Single Laboratory Validated Method for Determination of Cylindrospermopsin and Anatoxin-a in Ambient Freshwaters by Liquid Chromatography/ Tandem Mass Spectrometry (LC/MS/MS)

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DISCLAIMER

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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 cylindrospermopsin and anatoxin-a (combined intracellular and extracellular) in ambient freshwater. Single laboratory accuracy and precision data have been generated in reagent water and ambient freshwaters for cyanotoxins listed in the table below.

Analyte	Chemical Abstract Services Registry Number (CASRN)
Anatoxin-a	64285-06-9
Cylindrospermopsin	143545-90-8

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 are 0.23 µg/L for cylindrospermopsin and 0.097 µg/L for anatoxin-a (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 sample matrix, fortification concentration, and instrument performance. DLs for analytes in this method are 0.065 µg/L for cylindrospermopsin and 0.049 µg/L for anatoxin-a (Table 5).

1.5 This method is intended for use by analysts skilled in 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 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 preparation steps (Sect.0, 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 and internal standard must be adequately resolved chromatographically in order to

prevent misidentification of phenylalanine as anatoxin-a. 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 that acceptable method performance can be verified in a real sample matrix (Sect. 9.3.5).

NOTE: The above section is intended as an abbreviated summation of method flexibility. Sections 4-12 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 4-12, Sections 4-12 supersede Section 1.6.

2. SUMMARY OF METHOD

In the field, samples are added to bottles or vials containing sodium bisulfate (acidic microbial inhibitor). In the laboratory, aliquots (1 mL) of sample are subjected to three freeze/thaw cycles, the internal standard added, and filtered. Samples with significant cell densities may require centrifugation prior to filtration. An aliquot of the sample is injected into an LC equipped with an analytical column that is interfaced to an MS/MS. The analytes are separated and identified by comparing retention times and signals produced by unique mass transitions to retention times and mass transitions for calibration standards acquired under identical LC/MS/MS conditions. The concentration of each analyte is determined using the integrated peak area and the internal standard technique.

3. DEFINITIONS

3.1 ANALYSIS BATCH – A set of samples that is processed and 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 and/or the number of field samples.

3.2 CALIBRATION STANDARD (CAL) – A solution prepared from the primary dilution standard solution or stock standard solution, 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, and internal standard. 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 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.

3.7 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.

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

3.9 LABORATORY FORTIFIED BLANK (LFB) – A volume of reagent water or other blank matrix to which known quantities of the method analytes and all the 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.

3.10 LABORATORY FORTIFIED SAMPLE MATRIX (LFSM) – A preserved field sample to which known quantities of the 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. The background concentrations of the analytes in the sample matrix must be determined in a separate sample and the measured values in the LFSM corrected for background concentrations.

3.11 LABORATORY FORTIFIED SAMPLE MATRIX DUPLICATE (LFSMD) – A duplicate of the field sample used to prepare the LFSM. The LFSMD is fortified, processed, 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.

3.12 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 preservative, and internal standard 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, the reagents, or the apparatus.

3.13 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]

3.14 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.

3.15 PRECURSOR ION – For the purpose of this method, the precursor ion is the protonated molecule ($[M+H]^+$) 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.

3.16 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.

3.17 PRIMARY ION (P) TRANSITION – Primary MS/MS transition used to quantitate the method analyte.

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 second source stock standard solution is used to fortify the QCS at a known concentration. 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 SECONDARY ION (S) TRANSITION – Secondary MS/MS transition used to confirm the presence of the method analyte but not used in quantitation.

3.23 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.

4. INTERFERENCES

4.1 All glassware must be meticulously cleaned. Wash glassware 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.

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 in the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature of the water. High levels of humic and/or fulvic material can cause signal enhancement or suppression in the electrospray ionization source. [3-4]

4.4 Relatively large quantities of the preservative (Sect. 8) are added to sample bottles. The potential exists for trace-level organic contaminants in this reagent. Interferences from this source should be monitored by analysis of laboratory reagent blanks (Sect. 9.3.1), particularly when new lots of reagents are acquired.

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.[5] Additional references to laboratory safety are available.[6-8]

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 – 40-mL, or larger, amber glass bottles fitted with polytetrafluoroethylene (PTFE)-lined screw caps or amber plastic vials/bottles (polypropylene or polyethylene terephthalate glycol (PETG).

6.2 BULK COLLECTION CONTAINER – 40-mL (or larger depending on sample volume collected) clear or amber glass bottles or clear or amber plastic (polypropylene or PETG) bottles. One bulk container per field sample is required.

6.3 STANDARD CONTAINERS – Amber 12-mL 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).

6.4 MICROCENTRIFUGE VIALS - Two-mL amber polypropylene microcentrifuge Safe-Lock vials (Eppendorf #022363379).

6.5 SYRINGES AND PIPETTE

6.5.1 Glass microsyringes are recommended for aliquoting standards. Suggested sizes include 5, 10, 25, 50, 100, 250, 500, and 1000 microliters (µL).

6.5.2 Adjustable manual pipette with polypropylene tips are recommended for aliquoting aqueous samples. Suggested capacity of 1 milliter (mL).

6.6 DISPOSABLE PASTEUR PIPETTES – 5 ¾-inch or 9-inch borosilicate glass, used to transfer centrifuged samples to the filter and for sample preparation (Fisher Cat. No. 13-678-20B, 13-678-20C, or equivalent).

6.7 DISPOSABLE SYRINGES – 10-mL, polypropylene, Luer Lock syringes for use in filtering standards and samples (Fisher Cat No. 03-377-23, or equivalent).

6.8 SYRINGE FILTERS – 13 mm, 0.2-µm pore size PVDF filters (Fisher Cat No. 09-910-13, or equivalent).

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

6.11 CENTRIFUGE – Capable of centrifugation at 5,000 rpm of 2-mL microcentrifuge vials.

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

6.12.1 LC SYSTEM – Instrument capable of reproducibly injecting up to 50-µL aliquots, and performing binary linear gradients at a constant flow rate near the flow rate used for development of this method (0.25 mL/min). Usage of a column heater is optional.

6.12.2 TANDEM MASS SPECTROMETER – The mass spectrometer must be capable of positive ion electrospray ionization (ESI) near the suggested LC flow rate of 0.25 mL/min. The system must be capable of performing MS/MS to produce unique product ions for method analytes. 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.

6.12.3 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.

6.12.4 ANALYTICAL COLUMN – C_{18} column (2.1 x 150 mm) packed with 5 µm C_{18} solid phase particles (Thermo Scientific Betasil #70105-152130). Any equivalent column that provides adequate resolution, peak shape, capacity, accuracy, and precision (Sect. 1.6 and 9.2) may be used.

6.12.5 ANALYTICAL GUARD COLUMN – Not used during method development but may be used to prolong the life of the column.

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 ACETIC ACID, (CH3COOH, CASRN 64-19-7) – Glacial, HPLC grade (Fisher Cat. No. A35-500, or equivalent). Added to eluent as a mobile phase modifier.

7.1.4 100 mM ACETIC ACID – To prepare 1 L, add 5.8 mL glacial acetic acid to 1 L of reagent water.

7.1.5 SODIUM BISULFATE, ($NaHSO_4$, CASRN 7681-38-1) – ~95% (Sigma Cat. No. 71656, or equivalent). Used to inhibit microbial growth in water samples.

7.1.6 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. PDS and stock standards were found to be stable for a minimum of six 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) SOLUTION – L-Phenylalanine-d_5 (CASRN 56253-90-8), purchased as neat material from Cambridge Isotopes (or equivalent), is used as the internal standard. If isotopically labeled target analytes are available, they may be used. Depending on the source and purity, labeled internal standards may contain a small percentage of the corresponding native analyte. Therefore, the analyst must demonstrate that the labeled internal standards do not contain the unlabeled analytes at a concentration >1/3 of the MRL when added at the selected concentration to samples.

7.2.1.1 IS STOCK STANDARD SOLUTION (1000 ng/µL) – The IS stock standard solution is prepared by diluting 10 mg of the IS in 10 mL of methanol. This IS stock standard was stored at -15 ºC or less in amber glass screw cap vials.

7.2.1.2 IS PRIMARY DILUTION STANDARD – (IS PDS; 0.5 ng/µL) – The IS PDS is prepared at 0.5 ng/µL by diluting 5 µL of the IS stock standard in 10 mL of methanol. Ten µL of this 0.5 ng/µL solution is used to fortify the final 1-mL samples and standards (Sect. 11.1.7). This will yield an IS concentration of 5 µg/L in the 1-mL samples and standards. The IS PDS was stored at -15 ºC or less in amber glass screw cap vials. The IS concentration may be adjusted to accommodate instrument sensitivity.

7.2.2 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).

7.2.2.1 ANALYTE STOCK STANDARD SOLUTIONS (20-59 µg/mL) – Obtain the analytes listed in the table in Section 1.1 as neat materials. To prepare stock standards from neat material, individually weigh the appropriate mass of the solid standards into tared 10-mL volumetric flasks and dilute to volume with methanol/reagent water (1:1) to obtain analyte concentrations of approximately 20 µg/mL for cylindrospermopsin and

59 µg/mL anatoxin-a. Alternatively, purchase commercially available stock standard solutions of the analytes, if available. Concentrations may be adjusted for instrument sensitivity. These stock standards were stored at -15 ºC or less in amber glass screw cap vials.

NOTE: Anatoxin-a may not be available as a solution or neat material. If another form of anatoxin-a is used to prepare stock solutions (for example anatoxin-a fumarate), the analyst should correct for the mass difference. For example,

$$\text{Corrected mass} = \frac{\text{MW}_{\text{anatoxin - a}}}{\text{MW}_{\text{anatoxin - a fumarate}}} \times \text{Measured mass}$$

7.2.2.2 ANALYTE PRIMARY DILUTION STANDARD (PDS) SOLUTION (0.059 -0.15 µg/mL) – 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 1:1 methanol:reagent water at concentrations of 0.15 µg/mL for cylindrospermopsin and 0.059 µg/mL for anatoxin-a. The Analyte PDS is prepared by dilution of the combined Analyte Stock Standard Solutions (Sect. 7.2.2.1) and is used to prepare CAL standards, and fortify LFBs, LFSMs, and LFSMDs with the method analytes. The Analyte PDS was stored at -15 ºC or less in amber glass screw cap vials.

7.2.3 CALIBRATION (CAL) STANDARDS – The preparation of CAL standards requires the use of reagent water containing the sodium bisulfate preservative at 1.0 g/L as a matrix. Prepare at least five calibration standards over the concentration range of interest by adding aliquots of Analyte PDS with the reagent water containing 1.0 g/L sodium bisulfate and diluting to 1 mL. The lowest calibration standard must be at or below the MRL. Add a constant amount of the IS PDS to each 1-mL calibration standard. CAL standards may also be used as CCCs (Sect. 9.3.2). CAL standards must be discarded and replaced every 28 days.

8. SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 SAMPLE BOTTLE PREPARATION

8.1.1 Collect a minimum of a 40-mL (larger sample volumes may be collected if desired) sample in plastic or glass vials/bottles.

8.1.2 The preservation reagent, listed in the table below, is added to each sample bottle as a solid prior to shipment to the field (or prior to sample collection).

Compound	Amount	Purpose
Sodium bisulfate	1.0 g/L	Acidic microbial inhibitor

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 preservations agents is not permitted.)

8.2.1 Collect bulk sample water in a glass, PETG or polypropylene (Sect. 6.2) container of sufficient volume to obtain the sample.

8.2.2 Gently shake the bulk sample collection bottle at least 25 times to aid in homogenizing the sample. Immediately pour a portion of the sample water into the bottle containing the preservative (ensure the amount of preservative is appropriate for the volume of water aliquoted). Do not overflow the bottle containing the preservative. Samples do not need to be collected headspace free. The LFSM and the LFSMD may be drawn from the field sample by the laboratory, before processing, since not all of the field sample is consumed during processing and analysis. (Sect. 11.1.2).

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. Keep the sample sealed from time of collection until processing.

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 processing, 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 HOLDING TIMES – Water samples should be analyzed as soon as possible after collection but must be processed and analyzed within 28 days of collection. Sample holding time data are presented in Table 8.

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, 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 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 filter, 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.

9.2.2 INITIAL DEMONSTRATION OF PRECISION (IDP) – Prepare and analyze four to seven replicate LFBs fortified near the midrange of the initial calibration curve according to the procedure described in Section 11. The sample preservative as described in Section 8 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.

9.2.4 MINIMUM REPORTING LEVEL (MRL) CONFIRMATION – Establish a target concentration for the MRL based on the intended use of the method. 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 the MRL following the procedure outlined below.

9.2.4.1 Fortify, process, and analyze seven replicate LFBs at the proposed MRL concentration. These LFBs must contain the method preservative described in Section 8. 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 + HRPIR) 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.

NOTE: These equations are only valid for seven replicate samples.

9.2.5 CALIBRATION CONFIRMATION – Analyze a QCS as described in Section 9.3.11 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.

9.2.6.1 Replicate analyses for this procedure 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 must also be added to these samples. Analyze the seven replicates through all steps of Section 11.

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.

9.2.6.2 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 analysis batch (Sect. 3.1) 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 analysis 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, that is carried through all the processing steps in Section 11, is required with each analysis batch (Sect. 3.1). 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. 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 analysis 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 standard(s). If IS areas in a chromatographic run do not meet these criteria, inject a second aliquot of that standard or sample.

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 aliquot 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, another aliquot of the sample may need to be processed provided the sample is still within the holding time. Otherwise, report results obtained from the reinjected aliquot, but annotate as suspect.

9.3.5 LABORATORY FORTIFIED SAMPLE MATRIX (LFSM) – Analysis of an LFSM is required in each analysis 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. 9.3.6). 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.

9.3.5.1 Within each analysis batch (Sect. 3.1), 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. 7.2.2.2). 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 the 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.

9.3.5.2 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.

9.3.5.3 Analyte recoveries may exhibit matrix bias. For samples fortified at or above their native concentration, recoveries should range between 70-130%, 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 the results are suspect due to matrix effects.

9.3.6 LABORATORY FORTIFIED SAMPLE MATRIX DUPLICATE (LFSMD) – Within each analysis batch (not to exceed 20 field samples, Sect. 3.1), a minimum of one LFSMD must be analyzed. Duplicates check the precision associated with sample collection, preservation, storage, and laboratory procedures.

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

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

9.3.8 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 the results are suspect due to matrix effects.

9.3.9 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 the heterogeneity of the cyanobacterial bloom and not as a measure of sample collection or laboratory precision.

9.3.10 SECONDARY ION CRITERIA – The analyte areas for the secondary ion (S) transition and the primary ion (P) transition (P used for quantitation) must be monitored in all analyses and P/S ratios must be calculated for each analyte.

9.3.10.1 The P/S ratios must calculate to be within ± 30% of the average P/S ratio calculated from the most recent calibration (Sect. 10.2). Greater variability may be observed when the analyte concentrations are at or near the MRL (within a factor of two times the MRL concentration). At these concentrations, the P/S ratio must be within ± 50% of the average P/S ratio.

9.3.10.2 If the P/S ratios fall outside the designated range, inject a second aliquot of that standard or sample. If analysis of the second aliquot produces acceptable P/S criteria, results of the second aliquot may be reported.

9.3.10.3 If the P/S ratio of the re-analyzed second aliquot still does not meet the criterion, check the calibration by analyzing the most recent CCC. If the P/S criterion is met in the CCC but not the sample, the sample results must be reported as suspect. If the P/S criterion is not met in the CCC, then corrective action (Sect. 10.3.4) must be taken to address the issue.

9.3.11 QUALITY CONTROL SAMPLES (QCS) – As part of the IDC (Sect. 9.2), each time a new Analyte PDS (Sect. 7.2.2.2) 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 calculated value for each analyte must be within ±30% of the expected value.

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 Optimize the precursor ion (Sect. 3.15, $[M+H]^+$) for each method analyte by infusing approximately 2 µg/mL of each analyte (prepared in the initial mobile phase conditions) directly into the MS at the chosen LC mobile phase flow rate (approximately 0.25 mL/min). This tune can be done on a mix of method analytes. MS parameters (voltages, temperatures, gas flows, etc.) are varied until optimal analyte responses are determined. Method analytes may have different optima requiring some compromise between the optima. See Table 2 for ESI-MS conditions used in method development.

10.2.1.2 Optimize two product ions (Sect. 3.18) for each analyte, by infusing approximately 2 µg/mL of each analyte directly into the MS at the chosen LC mobile phase flow rate (approximately 0.25 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. One of these product ions (typically the most abundant) is used as the primary ion transition (P) and the other product ion as the secondary ion transition (S). The primary ion transition is used as the quantitative transition and the secondary ion transition is used for confirmation purposes (Sect. 9.3.10). A secondary ion transition is not required for the IS.

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- to high-level calibration standard under optimized LC/ESI-MS/MS conditions to obtain the retention times of each method analyte. Product ions (quantitation ions and confirmation ions) chosen during method development are in Table 4, although these may be instrument dependent.

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 each peak for optimum precision.

10.2.5 Prepare a set of at least five CAL standards as described in Section 7.2.3. 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 CALCULATION OF PRIMARY/SECONDARY ION RATIOS (P/S) – From the initial calibration data, establish average P/S ratios by dividing the primary ion transition area by the secondary ion area for each analyte. These ratios will be used to confirm the results as described in Section 9.3.10.

10.2.8 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. In addition, the P/S criteria described in Section 9.3.10 must be met for each calibration standard. 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.

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 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.

10.3.2 Calculate the P/S ratio as described in Section 9.3.10. The CCC must meet the P/S criteria in Section 9.3.10.

10.3.3 Calculate the concentration of each analyte in the CCC. 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% of the true value. 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 shows no detection for that method analyte, non-detects may be reported without re-analysis.**

10.3.4 CORRECTIVE 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 system 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 SAMPLE PREPARATION

11.1.1 Samples are preserved, collected and stored as presented in Section 8. All field and QC samples, including the LRB and LFB, must contain the preservative listed in Section 8. Verify that the sample pH is 2 ± 0.5 due to the addition of sodium bisulfate during sample collection. If the sample pH does not meet this requirement, discard the sample. If the sample pH is acceptable, proceed with the analysis.

11.1.2 Immediately, before processing, gently shake the sample at least twenty-five times to homogenize. Aliquot an appropriate amount of each field sample or QC sample into amber 2-mL microcentrifuge vials (Sect. 6.4). For example, aliquot 990 µL for field samples but

only 940 µL for an LFSM spiked with 50 µL of the Analyte PDS. (10 µL of IS PDS is added in Sect. 11.1.7 for a total volume of 1-mL.) The LFSM and LFSMD aliquots may be drawn from the remaining, unconsumed, field sample.

11.1.3 Fortify LFBs, LFSMs, or LFSMDs, with an appropriate volume of Analyte PDS (Sect. 7.2.2.2). Cap and invert each sample several times to mix.

11.1.4 Freeze all field and QC samples at -20 °C or less for 30 min or until completely frozen.

11.1.5 Thaw all field and QC samples at 40 ºC or less for 5 min or until completely thawed.

11.1.6 Repeat steps Sections 11.1.4 and 11.1.5 two more times.

11.1.7 Add 10 µL of the IS PDS (Sect. 7.2.1.2) and mix well.

11.1.8 Centrifuge all field and QC samples at 5,000 rpm for 5 min to pellet any cyanobacterial cells that may be present in the samples.

11.1.9 Filter the supernatants using 0.2 µm PVDF filters and disposable syringes. Place each filtered solution in an autosampler vial and cap. The filters used for calibration standards and samples must be of the same lot. If a new lot of filters is used for subsequent Analysis Batches, the analyst must ensure all QC requirements are still met.

11.2 SAMPLE ANALYSIS

11.2.1 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.

11.2.2 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.

11.2.3 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.

11.2.4 Begin analyzing field samples, including QC samples, at their appropriate frequency by injecting the same size aliquots (50 µL was used in method development), under the same conditions used to analyze the CAL standards.

11.2.5 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.

11.2.6 The analyst must not extrapolate beyond the established calibration range. If an analyte result exceeds the range of the initial calibration curve, the sample may be diluted using reagent water containing the preservative and the appropriate amount of internal standard added to match the original level. Re-inject the diluted sample. Incorporate the dilution factor into final concentration calculations. The resulting data must be annotated as a dilution, and the reported MRLs must reflect the dilution factor.

12. DATA ANALYSIS AND CALCULATION

12.1 Complete chromatographic resolution is not necessary for accurate and precise measurements of analyte concentrations using MS/MS. However, baseline resolution must be achieved for anatoxin-a and the IS in order to prevent misidentification of phenylalanine as anatoxin-a in samples. Phenylalanine and anatoxin-a are isobaric and the MS/MS transitions used for quantitation of anatoxin-a are also observed in the fragmentation pattern of phenylalanine. 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 concentrations using the multipoint calibration established in Section 10.2. Do not use daily calibration verification data to quantitate analytes in samples.

12.3 Prior to reporting data, the chromatogram should be reviewed for any incorrect peak identification, poor integration or failing P/S ratio criterion. See Figure 1 has an example chromatogram obtained under method conditions.

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 (Table 6) and lake water (Table 7).

13.2 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, 8, 14, 21 and 28 are presented in Table 8.

13.3 Performance of the method was evaluated in 16 different ambient water sources across the U.S. The box plots in Figure 2 show that QC criteria (dashed lines) were consistently met for both analytes in fortified QC samples (LFBs and LFSMs) except for the two QC failures shown as outliers (green triangles) in the box plots. The two anatoxin-a QC failures were due to matrix effects observed in the LFSM. Results in Figure 2 are compiled from 104 laboratory fortified blanks (LFBs) and 167 laboratory fortified sample matrices (LFSMs). The QC samples were spiked at concentrations spanning the calibration range, including the reporting limits which were 0.30 µg/L for cylindrospermopsin and 0.12 µg/L for anatoxin-a. Some of the matrices collected contained significant cyanobacterial blooms, including a few cyanobacterial scum samples.

14. POLLUTION PREVENTION

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

1. Winslow, S.D., Pepich, B.V., Martin, J.J., Hallberg, G.R., Munch, D.J., Frebis, C.P., Hedrick, E.J., Krop, R.A. “Statistical Procedures for Determination and Verification of Minimum Reporting Levels for Drinking Water Methods.” *Environ. Sci. Technol.* 2004, **40**, 281-288.

2. Glaser, J.A., D.L. Foerst, G.D. McKee, S.A. Quave, W.L. Budde, "Trace Analyses for Wastewaters." *Environ. Sci. Technol.* 1981, **15,** 1426-1435.

3. Leenheer, J.A., Rostad, C.E., Gates, P.M., Furlong, E.T., Ferrer, I. “Molecular Resolution and Fragmentation of Fulvic Acid by Electrospray Ionization/Multistage Tandem Mass Spectrometry.” *Anal. Chem.* 2001, **73**, 1461-1471.

4. Cahill, J.D., Furlong E.T., Burkhardt, M.R., Kolpin, D., Anderson, L.G. “Determination of Pharmaceutical Compounds in Surface- and Ground-Water Samples by Solid-Phase Extraction and High-Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry.” *J. Chromatogr. A*, 2004, **1041**, 171-180.

5. “Biosafety in Microbiological and Biomedical Laboratories”, 5th edition, *Appendix I—Guidelines for Work with Toxins of Biological Origin***.** A web-based resource available from U.S. Department of

Health and Human Services**,** Public Health Service Centers for Disease Control and Prevention, National Institutes of Health.

"Prudent Practices in the Laboratory: Handling and Disposal of Chemicals," a web-based resource available from National Academies Press (1995).

6. "OSHA Safety and Health Standards, General Industry," (29CFR1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, July 2001).

7. "Safety in Academic Chemistry Laboratories," a web-based resource available from American Chemical Society Publication, Committee on Chemical Safety, 7th Edition.

17. TABLES, DIAGRAMS, FLOWCHARTS AND VALIDATION DATA

TABLE 1. LC METHOD CONDITIONS

Time (min)	% 100 mM Acetic acid	% Methanol
Initial	100	0
7.0	20	80
12.0	20	80
12.1	100	0
40.0	100	0

Thermo Scientific Betasil C_{18} column, 5 µm, 2.1 x 150 mm
Flow rate of 0.25 mL/min
50 µL partial loop injection into a 250 µL loop

TABLE 2. ESI-MS/MS METHOD CONDITIONS

ESI Parameters	Settings
Polarity	Positive ion
Capillary needle voltage	4 kV
Cone gas flow	80 L/h
Nitrogen desolvation gas	800 L/h
Desolvation gas temp.	300 °C

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

Peak ID	Analyte	Method Analyte Source[a]	RT (min)
1	Cylindrospermopsin	Santa Cruz Biotechnology	6.39
2	Anatoxin-a	Abcam	6.57
3	L-Phenylalanine-d_5	Cambridge Isotopes	7.34

[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]

Analyte	Precursor Ion[b] (*m/z*)	Quantitation Ion (*m/z*)	Confirmation Ion[b,c] (*m/z*)	Cone Voltage (v)	Collision Energy[c] (v)
Cylindrospermopsin	416	194	176	35	35
Anatoxin-a	166	149	131	25	15
L-Phenylalanine-d_5	171	125	N/A	18	10

[a] An LC/MS/MS chromatogram of the analytes is shown in Figure 1.

[b] 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* 416.1→194.1). These precursor and product ion masses (with one decimal place) should be used in the MS/MS method for all analyses.

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

TABLE 5. DLs AND LCMRLs IN REAGENT WATER

Analyte	Fortified Conc. (µg/L)[a]	DL[b] (µg/L)	LCMRL[c] (µg/L)
Cylindrospermopsin	0.15	0.065	0.23
Anatoxin-a	0.059	0.049	0.097

[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 1.

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

Analyte	Fortified Conc. (µg/L)	Mean % Recovery	% RSD	Fortified Conc. (µg/L)	Mean % Recovery	% RSD
Cylindrospermopsin	0.30	103	8.4	3.75	101	2.4
Anatoxin-a	0.12	106	7.0	1.47	97.7	2.6
L-Phenylalanine-d_5	5.14	98.3	3.8	5.14	99.8	3.0

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

Analyte	Fortified Conc. (µg/L)	Mean % Recovery	% RSD	Fortified Conc. (µg/L)	Mean % Recovery	% RSD
Cylindrospermopsin	0.30	103	7.4	3.75	99.9	8.0
Anatoxin-a	0.12	101	1.9	1.47	93.3	5.2
L-Phenylalanine-d_5	5.14	99.6	3.7	5.14	102	5.3

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

Analyte	Fortified Conc. (µg/L)	Day 0	Day 0	Day 8	Day 8	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
Cylindrospermopsin	3.75	93.0	5.4	93.7	6.7	97.0	8.0	83.2	6.4	92.6	5.9
Anatoxin-a	1.47	83.4	7.1	85.5	7.9	87.3	6.4	77.8	6.6	91.1	8.3

FIGURE 1.
CHROMATOGRAM OF A CALIBRATION STANDARD AT CONCENTRATIONS OF 3.75 µg/L FOR CYLINDROSPERMOPSIN AND 1.47 µg/L FOR ANATOXIN-A

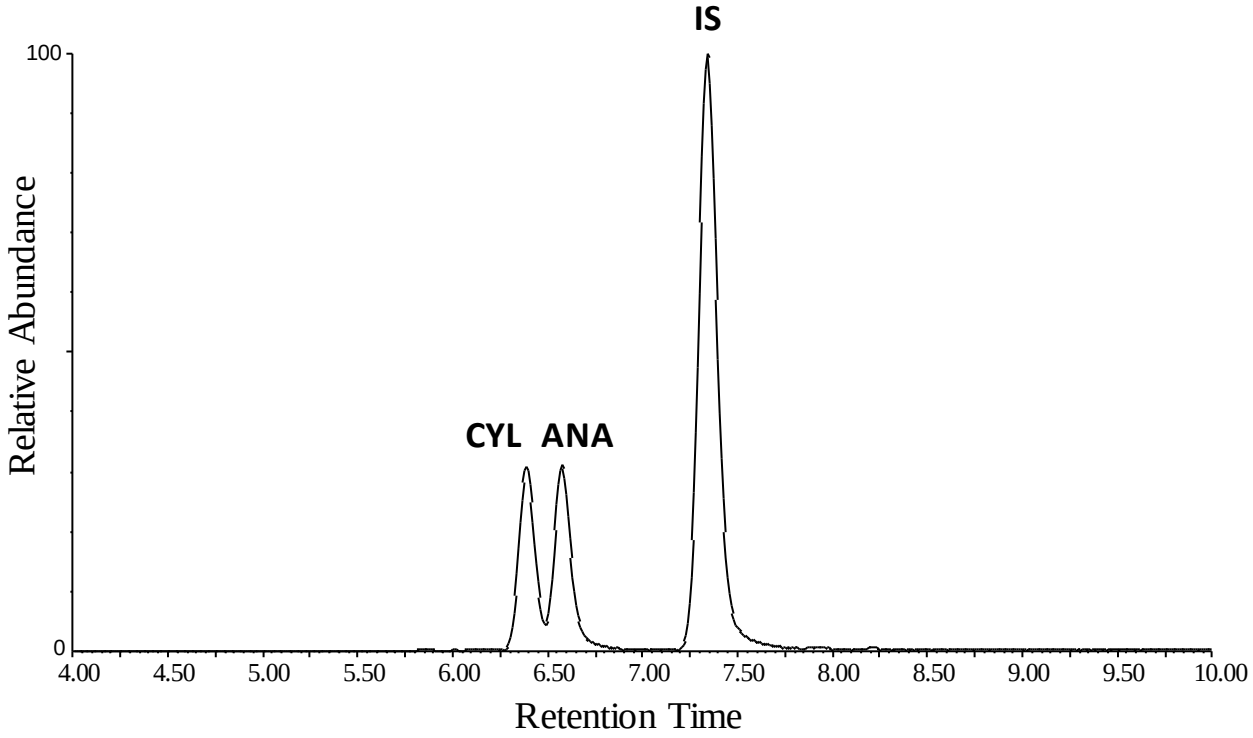

FIGURE 2.

BOX PLOTS SHOWING DISTRIBUTION OF RECOVERIES OBTAINED FROM QC SAMPLES (LFBs AND LFSMs) IN AMBIENT WATERS FROM 16 DIFFERENT WATER BODIES ACROSS THE U.S. (SECT. 13.3). GREEN TRIANGLES REPRESENT THE TWO QC FAILURES OUT OF 271 QC SAMPLES. CYL = cylindrospermopsin; ANA=anatoxin-a; RL= reporting limit