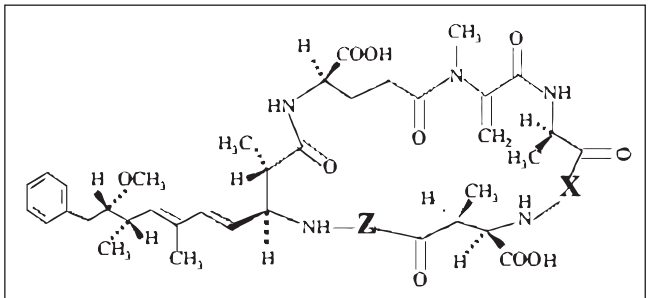

Figure 1. The common structure of microcystins. *X* and *Y* are the variable amino acids. The most common microcystins contain the amino acids leucine (L) and arginine (R) in these variable positions, including microcystin-LR (MC-LR): *X* = L, *Z* = R, and microcystin-RR (MC-RR).

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cation and sensitive quantitation of microcystins.

Several analytical techniques that have been developed for analysis of microcystins include the mouse bioassay (10), phosphatase inhibition assay (11–14), enzyme-linked immunosorbent assay (ELISA) (13–17), reverse-phase high-performance liquid chromatography (RP-HPLC) (13,18–26), or capillary electrophoresis (21,27,28) combined with UV or mass spectrometric (MS) detection. These techniques have specific shortcomings. The mouse bioassay requires sacrificing many animals and, as a result, is expensive. It is also non-specific and time-consuming (8 days). The phosphatase inhibition assay is based on the inhibition of microcystins and nodularin of the activity of serine–threonine protein phosphate enzymes. Different species of microcystins have various effects on the activity of protein phosphatase enzymes. The assay also has cross-reactions with other chemicals, such as okadaic acids. In addition, the cyanobacterial sample itself may have phosphatase activity, which causes unreliable identification of microcystins. The ELISA based on the polycolonal or monoclonal antibodies for microcystins is highly sensitive, and the commercially available kits are easy to use. However, the reactivity of the antibodies with microcystin variants varies and, thus, does not consistently correlate with their toxicity. The assays described previously cannot identify the species of microcystin variants. Therefore, liquid separation methods, including chromatography and capillary electrophoresis with photodiode array or UV detection methods, were developed (13,19,21). The mixture of a sample is separated, and the retention time or migration time of individual peaks is matched with those of standards for identification of the compounds in samples. The standards of microcystin variants are limited. Thus, it is difficult to analyze different microcystin variants in an unknown sample.

HPLC with MS detection can provide both structural information and quantitation data for microcystins. Recently, several liquid chromatography (LC)–MS methods have been reported, including various ionization techniques, such as frit–fast atom bombardment (FAB) (29), electrospray (18,20–23,30), and different mass analyzers, such as quadrupole, ion trap, and time of flight (TOF). Frit–FAB LC–MS produced not only molecular ions but also characteristic fragmental ions, but this technique has low sensitivity, requiring microgram amounts of the analytes. Electrospray ionization (ESI)-MS has become more appealing because of its ability to efficiently ionize small and large molecules and because it is the best suitable interface for LC–MS. The available LC–MS techniques are based on conventional LC with single quadrupole, triple quadrupole, or ion trap MS (20–22). They can provide sensitive analysis of microcystins. However, these techniques require a priori knowledge of the expected compounds in order to apply classic multiple-reaction monitoring (MRM) or product-ion scan methods.

The main challenge in the determination of microcystins in water is that microcystins, with many different variants, are present at trace levels in lake and river water. To reliably determine and provide specific identification of the microcystin variants detected, integrated solid-phase microextraction (SPME) with microbore high-performance liquid chromatography (micro-HPLC)–electrospray ionization (ESI)-quadrupole time-of-flight (QTOF)-mass spectrometry (MS) and information-dependent acquisition (IDA) technique was developed. The SPME directly extracts and concentrates the analytes from a water sample onto a fiber, and the micro-HPLC–ESI-QTOF-MS with IDA detection quantitates and identifies any compounds that are present in the extracts without a priori knowledge of these compounds. In addition, unlike the conventional solid-phase extraction (SPE) and liquid–liquid extraction, the SPME method simplifies the extraction procedures and minimizes the use of organic solvents, reducing the cost of disposal (31–33). In this paper, the development and validation of an SPME with HPLC–QTOF-MS with IDA technique for quantitation and identification of microcystins in water is reported.

Experimental

Chemicals and reagents

Microcystin standards (MC-LR and MC-RR) were supplied by Alexis Corporation (Lausen, Switzerland). Standard stock solutions of MC-LR at a concentration of 500 µg/mL and MC-RR at 100 µg/mL were prepared in methanol and stored in glass-stopped bottles at –20°C. Standard working solutions at the appropriate concentrations of MC-LR and MC-RR were freshly diluted with methanol from the stock solutions.

HPLC-grade methanol, formic acid, and acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Analytical-grade trifluoroacetic acid was obtained from Sigma-Aldrich (St. Louis, MO). Water was purified by a Millipore (Bedford, MA) Milli-Q water system.

Instrumentation and procedures

SPME procedure

Four commercially available SPME fibers, 100µM polydimethylsiloxane (PDMS), 60µM PDMS–divinylbenzene (DVB), 65µM carbowax (CW)–DVB, and 50µM CW–templated resin (TPR), were purchased from Supelco (Bellefonte, PA). Each fiber was preconditioned by immersion in methanol with stirring for 1 h, followed by 50% methanol containing 0.01% formic acid for 1 h, before it was used for extraction.

To carry out the SPME extraction, a sample (12.00 mL) was placed in a 15-mL screw-cap glass vial, adjusted with 0.6M HCl to pH 3, followed by addition of 1% methanol (v/v) and addition of sodium chloride (3.6 g). The sample vial was then closed and clamped on the magnetic stirrer (10- × 3-mm Teflon-coated stir bar). After mixing for 4 min, the entire SPME fiber was immersed in the sample with continuous stirring at 1200 rpm for 40 min. To desorb the analytes adsorbed on the fiber, the SPME fiber was transferred to 100 µL of methanol in a conical glass insert placed in a 2-mL crimp vial and soaked for 3 min, allowing the analytes to dissolve into the methanol. The methanol extract was evaporated to dryness under nitrogen and was reconstituted in 20 µL of 50% methanol–water for subsequent analysis by HPLC–MS–MS. To eliminate carryover between samples, the fiber was washed by cleanup with methanol for 2 min and then with water for 2 min. The blanks between runs were prepared using the same procedures to check the carryover.

HPLC–QTOF-MS analysis

A capillary HPLC system (Agilent 1100 series, Agilent Technologies, Palo Alto, CA) was coupled to an Applied Biosystems/MDS Sciex QSTAR Pulsar i hybrid QTOF-MS (Applied Biosystems/MDS SCIEX, Concord, Otario, Canada) with an ionspray ionization source. The microcystins were separated using a BetaBasic-18 microcolumn (100 × 0.5 mm, 3-µm particle size) (Thermo Electron Corporation, Waltham, MA). Mobile phase A was composed of Milli-Q water and mobile phase B of methanol, both containing 0.01% (v/v) of formic acid. A solvent gradient program consisted of 50% of B for 3 min, 70% of B for 5 min, and 100% of B for 10 min. It was then held for 3 min, with a flow rate of 12 µL/min. Subsequently, the HPLC system was programmed to its initial condition over 0.1 min, followed by an 8-min re-equilibration prior to the next injection. The sample injection volume was 2 µL. The entire effluent from the microcolumn was directly transferred to the MS through the ionspray interface.

MS analysis of the analytes was performed using positive electrospray ionization in IDA mode. Both a full-scan mass spectrum and IDA-MS–MS product ion spectra were acquired in the two mass ranges of *m/z* 400 to 1200 and *m/z* 100 to 600. The two mass ranges were set to obtain structural information of the molecular and fragment ions without loss of sensitivity by effective use of cycle time. The optimization of MS detection was performed by infusion of 50 ng/mL of a mixture of MC-LR and MC-RR using a syringe pump. The optimal instrumental conditions were determined to be: ESI spray voltage, 4.8 kV; curtain gas setting, 25; gas 1 (sheath gas) setting, 50; first declustering potential, 40 V; focusing potential, 300 V; and second declustering potential, 15 V. The threshold of intensity was set at two counts. The number of dependent MS–MS scans before the next survey scan (TOF-MS scan) was set at two. Two MS–MS spectra were recorded for each component, one at lower collision energy (15 eV) and the other at a higher setting (40 eV), to obtain more fragment ions.

Calibration curves of MC-RR and MC-LR were obtained using the standard solutions from 1.0 to 250 µg/L in water. The calibration curves were based on the relationship between the concentrations of the analytes and the peak area of extracted ion chromatograms (EIC).

Lake-water sample preparation

A water sample was obtained from Dried Meat Lake (Camrose, Alberta, Canada) and stored in the refrigerator at 4°C until use. The water sample was sonicated at ambient temperature for 30 min to break down any algae present. After passing through a 0.45-µm glass filter, 12 mL of the water was used for SPME extraction of microcystins under the optimized conditions and for the recovery study. Three replicates of the lake water samples spiked with 50 and 100 µg/L of MC-RR and MC-LR were extracted and analyzed to obtain recoveries.

Results and Discussion

Optimization of SPME enrichment method

Microcystins are often present at trace levels in water samples, and it is impossible to detect them in water samples without preconcentration. This study integrates SPME with micro-HPLC–MS–MS and IDA detection to achieve simple and efficient enrichment with high sensitivity quantitation and structural information for identification.

The initial focus was on the development of the enrichment method using the SPME technique. The choice of appropriate fiber coating is essential for the SPME method development. Depending on the molecular weight and polarity of analytes to be extracted, the extraction efficiency of each fiber coating is different. Four different polymer-coated fibers (PDMS, PDMS–DVB, CW–DVB, and CW–TPR) were evaluated for their extraction efficiency using a pure water sample spiked with 250 µg/L of microcystins. PDMS is suitable for extraction of volatile compounds with molecular weight from 60 to 275. As expected, no microcystins were detected when the PDMS fiber was used. The CW–DVB and CW–TPR fibers were designed for absorbing analytes from a solution; though these fibers extracted some microcystins, the CW–TPR gave a better extraction efficiency, demonstrated by higher peak areas. Therefore, the CW–TPR fiber was used in the subsequent experiments.

The effect of sample pH on the extraction efficiency was studied by varying the pH of a spiked water sample (250 µg/L) from 2 to 7. Figure 2 shows the peak areas of MC-RR and MC-LR when the sample solutions were at pH 2 to 7. The highest signals were obtained when the samples were prepared at pH 2–4; thus, pH 3 was used for the rest of this study. The effect of sample pH on the extraction efficiency observed in Figure 2 was consistent with the pK_a of microcystins (~ 3.5). At pH 2 to 4, a major portion of the microcystins was protonated (the neutral form), resulting in stronger adsorption on the polymer surface of the fiber.

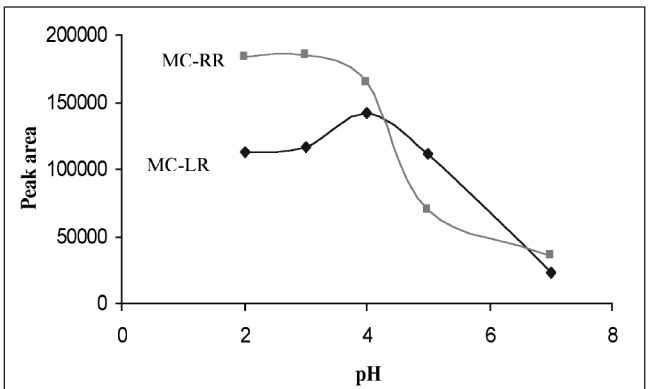

Figure 2. The effect of pH on the extraction efficiency.

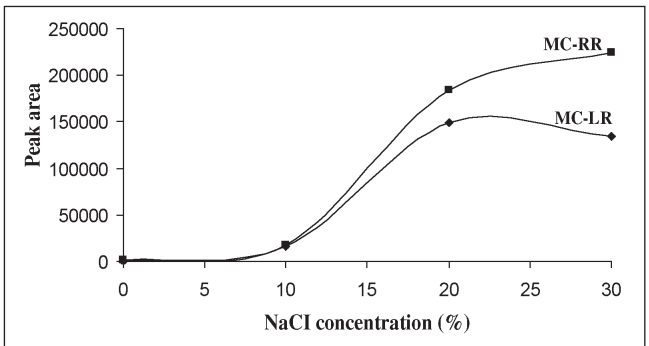

Figure 3. The effect of salt concentration on the extraction efficiency.

High salt concentrations in aqueous samples decrease the solubility of polar compounds in the sample matrix and promote their transfer to the fiber. In this study, sodium chloride was added to the sample to study the effect of ionic strength on the extraction efficiency. Figure 3 shows the extraction efficiency for both MC-LR and MC-RR when the salt content in the samples was varied from 0% to 30%. The efficiencies of the SPME extraction of microcystins were significantly improved with the addition of sodium chloride from 0% to 20%. The peak intensities reached their optimum and did not change significantly when the salt content was increased from 20% to 30%. Therefore, addition of 30% NaCl to samples was used to ensure efficient extraction for both MC-LR and MC-RR.

Microcystins have limited solubility in water. Therefore, the addition of a small amount of methanol to the water samples was evaluated in order to further improve the extraction efficiency. When the concentration of methanol in a water sample spiked with 250 µg/L of the standards was varied from 0.06% to 5% (v/v), the extracts were analyzed, and the peak areas were obtained and compared. The peak areas of MC-RR and MC-LR were improved, with an increase of methanol concentration in the samples from 0.06% to 1%. The highest peak areas of the analytes were obtained with the addition of 1% methanol to the sample, though a further increases of methanol concentration in the samples resulted in reduced peak areas. These results indicate that small amounts of methanol in a sample may increase the partition of microcystins in water with the membrane on the fiber. However, methanol can also dissolve the microcystins on the fiber when its concentration is sufficiently high in the sample.

The previously described parameters affect the adsorption efficiency, though the factors affecting desorption efficiency are also important. Therefore, the solvent and time used for desorption were also investigated. Two solvents used to desorb the analytes on the fiber were 100% methanol and HPLC mobile phase (50% methanol containing 0.01% formic acid). A better desorption efficiency for both MC-LR and MC-RR was obtained by using 100% methanol than by using 50% methanol containing 0.01% formic acid. No significant increases in response was observed when the desorption time was longer than 3 min. Thus, the fiber was desorbed in 100 µL of methanol for 3 min after SPME extraction.

Under the optimized conditions described, the extraction time profile obtained (as shown in Figure 4), in which water samples spiked with 250 µg/L of MC-RR and MC-LR, was used for the SPME enrichment. When the extraction time was varied from 10 to 20 min, a significant improvement in the peak areas for both compounds was observed. Figure 4 also shows that the optimum extraction time depended on individual analytes. Under the present conditions, with the CW–TPR fiber, MC-RR reached the extraction equilibrium in 20 min, whereas MC-LR needed 30 min. Based on the time profile (Figure 4), an extraction time of 40 min was chosen for further sample extraction.

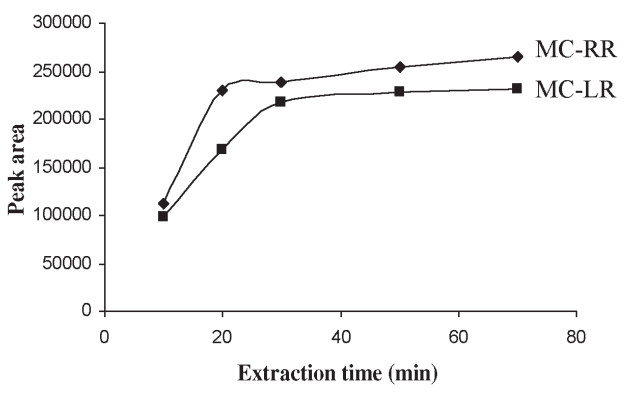

Figure 4. The effect of extraction time on the extraction efficiency.

Micro-HPLC–QTOF-MS with IDA determination of microcystins

Figure 5 shows the multiplex results from a single analysis of a standard water sample containing 50 µg/L microcystins using SPME with micro-HPLC–QTOF-MS with IDA detection. These results include: a total ion chromatogram (TIC) (or survey chromatogram) (Figure 5A); an extracted ion chromatogram, showing the well-separated peaks of MC-RR and MC-LC (Figure 5B); a full-scan spectrum of the peak at 10.6 min, the ion at *m/z* 512.8 corresponds to $[MC\text{-}RR+2H]^{2+}$ (Figure 5C); the product ion spectra of the peak at 10.6 min generated at collision energy

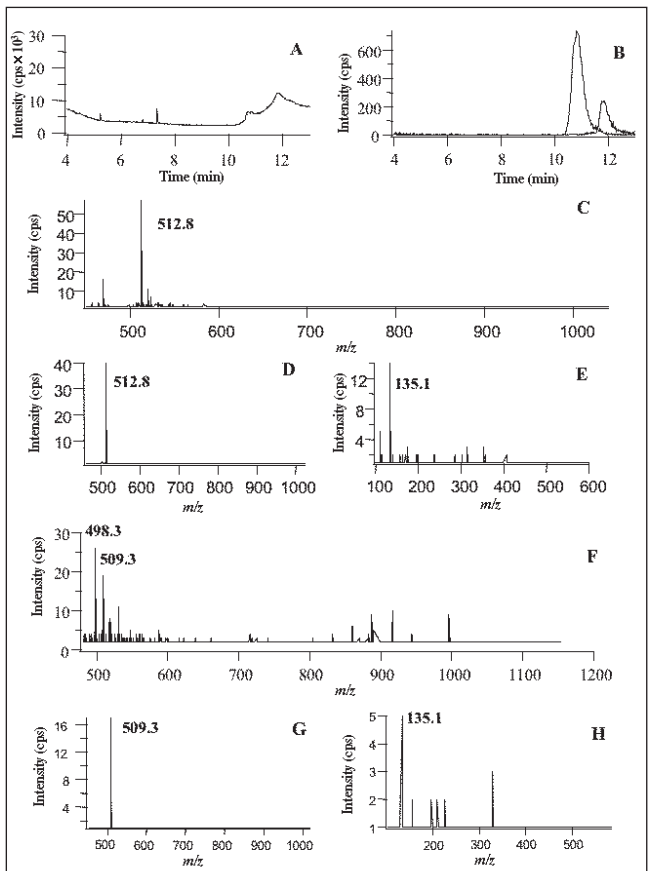

Figure 5. HPLC–MS–MS with IDA of 50 µg/L MC-RR and MC-LR TIC (A), an EIC of MC-RR and MC-LR (B), a full scan mass spectrum of MC-RR (C), MS–MS spectra of MC-RR generated at CE 15 eV (D) and CE 40 eV (E), a full scan mass spectrum of MC-LR (F), and MS–MS spectra of MC-LR generated at CE 15 eV (G) and CE 40eV (H). Chromatographic conditions: BetaBasic-18 column, 100 × 0.5 mm, 3-µm particle size. Mobile phase A was Milli-Q water and mobile phase B methanol, both containing 0.01% (v/v) formic acid. The gradient was: 0–3 min, 50% B; 5 min, 70% B; and 10 min, 100% B. The injection volume was 2 µL and the flow rate was 12 µL/min.

(CE) 15, *m/z* 512.8, $[MC\text{-}RR+2H]^{2+}$) (Figure 5D), and 40 eV (*m/z* 135.1, a characteristic fragment ion of microcystins) (Figure 5E). This confirms that the identity of the peak at 10.6 min was MC-RR. Similarly, a full-scan spectrum (Figure 5F) consisted of *m/z* 498.3, corresponding to $[MC\text{-}LR+2H]^{2+}$, and *m/z* 509.3, corresponding to $[MC\text{-}LR+H+Na]^{2+}$, of the peak at 11.8 min, and the product ion spectra (Figures 5G and 5H) generated at CE 15 showed *m/z* 509.3, corresponding to $[MC\text{-}LR+H+Na]^{2+}$, and at 40 eV showed *m/z* 135.1, the characteristic fragment ion of microcystins, respectively. These spectra were used to confirm the identity of this peak as MC-LR. The TIC showed very low intensity peaks, and the EIC showed well-separated and high-intensity peaks of the analytes, which were used for quantitation. Figure 5 demonstrates the capability of the IDA-QTOF-MS, which can simultaneously identify and quantitate analytes in an unknown sample without a priori knowledge of the compounds. This feature is particularly useful for surveillance of microcystins in real-water samples.

The micro-HPLC separation enables the effective use of microliter volumes of the extracts from the SPME for sensitive detection. The linear calibration curves and limits of detection (LOD) were further evaluated using the integrated SPME with micro-HPLC–QTOF-MS technique. Under the optimized conditions, calibration curves of MC-RR and MC-LR were obtained when spiked water samples containing 1, 5, 10, 50, 100, and 250 µg/L were extracted using the CW–TPR fiber and analyzed by the micro-HPLC–QTOF-MS with IDA detection. Figure 6 shows the calibration curves of MC-RR (Figure 6A) and MC-LR (Figure 6B),

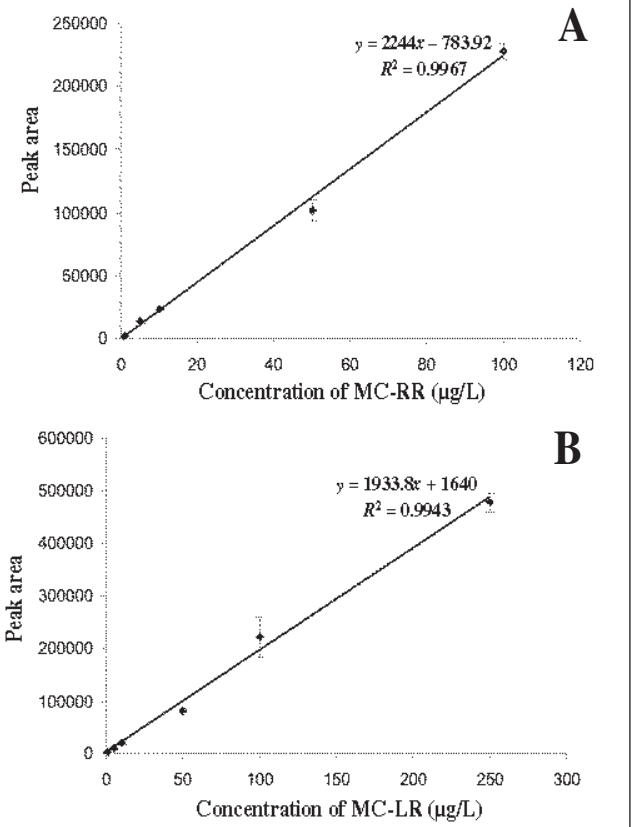

Figure 6. The calibration curves for MC-RR (A) and MC-LR (B).

demonstrating the linear relationship between the EIC peak area and the concentration of the analytes. The linear correlation coefficient was 0.997 for MC-RR and 0.994 for MC-LR.

The LOD (LOD at signal-to-noise = 3) was obtained when a spiked water sample containing 1 µg/L of the analytes was analyzed using the SPME with micro-HPLC–QTOF-MS technique. Figure 7 shows chromatograms of lake water (Figure 7A) and the spiked water sample with 1 µg/L of microcystins (Figure 7B). The LOD was determined to be 0.6 pg (0.3 µg/L) for MC-RR and 1.6 pg (0.8 µg/L) for MC-LR, when only 12 mL of a sample was used. In addition, the extract in 20 µL of 50% methanol can be reduced to a smaller volume, such as 5 µL, to further reduce the LOD because only 2 µL of the extract was injected for analysis.

Sample analysis

A lake-water sample collected from Dried Meat Lake was analyzed using this technique, and no microcystins were detected (Figure 7A). This lake-water sample was spiked with 50 and 100

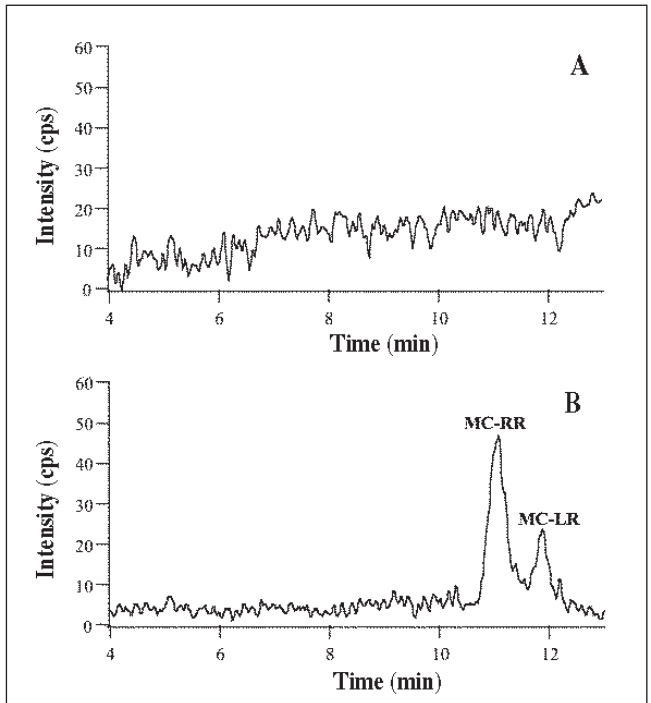

Figure 7. TIC of lake water sample analysis (A) and spiked water sample (B). Spiking concentration: 1 µg/L.

Table I. The Recovery and Precision (RSD) for the MC-RR and MC-LR in Lake Water Spiked at Two Different Levels

	Spiked level (µg/L)	Experimental (µg/L)	Recovery (%)	RSD (%)
MC-RR	0	0		
	50	43	86	9.5
	100	89	89	9.3
MC-LR	0	0		
	50	35.5	71	0.9
	100	76	76	7.2

µg/L MC-RR and MC-LR standards and used for evaluation of the recovery, accuracy, and precision of the method. Table I summarizes the recovery and RSD obtained from analysis of triplicates of the spiked-lake water samples. The recovery for MC-RR was > 86% with a relative standard deviation of < 9.5% and > 71% with < 7% for MC-LR. This demonstrates the potential application of the technique to the screening of microcystins in lake water.

Conclusion

The developed technique of SPME with micro-HPLC–QTOF-MS with IDA detection was demonstrated to be a simple, reliable, and sensitive method for the determination of dissolved microcystins in water. Only 12 mL of water was used to extract and concentrate the analytes by SPME. The SPME technique simplifies the enrichment process, eliminates the use of organic solvents, and reduces the cost of disposal.

The SPME with micro-HPLC–QTOF-MS technique provided excellent mass LOD of 0.6 pg for MC-RR and 1.6 pg for MC-LR, which was better than previously reported methods. The concentration LOD was at sub-µg/L levels, which is comparable with other SPE with LC–MS and LC–MS–MS techniques that typically use 500 mL of water samples and high volumes of organic solvents for elution (20). The extracts are generally analyzed by LC–MS with selected ion monitoring (SIM) and selected reaction monitoring (SRM)/MRM data acquisition techniques. The SIM or SRM/MRM data acquisition methods require a pre-analysis set of the ions, and is, thus, limited to the analysis of known compounds. The tandem mass spectrum of each compound for confirmation of the peak detected is obtained by a separate run. The SPME with micro-HPLC–QTOF-MS with IDA detection overcomes these problems and provides a simplified enrichment procedure and rich information for both quantitation and identification, including a survey chromatogram, a full scan mass spectrum, and MS–MS spectra at different collision energies of the analytes by a single analysis.

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