TN-1332

Application of EPA Method 545 for the Quantitation of Anatoxin-a and Cylindrospermopsin in Water Samples

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Introduction

Blue-green algae called cyanobacteria can produce toxins known as cyanotoxins, which include Anatoxin-a and Cylindrospermopsin. Cyanotoxins are harmful to humans and animals and potentially threaten the quality of drinking and surface water. Therefore, sensitive, accurate, and robust analytical methods are needed to ensure community drinking water safety. The US EPA (EPA-810F11001) has established Health Advisory Levels (HALs) in drinking water for children <6 years old that are 0.7 ng/mL for Cylindrospermopsin and 0.3 ng/mL for Microcystins. HALs for adults and school-age children (>6 years old) are 3.0 ng/mL for Cylindrospermopsin and 1.6 ng/mL for Microcystins in drinking water. The US EPA has not issued HALs for Anatoxin-a.

In this technical note, a simple, robust, reproducible, and rapid sample preparation was used to meet the requirements of the EPA Method 545. A Luna Omega 3 µm Polar C18 column was used to develop a chromatographic gradient that showed good retention from the void volume and achieved analyte baseline separation, while the sensitivity of the SCIEX QTRAP 4500 system enabled Limit of Quantitations (LOQs) of 0.10 ng/mL for Anatoxin-a and 0.20 ng/mL for Cylindrospermopsin. The method applicability was demonstrated in 4 different water samples. Pre-spikes at the LOQ and 2.5x LOQ levels showed accuracy within 25 % and %CV <15 %, meeting the requirements outlined in EPA Method 545.

LC Conditions

Column: Luna™ Omega 3 µm C18
Dimensions: 100 x 2.1 mm
Part No.: 00D-4784-AN
Mobile Phase: A: 0.2 % Acetic Acid in Water
B: Methanol

Gradient:

Time (min)	%B
0	2
1	20
6	60
8	60
8.2	2
10	2

Flow Rate: 0.35 mL/min
Injection Volume: 5 µL
Temperature: 40 °C
LC System: SCIEX® ExionLC™
Detection: MRM
Detector: SCIEX QTRAP® 4500

Sample Preparation

Internal standard (IS) Preparation
Intermediate and spiking stocks were prepared using Methanol / Water (1:1, v/v). Internal standards were spiked in the calibration standards and samples at final levels of 0.50 ng/mL for L-Phenylalanine-D_5 and 3.0 ng/mL for Uracil-D_4.

Standard Preparation
Intermediate and spiking stocks were prepared in Methanol / Water (1:1, v/v). The calibration standards were prepared in LC-MS grade water at concentrations ranging from 0.10–10 ng/mL for Anatoxin-a and 0.20–20 ng/mL for Cylindrospermopsin. Calibration standards were also spiked with the internal standards.

Pre-spiked Water Sample Preparation
Matrix spikes were prepared by aliquoting 850 µL of the water sample (RO lab water, drinking water, 2 river water samples), 50 µL of the analyte spiking solution, 50 µL of L-Phenylalanine-D_5 and 50 µL of Uracil-D_4 spiking solutions to yield a final volume of 1 mL. The solution was vortexed for 1 minute and filtered through a 13 mm PVDF CLARIFY Syringe Filter™ (hydrophilic, 0.22 µm, nonsterile, luer/slip, Part No.: AF8-7709-12). Next, blank samples were prepared using 1 mL of each water sample and filtered directly using the syringe filters without spiking. Internal standards were not spiked into the blank matrix samples. After filtration, samples were transferred to autosampler vials for LC-MS/MS analysis.

MRM Conditions

Polarity: Positive
Source Temperature: 500 °C
GS1: 55 psi
GS2: 55 psi
CUR: 35 psi
CAD: 8 psi
ISV: 4500 V

Table 1. MRM Parameters.

Compound	Q1 (m/z)	Q3 (m/z)	DP	EP	CE	CXP	Internal Standards
Anatoxin-a 1	165.9	149.1	52	9	19	12	L-Phenylalanine-D_5
Anatoxin-a 2	165.9	131.0	52	9	22	13	L-Phenylalanine-D_5
Cylindrospermopsin 1	416.1	194.1	106	5	44	10	Uracil-D_4
Cylindrospermopsin 2	416.1	336.1	106	5	31	7	Uracil-D_4
L-Phenylalanin-D_5	170.9	125.1	42	9	20	12	N/A
Uracil-D_4	115.0	98.0	40	9	24	10	N/A

Note: Quantifier transitions are designated by "1" and qualifier transitions are designated by "2."

Results and Discussion

The combination of the Luna™ Omega Polar 3 µm C18 column and gradient conditions resulted in good analyte retention from the void volume and baseline separation of the target compounds. The gradient runtime was extended to 10 min to improve the analyte separation and avoid co-elution of the analytes with matrix interferences. **Figure 1** shows the retention and chromatographic separation of the cyanotoxins and their internal standards.

The calibration curve was linear across the range of 0.10 to 10 ng/mL for Anatoxin-a and 0.20 to 20 ng/mL for Cylindrospermopsin. In addition, the curve had an r value >0.99 using a weighting factor of 1/x for both compounds (**Table 2**), meeting the EPA Method 545 requirements. Triplicate injections of each standard concentration were analyzed for the calibration curve and the average accuracy across each standard level ranged from 80 % to 120 %.

The method LOQs were 0.10 ng/mL for Anatoxin-a and 0.20 ng/mL for Cylindrospermopsin (**Figure 2** and **Table 4**). The Cylindrospermopsin LOQ was 3.5x lower than the US EPA HAL for children <6 years old. The US EPA has not issued HALs for Anatoxin-a. The accuracy, precision, and ion ratio were evaluated in the LOQ samples by spiking Anatoxin-a and Cylindrospermopsin into LC-MS grade water. To maintain statistical robustness, 3 individual LOQ samples were prepared, and each sample was analyzed in duplicate (n=6). The specific LOQ criteria evaluated included 2 selective MRM transitions, S/N ratio >10 for quantifier and qualifier transitions, accuracy within 30 %, %CV <15 %, and ion ratio tolerance within 30 %. Additional verification was performed by quantifying the LOQ samples against an external calibration curve. An accuracy of ±20 % and %CV <15 % were observed for both analytes, meeting the EPA Method 545 requirements.

Four different water samples (drinking water, RO lab water and 2 river water samples) were collected and processed to demonstrate the method applicability. Spiking concentrations were evaluated at the LOQ and 2.5x LOQ levels (**Table 3**). The spiking levels were 0.10 and 0.25 ng/mL for Anatoxin-a and 0.20 and 0.50 ng/mL for Cylindrospermopsin. The highest spiking level tested was lower than the Cylindrospermopsin HAL for children <6 years old. Similar to the LOQ experiment, statistical robustness was ensured by preparing each sample in triplicate and analyzing in duplicate (n=6) against the external solvent calibration curve. Accuracies within 25 % of the expected value and %CV values <15 % were observed at the 2 spiking levels in all 4 water samples, for both Anatoxin-a and Cylindrospermopsin (**Table 3**). XICs for the LOQ spiking level are shown in **Figure 3**. The unspiked samples did not show any detectable peaks, indicating that the cyanotoxins were not present in the original water samples.

Figure 1. Extracted Ion Chromatogram (XIC) of Anatoxin-a (1.0 ng/mL) and Cylindrospermopsin (2.0 ng/mL) Quantifier Transitions, L-Phenylalanine-D_5 (0.50 ng/mL) and Uracil-D_4 (3.0 ng/mL).

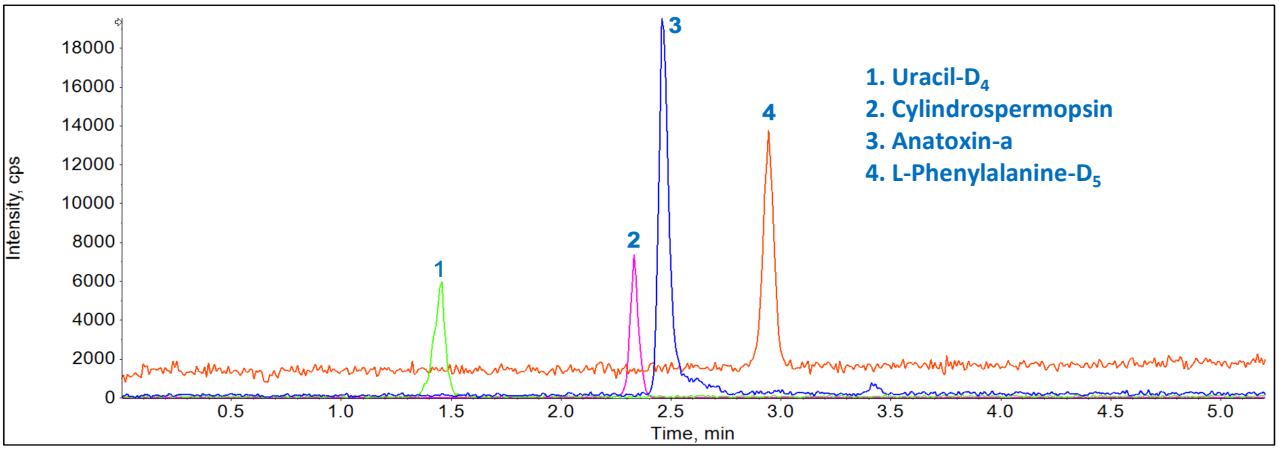


Table 2. Correlation Coefficient (r) and Accuracy Range Across the Calibration Curve for the Quantifier Ion.

Compound	Linear Range (ng/mL)	LOQ[1,2]	Correlation Coefficient (r)	Accuracy (%) Range of Calibration Standards[3]
Anatoxin-a	0.10-10	0.10	0.998	86.9-112
Cylindrospermopsin	0.20-20	0.20	0.995	89.6-111

Notes:
[1]The LOQ value was selected based on 2 selective MRM transitions, S/N ratio >10 for quantifier and qualifier ions, accuracy within ±30 %, %CV <15 %, and ion ratio tolerance within ±30 %.
[2]The LOQ samples were prepared in triplicate and analyzed in duplicate against the external solvent calibration curve.
[3]The accuracy range for the calibration curve was calculated based on the mean accuracy of each calibration standard in triplicate injections of the single sample.

Figure 2. XICs of Anatoxin-a and Cylindrospermopsin at their respective LOQs.

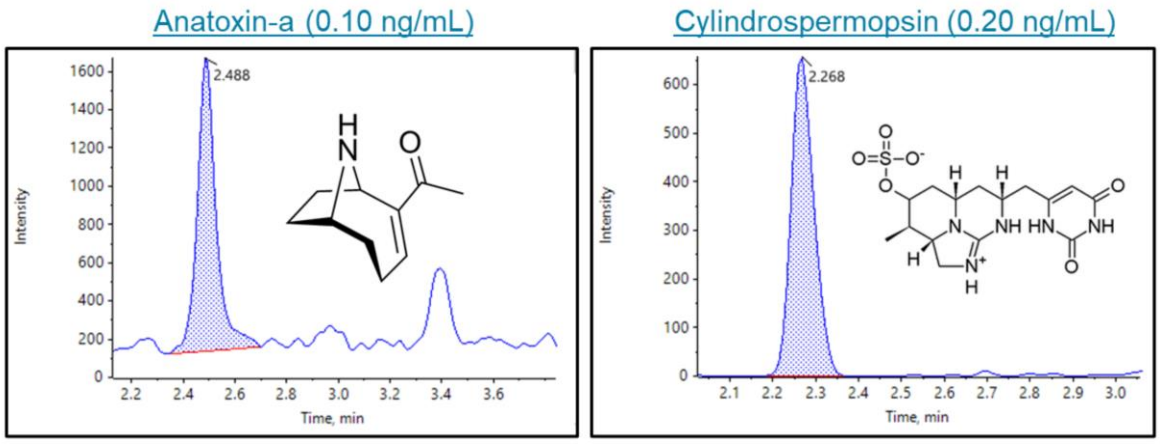
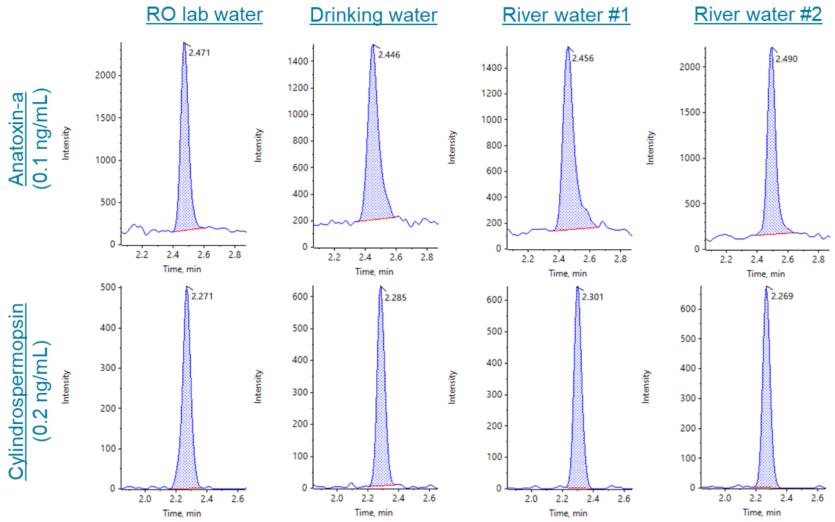


Table 2. Correlation Coefficient (r) and Accuracy Range Across the Calibration Curve for the Quantifier Ion.

Compound	Anatoxin-a		Cylindrospermopsin	
	Accuracy (%CV)	Accuracy (%CV)	Accuracy (%CV)	Accuracy (%CV)
	0.10 ng/mL	**0.25 ng/mL**	**0.20 ng/mL**	**0.50 ng/mL**
Drinking Water	75.6 (12)	92.5 (11)	88.2 (6.8)	96.9 (9.6)
RO Lab Water	74.5 (13)	93.5 (4.2)	87.2 (13)	96.4 (6.2)
River Water #1	80.5 (3.5)	96.4 (4.6)	87.8 (13)	97.5 (6.1)
River Water #2	83.9 (7.5)	94.4 (9.2)	92.7 (8.0)	100.5 (11)

Note: Each sample was prepared in triplicate and analyzed in duplicate.

Figure 3. XICs for Anatoxin-a (0.10 ng/mL) and Cylindrospermopsin (0.20 ng/mL) Spiked into Various Water Samples at the LOQ Level.



Conclusion

A robust, rapid and sensitive method to analyze Anatoxin-a and Cylindrospermopsin in water following EPA Method 545 was developed. The LOQs achieved on the QTRAP® 4500 system were 0.10 ng/mL for Anatoxin-a and 0.20 ng/mL for Cylindrospermopsin. The LOQs were 3.5-times lower than the US EPA HALs for Cylindrospermopsin for children <6 years old. An r value >0.99 was achieved for the linear range of 0.10–10 ng/mL for Anatoxin-a and 0.20–20 ng/mL for Xylindrospermopsin using a weighting factor of 1/x. Method applicability was demonstrated by spiking Anatoxin-a and Cylindrospermopsin into 4 different water samples and quantifying the cyanotoxins using a solvent-based calibration curve. Samples were spiked at the LOQ and 2.5x LOQ levels. Overall, the matrix spikes yielded accuracies of ±25 % and %CV values <15 % at the 2 spiking levels.

Luna™ Omega Ordering Information

3 µm Minibore Columns (mm)					SecurityGuard™ Cartridges (mm)
Phases	**30 x 2.1**	**50 x 2.1**	**100 x 2.1**	**150 x 2.1**	**4 x 2.0*/10pk**
Polar C18	00A-4760-AN	00B-4760-AN	00D-4760-AN	00F-4760-AN	AJ0-7600
PS C18	00A-4758-AN	00B-4758-AN	00D-4758-AN	00F-4758-AN	AJ0-7605
C18	—	00B-4784-AN	00D-4784-AN	00F-4784-AN	AJ0-7611
SUGAR	—	00B-4775-AN	00D-4775-AN	00F-4775-AN	AJ0-4496

for ID: 2.0 – 3.0 mm

*SecurityGuard Analytical Cartridges require holder, Part No.: KJ0-4282

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