Single Laboratory Validated Method for Determination of Microcystins and Nodularin in Ambient Freshwaters by Solid Phase Extraction and Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)

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1. SCOPE AND APPLICATION

1.1 This is a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for determination of microcystins and nodularin (combined intracellular and extracellular) in ambient freshwater. Accuracy and precision data have been generated in reagent water and ambient freshwaters for compounds listed in the table below.

Analyte	Chemical Abstract Services Registry Number (CASRN)
3-desmethylated-microcystin-LR (3-dm-MC-LR)	120011-66-7
3-desmethylated-microcystin-RR (3-dm-MC-RR)	202120-08-9
7-desmethylated-microcystin-LR (7-dm-MC-LR)	134842-07-2
microcystin-HilR (MC-HilR)	Not assigned
microcystin-HtyR (MC-HtyR)	Not assigned
microcystin-LA (MC-LA)	96180-79-9
microcystin-LF (MC-LF)	154037-70-4
microcystin-LR (MC-LR)	101043-37-2
microcystin-LW (MC-LW)	157622-02-1
microcystin-LY (MC-LY)	123304-10-9
microcystin-RR (MC-RR)	111755-37-4
microcystin-WR (MC-WR)	138234-58-9
microcystin-YR (MC-YR)	101064-48-6
nodularin-R (NOD)	118399-22-7

1.2 The Minimum Reporting Level (MRL) is the lowest analyte concentration that meets Data Quality Objectives (DQOs) that are developed based on the intended use of this method. The single laboratory lowest concentration MRL (LCMRL) is the lowest true concentration for which the future recovery is predicted to fall, with high confidence (99%), between 50 and 150% recovery. Single laboratory LCMRLs for analytes in this method range from 14-170 ng/L using Option A procedure (Sect. 11.4), and are listed in Table 5. The procedure used to determine the LCMRL is described elsewhere.[1]

1.3 Laboratories using this method will not be required to determine the LCMRL for this method, but will need to demonstrate that their laboratory MRL for this method meets the requirements described in Section 9.2.4.

1.4 Determining the Detection Limit (DL) for analytes in this method is optional (Sect. 9.2.6). Detection limit is defined as the statistically calculated minimum concentration that can be measured with 99% confidence that the reported value is greater than zero.[2] The DL is compound dependent and is dependent on extraction efficiency, sample matrix, fortification concentration, and instrument performance. DLs for analytes in this method range from 2.1-33 ng/L using Option A procedure (Sect. 11.4), and are listed in Table 5.

1.5 This method is intended for use by analysts skilled in solid phase extractions, operation of LC/MS/MS instruments, and the interpretation of associated data.

1.6 METHOD FLEXIBILITY – In recognition of technological advances in analytical systems and techniques, the laboratory is permitted to modify the evaporation technique, separation technique, LC column, mobile phase composition, LC conditions and MS and MS/MS conditions. Changes may not be made to sample collection and preservation (Sect. 8), sample extraction or intracellular toxin release steps (Sect. 11), or to quality control requirements (Sect. 9). Method modifications should be considered only to improve method performance. Modifications that are introduced in the interest of reducing cost or sample processing time, but result in poorer method performance, should not be used. Analytes must be adequately resolved chromatographically in order to permit the mass spectrometer to dwell on a minimum number of compounds eluting within a retention time window. Instrumental sensitivity (or signal-to-noise) will decrease if too many compounds are permitted to elute within a retention time window. In all cases where method modifications are proposed, the analyst must perform the procedures outlined in the initial demonstration of capability (IDC, Sect. 9.2), verify that all Quality Control (QC) acceptance criteria (Sect. 9) are met, and ensure that acceptable method performance can be verified in a real sample matrix (Sect. 9.3.6).

NOTE: The above section is intended as an abbreviated summation of method flexibility. Sections 6-12 provide detailed information of specific portions of the method that may be modified. If there is any perceived conflict between the general method flexibility statement in Section 1.6 and specific information in Sections 6-12, Sections 6-12 supersede Section 1.6.

2. SUMMARY OF METHOD

A water sample is filtered and intracellular toxins are released from cyanobacterial cells following two possible procedures chosen by visual transparency or cell density of the sample. Option A for clear to semi-transparent samples: A 100-mL water sample (fortified with a surrogate) is filtered and both the filtrate and filter are collected. The filter is placed in a solution of 80:20 methanol:reagent water (v/v)and held for at least one hour at -20 ºC to release intracellular toxins from cyanobacteria cells captured on the filter. The liquid is drawn off the filter and added back to the 100-mL aqueous filtrate. Option B for semi-transparent to opaque samples: A 10 mL water sample (fortified with a surrogate) is combined with 30 mL of methanol in a centrifuge tube and held for at least two hours

at -20 ºC to release intracellular toxins from cyanobacteria cells. The sample is centrifuged and the supernatant is filtered. The filtrate is diluted with reagent water to provide a sample appropriate for extraction. The filtered sample, containing intracellular and extracellular toxins (either Option A or B), is passed through a solid phase extraction (SPE) cartridge to extract the method analytes and surrogate. Analytes are eluted from the solid phase with a small amount of 90:10 methanol:reagent water (v/v). The extract is concentrated to dryness by evaporation with nitrogen in a heated water bath, and then adjusted to a 1-mL volume with 90:10 methanol:reagent water (v/v). A 10-µL injection is made into an LC equipped with a C_8 column that is interfaced to an MS/MS. Analytes are separated and identified by comparing the acquired mass spectra and retention times to reference spectra and retention times for calibration standards acquired under identical LC/MS/MS conditions. The concentration of each analyte is determined by internal standard calibration.

3. DEFINITIONS

3.1 ANALYSIS BATCH – A set of samples that is analyzed on the same instrument during a 24-hour period, including no more than 20 field samples, that begins and ends with the analysis of the appropriate Continuing Calibration Check (CCC) standards. Additional CCCs may be required depending on the length of the analysis batch or the number of field samples.

3.2 CALIBRATION STANDARD (CAL) – A solution prepared from the primary dilution standard solution or stock standard solution, the surrogate, and the internal standard. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.3 COLLISIONALLY ACTIVATED DISSOCIATION (CAD) – The process of converting the translational energy of the precursor ion into internal energy by collisions with neutral gas molecules to bring about dissociation into product ions.

3.4 CONTINUING CALIBRATION CHECK (CCC) – A calibration standard containing the method analytes, surrogate(s) and internal standard(s). The CCC is analyzed to verify the accuracy of the existing calibration for those analytes.

3.5 DETECTION LIMIT (DL) – The minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero. This is a statistical determination of precision (Sect. 9.2.6), and accurate quantitation is not expected at this level.[2]

3.6 EXTRACTION BATCH – A set of up to 20 field samples (not including QC samples) processed together (filtration, toxin release, extraction and evaporation) by the same person during a 24-hour work shift using the same lot of filters, SPE devices, solvents, surrogate, and fortifying solutions. Required QC samples include Laboratory Reagent Blank, Laboratory Fortified Blank, Laboratory Fortified Sample Matrix, and Laboratory Fortified Sample Matrix Duplicate.

3.7 FIELD DUPLICATES (FD) – Separate samples collected at the same time, shipped, and stored under identical conditions as the field sample. Analyses of FDs give a measure of the homogeneity of cyanotoxin concentrations within a cyanobacteria bloom.

3.8 INTERNAL STANDARD (IS) – A pure compound that is added to all standard solutions and samples in a known amount and used to measure the relative response of other method analytes that are components of the same solution. The internal standard must respond similarly to the method analytes, have no potential to be present in water samples, and not be a method analyte.

3.9 ION SUPPRESSION/ENHANCEMENT – An observable decrease or increase in analyte response in complex (field) samples as compared to the response obtained in standard solutions.

3.10 LABORATORY FORTIFIED BLANK (LFB) – A volume of reagent water or other blank matrix to which known quantities of method analytes and all preservation compounds are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

3.11 LABORATORY FORTIFIED SAMPLE MATRIX (LFSM) – A preserved field sample to which known quantities of method analytes are added in the laboratory. The LFSM is processed and analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. Background concentrations of the analytes in the sample matrix must be determined in a separate sample extraction and measured values in the LFSM corrected for background concentrations.

3.12 LABORATORY FORTIFIED SAMPLE MATRIX DUPLICATE (LFSMD) – A duplicate of the Field sample used to prepare the LFSM. The LFSMD is fortified, extracted, and analyzed identically to the LFSM. The LFSMD is used instead of the Field Duplicate to assess method precision when the occurrence of method analytes is infrequent.

3.13 LABORATORY REAGENT BLANK (LRB) – An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents and reagents, sample preservatives, surrogate(s) and internal standard(s) that are used in the analysis batch. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, reagents, or apparatus.

3.14 LOWEST CONCENTRATION MINIMUM REPORTING LEVEL (LCMRL) – The single laboratory LCMRL is the lowest true concentration for which a future recovery is expected, with 99% confidence, to be between 50 and 150% recovery.[1]

3.15 MINIMUM REPORTING LEVEL (MRL) – The minimum concentration that can be reported as a quantitated value for a method analyte in a sample following analysis. This defined concentration can be no lower than the concentration of the lowest calibration standard for that analyte and can only be used if acceptable QC criteria for this standard are met. A procedure for verifying a laboratory's MRL is provided in Section 9.2.4.

3.16 PRECURSOR ION – For the purpose of this method, the precursor ion is the protonated molecule ($[M+H]^+$ or $[M+2H]^{2+}$) of the method analyte. In MS/MS, the precursor ion is mass selected and fragmented by CAD to produce distinctive product ions of smaller *m/z* ratio.

3.17 PRIMARY DILUTION STANDARD (PDS) SOLUTION – A solution containing the analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

3.18 PRODUCT ION – For the purpose of this method, a product ion is one of the fragment ions produced in MS/MS by CAD of the precursor ion.

3.19 QUALITY CONTROL SAMPLE (QCS) – A solution of method analytes of known concentrations that is obtained from a source external to the laboratory and different from the source of calibration standards. The QCS is used to check calibration standard integrity.

3.20 REAGENT WATER – Purified water that does not contain any measurable quantity of the method analytes or interfering compounds at or above 1/3 the MRL.

3.21 SAFETY DATA SHEET (SDS) – Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.22 STOCK STANDARD SOLUTION (SSS) – A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

3.23 SURROGATE ANALYTE (SUR) – A pure chemical which chemically resembles method analytes and is extremely unlikely to be found in any sample. This chemical is added to a sample aliquot in known amount(s) before processing and is measured with the same procedures used to measure other method analytes. The purpose of the SUR is to monitor method performance with each sample.

4. INTERFERENCES

4.1 All glassware and plasticware must be meticulously cleaned. Wash glassware and plasticware with detergent and tap water, rinse with tap water, followed by a reagent water rinse. Non-volumetric glassware must be heated in a muffle furnace for a

minimum of 90 min at 400 °C. Volumetric glassware should be solvent rinsed and allowed to air dry or heated in an oven no hotter than 120 °C. Plasticware should be solvent rinsed and allowed to air dry.

4.2 Method interferences may be caused by contaminants in solvents, reagents (including reagent water), sample bottles and caps, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in chromatograms. All items must be routinely demonstrated to be free from interferences (less than 1/3 the MRL for each method analyte) under the conditions of the analysis by analyzing laboratory reagent blanks as described in Section 9.3.1. Subtracting blank values from sample results is not permitted.

4.3 Matrix interferences may be caused by contaminants that are co-extracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature of the water. Humic and/or fulvic material can be co-extracted during SPE and high levels can cause signal enhancement or suppression in the electrospray ionization source. [3-4] Also, high levels of humic and/or fulvic material can cause low recoveries on the SPE sorbent.

4.4 Although not observed during method development, suppression of analyte signals due to electrolyte-induced ionization caused by dissolved salts in the mobile phase has been reported in the literature.[5] Addition of ammonium formate to the mobile phase in this method aids in reducing the occurrence of this phenomenon.

4.5 Relatively large quantities of preservatives (Sect. 8.1.2) are added to sample bottles. The potential exists for trace-level organic contaminants in these reagents. Interferences from these sources should be monitored by analysis of laboratory reagent blanks (Sect. 9.3.1), particularly when new lots of reagents are acquired.

4.6 SPE cartridges can be a source of interferences. Analysis of laboratory reagent blanks can provide important information regarding the presence or absence of such interferences. Brands and lots of SPE devices should be tested to ensure that contamination does not preclude analyte identification and quantitation.

5. SAFETY

Each chemical should be treated as a potential health hazard, and exposure to these chemicals should be minimized. Each laboratory is responsible for maintaining an awareness of OSHA regulations regarding safe handling of chemicals used in this method. A reference file of SDSs should be made available to all personnel involved in the chemical analysis. Toxin decontamination/inactivation guidelines may be found in *Biosafety in Microbiological and Biomedical Laboratories*, 5th edition.[6] Additional references to laboratory safety are available.[7-9]

6. EQUIPMENT AND SUPPLIES

(Brand names and catalog numbers are included for illustration only, and do not imply endorsement of the product.)

6.1 SAMPLE CONTAINERS – 100-mL amber glass bottles fitted with polytetrafluoroethylene (PTFE)-lined screw caps or 125-mL amber polyethylene terephthalate glycol (PETG) bottles fitted with HDPE screw caps (Nalgene #322021-0125).

6.2 STANDARD CONTAINERS – Amber-12mL glass screw thread sample vials (Kimble #60815-1965 or equivalent) with black phenolic caps with PTFE-faced white rubber liners (Kimble #73802-15425 or equivalent). If available, small volume amber PETG bottles may also be used to prepare standards.

6.3 BULK COLLECTION CONTAINER – 500-mL (or larger) clear or amber PETG media bottles (Nalgene #2019-0500). One bulk container per field sample (the LFSM and LFSMD are drawn from the same bulk container as the field sample) is required.

6.4 SAMPLE FILTER APPARATUS (See Figure 1)

6.4.1 CONTAINERS FOR COLLECTING FILTRATE – 500-mL amber glass bottles (Fisher #02-542-4C or equivalent) and GL 45 bottle cap (Fisher #13247GL45 or equivalent; not shown in figure).

6.4.2 FILTER BASE O-RING – PTFE/silicone sealing ring (Kimble Chase #410171-4226 or equivalent).

6.4.3 BOTTLE CAP WITH HOLE – GL 45 bottle cap with hole for filter support base (Kimble #410170-4534, or equivalent).

6.4.4 SUPPORT BASE – 47 mm fritted glass support base for filtration (Kimble Chase #953752-5047 or equivalent).

6.4.5 HOSE BARB CONNECTOR – Barbed tubing adapter for filtration apparatus (Kimble Chase #736400-1413 or equivalent).

6.4.6 METAL CLAMP – 47 mm aluminum clamp (Kimble Chase #953753-0000 or equivalent).

6.4.7 FUNNEL – 47 mm, 300 mL glass funnel (Kimble Chase #953751-0000 or equivalent).

6.5 MEMBRANE FILTER – 47 mm Nuclepore polycarbonate filter membranes, pore size 0.8 μm, (Whatman #111109).

6.6 ROUND BOTTOM CULTURE TUBES – 15-mL round bottom glass culture tubes (Corning #9826-16X or equivalent) or other glassware suitable for use in releasing toxins from the filter.

6.7 CONICAL CENTRIFUGE TUBES – 15-mL conical glass centrifuge tubes (Corning #8082-15) or other glassware suitable for collection of the eluent from the solid phase after extraction.

6.8 CONICAL CENTRIFUGE TUBES – 50-mL conical plastic centrifuge tubes (Fisher #06-443-18) or other glassware suitable for freezing and centrifuging samples with high cyanobacterial cell densities during the toxin release procedure.

6.9 AUTOSAMPLER VIALS – Amber glass 2.0-mL autosampler vials (National Scientific #C4000-2W or equivalent) with caps containing PTFE-faced septa (National Scientific #C4000-53 or equivalent).

6.10 MICRO SYRINGES – Suggested sizes include 5, 10, 25, 50, 100, 250, 500 and 1000-µL syringes.

6.11 ANALYTICAL BALANCE – Capable of weighing to the nearest 0.0001 g.

6.12 CENTRIFUGE – Capable of centrifugation at 8,000 rpm and 4 ºC.

6.13 SOLID PHASE EXTRACTION (SPE) APPARATUS FOR USING CARTRIDGES

6.13.1 SPE CARTRIDGES – Waters Oasis HLB, 150 mg, 6 cc divinylbenzene N-vinylpyrrolidone copolymer (Waters # 186003365).

6.13.2 VACUUM EXTRACTION MANIFOLD

6.13.2.1 Manual Extraction – A manual vacuum manifold with Visiprep™ large volume sampler (Supelco #57030 and #57275 or equivalent) for cartridge extractions.

6.13.2.2 SAMPLE DELIVERY SYSTEM – Use of a transfer tube system (Supelco "Visiprep," #57275 or equivalent), which transfers sample directly from the sample container to the SPE cartridge is recommended.

6.14 EXTRACT CONCENTRATION SYSTEM – Extracts are concentrated by evaporation with nitrogen using a water bath set no higher than 60 °C (Meyer N-Evap, Model 111, Organomation Associates, Inc. or equivalent).

6.15 LABORATORY OR ASPIRATOR VACUUM SYSTEM – Sufficient capacity to maintain a vacuum of approximately 10 to 15 inches of mercury for extracting cartridges.

6.16 LIQUID CHROMATOGRAPHY (LC)/TANDEM MASS SPECTROMETER (MS/MS) WITH DATA SYSTEM

6.16.1 LC SYSTEM – Instrument capable of reproducibly injecting up to 10-µL aliquots, and performing binary linear gradients at a constant flow rate near the flow rate used for development of this method (0.3 mL/min). Usage of a column heater capable of heating to 65 °C is required to achieve suitable peak shape for the IS. If alternate ISs are used which meet the IS modification requirements (Sect. 7.2.1) and do not need column heating to achieve suitable peak shape, then a column heater is not required.

6.16.2 TANDEM MASS SPECTROMETER – The mass spectrometer must be capable of positive ion electrospray ionization (ESI) near the suggested LC flow rate of 0.3 mL/min. The system must be capable of performing MS/MS to produce unique product ions (Sect. 3.18) for method analytes within specified retention time segments. A minimum of 10 scans across the chromatographic peak is required to ensure adequate precision. Data demonstrated in Section 17 were collected using a triple quadrupole mass spectrometer.

6.16.3 DATA SYSTEM – An interfaced data system is required to acquire, store, reduce, and output mass spectral data. The computer software should have the capability of processing stored LC/MS/MS data by recognizing an LC peak within any given retention time window. The software must allow integration of the ion abundance of any specific ion within specified time or scan number limits. The software must be able to calculate relative response factors, construct linear regressions or quadratic calibration curves, and calculate analyte concentrations.

6.16.4 ANALYTICAL COLUMN – C_8 column (2.1 x 100 mm) packed with 2.6 µm C_8 solid phase particles (Phenomenex Kinetex #00D-4497-AN). Any equivalent column that provides adequate resolution, peak shape, capacity, accuracy, and precision (Sect. 1.6 and 9) may be used.

NOTE: This column has silanol groups which have the potential to impact retention times and area counts of the microcystins. Retention times of the microcystins have been found to slowly decrease with time on C_8 and C_{18} columns both during method development and in the literature.[10] In addition, during method development, area counts of some microcystins (especially arginine containing microcystins) increased as the number of ambient water extract injections increased. A potential reason for these drifts may be that active silanol sites on the column may be inactivated by the ambient water components; thereby allowing less binding of the microcystins to the silanol sites. Thus, sensitivity of the microcystins very slowly increased with time after repeated injections of ambient water extracts during method development. In any given batch, however, retention times and areas were very precise.

6.16.5 ANALYTICAL GUARD COLUMN (optional) – Phenomenex SecurityGuard Ultra Cartridges UHPLC C8 (#AJ0-8784, or equivalent).

7. REAGENTS AND STANDARDS

7.1 GASES, REAGENTS, AND SOLVENTS – Reagent grade or better chemicals should be used. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first determined that the reagent is of sufficiently high purity to permit its use without lessening the quality of the determination.

7.1.1 REAGENT WATER – Purified water which does not contain any measurable quantities of any method analytes or interfering compounds greater than 1/3 the MRL for each method analyte of interest.

7.1.2 METHANOL (CH_3OH, CASRN 67-56-1) – High purity, demonstrated to be free of analytes and interferences (Fisher #A456-4, Optima LC/MS grade or equivalent).

7.1.3 AMMONIUM FORMATE (CH_5O_2N, CASRN 540-69-2) – High purity, demonstrated to be free of analytes and interferences (LC/MS grade (Fluka #55674) or equivalent).

7.1.4 20 mM FORMATE BUFFER – To prepare 1 L, add 1.26 g ammonium formate to 1 L of reagent water. This solution is prone to volatility losses and should be replaced at least every 48 hours.

7.1.5 SAMPLE PRESERVATION REAGENTS – The following preservatives are solids at room temperature and may be added to the sample bottle before shipment to the field.

7.1.5.1 TRIZMA PRESET CRYSTALS, pH 7.0 (Sigma-Aldrich #T-7193 or equivalent) – Reagent grade. A premixed blend of Tris [Tris(hydroxy-methyl)aminomethane] and Tris HCL [Tris(hydroxymethyl)aminomethane hydrochloride]. Alternatively, a mix of the two components with a weight ratio of 15.5/1 Tris HCL/Tris may be used. These blends are targeted to produce a pH near 7.0 at 25 °C in reagent water. Trizma functions as a buffer (Sect. 8.1.2).

7.1.5.2 2-CHLOROACETAMIDE (CASRN 79-07-2) – Inhibits microbial growth and analyte degradation (Sigma-Aldrich #C0267 or equivalent).[11]

7.1.5.3 ETHYLENEDIAMINETETRAACETIC ACID, TRISODIUM SALT HYDRATE (Trisodium EDTA, CASRN 85715-60-2) – Inhibits metal-catalyzed hydrolysis of analytes. Trisodium salt is used instead of the

disodium salt because the trisodium salt solution pH is closer to the desired pH of 7 (Sigma #ED3SS or equivalent).

7.1.6 NITROGEN – DESOLVATION GAS – High purity compressed gas (e.g., nitrogen or zero-air) used for desolvation in the mass spectrometer. The specific type of gas, purity, and pressure requirements will depend on the instrument manufacturer's specifications. Nitrogen was used to generate the data in Section 17.

7.1.7 COLLISION GAS – High purity compressed gas (e.g., nitrogen or argon) used for CAD in the mass spectrometer. The specific type of gas, purity, and pressure requirements will depend on the instrument manufacturer's specifications. Argon was used to generate the data in Section 17.

7.2 STANDARD SOLUTIONS – When the purity of a compound is assayed to be 95% or greater, the weight can be used without correction to calculate concentration of the stock standard. The suggested concentrations are a description of concentrations used during method development, and may be modified to conform to instrument sensitivity. Standards for sample fortification generally should be prepared in the smallest volume that can be accurately measured to minimize addition of excess organic solvent to aqueous samples. Stock standards, PDSs and calibration standards were found to be stable for a minimum of three months during method development. Laboratories should use standard QC practices to determine when standards need to be replaced. The target analyte manufacturer's guidelines may be helpful when making the determination.

7.2.1 INTERNAL STANDARD (IS) SOLUTIONS – Cyclosporin-A, $^{13}C_2$, d_4, obtained from Toronto Research Chemicals as neat material, (Cat # C988901) is used as the IS. Although alternate IS standards may be used provided they are isotopically labeled compounds with similar functional groups as method analytes, the analyst must have documented reasons for using alternate IS standards (e.g., isotopically labeled microcystins become commercially available). In addition, alternate IS standards must meet QC requirements in Section 9.3.4.

7.2.1.1 IS STOCK SOLUTION (500 ng/µL) –The IS stock standard solution is prepared by diluting 0.5 mg of the IS in one mL of methanol. This IS stock standard is stored at -15 °C or less in amber glass screw cap vials.

7.2.1.2 IS PRIMARY DILUTION STANDARD – (IS PDS; 1.0 ng/µL) – The IS PDS is prepared at 1.0 ng/µL by diluting 20 µL of the IS stock standard in 10 mL of methanol. Ten µL of this 1.0 ng/µL solution is used to fortify the final 1-mL extracts (Sect. 11.6.5). This will yield an IS concentration of 10 µg/L in the 1-mL extracts. This IS PDS is stored at -15 °C or less in amber glass screw cap vials. The IS concentration may be adjusted to accommodate instrument sensitivity.

7.2.2 SURROGATE (SUR) ANALYTE STANDARD SOLUTIONS – Ethylated MC-LR, d_5 (C_2D_5-MC-LR), obtained from Tamarack Environmental Laboratories (now available through Cambridge Isotopes) as neat material, is used as the SUR. This isotopically labeled SUR standard was carefully chosen during method development because it contains similar functional groups as the method analytes. Although alternate SUR standards may be used provided they are isotopically labeled compounds with similar functional groups as method analytes, the analyst must have documented reasons for using alternate SUR standards. In addition, alternate SUR standards must meet QC requirements in Section 9.3.5.

7.2.2.1 SUR STOCK STANDARD; 100 µg/mL) – The SUR stock standard solution is prepared by diluting 0.1 mg of the SUR in one mL of methanol. This SUR stock standard is stored at -15 °C or less in amber glass screw cap vials.

7.2.2.2 SUR PRIMARY DILUTION STANDARD (SUR PDS; 5.0 ng/µL) – The SUR PDS was prepared at 5.0 ng/µL by diluting 500 µL of the SUR stock standard with 10 mL of methanol. This solution is used to fortify all QC and field samples. The PDS has been shown to be stable for at least one month when stored at -15 °C or less. Use 20 µL of this 5 ng/µL SUR PDS to fortify the 100 mL aqueous QC and field samples prior to extraction (Sect. 11.3.4). This will yield a concentration of 1000 ng/L of the SUR in 100-mL aqueous QC and field samples. The SUR concentration may be adjusted to accommodate instrument sensitivity.

7.2.3 ANALYTE STANDARD SOLUTIONS – Analyte standards may be purchased commercially as ampulized solutions or prepared from neat materials (see Table 3 for analyte sources used during method development).

7.2.3.1 ANALYTE STOCK STANDARD SOLUTION (10-100 µg/mL) – Neat cyanotoxins are typically purchased in quantities of 10-100 µg. Due to the small quantity and toxicity of these analytes, weighing the cyanotoxins is not feasible. If preparing from neat material, simply add 1 mL of methanol to the purchased neat material (10-100 µg) for a final concentration of 10-100 µg/mL. Repeat for each method analyte prepared from neat material. Alternatively, purchase commercially available stock standard solutions of the analytes, preferably in methanol, if available. These stock standards were stored at -15 °C or less in amber glass screw cap vials.

ANALYTE PRIMARY DILUTION STANDARD (PDS) SOLUTION (0.33-6.0 ng/µL) – The Analyte PDS contains all, or a portion, of method analytes at various concentrations in methanol. ESI and MS/MS response varies by compound; therefore, a mix of concentrations may be needed in the Analyte PDS. During method development, Analyte PDS solutions were prepared such that approximately the same instrument response was obtained for all analytes. The Analyte PDS was prepared in 10 mL of methanol at concentrations of 0.33-6.0 ng/µL as shown in the following table. The Analyte

PDS is prepared by dilution of the combined Analyte Stock Standard Solutions (Sect. 7.2.3.1) and is used to prepare CAL standards, and fortify LFBs, LFSMs, and LFSMDs with method analytes. The Analyte PDS was stored at -15 ºC or less in amber glass screw cap vials.

Analyte	Conc. of Analyte Stock Standard Solution (ng/µL)	Vol. of Analyte Stock Standard Solution (µL)	Final Conc. of Analyte in 10-mL PDS (ng/µL)
Nodularin	10.3	480	0.49
MC-YR	100	200	2.0
MC-HtyR	100	200	2.0
MC-RR	10.3	320	0.33
3-desmethylated-MC-RR	100	100	1.0
MC-LR	10.1	1890	1.9
MC-WR	100	600	6.0
7-desmethylated-MC-LR	9.4	2000	1.9
MC-HilR	25	800	2.0
3-desmethylated-MC-LR	100	400	4.0
MC-LA	100	200	2.0
MC-LY	100	200	2.0
MC-LW	100	200	2.0
MC-LF	100	200	2.0

7.2.4 CALIBRATION STANDARDS (CAL) – Prepare a series of at least five concentrations of calibration solutions in 90:10 methanol:reagent water (v/v), from dilutions of the Analyte PDS (Sect 7.2.3.2). The suggested concentrations in this section are a description of concentrations used during method development, and may be modified to conform with instrument sensitivity. Typical calibration standard concentration ranges are depicted in the table below. Larger concentration ranges will require more calibration points. The IS and SUR are typically added to CAL standards at constant concentration (see Note below). During method development, the concentration of the IS was 10 µg/L in each standard and the concentration of the SUR was 100 µg/L in each standard (1000 ng/L in the aqueous sample). The lowest concentration CAL standard must be at or below the MRL, which may depend on system sensitivity. CAL standards may also be used as CCCs (Sect. 9.3.2). During method development, CAL standards were stored at -4 ºC or less.

NOTE: Alternatively, a calibration curve may be used for quantitation of the SUR. Analysts may be hesitant to analyze undiluted extracts obtained from a water sample with high cyanobacterial cell densities. Dilution of extracts may be required or preferred causing the inability to accurately calculate SUR recoveries using average response factors. Generation of a calibration curve using a concentration range for the SUR will enable calculation of the SUR in diluted extracts.

Analyte	Cal standard Concentration Range, µg/L	Concentration Range in 100-mL aqueous sample, µg/L
Nodularin	1.5 – 247	0.015 – 2.47
MC-YR	6.0 – 1000	0.060 – 10.00
MC-HtyR	6.0 – 1000	0.060 – 10.00
MC-RR	0.99 – 165	0.0099 – 1.65
3-dm-MC-RR	3.0 – 500	0.030 – 5.00
MC-LR	5.7 – 945	0.057 – 9.45
MC-WR	18 – 3000	0.18 – 30.00
7-dm-MC-LR	5.6 – 940	0.056 – 9.40
MC-HilR	6.0 – 1000	0.060 – 10.00
3-dm-MC-LR	12 – 2000	0.12 – 20.00
MC-LA	6.0 – 1000	0.060 – 10.00
MC-LY	6.0 – 1000	0.060 – 10.00
MC-LW	6.0 – 1000	0.060 – 10.00
MC-LF	6.0 – 1000	0.060 – 10.00

8. SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 SAMPLE BOTTLE PREPARATION

8.1.1 Collect 100-mL samples in amber glass bottles or 125-mL amber PETG bottles (Sect. 6.1). It is recommended that more samples be collected than are needed to meet QC requirements in Section 9. This will allow laboratories some flexibility to analyze samples by the Option B procedure if samples fail to filter in the 8 h time period required in Option A.

8.1.2 Preservation reagents, listed in the table below, are added to each sample bottle as a solid prior to shipment to the field (or prior to sample collection).

Compound	Amount	Purpose
Trizma	7.75 g/L	buffering reagent (pH 7)
2-Chloroacetamide	2.0 g/L	antimicrobial
Ethylenediaminetetraacetic acid trisodium salt	0.35 g/L	inhibit binding of the targets to metals

8.1.3 Optional: If 125 mL amber bottles do not have visible 100-mL demarcations, a marker may be added to outside of the bottle to serve as a fill line and to prevent over-filling the bottle.

8.2 SAMPLE COLLECTION (The sample collection procedure in Section 8.2 is recommended but program requirements may involve alternate sample collection procedures, and it is incumbent upon the laboratory and field samplers to verify such requirements. Changes to preservation agents are not permitted.)

8.2.1 Collect approximately 500 mL of sample water in a 500-mL PETG container (Sect. 6.3).

8.2.2 Gently shake the 500-mL PETG container at least 25 times between sample draws to aid in homogenizing the sample. Immediately pour 100 mL of the sample water into the bottle containing preservatives. Do not completely fill the bottle as preservatives have been added at quantities appropriate for 100 mL samples. Samples do not need to be collected headspace free. Pour duplicate samples for the LFSM and LFSMD (if necessary to meet minimum QC requirements in Sections 9.3.6 and 9.3.7) from the same draw of water.

8.2.3 If a FD is desired, collect the FD from a second draw of water from the water body. Cyanobacterial blooms typically display heterogeneity in water bodies and collection of the first water sample will also disturb the bloom even further. Thus, the FD cannot be used as a measure of sample collection precision. However, if desired, the FD can be used as a measure of heterogeneity of the cyanobacterial bloom in the water body.

8.2.4 After pouring the sample, cap the sample bottle and agitate by hand until preservative is dissolved. Note that 2-chloroacetamide is slow to dissolve especially in cold water. Keep the sample sealed from time of collection until extraction.

8.3 SAMPLE SHIPMENT AND STORAGE – Samples must be chilled during shipment and must not exceed 10 °C during the first 48 hours after collection. Sample temperature must be confirmed to be at or below 10 °C when samples are received at the laboratory. Samples stored in the lab must be held at or below 6 °C until extraction, but should not be frozen.

NOTE: Samples that are significantly above 10 °C, at the time of collection, may need to be iced or refrigerated for a period of time, in order to chill them prior to shipping. This will allow them to be shipped with sufficient ice to meet the above requirements.

8.4 SAMPLE AND EXTRACT HOLDING TIMES – Water samples should be extracted as soon as possible after collection but must be extracted within 28 days of collection. Extracts must be stored at ≤ -4 °C and analyzed within 28 days after extraction. Sample and extract holding time data are presented in Tables 10 and 11.

9. QUALITY CONTROL

9.1 QC requirements include the Initial Demonstration of Capability (IDC) and ongoing QC requirements that must be met when preparing and analyzing field samples. This section describes QC parameters, their required frequencies, and performance criteria

that must be met in order to meet EPA quality objectives. These QC requirements are considered the minimum acceptable QC criteria. Laboratories are encouraged to institute additional QC practices to meet their specific needs.

9.1.1 METHOD MODIFICATIONS – The analyst is permitted to modify LC columns, LC conditions, evaporation techniques, internal standards, surrogate standards, and MS and MS/MS conditions. Each time such method modifications are made, the analyst must repeat the procedures of the IDC. **Modifications to LC conditions should still minimize co-elution of method analytes to reduce the probability of suppression/enhancement effects.**

9.2 INITIAL DEMONSTRATION OF CAPABILITY (IDC) – The IDC must be successfully performed for the Option A (Sect. 11.4) and Option B (Sect. 11.5) procedures prior to analyzing any field samples. Prior to conducting the IDC, the analyst must first generate an acceptable Initial Calibration following the procedure outlined in Section 10.2.

9.2.1 INITIAL DEMONSTRATION OF LOW SYSTEM BACKGROUND – Any time a new lot of filters, SPE cartridges, solvents, centrifuge tubes, disposable pipets, and autosampler vials are used, it must be demonstrated that an LRB is reasonably free of contamination and that criteria in Section 9.3.1 are met. If an automated extraction system is used, an LRB should be extracted on each port to ensure that all valves and tubing are free from potential contamination.

9.2.2 INITIAL DEMONSTRATION OF PRECISION (IDP) – Prepare, extract, and analyze four to seven replicate LFBs fortified near the midrange of the initial calibration curve according to the procedure described in Section 11. Sample preservatives, as described in Section 8.1.2, must be added to these samples. The relative standard deviation (RSD) of the results of replicate analyses must be less than 30%.

9.2.3 INITIAL DEMONSTRATION OF ACCURACY (IDA) – Using the same set of replicate data generated for Section 9.2.2, calculate average recovery. The average recovery of replicate values must be within ± 30% of the true value except for MC-WR and MC-LW which must be 50-130% of the true value.

9.2.4 MINIMUM REPORTING LEVEL (MRL) CONFIRMATION – Establish target concentrations for the MRL based on the intended use of the method for the Option A and Option B procedures. The MRL may be established by a laboratory for their specific purpose or may be set by a regulatory agency. Establish an Initial Calibration following the procedure outlined in Section 10.2. The lowest CAL standard used to establish Initial Calibration (as well as the low-level CCC, Section 10.3) must be at or below the concentration of the MRL. Establishing the MRL concentration too low may cause repeated failure of ongoing QC requirements. Confirm MRLs following the procedure outlined below using the Option A and Option B procedures.

9.2.4.1 Fortify, extract, and analyze seven replicate LFBs at the proposed MRL concentration. These LFBs must contain all method preservatives described in Section 8.1.2. Calculate the mean measured concentration (*Mean*) and standard deviation for these replicates. Determine the Half Range for the prediction interval of results (HR_{PIR}) using the equation below

$$HR_{PIR} = 3.963s$$

where

s = standard deviation

3.963 = a constant value for seven replicates.[1]

9.2.4.2 Confirm that the upper and lower limits for the Prediction Interval of Result ($PIR = Mean \pm HR_{PIR}$) meet the upper and lower recovery limits as shown below

The Upper PIR Limit must be ≤ 150% recovery.

$$\frac{Mean + HR_{PIR}}{Fortified\ Concentration} x\ 100\% \leq 150\%$$

The Lower PIR Limit must be ≥ 50% recovery.

$$\frac{Mean - HR_{PIR}}{Fortified\ Concentration} x\ 100\% \geq 50\%$$

9.2.4.3 The MRL is validated if both the Upper and Lower PIR Limits meet the criteria described above. If these criteria are not met, the MRL has been set too low and must be determined again at a higher concentration.

9.2.5 CALIBRATION CONFIRMATION – Analyze a QCS (if available) as described in Section 9.3.9 to confirm the accuracy of the standards/calibration curve.

9.2.6 DETECTION LIMIT DETERMINATION (optional) – While DL determination is not a specific requirement of this method, it may be required by various regulatory bodies associated with compliance monitoring. It is the responsibility of the laboratory to determine if DL determination is required based upon the intended use of the data.

Replicate analyses for this procedure should be done over at least three days (i.e., both the sample extraction and the LC/MS/MS analyses should be done over at least three days). Prepare at least seven replicate LFBs at a concentration estimated to be near the DL. This concentration may be estimated by selecting a concentration at two to five times the noise level. DLs in Table 5 were calculated from LFBs fortified at various concentrations as indicated in the table. Appropriate fortification concentrations will be dependent upon the sensitivity of

the LC/MS/MS system used. All preservation reagents listed in Section 8.1.2 must also be added to these samples. Analyze the seven replicates through all steps of Section 11. This procedure must be conducted for Option A and Option B procedures.

NOTE: If an MRL confirmation data set meets these requirements, a DL may be calculated from the MRL confirmation data, and no additional analyses are necessary.

Calculate the *DL* using the following equation

$$DL = s \times t_{(n-1, 1-\alpha=0.99)}$$

where

s = standard deviation of replicate analyses
$t_{(n\text{-}1,\ 1\text{-}\alpha=0.99)}$ = Student's t value for the 99% confidence level with n-1 degrees of freedom
n = number of replicates.

NOTE: Do not subtract blank values when performing DL calculations.

9.3 ONGOING QC REQUIREMENTS – This section summarizes ongoing QC criteria that must be followed when processing and analyzing field samples.

9.3.1 LABORATORY REAGENT BLANK (LRB) – An LRB is required with each extraction batch (Sect. 3.6) to confirm that potential background contaminants are not interfering with identification or quantitation of method analytes. If more than 20 field samples are included in a batch, analyze an LRB for every 20 samples. If the LRB produces a peak within the retention time window of any analyte that would prevent determination of that analyte, determine the source of contamination and eliminate the interference before processing samples. Background contamination must be reduced to an acceptable level before proceeding. Background from method analytes or other contaminants that interfere with the measurement of method analytes must be below 1/3 of the MRL. If method analytes are detected in the LRB at concentrations equal to or greater than this level, then all data for the problem analyte(s) must be considered invalid for all samples in the extraction batch. Blank contamination is estimated by extrapolation, if the concentration is below the lowest CAL standard. This extrapolation procedure is not allowed for sample results as it may not meet data quality objectives.

NOTE: Although quantitative data below the MRL may not be accurate enough for data reporting, such data are useful in determining the magnitude of background interference. Therefore, blank contamination levels may be estimated by extrapolation when the concentration is below the MRL.

9.3.2 CONTINUING CALIBRATION CHECK (CCC) – CCC standards are analyzed at the beginning of each analysis batch (Sect. 3.1), after every 10 field samples, and at the end of the analysis batch. See Section 10.3 for concentration requirements and acceptance criteria.

9.3.3 LABORATORY FORTIFIED BLANK (LFB) – An LFB is required with each extraction batch (Sect. 3.6). The fortified concentration of the LFB must be rotated between low, medium, and high concentrations from batch to batch. The low concentration LFB must be as near as practical to, but no more than two times, the MRL. Similarly, the high concentration LFB should be near the high end of the calibration range established during the initial calibration (Sect. 10.2). Results of low-level LFB analyses must be 50-150% of the true value. Results of medium and high-level LFB analyses must be 70-130% of the true value except for MC-WR and MC-LW which must be 50-130%. If LFB results do not meet these criteria for method analytes, then all data for the problem analyte(s) must be considered invalid for all samples in the extraction batch.

9.3.4 INTERNAL STANDARD(S) (IS) – The analyst must monitor peak areas of the IS(s) in all injections during each analysis day. Internal standard responses (as indicated by peak areas) for any chromatographic run must not deviate by more than ± 50% from average areas measured during the initial calibration for the internal standards. If IS areas in a chromatographic run do not meet these criteria, inject a second aliquot of that standard or extract.

9.3.4.1 If the reinjected aliquot produces an acceptable IS response, report results for that aliquot.

9.3.4.2 If the reinjected extract fails again, the analyst should check the calibration by reanalyzing the most recently acceptable CAL standard. If the CAL standard fails the criteria of Section 10.3, recalibration is in order per Section 10.2. If the CAL standard is acceptable, report results obtained from the reinjected extract, but annotate as suspect. Alternatively, if a duplicate sample was collected at the time of sample collection and is still within the holding time, extract the duplicate sample and re-analyze.

9.3.5 SURROGATE RECOVERY – The SUR standard is fortified into all samples, CCCs, LRBs, LFBs, LFSMs, LFSMDs, and FDs prior to extraction. It is also added to CAL standards. The SUR is a means of assessing method performance from extraction to final chromatographic measurement. Calculate recovery (%R) for the SUR using the following equation

$$\%R = \left(\frac{A}{B}\right) \times 100$$

where

A = measured SUR concentration for the QC or Field sample
B = fortified concentration of the SUR.

9.3.5.1 SUR recovery in extracts must be in the range of 60-130%. SUR recovery in CCCs must be 70-130%. A wider recovery range is allowed for the SUR in extracts due to SPE recoveries typically being 15-20% lower for this SUR during method development. When SUR recovery does not meet these criteria, check 1) calculations to locate possible errors, 2) standard solutions for degradation, 3) contamination, 4) instrument performance and 5) extraction procedure. Correct the problem and reanalyze the extract.

9.3.5.2 If the extract reanalysis meets the SUR recovery criterion, report only data for the reanalyzed extract.

9.3.5.3 If the extract reanalysis fails the 60-130% recovery criterion, the analyst should check the calibration by injecting the last CAL standard that passed. If the CAL standard fails the criteria of Section 10.3, recalibration is in order per Section 10.2. If the CAL standard is acceptable, report all data for that sample as suspect/SUR recovery to inform the data user that the results are suspect due to SUR recovery. Alternatively, if a duplicate sample was collected at the time of sample collection and is still within the holding time, extract the duplicate sample and re-analyze.

9.3.6 LABORATORY FORTIFIED SAMPLE MATRIX (LFSM) – Analysis of an LFSM is required in each extraction batch and is used to determine that the sample matrix does not adversely affect method accuracy. Assessment of method precision is accomplished by analysis of a LFSMD (Sect. 9.3.7). If a variety of different sample matrices are analyzed regularly method performance should be established for each. Over time, LFSM data should be documented by the laboratory for all routine sample sources.

9.3.6.1 Within each extraction batch (Sect. 3.6), a minimum of one Field sample is fortified as an LFSM for every 20 field samples analyzed. The LFSM is prepared by spiking a sample with an appropriate amount of the Analyte PDS (Sect. 0). Select a spiking concentration that is greater than or equal to the matrix background concentration, if known. Use historical data and rotate through low, mid and high concentrations when selecting a fortifying concentration. If high levels of method analytes are suspected, it may not be possible to spike the LFSM above the native amount. In this case, spike with the highest concentration within the calibration curve.

9.3.6.2 Calculate percent recovery (%*R*) for each analyte using the equation

$$\%R = \frac{(A - B)}{C} \times 100$$

where A = measured concentration in the fortified sample
B = measured concentration in the unfortified sample
C = fortification concentration.

9.3.6.3 Analyte recoveries may exhibit matrix bias. For samples fortified at or above their native concentration, recoveries should range between 60-140%, except for low-level fortification near or at the MRL (within a factor of two-times the MRL concentration) where 50-150% recoveries are acceptable. If the accuracy of any analyte falls outside the designated range, and laboratory performance for that analyte is shown to be in control in CCCs, the recovery is judged to be matrix biased. The result for that analyte in the unfortified sample is labeled suspect/matrix to inform the data user that results are suspect due to matrix effects.

9.3.7 LABORATORY FORTIFIED SAMPLE MATRIX DUPLICATE (LFSMD) – Within each extraction batch (not to exceed 20 field samples, Sect. 3.6), a minimum of one LFSMD must be analyzed. Duplicates check the precision associated with sample collection, preservation, storage, and laboratory procedures.

9.3.7.1 Calculate relative percent difference (RPD) for duplicate LFSMs (LFSM and LFSMD) using the equation

$$RPD = \frac{\left|LFSM - LFSMD\right|}{\left(LFSM + LFSMD\right)/2} \times 100$$

9.3.7.2 RPDs for duplicate LFSMs should be ≤ 50% for samples fortified at or above their native concentration. If the RPD of any analyte falls outside the designated range, and laboratory performance for that analyte is shown to be in control in the CCC, the recovery is judged to be matrix biased. The result for that analyte in the unfortified sample is labeled suspect/matrix to inform the data user that results are suspect due to matrix effects.

9.3.8 FIELD DUPLICATES (FD) – FDs may be collected and analyzed as a part of a sample batch, if desired. No QC criteria are being mandated as a part of this method because FDs can only be used as a measure of heterogeneity of the cyanobacterial bloom and not as a measure of sample collection or laboratory precision.

9.3.9 QUALITY CONTROL SAMPLES (QCS) – As part of the IDC (Sect. 9.2), each time a new Analyte PDS (Sect. 0) is prepared, and at least quarterly, analyze a QCS sample from a source different from the source of the CAL standards. If a second vendor is not available, then a different lot of the standard should be used. The QCS should be prepared and analyzed just like a CCC. Fortify the QCS near the midpoint of the calibration range. The expectation is that the calculated value for each analyte should be within ±30% of the expected value, but due to the lack of certified standards, calculated values within ±40% of the expected values are acceptable.

OPTIONAL: If available, certified reference materials are suggested for use in the QCS if not already being used in the Analyte PDS.

10. CALIBRATION AND STANDARDIZATION

10.1 Demonstration and documentation of acceptable initial calibration is required before any samples are analyzed. The MS tune check and initial calibration must be repeated each time a major instrument modification is made, or maintenance is performed.

10.2 INITIAL CALIBRATION

10.2.1 ESI-MS/MS TUNE

10.2.1.1 Calibrate the mass scale of the MS with the calibration compounds and procedures prescribed by the manufacturer.

10.2.1.2 Optimize the precursor ion (Sect. 3.16, $[M+H]^+$ or $[M+2H]^{2+}$) for each method analyte by infusing approximately 1-5 ng/µL of each analyte (prepared in the initial mobile phase conditions) directly into the MS at the chosen LC mobile phase flow rate (approximately 0.3 mL/min). This tune can be done on a mix of method analytes, provided analytes have different MS/MS transitions. MS parameters (voltages, temperatures, gas flows, etc.) are varied until optimal analyte responses are determined (see caution below). Method analytes may have different optima requiring some compromise between the optima. See Table 2 for ESI-MS conditions used in method development. Conditions may vary on different instruments, including whether the precursor ion is doubly charged or not. Precursor ions other than those listed may be selected.

CAUTION: Desolvation temperature was identified as a parameter that can affect the degree of analyte suppression observed in matrices. Desolvation temperature is applied in different ways to different instruments; a heated gas or a heated stainless steel capillary is used in ESI source designs. Thus, it is highly recommended that desolvation temperature be minimized and that temperatures of ≤400 °C be used for the heated gas source designs and ≤275 °C for heated capillary source designs (these recommended temperatures are based on LC conditions employed during development of this method).

10.2.1.3 Optimize the product ion (Sect. 3.18) for each analyte by infusing approximately 1-5 ng/µL of each analyte (prepared as PDS in methanol) directly into the MS at the chosen LC mobile phase flow rate (approximately 0.3 mL/min). This tune can be done on a mix of method analytes. MS/MS parameters (collision gas pressure, collision energy, etc.) are varied until optimal analyte responses are determined. See Table 4 for MS/MS conditions used in method development.

10.2.2 Establish LC operating parameters that optimize resolution and peak shape. Suggested LC conditions can be found in Table 1. LC conditions listed in Table 1 may not be optimum for all LC systems and may need to be optimized by the analyst.

10.2.3 Inject a mid-level CAL standard under LC/MS conditions to obtain retention times of each method analyte. Divide the chromatogram into retention time windows (segments) each of which contains one or more chromatographic peaks. During MS/MS analysis, fragment a small number of selected precursor ions ($[M+H]^+$ or $[M+2H]^{2+}$; Sect. 3.16) for the analytes in each window and choose the most abundant product ion. Product ions (also quantitation ions) chosen during method development are in Table 4. Product ions other than those listed may be selected. For maximum sensitivity in subsequent MS/MS analyses, minimize the number of transitions that are simultaneously monitored within each segment.

10.2.4 Inject a mid-level CAL standard under optimized LC/MS/MS conditions to ensure that each method analyte is observed in its MS/MS window and that there are at least 10 scans across the peak for optimum precision.

10.2.5 Prepare a set of at least five CAL standards as described in Section 7.2.4. The lowest concentration CAL standard must be at or below the MRL, which may depend on system sensitivity. It is recommended that at least four of the CAL standards are at a concentration greater than or equal to the MRL.

10.2.6 The LC/MS/MS system is calibrated using the internal standard technique. Use the LC/MS/MS data system software to generate a linear regression or quadratic calibration curve for each of the analytes. Curves may be concentration weighted, if necessary. Forcing zero as part of the calibration is not permitted.

10.2.7 CALIBRATION ACCEPTANCE CRITERIA – Validate the initial calibration by calculating the concentration of each analyte as an unknown against its regression equation. For calibration levels that are ≤ MRL, the result for each analyte should be within ± 50% of the true value. All other calibration points must calculate to be within ± 30% of their true value. If these criteria cannot be met, the analyst will have difficulty meeting ongoing QC criteria. It is recommended that corrective action is taken to reanalyze the CAL standards, restrict the range of calibration, or select an alternate method of calibration.

CAUTION: When acquiring MS/MS data, LC operating conditions must be carefully reproduced for each analysis to provide reproducible retention times. If this is not done, the correct ions will not be monitored at appropriate times. As a precautionary measure, chromatographic peaks in each window must not elute too close to the edge of the segment time window.

10.3 CONTINUING CALIBRATION CHECK (CCC) – Analyze a CCC to verify the initial calibration at the beginning of each analysis batch, after every tenth Field sample, and at the end of each analysis batch. LRBs, CCCs, LFBs, LFSMs, LFSMDs and FDs are not counted as samples. The beginning CCC of each analysis batch must be at or below the MRL in order to verify instrument sensitivity prior to any analyses. If standards have been prepared such that all low CAL points are not in the same CAL solution, it may be necessary to analyze two CAL standards to meet this requirement. Alternatively, analyte concentrations in the Analyte PDS may be customized to meet these criteria. Subsequent CCCs should alternate between a medium and high concentration CAL standard.

10.3.1 Inject an aliquot of the appropriate concentration CAL standard and analyze with the same conditions used during the initial calibration.

10.3.2 Determine that the absolute areas of the quantitation ions of the IS(s) are within 50-150% of the average areas measured during initial calibration. If any of the IS areas has changed by more than these amounts, adjustments must be made to restore system sensitivity. These adjustments may include cleaning of the MS ion source, or other maintenance as indicated in Section 10.3.4. Major instrument maintenance requires recalibration (Sect 10.2) and verification of sensitivity by analyzing a CCC at or below the MRL (Sect 10.3). Control charts are useful aids in documenting system sensitivity changes.

10.3.3 Calculate the concentration of each analyte and SUR in the CCC. The calculated amount for the SUR must be within ± 30% of the true value. Each analyte fortified at a level ≤ MRL must calculate to be within ± 50% of the true value. The calculated concentration of method analytes in CCCs fortified at all other levels must be within ± 30%. If these conditions do not exist, then all data for the problem analyte must be considered invalid, and remedial action must be taken (Sect. 10.3.4) which may require recalibration. Any Field or QC Samples that have been analyzed since the last acceptable calibration verification should be reanalyzed after adequate calibration has been restored, with the following exception. **If the CCC fails because the calculated concentration is greater than 130% (150% for the low-level CCC) for a particular method analyte, and Field sample extracts show no detection for that method analyte, non-detects may be reported without re-analysis.**

10.3.4 REMEDIAL ACTION – Failure to meet CCC QC performance criteria may require remedial action. Major maintenance, such as cleaning the electrospray probe, atmospheric pressure ionization source, mass analyzer, replacing the LC column, LC maintenance, etc., requires recalibration (Sect 10.2) and verification of sensitivity by analyzing a CCC at or below the MRL (Sect 10.3).

11. PROCEDURE

11.1 Ambient water samples may contain cyanobacterial cells at widely different densities. Thus, two procedural options are offered below: Option A – for samples with cell densities that can be filtered within 8 hours, and Option B – for samples with cell densities so high that direct filtration of the water sample is not practical. In most cases, the Option A procedure will be the procedure used. Visual inspection of the water sample and analyst experience will weigh heavily in determining the appropriate procedure to apply to samples. Note that Option B decreases the sensitivity of analysis and will require adjustment of the reported MRL for samples processed using this option.

11.2 This procedure may be performed manually or in an automated mode using a robotic or automatic sample preparation device. Data presented in Tables 5-11 demonstrate data collected by manual extraction. An automatic/robotic sample preparation system, designed for use with SPE cartridges, may be used if all QC requirements discussed in Section 9 are met. If an automated system is used to prepare samples, follow manufacturer's operating instructions, but all extraction and elution steps must be the same as in the manual procedure. Extraction and elution steps may not be changed or omitted to accommodate use of an automated system. If an automated system is used, LRBs should be rotated among the ports to ensure that all valves and tubing meet LRB requirements (Sect. 9.3.1).

NOTE: SPE cartridges described in this section are designed as single use items and must be discarded after use. They may not be reconditioned for reuse in subsequent analyses.

11.3 SAMPLE PREPARATION

11.3.1 Samples are preserved, collected and stored as presented in Section 8. All Field and QC Samples, including the LRB, and LFB, must contain the preservatives listed in Section 8.1.2. Before processing, gently shake the sample at least twenty-five times to homogenize, then verify that the sample pH is 7 ± 0.5. If the sample pH does not meet this requirement, discard the sample. If the sample pH is acceptable, proceed with the analysis. Visually inspect the sample cell density during pH verification and make a judgment as to whether the sample can be filtered following Option A (Sect 11.4; filterable within 8 h from the start of filtration) or Option B (Sect. 11.5). Samples that are observed during pH measurement to be semitransparent can typically be processed following the Option A procedure. If samples are not semi-transparent, but rather are thick and viscous, the samples are unlikely to filter within the required 8 h time period, therefore these samples must be processed with the Option B procedure.

11.3.2 If using the Option A procedure, weigh the sample bottle with collected sample to the nearest 1 g, after pH measurement, but before filtration. Measurement by volume will be used for the Option B procedure.

11.3.3 If using Option A, a 100-mL volume is used for the LRB, LFB, LFSM and LFSMD. If using Option B, a 10-mL volume is used for the LRB, LFB, LFSM and LFSMD.

11.3.4 Add an aliquot of the SUR PDS (Sect. 7.2.2.2) to each sample to be extracted, cap and invert to mix. During method development, a 20-µL aliquot of the 5.0 ng/µL SUR PDS (Sect. 7.2.2.2) was added to 100 mL for a final concentration of 1,000 ng/L in the aqueous sample using Option A. For Option B, a 20-µL aliquot of the 5.0 ng/µL SUR PDS (Sect. 7.2.2.2) was added to a 10-mL sample aliquot for a final concentration of 10,000 ng/L in the aqueous sample.

11.3.5 In addition to SUR and preservatives, if the sample is an LFB, LFSM, or LFSMD, add the necessary amount of Analyte PDS (Sect. 0). Cap and invert each sample to mix.

11.3.6 Proceed to the Option A (Sect. 11.4) or Option B (Sect. 11.5) procedure.

11.4 OPTION A PROCEDURE

11.4.1 INTRACELLULAR TOXIN RELEASE PROCEDURE

11.4.1.1 Filter the 100-mL water sample using a Nuclepore filter (Sect. 6.5) with the shiny side up; collect the filtrate into a 500 mL amber glass bottle (Sect. 6.4.1) for extraction in Sect. 11.6. During the filtration of the samples, turn off the hood lights to protect the sample from potential photodegradation.

NOTE: The entire 100-mL sample must be filtered within an 8 h period. If the sample cannot be filtered within an 8 h period and another replicate sample is not available to analyze by the Option B procedure, then allow the sample to finish filtering, if possible. However, the results from this sample must be flagged as suspect due to slow filtration.

11.4.1.2 Rinse sample bottle with 5 mL of 90:10 methanol:reagent water (v/v). Pour bottle rinsate into filter apparatus and combine the rinsate with the filtered water sample in Sect. 11.4.1.1.

11.4.1.3 Rinse the sides of the funnel with another 2.5 mL of 90:10 methanol:reagent water (v/v) and combine with the filtered water sample in Sect. 11.4.1.1.

NOTE: If a different type of filtration apparatus is used (than what is described in Section 6.4) that requires transfer of the filtrate from the receiving container to another container, additional solvent washes (90:10 methanol:water) will be necessary to prevent loss of analytes. Do not exceed 25% methanol in the final 100-mL sample to be extracted.

11.4.1.4 Using metal forceps remove the filter from the filter apparatus and fold the filter in half (top of the filter inward) while only touching the edges of the filter. Continue to fold the filter until it is small enough to fit into a glass test tube (Sect.6.6). Push the filter to the bottom of the glass test tube using a glass pipet.

11.4.1.5 Add 2 mL of 80:20 methanol:reagent water (v/v) to the test tube containing the filter (ensure that the filter is covered with liquid) and manually swirl the tube gently a few times.

11.4.1.6 Place the test tube containing the 2 mL filter solution and the filter in a freezer at -20 °C for a minimum 1 hour. Do not exceed 24 hours in the freezer. <u>If the filter is kept frozen for more than 2 hours, the 100-mL aqueous filtrate from Section 11.4.1.1 must be kept refrigerated at ≤6 °C until completion of the toxin release procedure</u>.

11.4.1.7 Remove the test tube from the freezer, swirl gently a few times, then draw off the 2 mL of liquid using a glass pipet. Transfer the 2 mL of liquid to the filtered 100 mL water sample collected in Section 11.4.1.1.

11.4.1.8 Rinse the filter and test tube by adding another 2 mL of 80:20 methanol:reagent water (v/v) to the test tube and swirl gently. Draw off the 2 mL of liquid using a glass pipet and transfer the 2 mL of liquid to the filtered 100 mL water sample collected in Section 11.4.1.1.

11.4.1.9 Rinse the filter a second time by adding another 1 mL of 80:20 methanol:reagent water (v/v) to the test tube and swirl gently. Draw off the 1 mL of liquid using a glass pipet and transfer the 1 mL of liquid to the filtered 100 mL water sample collected in Section 11.4.1.1. Swirl the 100 mL sample several times to homogenize the sample. Perform SPE as directed in Sect. 11.6.

11.4.2 SAMPLE VOLUME DETERMINATION – Weigh the empty sample bottle to the nearest 1 g and determine the sample weight by subtraction of the empty bottle weight from the original sample weight (Sect.11.3.2). Assume a sample density of 1.0 g/mL. The sample volume for the Option A procedure must be 75-115 mL. If the sample volume is not within this range, the sample results must be flagged as incorrect sample volume collected. The sample volume will be used in the final calculations of the analyte concentration (Sect. 12.2).

11.5 OPTION B PROCEDURE – Option B is a semi-quantitative procedure for samples difficult to filter due to high cell densities. Because of the need to aliquot, use, and analyze only a portion of the sample volume, quantitation of the method analytes will be affected. These effects arise from a lack of sample homogeneity as well as potential sample bottle adsorption losses. Results from this Option B procedure are expected to

be biased low; however, identity of the major congeners present in the sample can be determined.

11.5.1 INTRACELLULAR TOXIN RELEASE PROCEDURE

11.5.1.1 Using a glass graduated cylinder, place a 10 mL sample aliquot in a plastic centrifuge tube (Sect. 6.8), add 30 mL of methanol using the same graduated cylinder, cap and shake the tube gently, and freeze at -20 °C for a minimum of 2 hours and maximum of 24 hours.

11.5.1.2 Remove sample from the freezer and centrifuge at 8,000 rpm and 4 °C for 10 min. **NOTE**: Cyanobacterial cells may contain gas vesicles and depending on the age of the cells, some cells may remain buoyant even after the toxin release procedure and centrifugation.

11.5.1.3 Decant the supernatant into the filter apparatus and filter the supernatant using a Nuclepore filter (Sect. 6.5) with the shiny side up; collect the filtrate into a 500 mL amber glass bottle (Sect. 6.4.1) for extraction in Sect. 11.6. Try not to dislodge the pellet if possible. During filtration of the samples, turn off the hood lights to protect the sample from potential photodegradation.

NOTE: The entire supernatant must be filtered within an 8 h period. If the sample cannot be filtered within an 8 h period then allow the sample to finish filtering, if possible. However, the results from this sample must be flagged as suspect due to slow filtration.

11.5.1.4 Rinse the centrifuge tube with two 2-mL aliquots of 90:10 methanol:reagent water (v/v). Pour the centrifuge tube rinsate into filter apparatus (while not dislodging the pellet) and pass the rinsate through the filter into the water sample filtered in Sect. 11.5.1.3.

11.5.1.5 Add enough reagent water to the filtrate in the 500-mL amber bottle to bring the total volume up to approximately 150 mL (dilutes the methanol to levels that do not affect the SPE).

11.5.1.6 Proceed with SPE of the 150 mL sample in Section 11.6.

11.6 CARTRIDGE SPE PROCEDURE

11.6.1 CARTRIDGE CLEAN-UP AND CONDITIONING – DO NOT allow cartridge packing material to go dry during any of the conditioning steps. Rinse each cartridge with 15 mL of methanol. Next, rinse each cartridge with 15 mL of reagent water, without allowing the water to drop below the top edge of the packing. If the cartridge goes dry during the conditioning phase, the conditioning must be started over. Add 4-5 mL of reagent water to each cartridge, attach

sample transfer tubes (Sect. 6.13.2.2), turn on the vacuum, and begin adding filtered sample (containing the released intracellular toxins) to the cartridge.

11.6.2 SAMPLE EXTRACTION – Adjust the vacuum so that the approximate flow rate is 10-15 mL/min. Do not allow the cartridge to go dry before all the sample has passed through.

11.6.3 SAMPLE BOTTLE AND CARTRIDGE RINSE – After the entire sample has passed through the cartridge, rinse the sample bottles with 10 mL of reagent water and draw the rinse through the sample transfer tubes and the cartridges. Remove the sample transfer tubes from the cartridges, but keep the tubes in their respective bottles for the elution step in Section 11.6.4. Rinse the cartridges with another 5 mL of reagent water. Draw air or nitrogen through the cartridge for 10 min at high vacuum (10-15 in Hg).

11.6.4 SAMPLE BOTTLE AND CARTRIDGE ELUTION – Turn off and release the vacuum. Lift the extraction manifold top and insert a rack with collection tubes into the extraction tank to collect the extracts as they are eluted from the cartridges. Turn the vacuum back on, but ensure the vacuum does not exceed 10 in Hg during elution. Rinse the sample bottles with 5 mL of 90:10 methanol:reagent water (v/v) and elute the analytes from the cartridges by pulling the 5 mL of solution (used to rinse the bottles) through the sample transfer tubes and the cartridges. A soak time (draw a small amount of the elution solvent through the cartridge, release the vacuum), up to 5 min, is permitted, but not required, during the elution step to aid in recovery. Use a low vacuum such that the solvent exits the cartridge in a dropwise fashion. Repeat sample bottle rinse and cartridge elution with a second 5-mL aliquot of 90:10 methanol:reagent water (v/v).

11.6.5 EXTRACT CONCENTRATION – Concentrate the extract to dryness under a gentle stream of nitrogen in a heated water bath (60 °C). Care should be taken, as the extract approached dryness, to keep the nitrogen flow low to prevent blowing dried material out of the tube. Add 990 µL of 90:10 methanol:reagent water (v/v) and 10 µL of the IS PDS (Sect. 7.2.1.2) to the collection vial and vortex. Transfer an aliquot to an autosampler vial.

11.7 EXTRACT ANALYSIS

11.7.1 Establish operating conditions equivalent to those summarized in Tables 1-4 of Section 17. Instrument conditions and columns should be optimized prior to initiation of the IDC.

CAUTION: Diverting the first 6-8 minutes of the LC flow to waste is highly recommended. These extracts will contain small quantities of some of the preservatives which elute early in the chromatogram. Thus, diverting the early portion of the analysis will minimize fouling of the MS source.

11.7.2 Establish an appropriate retention time window for each analyte. This should be based on measurements of actual retention time variation for each method analyte in CAL standard solutions analyzed on the LC over the course of time. A value of plus or minus three times the standard deviation of the retention time obtained for each method analyte while establishing the initial calibration and completing the IDC can be used to calculate a suggested window size. However, the experience of the analyst should weigh heavily on the determination of the appropriate retention window size.

11.7.3 Establish a valid initial calibration following the procedures outlined in Sect. 10.2 or confirm that the calibration is still valid by running a CCC as described in Sect. 10.3. If establishing an initial calibration, complete the IDC as described in Section 9.2.

11.7.4 Begin analyzing field samples, including QC samples, at their appropriate frequency by injecting the same size aliquots (10 µL was used in method development), under the same conditions used to analyze the CAL standards.

11.7.5 At the conclusion of data acquisition, use the same software that was used in the calibration procedure to identify peaks of interest in predetermined retention time windows. Use the data system software to examine the ion abundances of the peaks in the chromatogram. Identify an analyte by comparison of its retention time with that of the corresponding method analyte peak in a reference standard.

11.7.6 The analyst must not extrapolate beyond the established calibration range. If an analyte peak area exceeds the range of the initial calibration curve, the extract may be diluted with 90:10 methanol:reagent water (v/v) and the appropriate amount of internal standard added to match the original level. Re-inject the diluted extract. Incorporate the dilution factor into the final concentration calculations. Acceptable SUR performance (Sect. 9.3.5) should be determined from the undiluted sample extract if analyzed. If the undiluted sample extract is not analyzed, the SUR recovery should be calculated from a standard calibration curve generated from a calibration curve containing a SUR concentration range. The resulting data should be documented as a dilution and MRLs should be adjusted accordingly. It is recommended that samples processed by Option B be analyzed after a 10-fold or 100-fold dilution as the undiluted sample is likely to foul the instrumentation and results are likely to be beyond the established calibration range.

12. DATA ANALYSIS AND CALCULATION

12.1 Complete chromatographic resolution is not necessary for accurate and precise measurements of analyte concentrations using MS/MS. Figure 2 demonstrates the chromatogram achieved using the method conditions. In validating this method,

concentrations were calculated by measuring the product ions listed in Table 4. Other ions may be selected at the discretion of the analyst.

12.2 Calculate analyte and SUR concentrations using the multipoint calibration established in Section 10.2. Do not use daily calibration verification data to quantitate analytes in samples. Adjust final analyte concentrations to reflect the actual sample volume determined in Section 11.4.2 (Option A) or the volume aliquoted in Section 11.5.1.1 (Option B).

12.3 Prior to reporting data, the chromatogram should be reviewed for any incorrect peak identification or poor integration.

12.4 Calculations must utilize all available digits of precision, but final reported concentrations should be rounded to an appropriate number of significant figures (one digit of uncertainty), typically two, and not more than three significant figures.

13. SINGLE LABORATORY METHOD PERFORMANCE

13.1 PRECISION, ACCURACY, AND MINIMUM REPORTING LEVELS – Tables for these data are presented in Section 17. LCMRLs and DLs for each method analyte are presented in Table 5. Precision and accuracy are presented for two water matrices: reagent water (Tables 6 and 8) and lake water (Table 7 and 9) using either Option A or Option B.

13.2 AQUEOUS SAMPLE STORAGE STABILITY STUDIES – An analyte storage stability study was conducted by fortifying the analytes into lake water samples that were collected, preserved, and stored as described in Section 8. Precision and mean recovery (n=4) of analyses, conducted on Days 0, 7, 14, 21 and 28 are presented in Table 10.

13.3 EXTRACT STORAGE STABILITY STUDIES – Extract storage stability studies were conducted on extracts obtained from lake water fortified with method analytes. Precision and mean recovery (n=4) of injections conducted on Days 0, 7, 14, 21, and 28 are reported in Table 11.

13.4 Performance of the method was evaluated in 14 different ambient water sources across the U.S. The box plots in Figure 3 show that QC criteria (dashed lines) were consistently met for 882 analyte measurements in 63 LFSMs except for the 33 analyte failures (96.3% QC pass rate) shown as outliers (green triangles) in the box plots. LFSM failures were due to matrix effects observed in the fortified matrices. Some of the matrices collected contained significant cyanobacterial blooms, including a few cyanobacterial scum samples for which the Option B Procedure was followed.

14. POLLUTION PREVENTION

14.1 This method utilizes SPE to extract analytes from water. It requires the use of very small volumes of organic solvent and very small quantities of pure analytes, thereby minimizing potential hazards to both the analyst and the environment as compared to the use of large volumes of organic solvents in conventional liquid-liquid extractions.

14.2 For information about pollution prevention applicable to laboratory operations described in this method, consult: *Less is Better, Guide to Minimizing Waste in Laboratories*, a web-based resource available from the American Chemical Society website.

15. WASTE MANAGEMENT

Analytical procedures described in this method generate relatively small amounts of waste since only small amounts of reagents and solvents are used. However, laboratory waste management practices must be conducted consistent with all applicable rules and regulations, and that laboratories protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Also, compliance is required with any sewage discharge permits and regulations, particularly the hazardous waste identification rules and land disposal restrictions.

16. REFERENCES

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17. TABLES, DIAGRAMS, FLOWCHARTS AND VALIDATION DATA

TABLE 1. LC METHOD CONDITIONS

Time (min)	% 20 mM Ammonium Formate	% Methanol
Initial	90	10
2.0	90	10
16.0	20	80
16.1	10	90
22.0	10	90
22.1	90	10
26.0	90	10

Phenomenex Kinetex C_8 column, 2.6 µm, 2.1 x 100 mm
Flow rate of 0.3 mL/min
Column temperature of 65 ^oC
10 µL partial loop injection into a 20 µL loop

TABLE 2. ESI-MS/MS METHOD CONDITIONS

ESI Parameter	Settings
Polarity	Positive ion
Capillary needle voltage	4 kV
Cone gas flow	50 L/h
Nitrogen desolvation gas	1000 L/h
Desolvation gas temp.	350 °C

TABLE 3. METHOD ANALYTE SOURCE AND RETENTION TIMES (RTs)

Peak ID	Analyte	Method Analyte Source[a]	RT (min)
1	Nodularin	National Research Council Canada	10.06
2	MC-YR	Enzo Life Sciences	10.15
3	MC-HtyR	Enzo Life Sciences	10.18
4	MC-RR	National Research Council Canada	10.55
5	3-desmethylated-MC-RR	Enzo Life Sciences	10.58
6	MC-LR	National Research Council Canada	10.67
7	MC-WR	Enzo Life Sciences	10.98
8	7-desmethylated-MC-LR	National Research Council Canada	11.08
9	MC-HilR	Enzo Life Sciences	11.19
10	3-desmethylated-MC-LR	Enzo Life Sciences	11.35
11	MC-LA	GreenWater Laboratories	11.47
12	MC-LY	Enzo Life Sciences	11.49
13	MC-LW	Enzo Life Sciences	12.45
14	MC-LF	Enzo Life Sciences	13.07
15	C_2D_5-MC-LR (SUR)	Tamarack Environmental	13.55
16	Cyclosporin-A, $^{13}C_2$, d_4 (IS)	Toronto Research Chemicals	17.19

[a] Data presented in this method were obtained using analytes purchased from these vendors. Other vendors' materials can be used provided the QC requirements in Section 9 can be met.

TABLE 4. MS/MS METHOD CONDITIONS[a,b]

Segment[c]	Analyte	Precursor Ion[d] *(m/z)*	Product Ion[d,e] (*m/z*)	Cone Voltage (v)	Collision Energy[f] (v)
1	Nodularin	825.4 $[M+H]^+$	134.9	45	55
1	MC-YR	523.4 $[M+2H]^{2+}$	134.9	20	20
1	MC-HtyR	1059.6 $[M+H]^+$	134.9	60	75
2	MC-RR	519.9 $[M+2H]^{2+}$	134.9	35	30
2	3-desmethylated-MC-RR	512.9 $[M+2H]^{2+}$	134.9	40	30
2	MC-LR	995.6 $[M+H]^+$	134.9	60	70
3	MC-WR	1068.6 $[M+H]^+$	134.9	60	75
3	7-desmethylated-MC-LR	981.5 $[M+H]^+$	134.9	75	65
3	MC-HilR	1009.6 $[M+H]^+$	134.9	70	65
3	3-desmethylated-MC-LR	981.5 $[M+H]^+$	134.9	70	65
3	MC-LA	910.5 $[M+H]^+$	134.9	40	50
3	MC-LY	1002.5 $[M+H]^+$	134.9	40	60
4	MC-LW	1025.5 $[M+H]^+$	134.9	45	65
4	MC-LF	986.5 $[M+H]^+$	134.9	40	60
4	C_2D_5-MC-LR (SUR)	1028.6 $[M+H]^+$	134.9	55	70
5	Cyclosporin-A, $^{13}C_2$, d_4 (IS)	1208.9 $[M+H]^+$	99.9	65	90

[a] An LC/MS/MS chromatogram of the analytes is shown in Figure 2.

[b] Conditions may vary on different instruments, including whether the precursor ion is doubly charged or not. The conditions in this table are suggested conditions. Other conditions and MS/MS transitions are permitted.

[c] Segments are time durations in which single or multiple scan events occur.

[d] During MS and MS/MS optimization, the analyst should determine the precursor and product ion masses to one decimal place by locating the apex of the mass spectral peak place (e.g., *m/z* 523.4→134.9 for MC-YR). These precursor and product ion masses (with one decimal place) should be used in the MS/MS method for all analyses.

[e] Ions used for quantitation purposes.

[f] Argon used as collision gas at a flow rate of 0.3 mL/min.

TABLE 5. DLs AND LCMRLs IN REAGENT WATER (Option A)

Analyte	Fortified Conc. (ng/L)[a]	DL[b] (ng/L)	LCMRL[c] (ng/L)
Nodularin	4.94	2.3	14
MC-YR	20.0	7.0	54
MC-HtyR	20.0	11	36
MC-RR	3.30	2.1	17
3-desmethylated-MC-RR	10.0	4.4	28
MC-LR	19.1	10	79
MC-WR	60.0	33	170
7-desmethylated-MC-LR	18.8	7.6	49
MC-HilR	20.0	13	62
3-desmethylated-MC-LR	40.0	13	89
MC-LA	20.0	6.9	22
MC-LY	20.0	10	50
MC-LW	20.0	7.6	39
MC-LF	20.0	6.5	23

[a] Spiking concentration used to determine DL.

[b] Detection limits were determined by analyzing seven replicates over three days according to Section 9.2.6.

[c] LCMRLs were calculated according to the procedure in reference 2.

TABLE 6. PRECISION AND ACCURACY DATA FOR METHOD ANALYTES FORTIFIED IN REAGENT WATER (n=4; Option A)

Analyte	Fortified Conc. (ng/L)	Mean % Recovery	% RSD	Fortified Conc. (ng/L)	Mean % Recovery	% RSD
Nodularin	247	108	2.9	49.4	107	1.2
MC-YR	1000	92.6	3.0	200	108	6.4
MC-HtyR	1000	99.7	2.1	200	105	6.2
MC-RR	165	95.2	4.1	33.0	106	5.3
3-desmethylated-MC-RR	500	102	3.8	100	107	3.9
MC-LR	954	105	1.9	191	100	6.9
MC-WR	3000	99.0	1.8	600	93.7	5.4
7-desmethylated-MC-LR	940	97.4	2.8	188	96.0	2.9
MC-HilR	1000	105	2.2	200	98.1	3.3
3-desmethylated-MC-LR	2000	99.6	2.0	400	94.8	6.3
MC-LA	1000	93.1	4.0	200	102	3.2
MC-LY	1000	93.4	2.8	200	99.5	4.3
MC-LW	1000	83.3	3.5	200	89.5	6.3
MC-LF	1000	92.2	2.9	200	97.4	2.1
C_2D_5-MC-LR (SUR)	1000	93.8	2.0	1000	91.7	2.1
Cyclosporin-A, $^{13}C_2$, d_4 (IS)	10000	87.7	6.9	10000	81.0	5.4

TABLE 7. PRECISION AND ACCURACY DATA FOR METHOD ANALYTES FORTIFIED IN LAKE WATER (n=4; Option A)

Analyte	Fortified Conc. (ng/L)	Mean % Recovery	% RSD	Fortified Conc. (ng/L)	Mean % Recovery	% RSD
Nodularin	247	93.0	1.2	49.4	95.6	3.0
MC-YR	1000	88.7	2.2	200	87.8	6.0
MC-HtyR	1000	112	1.2	200	109	12
MC-RR	165	91.8	1.4	33.0	84.9	2.7
3-desmethylated-MC-RR	500	94.1	1.7	100	95.7	3.4
MC-LR	954	110	2.4	191	105	5.1
MC-WR	3000	96.7	1.5	600	97.2	11
7-desmethylated-MC-LR	940	105	3.1	188	104	4.7
MC-HilR	1000	104	4.7	200	105	5.9
3-desmethylated-MC-LR	2000	106	3.5	400	110	5.3
MC-LA	1000	95.9	1.3	200	87.1	6.3
MC-LY	1000	95.6	2.0	200	92.4	4.8
MC-LW	1000	67.0	5.7	200	68.1	17
MC-LF	1000	88.9	2.0	200	86.8	7.0
C_2D_5-MC-LR (SUR)	1000	85.7	2.4	1000	83.5	6.0
Cyclosporin-A, $^{13}C_2$, d_4 (IS)	10000	89.9	5.0	10000	91.6	3.5

TABLE 8. PRECISION AND ACCURACY DATA FOR METHOD ANALYTES FORTIFIED IN REAGENT WATER (n=4; Option B)

Analyte	Fortified Conc. (µg/L)	Mean % Recovery	% RSD
Nodularin	2.50	95.1	6.6
MC-YR	10.0	94.6	7.2
MC-HtyR	10.0	86.8	14
MC-RR	1.80	87.4	12
3-desmethylated-MC-RR	5.00	83.4	13
MC-LR	10.0	90.5	10
MC-WR	30.0	90.4	11
7-desmethylated-MC-LR	9.40	89.8	6.9
MC-HilR	10.0	89.1	5.6
3-desmethylated-MC-LR	20.0	96.5	7.8
MC-LA	10.0	98.1	5.9
MC-LY	10.0	92.8	11
MC-LW	10.0	88.5	7.3
MC-LF	10.0	89.9	6.9
C_2D_5-MC-LR (SUR)	10.0	87.0	5.3
Cyclosporin-A, $^{13}C_2$, d_4 (IS)	10.0	94.2	12

TABLE 9. PRECISION AND ACCURACY DATA FOR METHOD ANALYTES FORTIFIED IN LAKE WATER (n=4; Option B)

Analyte	Fortified Conc. (µg/L)	Mean % Recovery	% RSD
Nodularin	2.50	92.6	7.7
MC-YR	10.0	94.4	6.9
MC-HtyR	10.0	85.4	3.9
MC-RR	1.80	89.7	6.2
3-desmethylated-MC-RR	5.00	91.8	8.3
MC-LR	10.0	89.7	5.7
MC-WR	30.0	86.3	6.5
7-desmethylated-MC-LR	9.40	87.7	6.3
MC-HilR	10.0	88.1	6.5
3-desmethylated-MC-LR	20.0	92.0	6.8
MC-LA	10.0	95.8	3.7
MC-LY	10.0	91.1	5.1
MC-LW	10.0	86.2	4.4
MC-LF	10.0	86.2	5.0
C_2D_5-MC-LR (SUR)	10.0	85.3	6.1
Cyclosporin-A, $^{13}C_2$, d_4 (IS)	10.0	91.3	13

TABLE 10. AQUEOUS SAMPLE HOLDING TIME DATA FOR LAKE WATER SAMPLES FORTIFIED WITH METHOD ANALYTES AND PRESERVED AND STORED ACCORDING TO SECTION 8 (n=4; Option A)

Analyte	Fortified Conc. (ng/L)	Day 0	Day 0	Day 7	Day 7	Day 14	Day 14	Day 21	Day 21	Day 28	Day 28
		Mean %Rec	% RSD	Mean %Rec	% RSD	Mean %Rec	% RSD	Mean %Rec	% RSD	Mean %Rec	% RSD
Nodularin	494.4	87.4	3.3	91.1	3.3	80.8	3.8	82.6	4.7	83.9	2.1
MC-YR	2000	92.6	1.8	100.4	1.6	87.5	2.0	88.6	2.9	86.6	4.8
MC-HtyR	2000	90.3	3.7	97.7	3.0	86.9	1.8	90.4	2.8	87.8	2.7
MC-RR	329.6	83.4	5.2	88.9	2.5	78.4	1.9	83.3	2.7	82.5	1.6
3-desmethylated-MC-RR	1000	91.8	2.3	100.7	2.5	88.9	3.0	93.8	3.7	91.9	1.7
MC-LR	2000	89.5	5.8	92.4	1.2	86.0	3.1	91.1	4.0	91.5	2.7
MC-WR	6000	91.9	6.1	99.4	1.0	91.0	1.9	95.5	5.0	91.4	1.9
7-desmethylated-MC-LR	1880	93.2	3.6	97.8	0.6	89.8	1.3	93.0	4.2	91.0	2.7
MC-HilR	2000	91.1	3.9	97.8	4.6	88.9	1.5	92.6	5.9	89.3	2.5
3-desmethylated-MC-LR	4000	98.0	3.4	105.5	2.4	97.2	1.1	96.2	3.1	96.2	2.1
MC-LA	2000	83.3	4.0	96.4	4.0	83.2	6.8	84.7	4.4	86.3	4.1
MC-LY	2000	92.1	3.2	95.7	3.8	80.2	4.0	89.4	6.4	91.3	1.4
MC-LW	2000	80.3	3.7	91.7	3.9	81.3	1.2	83.0	2.7	80.5	3.3
MC-LF	2000	85.4	1.4	92.2	0.8	85.4	0.6	89.1	1.8	88.5	2.1
C_2D_5-MC-LR (SUR)[a]	1664	82.8	4.7	89.7	3.7	84.9	2.3	85.6	4.2	87.4	2.4

[a] Surrogate was not added to samples until the day of extraction.

TABLE 11. EXTRACT HOLDING TIME DATA FOR SAMPLES FROM A LAKE WATER SOURCE, FORTIFIED WITH METHOD ANALYTES AND PRESERVED AND STORED ACCORDING TO SECTION 8 (n=4; Option A)

Analyte	Fortified Conc. (ng/L)	Day 0	Day 0	Day 7	Day 7	Day 14	Day 14	Day 21	Day 21	Day 28	Day 28
		Mean %Rec	% RSD	Mean %Rec	% RSD	Mean %Rec	% RSD	Mean %Rec	% RSD	Mean %Rec	% RSD
Nodularin	494.4	87.4	3.3	86.1	2.7	81.5	2.2	82.5	2.5	77.7	2.7
MC-YR	2000	92.6	1.8	98.0	4.1	84.9	2.0	89.1	2.0	81.4	1.1
MC-HtyR	2000	90.3	3.7	94.9	2.8	87.1	3.2	92.6	2.2	82.1	3.3
MC-RR	329.6	83.4	5.2	84.6	3.3	77.1	0.6	82.3	2.5	73.4	2.3
3-desmethylated-MC-RR	1000	91.8	2.3	98.8	3.5	90.5	1.8	91.6	2.6	85.2	2.6
MC-LR	2000	89.5	5.8	90.0	3.6	87.0	1.3	92.7	1.4	82.8	2.7
MC-WR	6000	91.9	6.1	98.5	4.0	92.6	1.4	99.2	3.6	89.5	0.5
7-desmethylated-MC-LR	1880	93.2	3.6	95.9	2.6	89.9	3.3	97.8	3.2	86.6	1.6
MC-HilR	2000	91.1	3.9	98.0	3.2	88.5	3.4	98.7	1.7	83.7	1.8
3-desmethylated-MC-LR	4000	98.0	3.4	102.7	4.2	97.8	0.9	101.5	2.2	92.5	2.1
MC-LA	2000	83.3	4.0	92.5	5.0	81.5	5.9	87.1	2.1	91.2	0.8
MC-LY	2000	92.1	3.2	90.1	4.9	82.2	3.3	91.7	4.6	83.0	2.6
MC-LW	2000	80.3	3.7	81.6	3.5	78.0	6.2	83.3	1.6	88.1	1.9
MC-LF	2000	85.4	1.4	86.9	5.9	83.9	3.3	86.1	3.0	82.6	3.2
C_2D_5-MC-LR (SUR)	1664	82.8	4.7	79.8	4.0	80.8	2.7	86.1	1.2	86.9	2.9

FIGURE 1.
DIAGRAM OF FILTER APPARATUS WITH PART NUMBERS (SECT. 6.4)

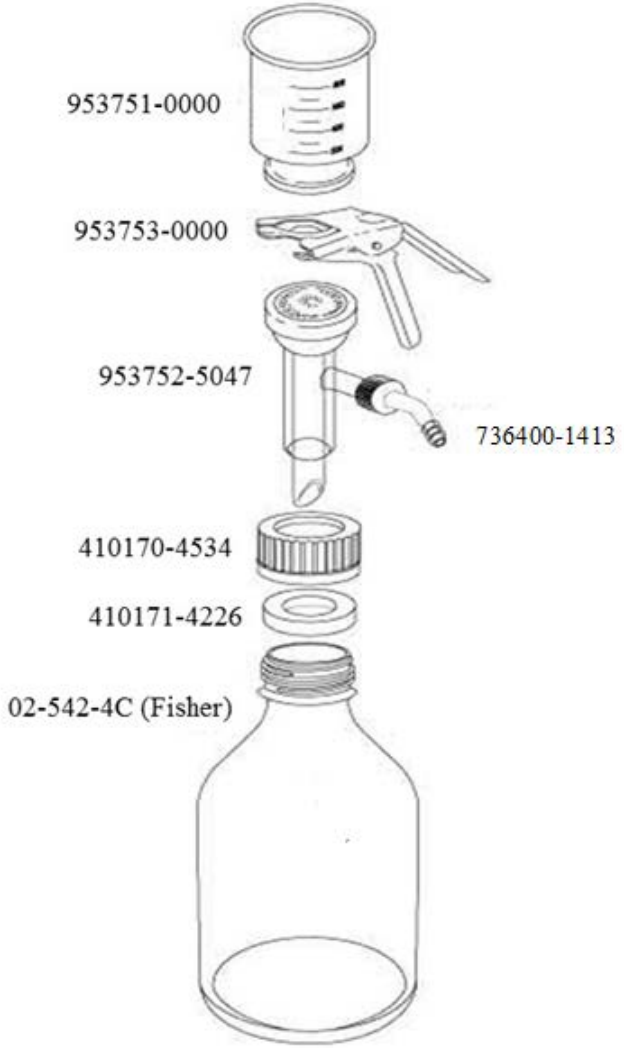

FIGURE 2.
EXAMPLE CHROMATOGRAMS (OVERLAID MS/MS SEGMENTS) OF A CALIBRATION STANDARD WITH ANALYTES AT MID-LEVEL CALIBRATION CONCENTRATIONS. SEE TABLE 3 FOR PEAK IDs.

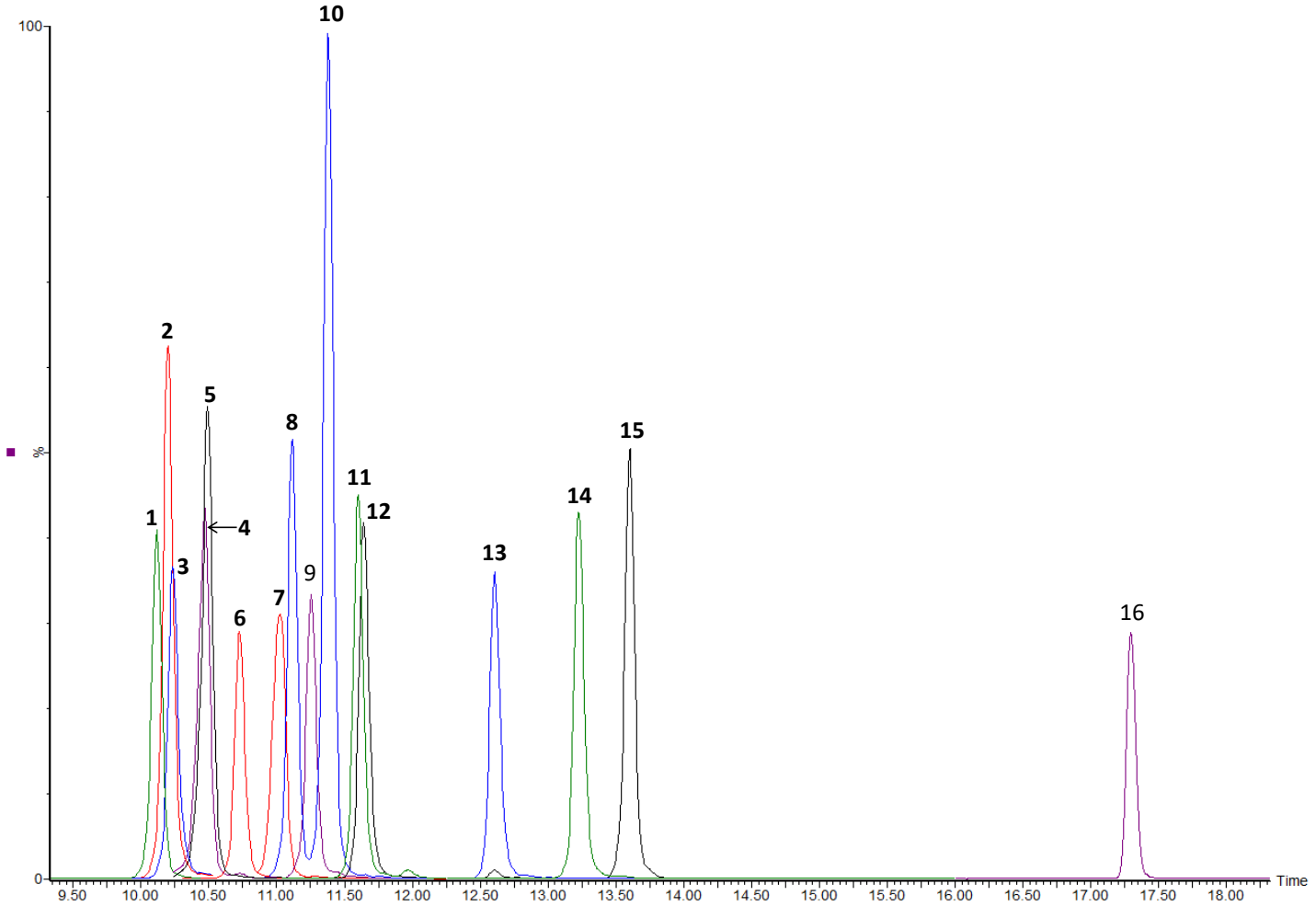

FIGURE 3.
BOX PLOTS SHOWING DISTRIBUTION OF LFSM RECOVERIES OBTAINED IN AMBIENT WATERS FROM 14 DIFFERENT WATER BODIES ACROSS THE U.S. (SECT. 13.4). GREEN TRAINGLES REPRESENT THE 33 ANALYTE QC FAILURES OUT OF 882 ANALYTE MEASUREMENTS.