
# Method 545: Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC/ESI-MS/MS)

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# 1 Scope and Application

## 1.1 Method

Method 545 is a liquid chromatography, electrospray ionization, tandem mass spectrometry (LC/ESI-MS/MS) method for the determination of the algal toxins, cylindrospermopsin and anatoxin-a, in finished drinking water. Method 545 requires the use of MS/MS in Multiple Reaction Monitoring (MRM) mode to enhance selectivity. This method is intended for use by analysts skilled in the operation of LC/ESI-MS/MS instrumentation and the interpretation of the associated data.

## 1.2 Analyte List

Method 545 is applicable for the measurement of the following analytes:

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

## 1.3 Supporting Data

### 1.3.1 Precision and Accuracy

Precision and accuracy data were generated in reagent water and finished drinking water for both ground water and surface water sources (Sect. 17, Tables 6 to 8).

### 1.3.2 Single Laboratory Lowest Concentration Minimum Reporting Levels

Single laboratory lowest concentration minimum reporting levels (LCMRLs) in this method were 0.018 and 0.063 micrograms per liter (µg/L) for anatoxin-a and cylindrospermopsin, respectively (Sect. 17, Table 5). The LCMRL is the lowest spiking concentration such that the probability of spike recovery in the 50% to 150% range is at least 99%. The procedure used to determine the LCMRL is described elsewhere.[1] Laboratories using this method are not required to determine LCMRLs, but they must demonstrate that the Minimum Reporting Level (MRL) for each analyte meets the requirements described in Section 9.1.4.

## 1.4 Method Flexibility

The laboratory is allowed to select LC columns, LC conditions, and MS conditions different from those used to develop the method. The two internal standards listed in this method must be used. However, if isotopically labeled target analytes become available (they were not available at the time of method development), they may be used to replace the internal standards listed in the method. Changes may not be made to sample collection and preservation (Sect. 8) or the quality control (QC) requirements (Sect. 9). Single quadrupole instruments are not permitted. Method modifications that improve method performance are allowed. Modifications that are considered in the interest of reducing cost or sample processing time, but result in poorer method performance, may not be used. Analytes should have sufficient chromatographic resolution to allow the mass spectrometer to acquire a minimum of 10 scans across a chromatographic peak. When method modifications are made, the analyst must perform the procedures outlined in the Initial Demonstration of Capability (IDC, Sect. 9.1) and verify that all QC acceptance criteria in the method (Sect. 17, Tables 11 and 12) are met. Additionally, the analyst must verify method performance in a representative sample matrix (Sect. 9.3.2).

## 2 Summary of Method

In the field, samples are added to bottles or vials containing ascorbic acid (dechlorinating agent) and sodium bisulfate (microbial inhibitor). In the laboratory, aliquots (1 mL) of sample are taken for analysis, and internal standards are added. 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 reference signals for procedural 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

**Analysis Batch** – A set of samples that are analyzed on the same instrument during a 24 hour period 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 the number of field samples.

**Calibration Standard** – A solution of the method analytes and internal standards that is prepared from the Primary Dilution Standards. The calibration standards are used to calibrate the instrument response with respect to analyte concentration.

**Continuing Calibration Check (CCC)** – A calibration standard that is analyzed periodically to verify the accuracy of the existing calibration.

**Field Duplicates (FD)** – Separate samples collected at the same time, shipped, and stored under identical conditions. Method precision, including the contribution from sample collection procedures, is estimated from the analysis of FDs. For the purposes of this method, Field Duplicates are necessary to conduct repeat analyses if the original field sample is lost, or to conduct repeat analyses in the case of QC failures associated with the analysis of the original field sample. Field Duplicates are used to prepare Laboratory Fortified Sample Matrix and Laboratory Fortified Sample Matrix Duplicate QC samples.

**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.

**Ion Suppression/Enhancement** – An observable decrease or increase in analyte response in complex (field) samples as compared to the response obtained in standard solutions.

**Laboratory Fortified Blank (LFB)** – A volume of reagent water, containing method preservatives, to which known quantities of the method analytes are added. The LFB is used during the IDC (Sect. 9.1) to verify method performance for precision and accuracy.

**Laboratory Fortified Sample Matrix (LFSM)** – A field sample containing preservatives to which known quantities of the method analytes are added in the laboratory. The LFSM is processed and analyzed as a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results.

**Laboratory Fortified Sample Matrix Duplicate (LFSMD)** – A Field Duplicate of the sample used to prepare the LFSM which is fortified and analyzed identically to the LFSM. The LFSMD is used instead of the Field Duplicate to assess method precision when the method analytes are rarely found at concentrations greater than the MRL.

**Laboratory Reagent Blank (LRB)** – A volume of reagent water that is processed exactly as a sample including exposure to all glassware, equipment, solvents and reagents, sample preservatives, and internal standards. The LRB is used to determine if the method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

**Lowest Concentration Minimum Reporting Level (LCMRL)** – The lowest true concentration for which the future recovery is predicted to fall between 50% to 150% with 99% confidence.[1]

**Material Safety Data Sheets (MSDS)** – Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire and reactivity data, storage instructions, spill response procedures, and handling precautions.

**Minimum Reporting Level (MRL)** – The minimum concentration that can be reported by a laboratory as a quantified value for the method analyte in a sample following analysis. This concentration must meet the criteria defined in Section 9.1.4 and must be no lower than the concentration of the lowest calibration standard for each method analyte.

**Multiple Reaction Monitoring (MRM)** – A mass spectrometric technique in which a precursor ion is first isolated, then subsequently fragmented into a product ion(s). Quantitation is accomplished by monitoring a specific product ion. As described in Section 10.1.2, MS parameters must be optimized for each precursor ion and product ion.

**Precursor Ion** – The gas-phase species corresponding to the method analyte that is produced in the ESI interface. In MS/MS, the precursor ion is mass selected and fragmented by collision-activated dissociation to produce distinctive product ions of smaller mass/charge (*m/z*) ratio.

**Primary Dilution Standard (PDS)** – A solution that contains the method analytes (or internal standards) prepared from Stock Standard Solutions. A PDS solution is diluted to prepare calibration standards and sample fortification solutions.

**Procedural Calibration Standard** – A calibration technique in which calibration standards are processed through the entire method, including sample preparation and addition of preservatives. For this method, reagent water is used as the aqueous medium.

**Product Ion** – One of the fragment ions that is produced in MS/MS by collision-activated dissociation of the precursor ion.

**Quality Control Sample (QCS)** – A solution containing the method analytes at a known concentration that is obtained from a source external to the laboratory and different from the source of calibration standards. The purpose of the QCS is to verify the accuracy of the primary calibration standards.

**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.

**Stock Standard Solution** – A concentrated standard solution that is prepared in the laboratory using assayed reference materials or that is purchased from a commercial source with a certificate of analysis.

# 4 Interferences

## 4.1 Clean Glassware

All glassware must be meticulously cleaned. Wash glassware with detergent and tap water, rinse with tap water, followed by reagent water. Non-volumetric glassware may be heated in a muffle furnace at 400 °C for two hours or solvent rinsed. Volumetric glassware should be solvent rinsed and allowed to air dry.

## 4.2 Reagent and Equipment Interferences

Method interferences may be caused by contaminants in solvents, reagents (including reagent water), sample bottles and caps, and other sample processing hardware. These interferences may lead to discrete artifacts or elevated baselines in the chromatograms or both. All laboratory reagents and equipment must be routinely demonstrated to be free from interferences (less than 1/3 the MRL for the target analytes) under the conditions of the analysis. This may be accomplished by analyzing LRBs as described in Section 9.2.1.

## 4.3 Sample Matrix Interferences

Matrix interferences may be caused by contaminants that are present in the sample. The extent of matrix interferences will vary considerably from source to source depending upon the nature of the water. Matrix components may directly interfere by producing a signal at or near the retention time of an analyte peak. Humic or fulvic material from environmental samples or both can cause enhancement or suppression in the electrospray ionization source or both. Total organic carbon (TOC) is an indicator of the humic content of a sample. Analysis of LFSMs (Sect. 9.2.5) provides evidence for the presence (or absence) of matrix effects.

## 4.4 IS Purity

Depending on the source and purity, labeled analogs used as internal standards may contain a small percentage of the corresponding native analyte. Such a contribution may be significant when attempting to determine MRLs. The labeled internal standards must meet the purity requirements stated in the IDC (Sect. 9.1.1).

# 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 MSDSs should be made available to all personnel involved in the chemical analysis. Guidance for the handling of chemicals in the workplace is available on the OSHA website.

# 6 Equipment and Supplies

References to specific brands or catalog numbers are included as examples only and do not imply endorsement of the product. Such reference does not preclude the use of other vendors or suppliers.

## 6.1 Sample Containers

Amber glass vials/bottles fitted with polytetrafluoroethylene (PTFE)-lined screw caps.

## 6.2 Autosampler Vials

Amber glass vials with PTFE/silicone septa.

## 6.3 Micro Syringes

Suggested sizes include 5, 10, 25, 50, 100, 250, 500, and 1000 microliters (µL).

## 6.4 Analytical Balance

Capable of weighing to the nearest 0.0001 gram (g).

## 6.5 Disposable Pasteur Pipettes

5 ¾-inch or 9-inch borosilicate glass, used to transfer samples to autosampler vials and for sample preparation (Fisher Cat. No. 13-678-20B, 13-678-20C, or equivalent).

## 6.6 Disposable Syringes

3-mL, polypropylene, Luer Lock syringes for use in filtering standards and samples (Fisher Cat No. 03-377-27, or equivalent).

## 6.7 Syringe Filters

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

## 6.8 Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry System (LC/ESI-MS/MS)

### 6.8.1 LC System

The LC system must provide consistent sample injection volumes and be capable of performing binary linear gradients at a constant flow rate.

### 6.8.2 Analytical Column

The method was developed using a Waters XSelect HSS T3 2.1 x 150 mm, 3.5-µm column (Waters Part No. 186006466). Any column that provides adequate resolution, peak shape, capacity, accuracy and precision (Sect. 9), and does not exacerbate suppression or enhancement of analyte responses may be used.

### 6.8.3 Electrospray Ionization Tandem Mass Spectrometer (ESI-MS/MS)

The mass spectrometer must be capable of electrospray ionization. The system must be capable of performing MS/MS to produce unique product ions for the method analytes within specified retention time segments. A minimum of 10 scans across the chromatographic peak is needed to ensure adequate precision.

### 6.8.4 MS/MS Data System

An interfaced data system is required to acquire, store and output MS data. The computer software must have the capability of processing stored data by recognizing a chromatographic peak within a given retention time window. The software must allow integration of the abundance of any specific ion between specified time or scan number limits. The software must be able to construct a linear

regression or quadratic regression calibration curve and calculate analyte concentrations using the internal standard technique.

# 7 Reagents and Standards

## 7.1 Gases, Reagents and Solvents

Reagent grade or better chemicals must be used. Unless otherwise indicated, all reagents will conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (ACS), where such specifications are available. Other grades may be used if the reagent is demonstrated to be free of analytes and interferences and all requirements of the IDC (Sect. 9.1) are met when using these reagents.

### 7.1.1 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.

### 7.1.2 Collision Gas

High purity compressed gas (e.g., nitrogen or argon) used in the collision cell of the mass spectrometer. The specific type of gas, purity and pressure requirements will depend on the instrument manufacturer's specifications.

### 7.1.3 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.

### 7.1.4 Methanol, (CH3OH, CASRN 67-56-1)

LC/MS Grade (Fisher Optima, Fisher Cat. No. A456, or equivalent).

### 7.1.5 L-Ascorbic Acid, (C6H8O6, CASRN 50-81-7)

≥99% (Sigma-Aldrich Cat. No. 255564, or equivalent). Added to reduce residual chlorine in finished waters.

### 7.1.6 Reagent Water

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

### 7.1.7 Sodium Bisulfate, ($NaHSO_4$, CASRN 7681-38-1)

~95% (Sigma Cat. No. 71656, or equivalent). Used to inhibit microbial growth in dechlorinated water samples.

## 7.2 Standard Solutions

The solution concentrations listed in this section were used to develop this method and are included only as examples. The concentrations chosen for standards are at the discretion of the analyst to further optimize the method (Sect. 1.4) as long as the IDC and ongoing QC requirements are met. PDS and calibration standards were found to be stable for, at least, one month 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 Standards

Two isotopically enriched internal standards must be used in the method. The following table lists the internal standards used in method development. If isotopically labeled target analytes are available, they may be used in place of the standards below.

| **Internal Standard** | **CASRN**[a] | **Catalog No.** |
|---|---|---|
| Uracil-$d_4$, neat material | 24897-55-0 | C/D/N Isotopes D-5135 |
| L-phenylalanine-$d_5$, neat material | 56253-90-8 | Cambridge Isotopes Labs DLM-1258-1 |

[a] CASRN = Chemical Abstract Registry Number.

#### 7.2.1.1 Internal Standard Stock Standards (1000 µg/mL)

Uracil-$d_4$ and L-phenylalanine-$d_5$ stock standard solutions are prepared by weighing 10 mg of the solid material into a tared 10-mL volumetric flask and diluting to volume with hot reagent water (uracil-$d_4$) and methanol/reagent water (1:1) (L-phenylalanine-$d_5$).

#### 7.2.1.2 Internal Standard Primary Dilution Standard (IS PDS) (0.250–1.00 µg/mL)

The IS PDS is prepared in methanol/reagent water (1:1) and is stored at a temperature <-15 °C. Use 20 µL of the IS PDS to fortify the final 1-mL samples. This will yield a final concentration of 5.00 ng/mL L-phenylalanine-$d_5$ and 20.0 ng/mL Uracil-$d_4$ in the samples. Analysts are permitted to use other IS PDS concentrations and volumes provided all samples and calibration standards contain the same final concentration of the internal standards and adequate signal is obtained.

| **Internal Standard** | **Conc. of IS Stock (µg/mL)** | **Final Conc. of IS PDS (µg/mL)** |
|---|---|---|
| Uracil-d4 | 1000 | 1.00 |
| L-phenylalanine-d5 | 1000 | 0.250 |

### 7.2.2 Method Analyte Standard Solutions

#### 7.2.2.1 Analyte Stock Standard Solution (1000 µg/mL)

Obtain the analytes listed in the table in Section 1.2 as ampoulized solutions or as neat materials. Prepare stock standards individually by weighing 10 mg of the solid standards into tared 10-mL volumetric flasks and diluting to volume with methanol/reagent water (1:1). If only limited quantities of neat material are available, stock standards may be prepared by adding a known volume of methanol/reagent water (1:1) directly to manufacturers' vials and calculating the concentration based on the mass provided by the manufacturer.

**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 (Analyte PDS) (1.00 µg/mL)

Prepare the Analyte PDS by diluting the Analyte Stock Standard solutions into methanol/reagent water (1:1). An example preparation of the Analyte PDS that was used to collect data presented in Section 17 is provided in the table below. Analyte PDS is used to prepare calibration standards, and to fortify LFBs, LFSMs, and LFSMDs with the method analytes.

| **Analyte Stock** | **Stock Concentration (µg/mL)** | **Analyte PDS Concentration (µg/mL)[a]** | **Calibration Range (ng/mL)[b]** |
|---|---|---|---|
| Cylindrospermopsin | 100 | 1.00 | 0.050–10.0 |
| Anatoxin-a | 1000 (*as fumarate*) | 1.00 (*as fumarate*) | 0.029–5.87[c] |

[a] Multiple dilutions of the Analyte PDS were used in the preparation of calibration standards.

[b] Calibration curve concentration ranges used in method development.

[c] Corrected for anatoxin-a.

### 7.2.3 Procedural Calibration (CAL) Standards

The preparation of calibration standards requires the use of reagent water containing all sample preservatives. Prepare at least five calibration standards over the concentration range of interest by adding aliquots of Analyte PDS with the reagent water matrix 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. The CAL standards may also be used as CCCs (Sect. 9.2.2).

# 8 Sample Collection, Preservation and Storage

## 8.1 Sample Bottle Preparation

### 8.1.1 Sample Containers

At a minimum, ten-milliliter amber glass bottles or vials with PTFE-lined screw caps. Collect additional samples to fulfill the QC requirements for the frequency of field duplicates, LFSM, and LFSMD QC samples (Sect. 9.2.5 and 9.2.6).

### 8.1.2 Addition of Preservatives

Preservation reagents, listed in the table below, are added to each sample container prior to shipment to the field (or prior to sample collection).

| **Compound** | **Amount** | **Purpose** |
|---|---|---|
| Sodium bisulfate | 1.0 g/L | Acidic microbial inhibitor |
| Ascorbic acid | 0.10 g/L | Reducing agent for chlorine |

## 8.2 Sample Collection

When sampling from a cold water tap, remove the aerator, open the tap, and allow the system to flush until the water temperature has stabilized (approximately three to five minutes). Invert the bottles or vials several times to mix the sample with the preservation reagents. Fill sample bottles or vials taking care not to flush out the preservatives. It is acceptable to leave head-space in the container.

## 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. Samples must be at or below 10 °C when they are received at the laboratory. In the laboratory, samples must be stored at or below 6 °C and protected from light until analysis. Samples must not be frozen.

## 8.4 Sample Holding Times

Results of the sample storage stability study (Sect. 17, Table 9) indicated that all compounds listed in the method have adequate stability for 28 days when collected, preserved, shipped and stored as described in Sections 8.1–8.3. Therefore, samples should be analyzed as soon as possible, but must be analyzed within 28 days.

# 9 Quality Control

QC requirements include the IDC and ongoing QC requirements. This section describes each QC parameter, its required frequency, and the performance criteria that must be met in order to satisfy EPA quality objectives. The QC criteria discussed in the following sections are summarized in Section 17, Tables 11 and 12. These QC requirements are considered the minimum acceptable QC.

## 9.1 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 generate an acceptable initial calibration following the procedure outlined in Section 10.2.

### 9.1.1 Demonstration of Low System Background

Analyze a LRB. Confirm that the blank is free of contamination as defined in Section 9.2.1.

#### 9.1.1.1 IS Purity

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 internal standards do not contain the unlabeled analytes at a concentration >1/3 of the MRL when added at the selected concentration to samples.

#### 9.1.1.2 Carry-over

The system should also be checked for carry-over by analyzing a LRB immediately following the highest CAL standard. If this sample does not meet the criteria outlined in Section 9.2.1, then carry-over is present and should be eliminated.

### 9.1.2 Demonstration of Precision

Prepare and analyze four to seven replicate LFBs. Fortify these samples near the midrange of the initial calibration curve. The method preservatives must be added to the LFBs as described in Section 8.1.2. The percent relative standard deviation (%RSD) of the results of the replicate analyses must be ≤20%.

$$\%\,\mathrm{RSD} = \frac{\text{Standard Deviation of Measured Concentrations}}{\text{Average Concentration}} \times 100$$

### 9.1.3 Demonstration of Accuracy

Using the same set of replicate data generated for Section 9.1.2, calculate the average percent recovery (%R). The average percent recovery for each analyte must be within ±30% of the true value.

$$\%R = \frac{\text{Average Measured Concentration}}{\text{Fortified Concentration}} \times 100$$

### 9.1.4 Minimum Reporting Level (MRL) Confirmation

Establish a target concentration for the MRL based on the intended use of the method. Analyze an initial calibration following the procedures in Section 10. The lowest calibration standard used to establish the initial calibration (as well as the low-level CCC) 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.

#### 9.1.4.1 Fortify and Analyze Seven Replicate LFBs at or Below the Proposed MRL Concentration.

The LFBs must contain the method preservatives as specified in Section 8.1.2. Calculate the mean (*Mean*) and standard deviation for these replicates. Determine the Half Range for the Prediction Interval of Results ($HR_{PIR}$) using the equation:

$$HR_{PIR} = 3.963s$$

Where *s* is the standard deviation and 3.963 is a constant value for seven replicates.[1]

#### 9.1.4.2 Confirm Upper and Lower Limits for the PIR

Confirm that the Upper and Lower limits for the Prediction Interval of Results (PIR = *Mean* +$HR_{PIR}$) meet the upper and lower recovery limits as shown below.

The Upper PIR Limit must be ≤150 percent recovery.

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

The Lower PIR Limit must be ≥50 percent recovery.

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

#### 9.1.4.3 MRL Criteria

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 confirmed again at a higher concentration.

**Note:** These equations are only valid for seven replicate samples.

### 9.1.5 Quality Control Sample (QCS)

Analyze a mid-level Quality Control Sample (Sect. 9.2.7) to confirm the accuracy of the primary calibration standards.

## 9.2 Ongoing QC Requirements

This section describes the ongoing QC elements that must be included when processing and analyzing field samples.

### 9.2.1 Laboratory Reagent Blank (LRB)

Analyze a LRB with each Analysis Batch. The LRB must contain the method preservatives at the same concentration as field samples. Background from method analytes or contaminants that interfere with the measurement of method analytes must be ≤1/3 of the MRL. If method analytes are detected in the LRB at concentrations greater than this level, then all data for the problem analyte(s) must be considered invalid for all samples in the Analysis Batch. Subtracting blank values from sample results is not permitted.

**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.

### 9.2.2 Continuing Calibration Check (CCC)

Analyze CCC standards at the beginning of each Analysis Batch, after every ten field samples, and at the end of the Analysis Batch. See Section 10.3 for concentration requirements and acceptance criteria for CCCs.

### 9.2.3 Laboratory Fortified Blank (LFB)

Since this method utilizes procedural calibration standards, which are fortified reagent waters, there is no difference between the LFB and the continuing calibration check standard. Consequently, the analysis of a separate LFB is not required as part of the ongoing QC; however, the acronym LFB is used for clarity in the IDC.

### 9.2.4 Internal Standards (IS)

The analyst must monitor the peak areas of the internal standards in all injections of the Analysis Batch. The internal standard responses (as indicated by peak areas) for any chromatographic run must not deviate by more than ±50% from the average areas measured during the initial calibration for the internal standards. If the IS areas in a chromatographic run do not meet these criteria, check the corresponding IS of the most recent CCC and proceed as follows:

#### 9.2.4.1 IS Failure in Sample but not CCC

If the IS criterion is met in the CCC but not in the sample, reanalyze the sample in the same or subsequent Analysis Batch. If the reanalyzed sample produces an acceptable IS response, report results for that injection. If the IS area count fails to meet the acceptance criterion in the repeated analysis but still passes the most recent CCC, report the sample results as “suspect/matrix.” Alternatively, analyze a FD or collect a new sample and reanalyze.

#### 9.2.4.2 IS Failure in Sample and CCC

If both the original sample and the CCC fail the IS criteria, take corrective action (e.g., Sect. 10.4). It might be helpful to check the integrity of the IS solution and the fortification technique before reanalyzing the sample in a subsequent Analysis Batch. After corrective action, re-inject the sample in a subsequent Analysis Batch. If the IS area fails to meet the acceptance criterion in the repeat analysis, but passes in the most recent CCC, report the sample results as “suspect/matrix.”

### 9.2.5 Laboratory Fortified Sample Matrix (LFSM)

Within each Analysis Batch, analyze a minimum of one LFSM for every 20 samples. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and subtracted from the measured values in the LFSM. If various sample matrixes are analyzed regularly, for example, drinking water processed from ground water and surface water sources, performance data should be collected for each source.

#### 9.2.5.1 Prepare LFSM

Prepare the LFSM by fortifying a Field Duplicate with an appropriate amount of the Analyte PDS. Generally, select a spiking concentration that is greater than or equal to the native concentration for most analytes. Selecting a duplicate aliquot of a sample that has already been analyzed aids in the selection of an appropriate spiking level. If this is not possible, use historical data when selecting a fortifying concentration.

#### 9.2.5.2 Calculate the %R

Calculate the %R 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, and
C = fortification concentration.

#### 9.2.5.3 Recoveries

Recoveries for samples fortified at concentrations near or at the MRL (within a factor of two times the MRL concentration) must be within ±50% of the true value. Recoveries for samples fortified at all other concentrations must be within ±30% of the true value. If the accuracy for any analyte falls outside the designated range, and the laboratory performance for that analyte is shown to be in control in the CCCs and in the LFB, the recovery is judged matrix biased. Report the result for the corresponding analyte in the unfortified sample as "suspect/matrix."

**Note:** In order to obtain meaningful percent recovery results, correct the measured values in the LFSM and LFSMD for the native levels in the unfortified samples, even if the native values are less than the MRL. This situation and the LRB background estimation are the only permitted uses of analyte results below the MRL.

### 9.2.6 Field Duplicate or Laboratory Fortified Sample Matrix Duplicate (FD or LFSMD)

Within each Analysis Batch, analyze a minimum of one Field Duplicate or one Laboratory Fortified Sample Matrix Duplicate. If the method analytes are not routinely observed in field samples, analyze an LFSMD rather than an FD.

#### 9.2.6.1 Relative Percent Difference Calculation

Calculate the relative percent difference (RPD) for duplicate measurements ($FD_1$ and $FD_2$) using the equation:

$$\text{RPD} = \frac{|\text{FD}_1 - \text{FD}_2|}{(\text{FD}_1 + \text{FD}_2)/2} \times 100$$

#### 9.2.6.2 RPDs for Field Duplicates

RPDs for Field Duplicates should be ≤30% for each analyte. Greater variability may be observed when Field Duplicates have analyte concentrations that are near or at the MRL (within a factor of two times the MRL concentration). At these concentrations, Field Duplicates must have RPDs that are ≤50%. If the RPD of an analyte falls outside the designated range, and the laboratory performance for the analyte is shown to be in control in the CCC and in the LFB, the precision is judged matrix influenced. Report the result for the corresponding analyte in the unfortified sample as "suspect/matrix."

#### 9.2.6.3 RPDs for LFSMD

If an LFSMD is analyzed instead of a Field Duplicate, calculate the RPD for the LFSM and LFSMD using the equation:

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

#### 9.2.6.4 RPDs for Duplicate LFSMs

RPDs for duplicate LFSMs should be ≤30% for each analyte. Greater variability may be observed when the matrix is fortified at analyte concentrations near or at the MRL (within a factor of two times the MRL concentration). LFSMs at these concentrations must have RPDs that are ≤50%. If the RPD of an analyte falls outside the designated range, and the laboratory performance for the analyte is shown to be in control in the CCC and in the LFB, the precision is judged matrix influenced. Report the result for the corresponding analyte in the unfortified sample as "suspect/matrix."

### 9.2.7 Quality Control Sample

As part of the IDC (Sect. 9.1), 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. Fortify the QCS near the midpoint of the calibration range. The acceptance criteria for the QCS are the same as the mid- and high-level CCCs (Sect. 10.3). If the accuracy for any analyte fails the recovery criterion, prepare fresh standard dilutions and repeat the QCS evaluation.

## 9.3 Method Modification QC Requirements

The analyst is permitted to modify the separation technique, LC column, mobile phase composition, LC conditions, and MS/MS conditions.

### 9.3.1 Repeat the Procedures of the IDC and Verify all QC

Each time method modifications are made, the analyst must repeat the procedures of the IDC (Sect. 9.1) and verify that all QC criteria can be met in ongoing QC samples (Sect. 9.2).

### 9.3.2 Document Method Performance

The analyst is also required to evaluate and document method performance for the proposed modifications in real matrixes that span the range of waters that the laboratory analyzes. This additional step is required because modifications that perform acceptably in the IDC, which is conducted in reagent

water, could fail ongoing method QC requirements in real matrixes. This is particularly important for methods subject to matrix effects, such as LC/MS-based methods. For example, a laboratory may routinely analyze drinking water from municipal treatment plants that process ground water, surface water, or a blend of surface and ground water. In this case, this modification requirement could be accomplished by assessing precision and accuracy (Sects. 9.1.2 and 9.1.3) in a surface water with moderate to high total organic carbon (for example, 2 mg/L or greater) and a hard ground water (for example, 250 mg/L as calcium carbonate ($CaCO_3$) equivalent, or greater).

#### 9.3.3 Document and Assess before Analyzing Field Samples

The results of Sections 9.3.1 and 9.3.2 must be appropriately documented by the analyst and independently assessed by the laboratory's QA officer prior to analyzing field samples. When implementing method modifications, it is the responsibility of the laboratory to closely review the results of ongoing QC, and in particular, the results associated with the LFSM (Sect. 9.2.5), FD (Sect. 9.2.6), CCCs (Sect. 10.3), and the internal standard area counts (Sect. 9.2.4). If repeated failures are noted, the modification must be abandoned.

## 10 Calibration and Standardization

Demonstration and documentation of acceptable MS calibration and initial analyte calibration are required before performing the IDC (Sect. 9.1) and prior to analyzing field samples. The initial calibration should be repeated each time a major instrument modification or maintenance is performed.

### 10.1 LC/ESI-MS/MS Calibration and Optimization

#### 10.1.1 Mass Calibration

Calibrate the mass spectrometer with the calibration compounds and procedures specified by the manufacturer.

#### 10.1.2 Optimizing MS Parameters

Each LC/ESI-MS/MS system will have different optimal conditions, which are influenced by the source geometry and system design. Due to the differences in design, follow the recommendations of the instrument manufacturer when tuning the instrument. During the development of this method, instrumental parameters were optimized for the precursor and product ions listed in Section 17, Table 3. Product ions other than those listed may be selected; however, the analyst is cautioned to avoid using ions with lower mass or common ions or both, that may not provide sufficient discrimination between the analytes of interest and co-eluting interferences.

##### 10.1.2.1 Optimize the Response of the Precursor Ion

Optimize the response of the precursor ion for each analyte by split infusion at the analytical flow rate using approximately 1 µg/mL of each analyte. Vary the MS parameters (source voltages, source and desolvation temperatures, gas flows, etc.) until optimal analyte responses are obtained. The target analytes may have different optimal instrument parameters, thus requiring some compromise on the final operating conditions. See Section 17, Table 2 for the ESI-MS/MS conditions used in method development.

#### 10.1.2.2 Optimize the Response of the Product Ion

Optimize the response of the product ion for each analyte by split infusion at the analytical flow rate using approximately 1 µg/mL of each analyte. Vary the MS/MS parameters (collision gas pressure, collision energy, etc.) until optimal product ion responses are determined.

### 10.1.3 Liquid Chromatography Instrument Conditions

Establish LC operating parameters that optimize resolution. Suggested LC operating conditions are described in Section 17, Table 1. Conditions different from those listed (for example, LC columns and mobile phase compositions) may be used if the QC criteria in Sections 9.1, 9.2, and 9.3 are met and chromatographic separation of the method analytes is achieved.

**Note:** Chromatographic separation as defined does not include isotopically enriched internal standards (if used), which are mass separated. Co-elution of the internal standards with their analogous method analytes helps mitigate matrix suppression or enhancement effects or both.

### 10.1.4 Establish LC/ESI-MS/MS Retention Times and MRM Segments

Inject a mid- to high-level calibration standard under optimized LC/ESI-MS/MS conditions to obtain the retention times of each method analyte. Divide the chromatogram into segments that contain one or more chromatographic peaks. For maximum sensitivity in subsequent MS/MS analyses, minimize the number of MRM transitions that are simultaneously monitored within each segment.

## 10.2 Initial Calibration

During method development, daily calibrations were performed; however, it is permissible to verify the calibration with daily CCCs. Calibration must be performed using peak areas and the internal standard technique. Calibration using peak heights or external standard calibration is not permitted.

### 10.2.1 Procedural Calibration Standards

Prepare a set of at least five calibration standards as described in Section 7.2.3. The analyte concentrations in the lowest calibration standard must be at or below the MRL. Field samples must be quantified using a calibration curve that spans the same concentration range used to collect the IDC data (Sect. 9.1), that is, analysts are not permitted to use a restricted calibration range to meet the IDC criteria and then use a larger dynamic range during analysis of field samples.

### 10.2.2 Calibration

Calibrate the LC/ESI-MS/MS and fit the calibration points with either a linear regression or quadratic regression (response vs. concentration). Weighting may be used. Forcing the calibration curve through the origin is not recommended. The MS/MS instruments used during method development were calibrated using weighted (1/x) quadratic curves. Internal standard assignments appropriate for each method analyte are presented in Section 17, Table 3. The MRM transitions for the internal standards are provided in Section 17, Table 4.

### 10.2.3 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 should calculate to be within ±30% of their true value. If these criteria cannot be met, the analyst will have difficulty meeting ongoing QC criteria. In this case, reanalyze the calibration standards or restrict the range of calibration. If the cause for failure to

meet the criteria is due to contamination or standard degradation, prepare fresh CAL standards and repeat the initial calibration.

### 10.3 Continuing Calibration Checks (CCCs)

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. The beginning CCC for each Analysis Batch must be at or below the MRL. This CCC verifies instrument sensitivity prior to the analysis of samples. Subsequent CCCs should alternate between a medium and high concentration CAL standard.

#### 10.3.1 Aliquot Injection and Analysis

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

#### 10.3.2 Verify Quantitation Ions

Verify that the absolute areas of the quantitation ions of each of the internal standards have not changed by more than ±50% from the average areas measured during the initial calibration. If this limit is exceeded, corrective action is necessary (Sect. 10.4).

#### 10.3.3 Calculate Concentration

Calculate the concentration of each method 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 the method analytes in CCCs fortified at all other levels must be within +30%. If these limits are exceeded, then all data for the failed analytes must be considered invalid. Any field samples analyzed since the last acceptable CCC that are still within holding time must be reanalyzed after an acceptable calibration has been restored.

### 10.4 Corrective Action

Failure to meet the CCC QC performance criteria requires corrective action. Acceptable method performance may be restored simply by flushing the column with 100% methanol. Following this and other minor remedial action, check the calibration with a mid-level CCC and a CCC at the MRL, or recalibrate according to Section 10.2. If internal standard and calibration failures persist, maintenance may be required, such as servicing the ESI-MS/MS system or replacing the LC column. These latter measures constitute major maintenance, and the analyst must return to the initial calibration step (Sect. 10.2).

## 11 Procedure

This section describes the procedures for sample preparation and analysis. Important aspects of this analytical procedure include proper sample collection and storage (Sect. 8), ensuring that the instrument is properly calibrated (Sect. 10), and that all required QC elements are included (Sect. 9).

### 11.1 Sample Preparation

#### 11.1.1 Triple Freeze and Thaw Process

If necessary, a triple freeze and thaw process may be used on samples prior to filtration and analysis in order to address the potential presence of intact algal cells in finished water samples. During method development, the process was evaluated by freezing samples for 1 h at -30°C, then thawing samples in a 40°C water bath for 5 min. The freeze and thaw process was repeated two more times prior to the addition of an IS and analysis. Water samples were frozen and thawed inside plastic syringes

(polyethylene barrel/polypropylene plunger) to allow for an efficient transfer and filtration of the sample. The results of the freeze and thaw process and a comparison of plastic syringes and glass vials can be seen in Section 17, Table 10.

#### 11.1.2 Dechlorinating and Preservation Agents

Samples are dechlorinated, preserved, collected and stored as described in Section 8. All field and QC samples must contain the dechlorinating and preservation agents listed in Section 8.1.2, including the LRB.

#### 11.1.3 Fortify with PDS

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 Measure, Mix, Filter, etc.

Measure 1 mL of each field or QC sample. Add the IS PDS (Sect. 7.2.1.2) and mix well. Filter each 1-mL solution using 0.2 µm PVDF filters and disposable syringes. Place the 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 LC/ESI-MS/MS Operating Conditions

Establish LC/ESI-MS/MS operating conditions equivalent to those summarized in Tables 1–4 of Section 17 as per the guidance in Section 10.1. Column choice and instrument parameters should be optimized prior to initiation of the IDC (Sect. 9.1).

#### 11.2.2 Establish Initial Calibration

Establish a valid initial calibration following the procedures in Section 10.2 or confirm that the calibration is still valid by analyzing a CCC (Sect. 10.3). If establishing an initial calibration for the first time, complete the IDC as described in Section 9.1 prior to analyzing field samples.

#### 11.2.3 Analyze Field and QC Samples

Analyze field and QC samples at appropriate frequencies in a properly sequenced Analysis Batch as described in Section 11.3.

### 11.3 The Analysis Batch

An Analysis Batch is a sequence of samples, analyzed within a 24-hour period, of no more than 20 field samples and includes all required QC samples (LRB, CCCs, the LFSM and LFSMD (or FD)). The required QC samples are not included in counting the maximum field sample total of 20. LC-MS/MS conditions for the Analysis Batch must be the same as those used during calibration.

#### 11.3.1 Analyze Initial CCC

After a valid calibration is established, begin every Analysis Batch by analyzing an initial low-level CCC at or below the MRL. This initial CCC must be within ±50% of the true value for each method analyte and must pass the IS area criterion (Sect. 10.3.2). The initial CCC confirms that the calibration is still valid. Failure to meet the QC criteria may indicate that recalibration is required prior to analyzing samples. After the initial CCC, continue the Analysis Batch by analyzing an LRB, followed by field and QC samples

at appropriate frequencies (Sect. 9.2). Analyze a mid- or high-level CCC after every ten field samples and at the end each Analysis Batch. Do not count QC samples (LRBs, FDs, LFSMs, LFSMDs) when calculating the required frequency of CCCs.

#### 11.3.2 Analyze Final CCC

A final CCC completes the Analysis Batch. The acquisition start time of the final CCC must be within 24 hours of the acquisition start time of the initial low-level CCC at the beginning of the Analysis Batch. More than one Analysis Batch within a 24-hour period is permitted.

# 12 Data Analysis and Calculations

## 12.1 Establish a Retention Time Window

Establish an appropriate retention time window for each analyte to identify them in the resulting chromatograms. Base this assignment on measurements of actual retention time variation for each compound in standard solutions over the course of time. The suggested variation is plus or minus three times the standard deviation of the retention time for each compound for a series of injections. The injections from the initial calibration and from the IDC (Sect. 9.1) may be used to calculate the retention time window. However, the experience of the analyst should weigh heavily on the determination of an appropriate range.

## 12.2 Identify Peaks of Interest

At the conclusion of data acquisition, use the same software settings established during the calibration procedure to identify peaks of interest in the predetermined retention time windows. Confirm the identity of each analyte by comparison of its retention time with that of the corresponding analyte peak in an initial calibration standard or CCC.

## 12.3 Calculate Analyte Concentrations

Calculate analyte concentrations using the multipoint calibration established in Section 10.2. Report only those values that fall between the MRL and the highest calibration standard.

## 12.4 Round Concentrations

Calculations must use 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.

## 12.5 Review

Prior to reporting the data, the chromatograms must be reviewed for any incorrect peak identifications or improper integration. The laboratory is responsible for ensuring that QC requirements have been met and that any appropriate qualifier is assigned.

## 12.6 Exceeding the Calibration Range

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 all preservatives 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.

# 13 Method Performance

Single laboratory method performance data were collected using a Waters Acquity liquid chromatograph coupled to a Micromass Quattro Premier XE triple quadrupole mass spectrometer.

## 13.1 Precision, Accuracy and LCMRL

Tables for single laboratory data are presented in Section 17. LCMRLs for each method analyte are presented in Section 17, Table 5. Precision and accuracy are presented for three water matrixes: reagent water (Sect. 17, Table 6); chlorinated (finished) groundwater (Sect. 17, Table 7); and moderate TOC chlorinated (finished) surface water (Sect. 17, Table 8).

## 13.2 Analyte Stability Study

Chlorinated (finished) surface water samples, inoculated with diluted local microbial rich ambient water and fortified with method analytes at 1.47–2.50 µg/L (Anatoxin-a/Cylindrospermopsin), were preserved as required in Section 8 and stored over a 28-day period. The percent change from the initial analyzed concentration, observed after 7, 14, 21 and 28 days storage, is presented in Section 17, Table 9.

# 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

The analytical procedures described in this method generate relatively small amounts of waste since only small amounts of reagents and solvents are used. The matrix of concern is finished drinking water. However, the Agency requires that laboratory waste management practices 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. In addition, 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." *Environmental Science & Technology* **2006**, *40*, 281.

# 17 Tables, Diagrams, Flowcharts and Validation Data

## Table 1. HPLC Conditions

HPLC[a]
Column: Waters XSelect® HSS T3, 2.1 x 150 mm, 3.5 µm
Column temperature: 30 °C
Column flow rate: 0.20 mL/min
Autosampler temperature: 10 °C
Injection volume: 50 µL
Elution: Step Gradient

| **Time (min)** | **%100 mM acetic acid in reagent water[b]** | **%MeOH** |
|---|---|---|
| 0.00 | 100 | 0 |
| 0.50 | 90 | 10 |
| 8.50 | 100 | 0 |
| 13.50 | 100 | 0 |

[a] Waters Acquity LC system

[b] Preparation of 100 mM acetic acid in reagent water: Combine 5.8 mL concentrated acetic acid and dilute to volume with reagent water in a 1 L volumetric flask.

## Table 2. Positive Mode ESI-MS/MS Method Conditions

| **MS Parameter** | **MS/MS[a]** |
|---|---|
| Polarity | Positive ion electrospray |
| Capillary Voltage, kV | 4.00 |
| Source Temperature, °C | 120 |
| N2 Desolvation Temperature, °C | 350 |
| N2 Desolvation Gas Flow, L/hr | 800 |
| Cone Gas Flow, L/hr | 100 |
| Extractor Lens, V | 2.00 |
| RF Lens, V | 0.1 |

[a] Micromass Quattro Premier XE triple quadrupole mass spectrometer

## Table 3. Analyte Retention Times, Ions, Cone Voltage, Collision Energy and IS Assignments

| Analyte | Ret. Time (min) | ESI Mode | Precursor Ion | Product Ion | Cone Voltage (V) | Collision Energy (eV) | Dwell Time (s) | Internal Standard |
|---|---|---|---|---|---|---|---|---|
| Cylindrospermopsin | 4.57 | ESI+ | 416.2 | 194.0 | 35 | 35 | 0.250 | Uracil-$d_4$ |
| Anatoxin-a | 5.74 | ESI+ | 165.8 | 148.8 | 25 | 15 | 0.250 | L-phenylalanine-$d_5$ |

## Table 4. IS Retention Times, Ions, Cone Voltage and Collision Energy

| Internal Standard | Ret. Time (min) | ESI Mode | Precursor Ion | Product Ion | Cone Voltage (V) | Collision Energy (eV) | Dwell Time (s) |
|---|---|---|---|---|---|---|---|
| Uracil-$d_4$ | 3.85 | ESI+ | 114.8 | 97.8 | 28 | 15 | 0.250 |
| L-phenylalanine-$d_5$ | 8.00 | ESI+ | 170.8 | 124.8 | 18 | 10 | 0.250 |

## Table 5. Lowest Concentration Minimum Reporting Levels (LCMRLs)

| Analyte | LCMRL (µg/L)[a] |
|---|---|
| Cylindrospermopsin | 0.063 |
| Anatoxin-a | 0.018 |

[a] LCMRLs were calculated according to the procedure in reference 1 with the following modification: Instead of evaluating seven replicates at four concentration levels, LCMRLs are now obtained by analyzing four replicates at seven concentration levels.

## Table 6. Precision and Accuracy in Fortified Reagent Water (n=7)

*Low Concentration*

| Analyte | Fortified Concentration (µg/L) | Avg. %Recovery | %RSD |
|---|---|---|---|
| Cylindrospermopsin | 0.100 | 109 | 11 |
| Anatoxin-a | 0.059 | 111 | 7.4 |

*High Concentration*

| Analyte | Fortified Concentration (µg/L) | Avg. %Recovery | %RSD |
|---|---|---|---|
| Cylindrospermopsin | 2.50 | 109 | 3.6 |
| Anatoxin-a | 1.47 | 109 | 2.9 |

## Table 7. Precision and Accuracy in Fortified Chlorinated Ground Water [a] (n=7)

*Low Concentration*

| Analyte | Fortified Concentration (µg/L) | Avg. %Recovery | %RSD |
|---|---|---|---|
| Cylindrospermopsin | 0.100 | 126 | 5.2 |
| Anatoxin-a | 0.059 | 95.4 | 5.3 |

*High Concentration*

| Analyte | Fortified Concentration (µg/L) | Avg. %Recovery | %RSD |
|---|---|---|---|
| Cylindrospermopsin | 2.50 | 98.0 | 2.2 |
| Anatoxin-a | 1.47 | 95.0 | 1.8 |

[a] Ground water physical parameters: total hardness = 325 milligrams/liter (mg/L) (as $CaCO_3$); free chlorine = 0.69 mg/L; total chlorine = 0.97 mg/L; conductivity = 798 µS.

## Table 8. Precision and Accuracy in Fortified Moderate TOC Chlorinated Surface Water [a] (n=7)

*Low Concentration*

| Analyte | Fortified Concentration (µg/L) | Avg. %Recovery | %RSD |
|---|---|---|---|
| Cylindrospermopsin | 0.100 | 117 | 10 |
| Anatoxin-a | 0.059 | 96.9 | 6.2 |

*High Concentration*

| Analyte | Fortified Concentration (µg/L) | Avg. %Recovery | %RSD |
|---|---|---|---|
| Cylindrospermopsin | 2.50 | 108 | 1.6 |
| Anatoxin-a | 1.47 | 100 | 2.8 |

[a] Surface water physical parameters: total hardness = 142 milligrams/liter (mg/L) (as $CaCO_3$); free chlorine = 0.64 mg/L; total chlorine = 1.14 mg/L; conductivity = 344 µS; TOC = 3.04 ppm.

## Table 9. Aqueous Sample Holding Time Data

For samples from chlorinated surface water [a], fortified with method analytes and preserved and stored according to method section 8 (n=3).

| Analyte | Fortified Concentration (µg/L) | Day | Average Measured Concentration (µg/L) | % Change from Day 0[b] | % RSD |
|---|---|---|---|---|---|
| Cylindrospermopsin | 2.50 | 0 | 2.57 | Not applicable. | 3.3 |
| Anatoxin-a | 1.47 | 0 | 1.49 | Not applicable. | 2.6 |
| Cylindrospermopsin | 2.50 | 7 | Not applicable. | -5.4 | 2.4 |
| Anatoxin-a | 1.47 | 7 | Not applicable. | 0.67 | 4.4 |
| Cylindrospermopsin | 2.50 | 14 | Not applicable. | -6.2 | 1.5 |
| Anatoxin-a | 1.47 | 14 | Not applicable. | -7.4 | 2.4 |
| Cylindrospermopsin | 2.50 | 21 | Not applicable. | -5.1 | 1.2 |
| Anatoxin-a | 1.47 | 21 | Not applicable. | -0.67 | 0.91 |
| Cylindrospermopsin | 2.50 | 28 | Not applicable. | -1.6 | 3.5 |
| Anatoxin-a | 1.47 | 28 | Not applicable. | -1.3 | 2.1 |

[a] Surface water physical parameters: total hardness = 142 milligrams/liter (mg/L) (as $CaCO_3$); free chlorine = 0.64 mg/L; total chlorine = 1.14 mg/L; conductivity = 344 µS; TOC = 3.04 ppm.

[b] % Change from Day 0 calculation: (Day *x* mean concentration – Day 0 mean concentration) / Day 0 mean concentration * 100%, where *x* is the analysis day.

## Table 10. Comparison of Fortified Reagent Water Samples

Comparison of fortified reagent water samples in contact with plastic and glass during a triple freeze and thaw process [a] (n=4).

*Plastic Contact*

| Analyte | Fortified Concentration (µg/L) | Avg. %Recovery | %RSD |
|---|---|---|---|
| Cylindrospermopsin | 2.50 | 110 | 4.3 |
| Anatoxin-a | 1.47 | 94.1 | 1.0 |

*Glass Contact*

| Analyte | Fortified Concentration (µg/L) | Avg. %Recovery | %RSD |
|---|---|---|---|
| Cylindrospermopsin | 2.50 | 111 | 4.1 |
| Anatoxin-a | 1.47 | 98.4 | 2.4 |

[a] Samples were frozen at –30°C for 1 h, then thawed in a 40°C water bath for 5 min. The process was repeated two more times prior to the addition of an IS and analysis.

## Table 11. Initial Demonstration of Capability (IDC) Quality Control Requirements

| Method Reference | Requirement | Specification and Frequency | Acceptance Criteria |
|---|---|---|---|
| Section 9.1.1 | Demonstration of low system background | Analyze a Laboratory Reagent Blank (LRB) prior to any other IDC steps and after the highest CAL standard to check for carry-over. | Demonstrate that all method analytes are ≤1/3 of the Minimum Reporting Level (MRL) and that possible interferences from reagents and glassware do not prevent the identification and quantitation of method analytes. |
| Section 9.1.2 | Demonstration of precision | Prepare and analyze 4–7 replicate Laboratory Fortified Blanks (LFBs) fortified near the midrange concentration. | Percent relative standard deviation must be ≤20%. |
| Section 9.1.3 | Demonstration of accuracy | Calculate average percent recovery for replicates used in Section 9.1.2. | Average percent recovery within ±30% of the true value. |

| Method Reference | Requirement | Specification and Frequency | Acceptance Criteria |
|---|---|---|---|
| Section 9.1.4 | MRL confirmation | Fortify and analyze 7 replicate LFBs at the proposed MRL concentration. Confirm that the Upper Prediction Interval of Results (PIR) and Lower PIR (Sect. 9.1.4.1 and Sect. 9.1.4.2) meet the recovery criteria. | Upper PIR ≤150%; Lower PIR ≥50% |
| Section 9.1.5 | Quality Control Sample (QCS) | Analyze mid-level QCS. | Results must be within ±30% of the true value. |

## Table 12. Ongoing Quality Control Requirements

| Method Reference | Requirement | Specification and Frequency | Acceptance Criteria |
|---|---|---|---|
| Section 10.2 | Initial calibration | Use the internal standard calibration technique to generate a linear or quadratic calibration curve. Use at least five calibration concentrations. Validate the calibration curve as described in Section 10.2.3. | When each calibration standard is calculated as an unknown using the regression equation, the lowest level standard must be within ±50% of the true value. All other points must be within ±30% of the true value. |
| Section 9.2.1 | Laboratory Reagent Blank (LRB) | Analyze one LRB with each Analysis Batch. | Demonstrate that all method analytes are ≤1/3 the Minimum Reporting Level (MRL), and that possible interferences from reagents and glassware do not prevent identification and quantitation of method analytes. |

| Method Reference | Requirement | Specification and Frequency | Acceptance Criteria |
|---|---|---|---|
| Section 10.3 | Continuing Calibration Check (CCC) | Verify initial calibration by analyzing a low-level CCC at the beginning of each Analysis Batch. Subsequent CCCs are required after every 10 field samples, and at the end of the Analysis Batch. | The lowest level CCC must be within ±50% of the true value. All other points must be within ±30% of the true value. Internal standards must be ±50% of the average peak areas in the initial calibration. Results for field samples that are not bracketed by acceptable CCCs are invalid. |
| Section 9.2.4 | Internal standard (IS) | Internal standards are added to all standards and samples. | Peak area counts for each IS must be within ±50% of the average peak areas in the initial calibration. |
| Section 9.2.5 | Laboratory Fortified Sample Matrix (LFSM) | Analyze one LFSM per Analysis Batch. Fortify the LFSM with method analytes at a con-centration greater than the native concentrations. | For LFSMs fortified at concentrations ≤2 x MRL, the calculated recovery must be within +50% of the true value. At concentrations greater than the 2 x MRL, the recovery must be ±30% of the true value. |
| Section 9.2.6 | Laboratory Fortified Sample Matrix Duplicate (LFSMD) or Field Duplicate (FD) | Analyze at least one LFSMD or FD with each Analysis Batch. | For LFSMDs or FDs, the calculated relative percent difference must be ≤30%. (≤50% if concentration ≤2 x MRL.) |
| Section 9.2.7 | Quality Control Sample (QCS) | Analyze mid-level QCS at least quarterly. | Results must be ±30% of the true value. |

Figure 1. Example Chromatogram of ESI (+) Transitions for Method 545 Analytes