HHS Public Access

Author manuscript

Harmful Algae. Author manuscript; available in PMC 2020 June 09.

Published in final edited form as:

Harmful Algae. 2018 June ; 76: 47–57. doi:10.1016/j.hal.2018.04.009.

Optimization of extraction methods for quantification of microcystin-LR and microcystin-RR in fish, vegetable, and soil matrices using UPLC–MS/MS

Manjunath Manubolu[a,b,*], **Jiyoung Lee**[b,c], **Kenneth M. Riedl**[c], **Zi Xun Kua**[a], **Lindsay P. Collart**[a,d], **Stuart A. Ludsin**[a]

[a]Aquatic Ecology Laboratory, Department of Evolution, Ecology and Organismal Biology, The Ohio State University, Columbus, OH, USA

[b]Division of Environmental Health Sciences, College of Public Health, The Ohio State University, Columbus, OH, USA

[c]Department of Food Science and Technology, The Ohio State University, Columbus, OH, USA

[d]Department of Microbiology, Oregon State University, Corvallis, OR, USA

Abstract

Human-driven environmental change has increased the occurrence of harmful cyanobacteria blooms in aquatic ecosystems. Concomitantly, exposure to microcystin (MC), a cyanobacterial toxin that can accumulate in animals, edible plants, and agricultural soils, has become a growing public health concern. For accurate estimation of health risks and timely monitoring, availability of reliable detection methods is imperative. Nonetheless, quantitative analysis of MCs in many types of biological and environmental samples has proven challenging because matrix interferences can hinder sample preparation and extraction procedures, leading to poor MC recovery. Herein, controlled experiments were conducted to enhance the use of ultra-performance liquid-chromatography tandem-mass spectrometry (UPLC–MS/MS) to recover MC-LR and MC-RR at a range of concentrations in seafood (fish), vegetables (lettuce), and environmental (soil) matrices. Although these experiments offer insight into detailed technical aspects of the MC homogenization and extraction process (i.e., sonication duration and centrifugation speed during homogenization; elution solvent to use during the final extraction), they centered on identifying the best (1) solvent system to use during homogenization (2–3 tested per matrix) and (2) single-phase extraction (SPE) column type (3 tested) to use for the final extraction. The best procedure consisted of the following, regardless of sample type: centrifugation speed = 4200 × *g*; elution volume = 8 mL; elution solvent = 80% methanol; and SPE column type = hydrophilic–lipophilic balance (HLB), with carbon also being satisfactory for fish. For sonication, 2 min, 5 min, and 10 min were optimal for fish, lettuce, and soil matrices, respectively. Using the recommended HLB column, the solvent systems that led to the highest recovery of MCs were methanol:water:butanol for fish, methanol:water for lettuce, and EDTA-$Na_4P_2O_7$ for soils. Given that the recommended

*Corresponding author at: Aquatic Ecology Laboratory, Department of Evolution, Ecology and Organismal Biology, The Ohio State University, Columbus, OH, USA. manubolu.1@osu.edu (M. Manubolu).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.hal.2018.04.009.

procedures resulted in average MC-LR and MC-RR recoveries that ranged 93 to 98%, their adoption for the preparation of samples with complex matrices before UPLC–MS/MS analysis is encouraged.

Keywords

Cyanotoxin; Eutrophication; *Microcystis*; Harmful algal bloom; Lake Erie

1. Introduction

Cyanobacteria blooms have increased in aquatic ecosystems worldwide, owing to human-driven eutrophication, with their prominence expected to increase with continued climate change (Michalak et al., 2013; Paerl and Huisman, 2008). This increase is problematic because excessive cyanobacteria can both directly and indirectly degrade ecosystem services vital to humans (Saqrane and Oudra, 2009). One prominent negative feature of cyanobacteria blooms is the production of toxins that can reduce the availability of clean, safe water for recreation (e.g., swimming), human and livestock consumption, and crop irrigation (Brooks et al., 2016; Manubolu et al., 2014; Lee et al., 2017b; Saqrane and Oudra, 2009). Among the many types of cyanotoxins that have been documented, microcystin (MC), a liver toxin, is the most frequently occurring in freshwater environment, and hence, has been widely investigated (An and Carmichael, 1994). While MC contamination of drinking water and recreational waters can pose an immediate threat to humans (Brooks et al., 2016; Lee et al., 2017b; Saqrane and Oudra, 2009), food consumption also can be an exposure route, as MCs can accumulate in the edible tissues of animals and plants (Li et al., 2014; Qian et al., 2017; Saqrane and Oudra, 2009; Schmidt et al., 2013; Vasconcelos, 1999). In addition, because MCs can negatively affect the development, growth, and survival of both fish and plants (Jacquet et al., 2004; Lee et al., 2017a; Oberemm et al., 1999; Prieto et al., 2011; Saqrane et al., 2008), their common occurrence in eutrophic water bodies and surface waters used for irrigation (e.g., retention ditches, ponds, reservoirs) in many parts of the world can pose a threat to the production of edible seafood and terrestrial crops (Ferrao and Kozlowsky-Suzuki, 2011; Zanchett and Oliveira-Filho, 2013). For these reasons, interest in accurately quantifying cyanotoxins in the tissues of edible animals and plants, as well as agricultural soils, has increased (Bouaïcha and Corbel, 2016; Corbel et al., 2014; Li et al., 2014).

With the growing prominence of MC-producing cyanobacteria blooms in aquatic ecosystems worldwide (Schmidt et al., 2013), the need to develop accurate, robust, and ideally inexpensive methods for quantifying MCs in biological and environmental samples has become paramount. Unfortunately, reliable quantification of MCs in such sample types has proven difficult, owing to matrix interferences associated with complex tissues and soils that can lead to reduced recovery of MCs, and hence their underestimation (Geis-Asteggiante et al., 2011b). Most problematic have been difficulties associated with (1) freeing bound MCs from the tissue or soil matrix during the homogenization process, and (2) removing non-MC materials and molecules that can lead to false positive results during the final phase of the MC extraction process (termed “sample cleanup”).

These difficulties highlight the need for testing of various materials and methods used during the pre-analytical, sample preparation phase. For example, during the homogenization phase, different solvent systems can be used to extract MCs into solution and the duration of sonication can be varied to optimize the lysing of tissue membranes (to free bound MCs). During later phases of MC extraction, decisions need to be made about what centrifugation speed to use to concentrate MCs before the final extraction, what solid-phase extraction (SPE) filtration column to use to remove non-MC materials/molecules, and what elutant to use to reconstitute the MCs for analysis after cleaning them (Silva-Stenico et al., 2009). Because the properties (e.g., degree of organic material, membrane structure, polarity) of animal, plant, and soil matrices differ, one might expect the optimal sample preparation protocol to also vary among these sample types.

Towards identifying an efficient, effective approach for quantifying MC-LR and MC-RR in animal, plant, and soil samples using ultra-performance liquid-chromatography tandem-mass spectrometry (UPLC-MS/MS), a set of controlled laboratory experiments was conducted. These experiments focused on two key aspects of the sample preparation phase during which extracting bound MCs and removing non-MC materials can be difficult (Fig. 1). First, to improve the ability to extract bound MCs during the homogenization phase, the efficacy of several commonly applied solvents systems was tested (2 to 3 per matrix type; see below for details). Second, because SPE is critical to minimizing LC-MS matrix interferences that are caused by non-MC materials (i.e., cleaning up the sample) (Frazier et al., 1998; Rodriguez-Mozaz et al., 2007), especially when MCs are in low abundance, the performance of three SPE filtration column types (activated charcoal; hydrophilic-lipophilic balance, HLB; and carbon, C18) was tested at environmentally relevant concentrations (Merel et al., 2013). To determine the best combination of homogenization solvent system and SPE column, a full-factorial laboratory experiment was conducted (i.e., 2–3 solvent systems crossed with 3 SPE columns) for each sample type. These experiments were conducted using common sonication times, centrifugation speeds, and SPE elution solvents from a related set of experimental trials.

The experiments focused on maximizing the recovery of MC-LR and MR-RR, which are highly toxic and the most abundant MC congeners in many aquatic ecosystems (Yang et al., 2013). For these reasons, these two congeners have been of concern to regulatory and management agencies worldwide (Geneva, 1998; Giddings et al., 2012; Meriluoto et al., 2005). This research also helps to fill an information gap as comparative studies that have tested the ability to recover multiple MC congeners in fish, plants, and soil samples using UPLC-MS/MS are sparse (Ame et al., 2010; Pekar et al., 2016; Yongtao and Joshua, 2015). Finding recovery rates ranging 93% to 98%, this study offers much-needed guidance on how to prepare animal and plant tissues, as well as soil samples, for the maximal recovery and detection of MC-LR and MC-RR using UPLC–MS/MS.

2. Material and methods

2.1. Sample preparation and spiking

Described below are the early steps of the sample homogenization process and the procedure for spiking samples with MC-LR and MC-RR for eventual extraction and detection with

UPLC–MS/MS. All hardware, glassware, and utensils (e.g., cleaver, fillet knife, blender, mortar and pestle, etc.) were thoroughly cleaned with soap and hot water, rinsed with distilled water, and wiped down with pesticide-quality acetone before and after use.

2.1.1. Fish—Walleye (*Sander vitreus*) muscle tissue was used to assess MC-LR and MC-RR recovery levels in fish. The walleye used in this study (n = 2) were collected during September 29 through October 6, 2015 from Ohio waters of western Lake Erie (USA) via gillnet surveys conducted by the Ohio Department of Natural Resources-Division of Wildlife (ODNR-DOW). Upon collection, individuals were filleted, with the fillets wrapped in analytical-grade aluminum foil before being frozen at −80 °C until homogenization.

The homogenization protocol for the samples of fish muscle followed procedures used by the Ohio Environmental Protection Agency (Ohio, 2007; Schmidt et al., 2013). In brief, the fillets were chopped into small chunks using a meat cleaver and then combined with dry ice (~50% of the tissue volume) before being ground in a meat grinder (Power Zen 125, Fisher Scientific, Pittsburgh, PA, USA). Each sample was passed through the meat grinder twice to ensure complete pulverization. Samples were ground to a powder using a mortar and pestle, with dry ice added as necessary to keep the samples frozen. Samples were split into 1 g aliquots (n = 3 per MC concentration per solvent system per SPE column) after processing, which were then wrapped in aluminum foil and frozen at −80 °C until spiking with MCs (see below).

With exception of aliquots used as controls, each sample of homogenate used in this study was spiked with MC-LR and MC-RR (Beagle Bioproducts, Columbus, OH, USA) that was greater than 95% pure. To do so, samples were thawed and MC-LR and MC-RR were added in a concentration of 0.25 μg g^{-1} or 1 μg g^{-1} of wet mass (thawed fish tissue). The spiked samples were then incubated in glass vials (13 × 100 mm, 8mL capacity, Fisher scientific, Pittsburgh, PA, USA) at room temperature for 20 h in the dark, to allow the formation of covalently bound MC complexes (Craig et al., 1996). After spiking and incubation, a tissue probe homogenizer (Power Zen 125, Fisher Scientific, Pittsburgh, PA, USA) was used to further homogenize the sample.

2.1.2. Lettuce—The greenhouse-grown lettuce used in this study was purchased from a local grocery store in Columbus, OH, USA (cultivated by Queen Victoria, CA, USA). Samples of fresh lettuce were ground using a mortar and pestle, portioned into 1 g aliquots, and then placed into glass test tubes (13 × 100 mm, 8 mL capacity, Fisher Scientific, Pittsburgh, PA USA). Each aliquot was spiked with MC-LR and MC-RR, incubated, and re-homogenized using the same methods described for the fish samples above.

2.1.3. Soil—The soil used in this study was purchased from Professional Growing Mix (Sung Gro Horticulture, Agawam, MA, USA). Its composition consisted of Canadian Sphagnum Peat Moss (80%) and Coarse Perlite (20%). After homogenizing the soil samples with a mortar and pestle, 1 g subsamples were placed into 13 × 100 mm (8 mL) glass test tubes (Fisher Scientific, Pittsburgh, PA, USA). Afterwards, each sample was spiked with MC-LR and MC-RR and incubated for 20 h at room temperature, as described above (see Section 2.1.1).

2.2. Optimization of extraction conditions

2.2.1. Homogenization solvent system—Towards maximizing the movement of bound MCs from the homogenate into solution, the efficacy of three solvent systems was tested for both fish and lettuce, whereas only two systems were tested for the soil samples (Fig. 1). For the fish samples, three common solvent systems were tested, including 80:20 methanol: water (hereafter, 80% methanol) (Smith and Boyer, 2009), 15:4:1 (75:20:5 vol:vol:vol) methanol:water:butanol (Cadel-Six et al., 2014; Ernst et al., 2005; Jia et al., 2014; Xie and Park, 2007b; Zhang et al., 2009), and 5% acetic acid and 0.01 M EDTA (hereafter, AA-EDTA Dai et al., 2008; Guo et al., 2014; Ríos et al., 2013; Schmidt et al., 2013; Wu et al., 2010). For lettuce, two previously used solvent systems, 50:50 methanol:water (hereafter, 50% methanol) (Gutiérrez-Praena et al., 2014) and 80:19:1 methanol:water: trifluoroacetic acid (Li et al., 2014), as well as AA-EDTA were tested. Finally, for the soil samples, solvent systems consisting of $Na_4P_2O_7$ and 0.1 M EDTA (hereafter, EDTA-$Na_4P_2O_7$; Chen et al., 2006; Corbel et al., 2016; Li and Pan, 2015) and AA-EDTA were compared, the latter of which had yet to be used for soil samples.

To extract the MCs from the homogenate, 10 mL of a solvent was added to each homogenized sample and incubated for 10 min in the original glass test tube at room temperature. Afterwards, the homogenized extract was sonicated in its original test tube in a water bath (Branson 2510, Danbury, CT, USA) for 2, 5, and 10 min for fish, lettuce, and soil samples respectively. These sonication times were determined experimentally (Fig. S1). After sonication, the extracts were centrifuged (Thermo Sorvall™ Legend™ XT/XF) at 4500 × *g* for 20 min, with the centrifuge speed determined experimentally (Fig. S2). After centrifugation, the resulting supernatant was collected. This entire process (i.e., adding 10 mL of the homogenization solvent through collecting the supernatant after centrifugation) was repeated three times for each sample, with the resulting supernatants pooled afterwards. The samples were now ready for the final sample cleanup using an SPE column.

2.2.2. SPE cleanup procedure—Three SPE column types were tested (i.e., HLB, C18, and activated-charcoal; Fig. 1) to assess their ability to remove interfering compounds. The SPE system consisted of a 24-port vacuum manifold (Sigma-Aldrich, St. Louis, MO, USA). The HLB (Oasis, PRiME, part # 186008718) and Sep-pak C18 (end-capped, bonded phase, silica-based; part # 186004620) SPE columns used were purchased from Waters Corporation (Milford, MA, USA), whereas the activated-charcoal SPE columns (part # 12252201) were purchased from Agilent Technologies (Santa Clara, CA, USA). The bed size of all SPE columns was 500 mg (6 mL capacity). The SPE columns were preconditioned with 6 mL of methanol, followed by 6 mL of ultrapure water. Samples (approximately 30 mL) were applied to the column slowly, and then rinsed afterwards with 20% methanol. Samples were then eluted from column using 25 mL of 80% methanol, with the methanol concentration of this elution solvent being determined experimentally (Fig. S3). The final eluent was evaporated to dryness under a gentle stream of nitrogen (N2) gas, with the residue suspended in 1 mL of 80% methanol and then subsequently filtered through 0.2 mm polytetrafluoroethylene (Ultrafree®-MC, Sigma, Bedford, MA, USA).

2.3. Chemicals and reagents

All of the chemicals and reagents used in this study were purchased from Sigma-Aldrich (Prague, Czech Republic), with exception of MC-LR and MC-RR (Beagle Bioproducts, Columbus, OH, USA). All of the extraction and sample reconstitution solvents were LC-MS grade. Ultrapure water (Milli Q, Millipore, Bedford, MA, USA) was used throughout the entire analytical process.

2.4. UPLC-MS/MS method development

A UPLC chromatograph (1200 SL series, Agilent Technologies, Santa Clara, CA) that was interfaced with a triple quadrupole mass spectrometer (QTrap 5500, ABSciex, Concord, Canada) *via* an electrospray ionization (ESI) source was used. Reversed-phase chromatography was performed on an Eclipse plus C18 column (4.6 mm × 100 mm, particle size 1.8 μm) (Agilent Technologies, Santa Clara, CA).

Injection of 1 μL analytical volume was found suitable or maximal sensitivity while avoiding suppression (as indicated by the signal-to-noise ratio). A flow rate at 1 mL min^{-1} was maintained with an initial mobile phase composition of 95% (A) water with 0.1% (v/v) formic acid and 5% (B) acetonitrile with 0.1% formic acid. Both MCs were eluted within 6 min. During this time, the composition was changed linearly to 95% B, which was then held for 1 min. Starting conditions were re-established over an additional 3 min. The column oven temperature was maintained at 40 °C. Analyst software (version 1.4.1) was used for data acquisition and integration.

As the UPLC–MS/MS data were acquired on a triple quadrupole instrument, both positive and negative ionization modes were used in a single analysis without substantial negative impact on the duty cycle (polarity switches occurred less than 50 ms). The highest intensity Multiple Reaction Monitoring (MRM) transition of each cyanotoxin was used for quantitation with the optimal settings: source temperature 700 °C; electrospray voltage +4500 V; curtain gas (N_2) pressure 30 PSI; declustering potential of 100V; and collision-induced dissociation affected with N_2 gas.

Both MC congeners were quantified using the peak areas of chromatograms from samples, as well as external calibration curves and a correction based on recovery (see below). For MC-LR, a single transition in a negative MS/MS mode was used $[MC\text{-}LR - H]^-$, *m/z* 993.5 > 975.5, whereas a positive MRM mode was optimal for MC-RR using double charged precursor $[MC\text{-}RR+2H]^{+2}$, *m/z* 519.8 ≥ 135, 447.5, 620.4 and 887.5. These settings were applied to quantify MC-LR and MC-RR for all three sample types (Fig. S4).

2.5. Calibration and recovery level estimation

To correct our estimate of MC-LR and MC-RR for incomplete recovery, as well as to assess matrix effects, a calibration curve was developed for each MC congener. These calibration curves were derived by measuring toxins in standard solutions consisting of 80% methanol (neat solvent solution) and a known concentration of MC-LR and MC-RR (0.125, 0.25, 0.5, and 1 μg g^{-1}). By comparing the recovered level of MC-LR and MC-RR in each fish, lettuce, or soil sample to the known level (0.25 ug g^{-1} or 1 ug g^{-1}), which was corrected

using the calibration curves developed from standard solutions, matrix interference could be assessed. Matrix enhancement would result recovery rates of >100%, whereas signal suppression would result in values <100%.

2.6. Analytical detection limits

The quantifier selected to determine limits of detection (LOD) and limits of quantitation (LOQ) was the MS/MS transition with the highest signal to noise ratio. The peak height of each quantifying MS/MS transition for both MC-LR and MC-RR for all three sample types are provided in Table S1. Signal:noise (S:N) values of 3 and 10 were used to define the LOD and LOQ, respectively. Both congeners were sufficiently low for use in this study, as their average (±1 standard deviation, SD) LOD were less than 0.026 ±0.009 μg g^{-1} and their average LOQ were less than 0.072 ± 0.022 μg g^{-1}, regardless of sample type (Table S1).

2.7. Statistical analyses

Three-way analysis of variance (ANOVA) was used to evaluate how MC recovery levels varied with the different extraction/cleanup protocols. Six ANOVAs were conducted, one for each combination of sample type (fish, lettuce, and soil) and MC congener (MC-LR and MC-LR). The homogenization solvent system (n = 3 types for fish and lettuce; n = 2 types for soil), SPE column type (n = 3 types for all sample types), and the MC-spiking level (n = 2 levels for all sample types) were included as main factors in the model, with all two-way and the three-way interaction also included. By including MC-spiking level in these analyses, the effectiveness of extracting different levels of MCs could be assessed. This knowledge is important, given that MC levels in fish tissues have been shown to vary in nature (Jia et al., 2014; Schmidt et al., 2013; Xue et al., 2016). All treatments (n = 18 for fish and lettuce; n=12 for soil) were replicated in triplicate in a balanced design. Because two independent ANOVAs were used to explore the recovery of MC-LR and MC-RR within a sample type, a Bonferroni-corrected *p*-value was used to denote significance ($p = 0.05/2 = 0.025$). This correction reduced the chance of causing a Type I error. For those factors that were significant ($p < 0.025$), a Tukey honestly significant difference (hsd) test was used to identify which treatments differed. All data used in the ANOVAs were normally distributed (Kolmogorov-Smirnov normality tests, all $p \geq 0.20$). All analyses were conducted using Statistica Software (v13, TIBCO®, Palo Alto, CA, USA).

3. Results

3.1. MC recovery from fish tissues

Both MC-LR and MC-RR were simultaneously recovered from homogenized fish muscle tissues with a high degree of success (Table 1, Fig. 2). While the homogenization solvent system, SPE filtration column, and spiking level each significantly affected MC recovery levels, their effects were interactive. Thus, only some combinations of these attributes led to high levels of MC recovery (Table 1). Average recovery levels were highest when a 75:20:5 methanol:water:butanol solvent was used during the homogenization process, but only when used in conjunction with the HLB column (≥94% recovery) or the charcoal column (≥93% recovery) (Fig. 2); no differences were found between these column types, or between MC-spiking levels, for either MC congener when this solvent system was used. By contrast,

when either of the other homogenization solvents was used (i.e., 80% methanol or AA-EDTA), or a C18 SPE column was used, average MC recovery levels were significantly lower, ranging 43% to 78% (Table 1, Fig. 2).

Three-way ANOVA results showed that the homogenization solvent had the largest effect on MC recovery levels in fish tissues. Based on ratios of F-values, the effect of homogenization solvent was 2.2-fold (MC-LR) to 3-fold (MC-RR) larger than that of the SPE filtration column and 5-fold to 8-fold larger than that of the MC-spiking level (Table 1).

3.2. MC recovery from plant tissues

Similar to fish samples, MC-LR and MC-RR could be recovered with a high degree of success from lettuce samples (Table 1, Fig. 3). Unlike fish, however, average recovery levels were highest when a 50% methanol solvent was used during the homogenization process. Further, this solvent was only successful when used in conjunction with an HLB filtration column (≥93% recovery); the use of an activated-charcoal or C18 column led to significantly lower MC-recovery levels (i.e., 52%–77%; Fig. 3). Likewise, using 80:19:1 methanol:water:trifluoroacetic acid or AA-EDTA as homogenization solvents led to significantly lower MC-recovery levels (i.e., 42% to 74%; Fig. 3).

As with the fish samples, the homogenization solvent had the largest effect on MC-recovery levels in lettuce samples. In the three-way ANOVAs, the F-value of the homogenization solvent was 3.2-fold (MC-LR) to 1.7-fold (MC-RR) larger than that of the SPE filtration column and 6.2-fold to 14.6-fold larger than that of the MC-spiking level (Table 1).

3.3. MC recovery from soil samples

The recovery of MC-LR and MC-RR in soil samples varied among the experimental conditions in a similar manner as the fish and lettuce samples. Average recovery levels were highest when an EDTA-$Na_4P_2O_7$ homogenizing solvent was used, but only in combination with a HLB column (>95% recovery). When the activated-charcoal or C18 columns were used, recovery levels ranged lower (58% to 84%; Fig. 4). Likewise, when AA-EDTA was used as a homogenizing solvent, the average recovery of both MC congeners was significantly lower (ranging 40% to −69%), regardless of SPE column type (Table 1).

As with the biological samples, the homogenization solvent had the largest effect on MC recovery levels in soil samples. The effect of the homogenization solvent was 1.9-fold (MC-LR) to 1.3-fold (MC-RR) larger than that of the SPE filtration column and 10-fold larger than that of the spiking level (see F-values in Table 1).

4. Discussion

Reliable quantification of MCs in many biological and environmental sample types has proven difficult, owing to interferences imposed by sample matrices that can hamper extraction procedures and lead to poor MC recovery (Smith and Boyer, 2009). Controlled experiments were conducted to more reliably extract and recover MCs from fish, plant, and soil samples, all of which have complex matrices. These experiments quantified the independent and combined effects of different homogenization solvent systems and SPE

filtration columns on the simultaneous recovery of MC-LR and MC-RR. Since MC concentrations in biological and environmental samples can vary considerably in nature (e.g.,. Jia et al., 2014; Schmidt et al., 2013; Xue et al., 2016), we tested the effects of both factors (homogenization solvent, SPE column) on MC recovery at low (0.25 μg/L) and high (1.0 μg/L) spiking levels.

The procedures used herein, which included experimentally-derived sonication times, centrifugation times, and elution solvent systems (Fig. S1–S3), led to MC-LR and MC-RR recovery levels that ranged 93% to 98%. These levels are comparable to or better than previously reported values (Table 2). Analyses of the experimental data revealed that an HLB column was most appropriate for all three sample types (with an activated-charcoal column also being satisfactory for fish samples). Nevertheless, these analyses showed that different homogenization solvent systems should be used, with methanol:water:butanol, methanol:water, and EDTA-$Na_4P_2O_7$ leading to the highest MC recovery levels for fish, lettuce, and soils, respectively. In addition to providing quantitative support for these conclusions below, guidelines are offered on how to prepare samples with complex matrices to maximize the simultaneous recovery of MC-LR and MC-RR with UPLC–MS/MS.

4.1. MC recovery from fish tissues

While previous studies have shown that the homogenization solvent can influence recovery levels of MCs fish samples (Table 2) (Christoffersen and Kaas, 2000; Dai et al., 2008; Geis-Asteggiante et al., 2011a; Moreno et al., 2005), the magnitude of this effect in this study was not anticipated. One important finding from this study was the superior performance of the 15:4:1 methanol:water: butanol solvent relative to the 80% methanol or AA-EDTA solvent systems. This finding was a bit surprising, given that the previously published studies that used 15:4:1 methanol:water:butanol as a solvent had MC recovery rates that did not exceed 88% (see Table 2). In addition, the single study that used 80% methanol as a solvent reported MC recovery levels that ranged 80% to 99% (Smith and Boyer, 2009), with all studies that used the AA-EDTA solvent system reporting maximum MC recovery rates ranging from 90% to 110% (Dai et al., 2008; Guo et al., 2014; Ríos et al., 2013; Schmidt et al., 2013; Wu et al., 2010).

Based on the published literature and the results presented herein, the recovery of MC-LR and MC-RR from fish tissues depends on many factors, not just the homogenization solvent system used. The significant solvent system X SPE column interaction term in the three-way ANOVAs used to analyze the data support this conclusion. Thus, to achieve maximal MC recovery, a15:4:1 methanol:water:butanol solvent system needs to be paired with a proper SPE column (HLB or activated charcoal) during the cleanup phase. When a different (i.e., C18) SPE column was used with this homogenization solvent system, MC recovery levels dropped from ≥ 93% to levels that ranged 43% to 78%.

The recommendations emanating from the experimental trials conducted with fish tissues are supported by the literature. Jia et al. (2014) and Xie and Park (2007a,b) have recommended the use of a15:4:1 methanol:water:butanol solvent system, albeit based on lower MC-recovery levels (see Table 2). In addition, the use of a HLB column with animal tissues has been suggested (Dai et al., 2008; Rita et al., 2014; Xie and Park, 2007a). Even so,

systematic investigations of the impact of SPE column types on MC recovery are lacking in the literature, with Cadel-Six et al. (2014) being the only other study to compare HLB column performance to another column type (Strata X 60 column, polymer based).

While these results strongly point the use of a 15:4:1 methanol: water:butanol solvent system in conjunction with an HLB or activated-charcoal SPE column, other methods also need to be taken into account. This notion is supported by two published studies that used this combination but only achieved MC recovery rates as high as 81% to 86% (Cadel-Six et al., 2014; Xie and Park, 2007b). The reasons for the higher recovery rates in this study relative to these two others is uncertain, but may emanate from the use of optimized sonication times, centrifugation times, and elution solvent systems (see Fig. S1–S3). Likewise, the differential use of these kinds of conditions during the sample preparation phase may help explain why the 80% methanol solvent system performed better in other studies that our own when paired with a C18 SPE column (MC recoveries ranged 80% to 99%; Smith and Boyer, 2009) or the AA-EDTA solvent system when paired with an activated charcoal SPE column (Schmidt et al., 2013).

4.2. MC recovery from plant tissues

Relative to fish, fewer studies have used UPLC–MS/MS to quantify MC levels in plant tissues (Table 2). Of the five published studies that were found (Table 2), each used a different homogenization solvent system, including pure water (do Carmo Bittencourt-Oliveira, 2016), 100% methanol (Järvenpää et al., 2007), 75% methanol (Corbel et al., 2016), 50% methanol (Gutiérrez-Praena et al., 2014), and 80:19:1 methanol:water:trifluoroacetic acid (Li et al., 2014). Similar to the studies conducted with fish, none systematically compared homogenization solvent systems, thus highlighting the importance of this investigation. In addition, 3 of these 5 studies did not quantify MC recoveries, thus limiting ability of practitioners to know which procedures are most appropriate to use.

Of those studies reporting MC recovery rates, their results were variable (Table 2). For example, the use of 80:19:1 methanol:water: trifluoroacetic acid as a homogenization solvent resulted in MC recovery rates ranging 61% to 117% (Li et al., 2014). This variation is no doubt partly due to their crossing this solvent with two different SPE column types (HLB and C18; see Table 2), as similar variation was found in this study when this solvent system was used different columns (see Fig. 3). Because the use of 50% methanol as a homogenization solvent led to higher recoveries in this study than with the other two solvent systems (80:19:1 methanol:water:trifluoroacetic acid and AA-EDTA), its use is recommended in future applications. Unfortunately, the only other published study (i.e., Gutiérrez-Praena et al., 2014) that used this solvent system did not report MC recovery levels nor did it use the recommended HLB column.

Comparative research aimed at assessing the impact of SPE filtration columns on MC recovery in plant tissues has been equally as limited, with Li et al., (2014) offering the only contrast. In that study, the ability to recover MC-LR, MC-RR, and MC-YR using C18 and HLB columns was tested in lettuce and nine other plants. In contrast to the results presented herein, Li et al. (2014) showed that the C18 column performed better than the HLB column

for MC-LR (>95% recovery), with both not performing as well for MC-RR (8085%). For this reason, plus the fact that the C18 column is less expensive than the HLB column, Li et al. (2014) recommended the C18 column. Unfortunately, an obvious explanation for the disparity between this study and Li et al. (2014) is lacking. Perhaps it is related to the solvent system used, as the optimal solvent system in this study (50% methanol) was not tested by Li et al. (2014). Alternatively, perhaps the disparity relates to different centrifugation, sonication, or elution procedures being used by Li et al. (2014), which improved the functionality of the C18 column. Regardless, given the consistently high recovery rates in plant samples analyzed herein, the use of 50% methanol as a solvent with a HLB SPE column is recommended for lettuce samples. Most certainly, investigations that assess the performance of this combination with other plant types is encouraged.

4.3. MC recovery from soils

Even fewer comparative studies have quantified MCs in soils than for plants and fish (Table 2). Of the four studies found in the published literature, one used water as a homogenization solvent (Järvenpää et al., 2007), with the others using EDTA-$Na_4P_2O_7$ (Chen et al., 2006; Corbel et al., 2016; Li and Pan, 2015). While water did lead to subpar MC recovery levels (Järvenpää et al., 2007), levels exceeding 90% were documented in the two studies that used the EDTA-$Na_4P_2O_7$ solvent and reported MC recovery rates (Chen et al., 2006; Li and Pan, 2015). Thus, the superior performance of EDTA-$Na_4P_2O_7$ in this study is supported by the literature. Moreover, this finding is encouraging considering the hydrophilic nature of MC-LR and MC-RR, which make them typically more difficult to extract from sediments and soils than biological samples (Chen et al., 2006; Tsuji et al., 2001). This ability to reliably recover MC-LR and MC-RR in soils is especially relevant, as the research has suggested that MCs can persist in agricultural soils for weeks, if MC-tainted water is used to fertilize crops (Lee et al., 2017a).

Interestingly, the experimental trials conducted herein demonstrated that the HLB SPE column performed best, the activated-charcoal column performed worst, and the C18 column was intermediate. Thus, use of an HLB column with EDTA-$Na_4P_2O_7$ as a homogenization solvent system appears optimal for quantifying MC-LR and MC-RR in soils, when used in conjunction with recommended sonication, centrifugation, and elution methods (Fig. S1–S4). Even so, the use of a different type of C18 column could work under some conditions, as both Chen et al. (2006) and Li and Pan (2015) successfully used this column type with an EDTA-$Na_4P_2O_7$ solvent. This finding again points to the notion that procedural conditions and decisions beyond the *general* homogenization solvent and SPE column type can influence the success of MC recovery in environmental samples. For this reason, practitioners should (1) take care when making decisions about what exact C18 SPE column (for example) is used, and (2) take the time to report the chemistry of their SPE cartridges, including the exact model number(s) used.

5. Study limitations

Despite this study partially filling an information gap by offering improved methods for extracting and quantifying MCs in three different and challenging matrix types, this study is limited in two key ways. These limitations are discussed below.

5.1. Applicability to other MC congeners

An important potential limitation to this study is its focus on only MC-LR and MC-RR. These MC congeners were chosen for study because of their known high toxicity and abundance in many north-temperate aquatic ecosystems worldwide, owing to the dominance of *Microcystis* spp. in typical harmful algal blooms (Beversdorf et al., 2013). Other MC congeners, however, can be more abundant in nature at times, including more hydrophobic ones such as MC-LF and MC-LW, which potentially could be equally as toxic, if not more toxic, than MC-RR (Fischer et al., 2010; Vesterkvist et al., 2012). Likewise, desmethylated MC variants can be common in blooms in some ecosystems, especially those dominated by *Planktothrix* spp. (Fastner et al., 1999; Messineo et al., 2009). Because MC-LF, MC-LW, and desmethyl MC hydrophobicity is expected to impede their recovery (Lawton et al., 1994), yet data on the recovery of these variants is lacking, future assessment of whether the methods developed herein apply to these MC congeners is encouraged. Ideally, this assessment would be conducted on “naturally toxic” samples that have known concentrations of bound MCs.

5.2. MC-LR and MC-RR detection at low concentration

A noteworthy caveat relates to the ability of this study’s UPLC–MS/MS to detect MC-LR and MC-RR at extremely low concentrations. For MC-LR, although multiple electrospray ionization transitions were obtained, only a single (peak) quantifier was used to determine the MC-LR concentration. The choice to use a single quantifier was based on it being >20-fold more sensitive than the potential qualifiers, and the LOQs achieved were reasonably low (e.g., 0.016 ± 0.005 ug g^{-1} for fish; see Table S1). By contrast, four transitions (confirmation ions) were used for MC-RR. Unfortunately, even after extensively optimizing the MC extraction and LC–MS/MS conditions, LOQs below 0.035 ± 0.009 ug g^{-1} could not be achieved for any of our sample types (see Table S1). Thus, at levels below these limits, the analytical approach used herein could only serve as a useful screening tool for MC speciation in contaminated environmental samples.

Despite these limitations for MC-LR and MC-RR quantification, the achieved LODs and LOQs were sufficiently low to reliably quantify MC-LR and MC-RR in samples analyzed as part of this study. In addition, they were quite comparable to the LOQs reported by other published studies (e.g., Dai et al., 2008; Li et al., 2014). Even so, the use of two or more transitions (confirmation ions) is strongly recommended in any future applications of this type, as it could improve the ability to reliably detect and quantify MCs at low concentration (Kohoutek et al., 2010). In addition, continued research into methods to improve the quantification capabilities of both MC-LR and MC-RR using UPLC–MS/MS are encouraged, especially if target detection levels (0.04 ug kg^{-1}) by the World Health Organization are going to be achieved (Toxins, 2003).

6. Summary & conclusions

In summary, this experimental approach used herein led to improved methods to simultaneously extract, recover, and analyze MC-LR and MC-RR from samples with complex matrices, including fish, plant (lettuce), and soils. Regardless of sample type, the best protocol had a centrifugation speed of 4200 × *g*, an elution volume of 8 mL, and an elution solvent that consisted of 80% methanol. A HLB SPE column was found to be the most suitable sorbent to use during the sample cleanup phase of the extraction process for all three sample types, with an activated-charcoal column also producing high MC-LR and MC-RR recovery levels in fish samples. Most important, however, was the choice of the solvent system, with 15:4:1 methanol:water:butanol, 50% methanol, and EDTA-$Na_4P_2O_7$ solvents performing the best for fish, plants, and soils, respectively. When this suite of methods was used in combination with sonication times of 2, 5, and 10 min for fish, lettuce, and soil matrices, respectively, average MC-LR and MC-RR recovery rates ≥93% could be consistently achieved.

Collectively, this study's findings will aid efforts to measure the impact of cyanobacteria blooms on human health and some of the vital services that ecosystems provide. With improved methods to extract and quantify MC-LR and MC-RR in environmental and biological samples with complex matrices, agencies and decision-makers will be better positioned to assess whether cyanobacteria blooms are causing lost seafood or crop production in eutrophic water bodies or areas of the world where cyanobacteria-laden surface waters are used for crop irrigation (Ferrao and Kozlowsky-Suzuki, 2011; Zanchett and Oliveira-Filho, 2013). Likewise, these optimized extraction methods could be used in conjunction with behavioral research to explore whether MC exposure is leading to increased human health risk through the accumulation of MCs in the tissues of edible animals and plants that have been exposed to contaminated water. Given the potential benefits to assessing human welfare in the face of continued human-driven environmental change, continued research that assesses the general applicability of this study's methods (e.g., test more hydrophobic MCs, different soil and plant types, and naturally toxic samples) is strongly encouraged.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Research support was provided by Ohio Department of Higher Education (to SAL, JL, and KMR). We thank (1) The Ohio State University's (OSU's) College of Arts and Sciences for providing in-kind technical support for this project, (2) Dr. Qinghua Sun (Environmental Health Sciences, OSU) for providing access to laboratory equipment, (3) the ODNR-DOW for collecting the fish used in this study, and (4) OSU's Comprehensive Cancer Center (NIH P30 CA016058) for allowing us to analyze samples within the Nutrient and Phytochemical Analytic Shared Resource.[CG]

References

Ame MV, Galanti LN, Menone ML, Gerpe MS, Moreno VJ, Wunderlin DA, 2010 Microcystin-LR, –RR, –YR and –LA in water samples and fishes from a shallow lake in Argentina. Harmful Algae 9 (1), 66–73.

An J, Carmichael WW, 1994 Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. Toxicon 32 (12), 1495–1507. [PubMed: 7725318]

Beversdorf LJ, Miller TR, McMahon KD, 2013 The role of nitrogen fixation in cyanobacterial bloom toxicity in a temperate, eutrophic lake. PLoS One 8 (2), e56103. [PubMed: 23405255]

Bouaïcha N, Corbel S, 2016 Cyanobacterial toxins emerging contaminants in soils: a review of sources, fate and impacts on ecosystems, plants and animal and human health In: Larramendy ML, Soloneski S (Eds.), Soil Contamination – Current Consequences and Further Solutions. InTech, Rijeka (p. Ch. 06).

Brooks BW, Lazorchak JM, Howard MDA, Johnson M-VV, Morton SL, Perkins DAK, Reavie ED, Scott GI, Smith SA, Steevens JA, 2016 Are harmful algal blooms becoming the greatest inland water quality threat to public health and aquatic ecosystems? Environ. Toxicol. Chem 35 (1), 6–13. [PubMed: 26771345]

Cadel-Six S, Moyenga D, Magny S, Trotereau S, Edery M, Krys S, 2014 Detection of free and covalently bound microcystins in different tissues (liver, intestines, gills, and muscles) of rainbow trout(Oncorhynchus mykiss) by liquid chromatography–tandem mass spectrometry: method characterization. Environ. Pollut 185 (Supplement C), 333–339. [PubMed: 24316797]

Cazenave J, Wunderlin DA, de los Ángeles Bistoni M, Amé MV, Krause E, Pflugmacher S, Wiegand C, 2005 Uptake, tissue distribution and accumulation of microcystin-RR in Corydoras paleatus, Jenynsia multidentata and Odontesthes bonariensis: a field and laboratory study. Aquat. Toxicol 75 (2), 178–190. [PubMed: 16157397]

Chen W, Song L, Gan N, Li L, 2006 Sorption, degradation and mobility of microcystins in Chinese agriculture soils: risk assessment for groundwater protection. Environ. Pollut 144 (3), 752–758. [PubMed: 16632129]

Christoffersen K, Kaas H, 2000 Toxic cyanobacteria in water. A guide to their public health consequences, monitoring, and management. Limnol. Oceanogr 45 (5) 1212–1212.

Corbel S, Mougin C, Bouaïcha N, 2014 Cyanobacterial toxins: modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops. Chemosphere 96, 1–15. [PubMed: 24012139]

Corbel S, Mougin C, Nélieu S, Delarue G, Bouai'cha N, 2016 Evaluation of the transfer and the accumulation of microcystins in tomato (Solanum lycopersicum cultivar MicroTom) tissues using a cyanobacterial extract containing microcystins and the radiolabeled microcystin-LR (14 C-MC-LR). Sci. Total Environ 541, 1052–1058. [PubMed: 26473707]

Craig M, Luu HA, McCready TL, Williams D, Andersen RJ, Holmes CF, 1996 Molecular mechanisms underlying the interaction of motuporin and microcystins with type-1 and type-2A protein phosphatases. Biochem. Cell Biol 74 (4), 569–578. [PubMed: 8960363]

Dai M, Xie P, Liang G, Chen J, Lei H, 2008 Simultaneous determination of microcystin-LR and its glutathione conjugate in fish tissues by liquid chromatography-tandem mass spectrometry. J. Chromatogr. B 862 (1–2), 43–50.

do Carmo Bittencourt-Oliveira M, Cordeiro-Araújo MK, Chia MA, de Toledo Arruda-Neto JD, de Oliveira ÊT, dos Santos F, 2016 Lettuce irrigated with contaminated water: photosynthetic effects, antioxidative response and bioaccumulation of microcystin congeners. Ecotoxicol. Environ. Saf 128, 83–90. [PubMed: 26896895]

Ernst B, Dietz L, Hoeger SJ, Dietrich DR, 2005 Recovery of MC-LR in fish liver tissue. Environ. Toxicol 20 (4), 449–458. [PubMed: 16007663]

Fastner J, Erhard M, Carmichael WW, Sun F, Rinehart KL, Rönicke H, Chorus I, 1999 Characterization and diversity of microcystins in natural blooms and strains of the genera Microcystis and Planktothrix from German freshwaters. Arch. Hydrobiol 145 (2), 147–163.

Ferrao AD, Kozlowsky-Suzuki B, 2011 Cyanotoxins: bioaccumulation and effects on aquatic animals. Mar. Drugs 9 (12), 2729–2772. [PubMed: 22363248]

Fischer A, Hoeger SJ, Stemmer K, Feurstein DJ, Knobeloch D, Nussler A, Dietrich DR, 2010 The role of organic anion transporting polypeptides (OATPs/SLCOs) in the toxicity of different microcystin congeners in vitro: a comparison of primary human hepatocytes and OATP-transfected HEK293 cells. Toxicol. Appl. Pharmacol 245 (1), 9–20. [PubMed: 20171238]

Frazier K, Colvin B, Styer E, Hullinger G, Garcia R, 1998 Microcystin toxicosis in cattle due to overgrowth of blue-green algae. Vet. Hum. Toxicol 40 (1), 23–24. [PubMed: 9467204]

Geis-Asteggiante L, Lehotay SJ, Fortis LL, Paoli G, Wijey C, Heinzen H, 2011a Development and validation of a rapid method for microcystins in fish and comparing LC-MS/MS results with ELISA.Anal. Bioanal. Chem401 (8), 2617–2630. [PubMed: 21881880]

Geis-Asteggiante L, Lehotay SJ, Fortis LL, Paoli G, Wijey C, Heinzen H, 2011b Development and validation of a rapid method for microcystins in fish and comparing LC-MS/MS results with eLiSA. Anal. Bioanal. Chem 401 (8), 2617–2630. [PubMed: 21881880]

Geneva I, 1998 Cyanobacterial Toxins: Microcystin-LR in Drinking Water..

Giddings M, Aranda-Rodriguez R, Yasvinski G, Watson S, Zurawell R, 2012 Canada: Cyanobacterial Toxins; Drinking and Recreational Water Quality Guidelines. Current Approaches to Cyanotoxin Risk Assessment, Risk Management and Regulations in Different Countries. Federal Environment Agency (Umweltbundesamt), Germany, pp. 29–39. hhttp://www.umweltdaten.de/publi-kationen/fpdf-l/4390.pdfi.

Greer B, Maul R, Campbell K, Elliott CT, 2017 Detection of freshwater cyanotoxins and measurement of masked microcystins in tilapia from Southeast Asian aquaculture farms. Anal. Bioanal. Chem 409(16), 4057–4069. [PubMed: 28429062]

Guo X, Xie P, Chen J, Tuo X, Deng X, Li S, Yu D, Zeng C, 2014 Simultaneous quantitative determination of microcystin-LR and its glutathione metabolites in rat liver byliquid chromatography-tandem mass spectrometry. J. Chromatogr. B 963, 54–61.

Gutiérrez-Praena D, Campos A, Azevedo J, Neves J, Freitas M, Guzmán-Guillén R, Cameán AM, Renaut J, Vasconcelos V, 2014 Exposure of Lycopersicon Esculentum to microcystin-LR: Effects in the leaf proteome and toxin translocation from water to leaves and fruits. Toxins 6 (6), 1837–1854. [PubMed: 24921194]

Ibelings BW, Bruning K, De Jonge J, Wolfstein K, Pires LD, Postma J, Burger T, 2005 Distribution of microcystins in a lake foodweb: no evidence for biomagnification. Microb. Ecol 49 (4), 487–500. [PubMed: 16052377]

Järvenpää S, Lundberg-Niinistö C, Spoof L, Sjövall O, Tyystjärvi E, Meriluoto J, 2007 Effects of microcystins on broccoli and mustard, and analysis of accumulated toxin by liquid chromatography-mass spectrometry. Toxicon 49 (6), 865–874. [PubMed: 17275870]

Jacquet C, Thermes V, De Luze A, Puiseux-Dao S, Bernard C, Joly J-S, Bourrat F, Edery M, 2004 Effects of microcystin-LR on development of medaka fish embryos (Oryzias latipes). Toxicon 43 (2), 141–147. [PubMed: 15019473]

Jia J, Luo W, Lu Y, Giesy JP, 2014 Bioaccumulation of microcystins (MCs) in four fish species from Lake Taihu, China: assessment of risks to humans. Sci. Total Environ 487 (Supplement C), 224–232. [PubMed: 24784747]

Karlsson KM, Spoof LE, Meriluoto JA, 2005 Quantitative LC-ESI-MS analyses of microcystins and nodularin-R in animal tissue—Matrix effects and method validation. Environ. Toxicol 20 (3), 381–389. [PubMed: 15892039]

Kohoutek J, Adamovsky O, Oravec M, Simek Z, Palikova M, Kopp R, Blaha L, 2010 LC-MS analyses of microcystins in fish tissues overestimate toxin levels-critical comparison with LC-MS/MS. Anal. Bioanal. Chem 398 (3), 1231–1237. [PubMed: 20535609]

Lawton LA, Edwards C, Codd GA, 1994 Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst 119 (7), 1525–1530. [PubMed: 7943743]

Lee S, Jiang X, Manubolu M, Riedl K, Ludsin SA, Martin JF, Lee J, 2017a Fresh produce and their soils accumulate cyanotoxins from irrigation water: implications for public health and food security. Food Res. Int 102, 234–245. [PubMed: 29195944]

Lee J, Lee S, Jiang X, 2017b Cyanobacterial Toxins in Freshwater and Food: Important Sources of Exposure to Humans. Annu. Rev. Food Sci. Technol 8, 281–304. [PubMed: 28245155]

Li H, Pan G, 2015 Simultaneous removal of harmful algal blooms and microcystins using microorganism-and chitosan-modified local soil. Environ. Sci. Technol 49 (10), 6249–6256. [PubMed: 25901393]

Li YW, Zhan XJ, Xiang L, Deng ZS, Huang BH, Wen HF, Sun TF, Cai QY, Li H, Mo CH, 2014 Analysis of trace microcystins in vegetables using solid-phase extraction followed by high performance liquid chromatography triple-quadrupole mass spectrometry. J. Agric. Food Chem 62 (49), 11831–11839. [PubMed: 25393522]

Manubolu M, Madawala SR, Dutta PC, Malmlof K, 2014 Invitro biodegradation of cyanotoxins in the rumen fluid of cattle. BMC Vet. Res 10, 110. [PubMed: 24885733]

Merel S, Walker D, Chicana R, Snyder S, BaurÃs E, Thomas O, 2013 State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environ. Int 59, 303–327. [PubMed: 23892224]

Meriluoto J, Codd G, Reilly M, Metcalf JS, Spoof L, Sjövall O, Hall T, Izydorczyk K, Lindholm T, Kull T, 2005 TOXIC: Cyanobacterial Monitoring and Cyanotoxin Analysis. .

Messineo V, Bogialli S, Melchiorre S, Sechi N, Lugliè A, Casiddu P, Mariani MA, Padedda BM, Corcia AD, Mazza R, Carloni E, Bruno M, 2009 Cyanobacterial toxins in Italian freshwaters. Limnol. Ecol. Manage. Inland Waters 39 (2), 95–106.

Michalak AM, Anderson EJ, Beletsky D, Boland S, Bosch NS, Bridgeman TB, Chaffin JD, Cho K, Confesor R, DaloÄŸlu I, DePinto JV, Evans MA, Fahnenstiel GL, He L, Ho JC, Jenkins L, Johengen TH, Kuo KC, LaPorte E, Liu X, McWilliams MR, Moore MR, Posselt DJ, Richards RP, Scavia D, Steiner AL, Verhamme E, Wright DM, Zagorski MA, 2013 Record-setting algal bloom in Lake Erie caused by agricultural and meteorological trends consistent with expected future conditions. Proc. Natl. Acad. Sci 110 (16), 6448–6452. [PubMed: 23576718]

Moreno IM, Molina R, Jos A, Pico Y, Cameán AM, 2005 Determination of microcystins in fish by solvent extraction and liquid chromatography. J. Chromatogr. A 1080 (2), 199–203. [PubMed: 16008059]

Oberemm A, Becker J, Codd G, Steinberg C, 1999 Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians. Environ. Toxicol 14 (1), 77–88.

Ohio EPA, 2007 Preparation of Tissue Samples for Extraction of Organic and Metals Determination Ohio EPA method 581.10, Cincinnati, OH, USA.

Paerl HW, Huisman J, 2008 Blooms like it hot. Science 320 (5872), 57–58. [PubMed: 18388279]

Pekar H, Westerberg E, Bruno O, Laane A, Persson KM, Sundstrom LF, Thim AM, 2016 Fast, rugged and sensitive ultra high pressure liquid chromatography tandem mass spectrometry method for analysis of cyanotoxins in raw water and drinking water-first findings of anatoxins, cylindrospermopsins and microcystin variants in Swedish source waters and infiltration ponds.J. Chromatogr. A 2016 (15), 265–276.

Prieto A, Campos A, Camean A, Vasconcelos V, 2011 Effects on growth and oxidative stress status of rice plants (Oryza sativa) exposed to two extracts of toxin-producing cyanobacteria (Aphanizomenon ovalisporum and Microcystis aeruginosa). Ecotoxicol. Environ. Saf 74 (7), 1973–1980. [PubMed: 21723604]

Qian Z-Y, Li Z-G, Ma J, Gong T.-t., Xian Q-M, 2017 Analysis of trace microcystins in vegetables using matrix solid-phase dispersion followed by high performance liquid chromatography triple-quadrupole mass spectrometry detection. Talanta 173 (Supplement C), 101–106. [PubMed: 28602183]

Ríos V, Moreno I, Prieto AI, Puerto M, Gutiérrez-Praena D, Soria-Díaz ME, Cameán AM, 2013 Analysis of MC-LR and MC-RR in tissue from freshwater fish (Tinca tinca) and crayfish (Procambarus clarkii) in tench ponds (Cíceres, Spain) by liquid chromatography–mass spectrometry (LC-MS). Food Chem. Toxicol 57, 170–178. [PubMed: 23537600]

Rita DP, Valeria V, Silvia BM, Pasquale G, Milena B, 2014 Microcystin contamination in sea mussel farms from the italian southern adriatic coast following cyanobacterial blooms in an artificial reservoir. J. Ecosyst 2014, 11.

Rodriguez-Mozaz S, Lopez de Alda MJ, Barcelo D, 2007 Advantages and limitations of on-line solid phase extraction coupled to liquid chromatography-mass spectrometry technologies versus biosensors for monitoring of emerging contaminants in water. J. Chromatogr. A 8, 1–2.

Saqrane S, Oudra B, 2009 CyanoHAB occurrence and water irrigation cyanotoxin contamination: ecological impacts and potential health risks. Toxins 1 (2), 113–122. [PubMed: 22069535]

Saqrane S, Ghazali IE, Oudra B, Bouarab L, Vasconcelos V, 2008 Effects of cyanobacteria producing microcystins on seed germination and seedling growth of several agricultural plants. J. Environ. Sci. Health Part B 43 (5), 443–451.

Schmidt J, Shaskus M, Estenik J, Oesch C, Khidekel R, Boyer G, 2013 Variations in the microcystin content of different fish species collected from a eutrophic lake. Toxins 5 (5), 992–1009. [PubMed: 23676698]

Silva-Stenico ME, Cantflsio, Neto R, Alves I.n.R., Moraes LAB, Shishido T.n.K., Fiore M.F.t., 2009 Hepatotoxin microcystin-LR extraction optimization. J. Braz. Chem. Soc 20, 535–542.

Smith J, Boyer G, 2009 Standardization of Microcystin Extraction from Fish Tissues: A Novel Internal Standard as a Surrogate for Polar and Non-polar Variants. .

Toxins C, 2003 Microcystin-LR in Drinking-Water. Background Document for Development of WHO Guidelines for Drinking-Water Quality. World Health Organization.

Tsuji K, Masui H, Uemura H, Mori Y, Harada K. i., 2001 Analysis of microcystins in sediments using MMPB method. Toxicon 39 (5), 687–692. [PubMed: 11072048]

Vasconcelos VM, 1999 Cyanobacterial toxins in Portugal: effects on aquatic animals and risk for human health. Braz. J. Med. Biol. Res 32, 249–254. [PubMed: 10347780]

Vesterkvist P, Misiorek J, Spoof L, Toivola D, Meriluoto J, 2012 Comparative cellular toxicity of hydrophilic and hydrophobic microcystins on caco-2Cells. Toxins 4 (11), 1008. [PubMed: 23202304]

Wu L, Xie P, Chen J, Zhang D, Liang G, 2010 Development and validation of a liquid chromatography-tandem mass spectrometry assay for the simultaneous quantitation of microcystin-RR and its metabolites in fish liver. J. Chromatogr. A 1217 (9), 1455–1462. [PubMed: 20060532]

Xie L, Park H-D, 2007a Determination of Microcystins in FishTissues UsingHPLC with a Rapid and Efficient Solid Phase Extraction. .

Xie L, Park H-D, 2007b Determination of microcystins in fish tissues using HPLC with a rapid and efficient solid phase extraction. Aquaculture 271 (1–4), 530–536.

Xue Q, Su X, Steinman AD, Cai Y, Zhao Y, Xie L, 2016 Accumulation of microcystins in a dominant Chironomid Larvae (Tanypus chinensis) of a large, shallow and eutrophic Chinese lake. Lake Taihu. 6, 31097.

Yang B, Xu J-Z, Ding T, Wu B, Jing S, Ding -j S, Chen H-L, Sheng C-Y, Jiang Y, 2009 A novel method to detect seven microcystins in hard clam and corbicula fluminea by liquid chromatography-tandem mass spectrometry. J. Chromatogr. B 877 (29), 3522–3528.

Yang F, Zhou Y, Sun R, Wei H, Li Y, Yin L, Pu Y, 2013 Biodegradation of microcystin-LR and-RR by a novel microcystin-degrading bacterium isolated from Lake Taihu. Biodegradation 25 (3), 447–457. [PubMed: 24179091]

Yongtao L, Joshua SW, 2015 Rapid and Accurate Analysis of Microcystins Using UPLC/MS/MS. Inc NEMC, Chicago, IL.

Zanchett G, Oliveira-Filho EC, 2013 Cyanobacteria and cyanotoxins: from impacts on aquatic ecosystems and human health to anticarcinogenic effects. Toxins (Basel) 5 (10), 1896–1917. [PubMed: 24152991]

Zhang H-J, Zhang J-Y, Hong Y, Chen Y-X, 2007 Evaluation of organ distribution of microcystins in the freshwater phytoplanktivorous fish Hypophthalmichthys molitrix. J. Zhejiang Univ.-Sci. B 8 (2), 116–120. [PubMed: 17266187]

Zhang D, Xie P, Liu Y, Qiu T, 2009 Transfer, distribution and bioaccumulation of microcystins in the aquatic food web in Lake Taihu, China, with potential risks to human health. Sci. Total Environ 407 (7), 2191–2199. [PubMed: 19185334]

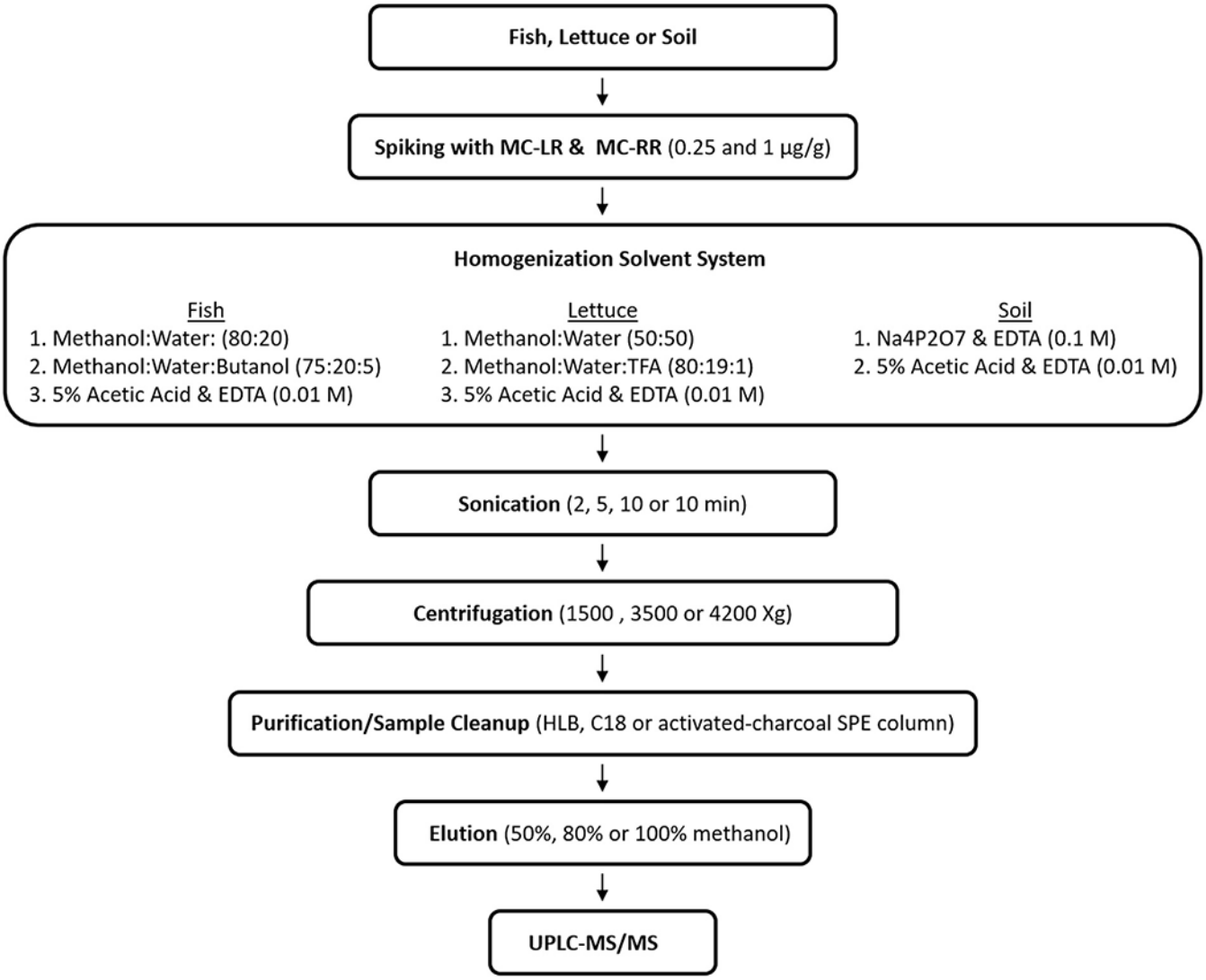

Fig. 1.

Schematic showing how samples with complex matrices (fish, lettuce, and soil) were prepared for microcystin (MC) quantification using UPLC-MS/MS in this study's controlled experiments. An experiment was conducted with each sample type to optimize the recovery of MC-LR and MC-RR (spiking levels of 0.25 and 1 µg/g wet mass). These experiments sought to identify the best (1) solvent system to use during homogenization (2–3 tested per sample type) and (2) single-phase extraction (SPE) column type (3 tested) to use in the final purification ("sample cleanup") step. These experiments also offered insight into other technical aspects of the MC homogenization and extraction process, including sonication and centrifugation speed during homogenization, as well as the elution solvent to use during the final extraction.

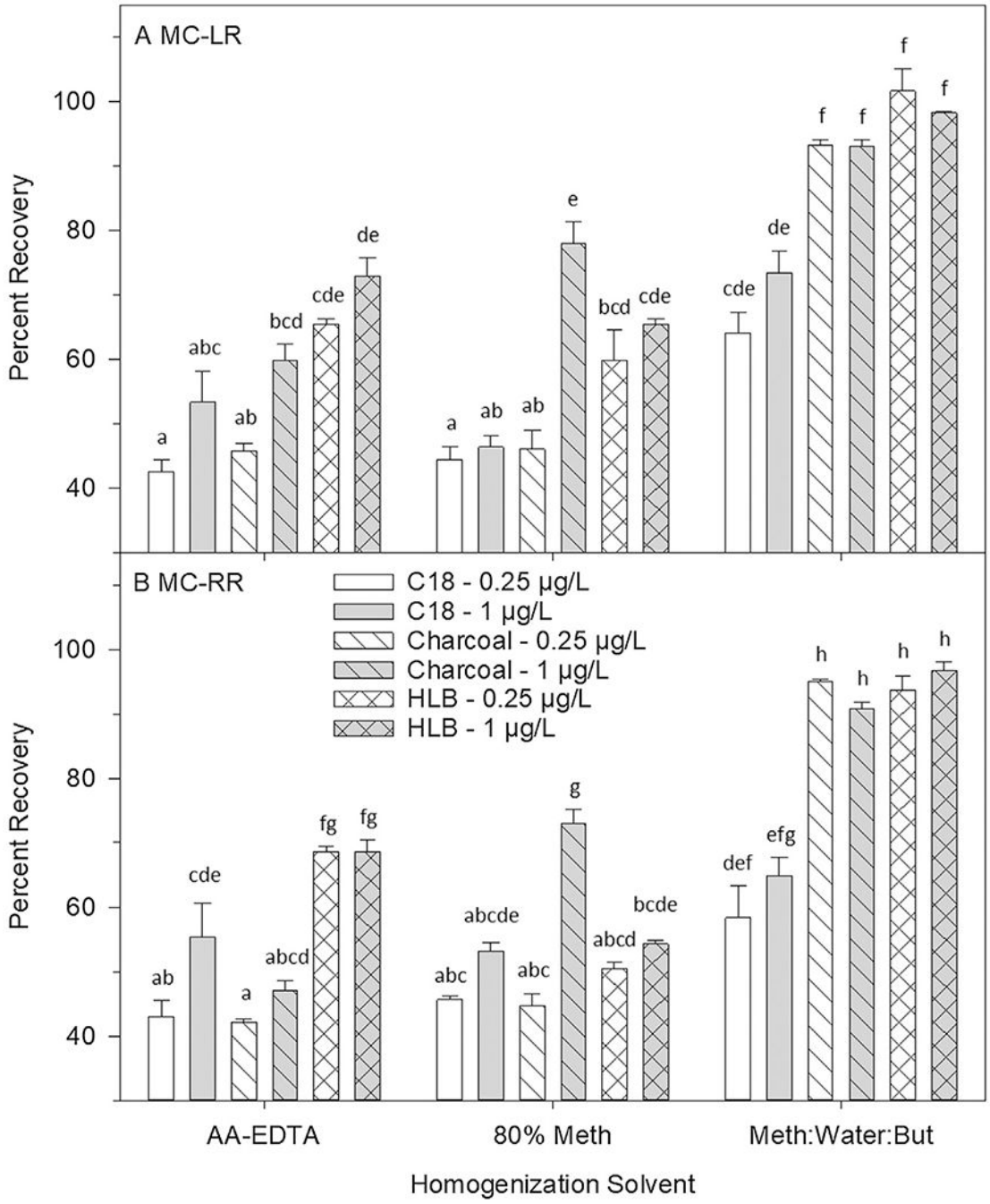

Fig. 2.

Mean (±1 standard error) percent recovery of A) MC-LR and B) MC-RR using UPLC–MS/MS in fish muscle tissues using different combinations of homogenization solvents (5% acetic acid & 0.01 M EDTA, AA-EDTA; 80:20 methanol:water, 80% Meth; and 15:4:1 (75:20:5 vol:vol:vol) methanol:water:butanol, Meth:Wat:But), SPE columns (carbon, C18; activated-charcoal; and hydrophilic-lipophilic balance, HLB), and microcystin (MC) spiking levels (0.25 and 1 µg/g wet mass). Bars with letters in common above them do not

statistically differ (three-way ANOVA, Tukey's honestly significant different test; alpha-level = 0.025).

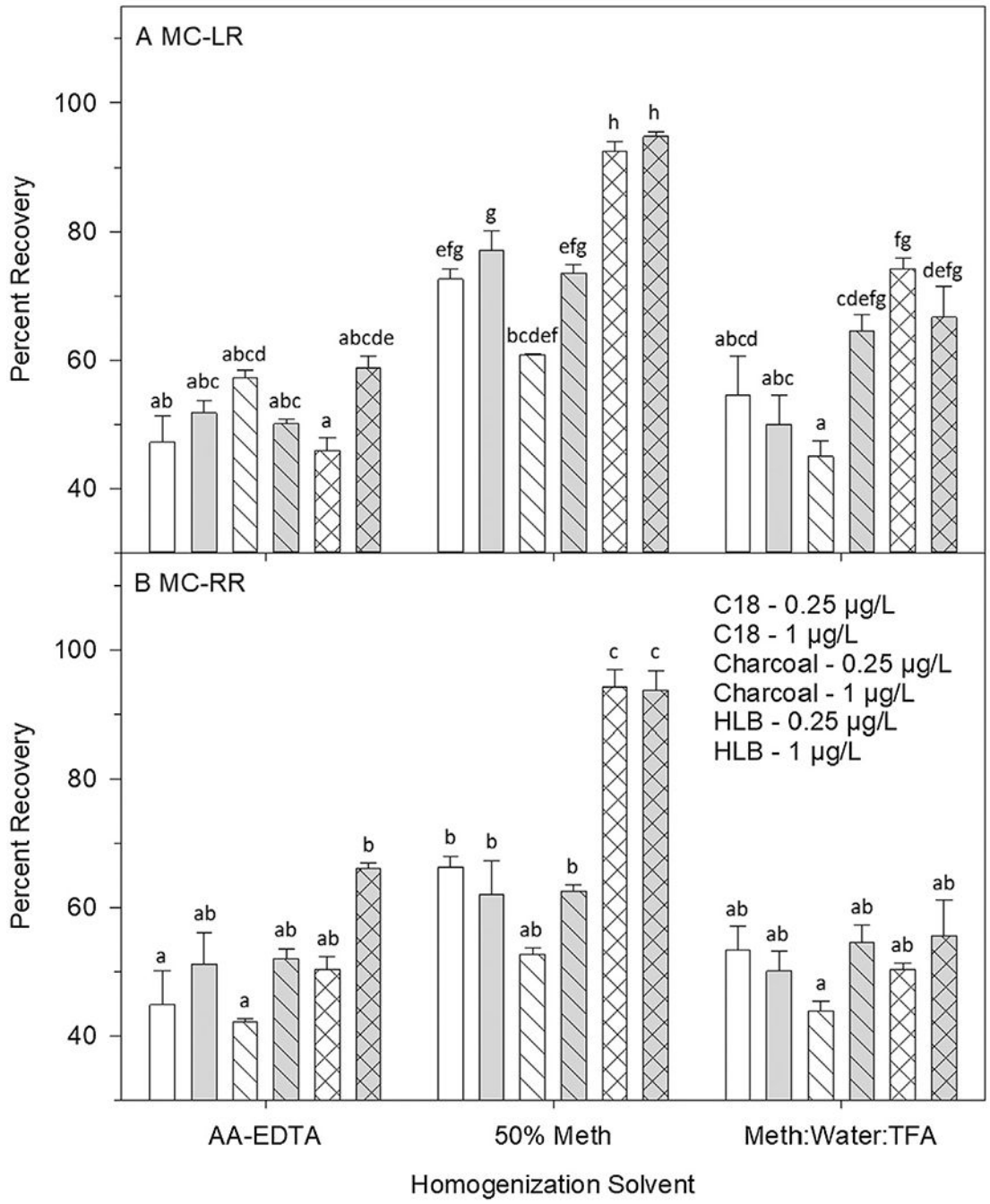

Fig. 3.

Mean (±1 standard error) percent recovery of A) MC-LR and B) MC-RR using UPLC–MS/MS in lettuce samples using different combinations of homogenization solvents (5% acetic acid & 0.01 M EDTA, AA-EDTA; 50:50 methanol:water, 50% Meth; and 80:19:1 methanol:water:trifluoroacetic acid, Meth:Wat:TFA), SPE columns (carbon, C18; activated-charcoal; and hydrophilic–lipophilic balance, HLB), and microcystin (MC) spiking levels (0.25 and 1 µg/g wet mass). Bars with letters in common above them do not statistically differ (three-way ANOVA, Tukey's honestly significant different test; alpha-level = 0.025).

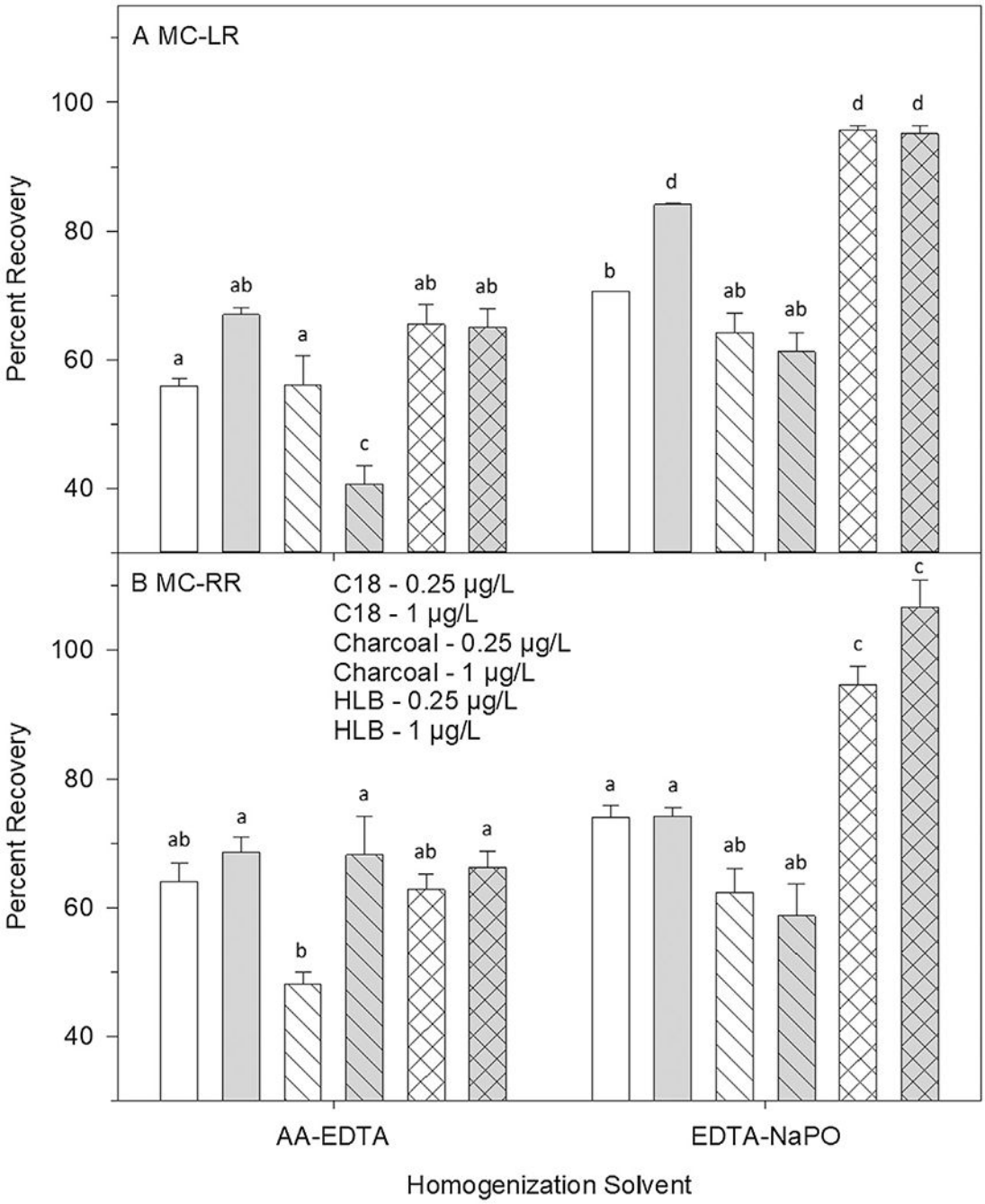

Fig. 4.

Mean (±1 standard error) percent recovery of A) MC-LR andB) MC-RR using UPLC–MS/MS in soil samples using different combinations of homogenization solvents (5% acetic acid & 0.01 M EDTA, AA-EDTA; and $Na_4P_2O_7$ and 0.1 M EDTA, EDTA-$Na_4P_2O_7$), SPE columns (carbon, C18; activated-charcoal; and hydrophilic–lipophilic balance, HLB), and microcystin (MC) spiking levels (0.25 and 1 µg/g wet mass). Bars with letters in common

above them do not statistically differ (three-way ANOVA, Tukey's honestly significant different test; alpha-level = 0.025).

Table 1.

Three-way ANOVA results from the analysis of the effect of homogenization solvent (Solvent), solid-phase extraction (SPE) filtration column type, and microcystin spiking level (Spike) on the recovery of MC-LR and MC-RR from fish muscle (left column), lettuce (middle column), and soil (right column) matrices. Main factors and interactions that are significant ($p < 0.05$) are in bold.

	Fish MC-LR					**Lettuce MC-LR**					**Soil MC-LR**				
Effect	**df**	**SS**	**MS**	**F**	**p**	**df**	**SS**	**MS**	**F**	**p**	**df**	**SS**	**MS**	**F**	**p**
Intercept	1	241,748	241,748	11,193	**<0.0001**	1	215,712	215,712	9196	**<0.0001**	1	168,647	168,647	9775	**<0.0001**
Solvent	2	11,236	5618	260	**<0.0001**	2	6839	3420	146	**<0.0001**	1	3648	3648	211	**<0.0001**
Spike	1	1000	1000	46	**<0.0001**	1	232	232	10	**0.0033**	1	7	7	0	0.5335
SPE	2	5020	2510	116	**<0.0001**	2	2167	1083	46	**<0.0001**	2	3700	1850	107	**<0.0001**
Solvent*Spike	2	311	156	7	**0.0023**	2	39	19	1	0.4477	1	55	55	3	0.0878
Solvent*SPE	4	846	212	10	**<0.0001**	4	1269	317	14	**<0.0001**	2	456	228	13	**0.0001**
Spike*SPE	2	335	167	8	**0.0016**	2	124	62	3	0.0842	2	697	349	20	**<0.0001**
Solvent*Spike*SPE	4	625	156	7	**0.0002**	4	933	233	10	**<0.0001**	2	66	33	2	0.1692
Error	36	778	22			36	844	24			24	414	17		
	Fish MC-RR					**Lettuce MC-RR**					**Soil MC-RR**				
Effect		**SS**	**MS**	**F**	**p**		**SS**	**MS**	**F**	**p**		**SS**	**MS**	**F**	**p**
Intercept	1	218,661	218,661	14,421	**<0.0001**	1	182,568	182,568	6208	**<0.0001**	1	180,175	180,175	5364	**<0.0001**
Solvent	2	10,385	5193	342	**<0.0001**	2	5143	2572	87	**<0.0001**	1	2131	2131	63	**<0.0001**
Spike	1	643	643	42	**<0.0001**	1	408	408	14	**0.0007**	1	340	340	10	**0.0040**
SPE	2	3230	1615	107	**<0.0001**	2	2940	1470	50	**<0.0001**	2	3247	1623	48	**<0.0001**
Solvent*Spike	2	306	153	10	**0.0003**	2	191	96	3	0.0504	1	95	95	3	0.1064
Solvent*SPE	4	3226	807	53	**<0.0001**	4	2053	513	17	**<0.0001**	2	1958	979	29	**<0.0001**
Spike*SPE	2	144	72	5	**0.0147**	2	258	129	4	**0.0197**	2	64	32	1	0.4001
Solvent*Spike*SPE	4	581	145	10	**<0.0001**	4	114	29	1	0.4356	2	394	197	6	**0.0084**
Error	36	546	15			36	1059	29			24	806	34		

Table 2.

Homogenization solvent systems, solid-phase extraction (SPE) column types, and percent recovery of microcystins (Rec.%) in studies that have measured microcystins in fish, plants, and soils. AA = 5% acetic acid, EDTA = 0.01 M ethylenediaminetetraacetic acid; MeOH = methanol; BuOH = butanol; and TFA = trifluoroacetic acid.

Sample	Homogenization Solvent	SPE column	Detection	MC Congeners	Rec.%	Citation
Fish						
Muscle	BuOH: MeOH: H_2O (1:4:15)	HLB	LC–MS	LR,RR	85	Xie and Park (2007a)
Liver muscle	BuOH: MeOH: H_2O (1:4:15)	Reversed phase ODS	HPLC	LR, RR, YR	48–86	Jia et al. (2014)
Liver & muscle	BuOH: MeOH: H_2O (1:4:15)	Strata X 60 and HLB	LC–MS	LR	36–81	Cadel-Six et al. (2014)
Muscle	BuOH: MeOH: H_2O (1:4:15)	C18	LC–MS	LR, RR, YR	66–88	Zhang et al. (2009)
Liver	BuOH: MeOH: H_2O (1:4:15)	C18	HPLC	LR	45–81	Ernst et al. (2005)
Muscle	100% MeOH	HLB	LC–MS	RR, YR, LR, LA, LY, LW, LF	47–93	Yang et al. (2009)
Liver	85% MeOH	ND	LC–MS	LR, RR, YR	68–105	Moreno et al. (2005)
Liver & muscle	80% MeOH	C18	LC–MS	LR, RR, LA	80–99	Smith and Boyer (2009)
Liver	70% MeOH	C18	LCMS	LR	68–96	Ibelings et al. (2005)
Liver	75% MeOH	C18	LC–MS	LR, RR, LF, LW	57	Karlsson et al. (2005)
Liver & muscle	75% MeOH	HLB	HPLC	LR, RR	74–88	Zhang et al. (2007)
Muscle	75% MeOH	ND	LC–MS	LR, RR, LA, YR	71–80	Lawrence and Menard (2001)
Liver & muscle	75% MeOH–1% TFA	C18	HPLC	RR	80	Cazenave et al. (2005)
Liver & muscle	75% MeOH (0.5% AA)	HLB	UPLC–MS/MS	LR, YR, RR, LA, LY, LF	76–87	Greer et al. (2017)
Liver	EDTA-$Na_4P_2O_7$-AA	HLB	LC–MS	LR, LR-GSH	90	Dai et al. (2008)
Muscle	AA-EDTA	Charcoal SPE	LC–MS/MS	LR	54–106	Schmidt et al. (2013)
Liver & muscle	EDTA-$Na_4P_2O_7$-AA	HLB	LC–MS	RR, –LR, YR	80–110	Ríos et al. (2013)
Liver	EDTA-$Na_4P_2O_7$-AA	HLB	LC–MS	LR, LR-GSH, LR-Cys	70–98	Guo et al. (2014)
Liver	AA-EDTA	Cation-exchange SPE	LC–ESI–MS	RR	94–99	Wu et al. (2010)
Muscle	Acidified acetonitrile:H_2O (3:1)	C18	LC–MS	RR, YR, LR, WR, LA, LY, LW, LF	70–100	Geis-Asteggiante et al. (2011a)
Plant						
Lettuce leaf	100% H_2O	NA	LC–MS	LR, RR	NA	do Carmo Bittencourt-Oliveira (2016)
Broccoli	100% MeOH	Super clean LC-18	LC–MS	LR, RR, YR	56–81	Järvenpää et al. (2007)
Plant –Tissue	50% MeOH	C-18	LC–MS	LR	NA	Gutiérrez-Praena et al. (2014)
Plant tissue	75% MeOH	C-18	UPLC–MS/MS	LR	NA	Corbel et al. (2016)

Sample	Homogenization Solvent	SPE column	Detection	MC Congeners	Rec.%	Citation
Plant –Tissue	MeOH:H_2O:TFA (80:19:1)	C-18 & HLB	LC–MS/MS	LR, RR, YR	61–117	Li et al. (2014)
Soil						
Soil	H_2O	C18	HPLC	LR, RR, YR	56–81	Järvenpää et al. (2007)
Soil & sediment	EDTA-$Na_4P_2O_7$	Sep-pak ODS	HPLC	LR, RR, [Dha7]LR	90	Chen et al. (2006)
Soil	EDTA-$Na_4P_2O_7$	C18	UPLC–MS/MS	LR	NA	Corbel et al. (2016)
Soil	EDTA-$Na_4P_2O_7$	C-18	UPLC–MS/MS	LR, RR	91–103	Li and Pan (2015)

*NA: not available.