Rapid quantification of Anatoxin-a and Cylindrospermopsin in water samples using EPA Method 545

Achieving parts-per-trillion (ppt) sensitivity using the SCIEX 7500 system

Sabarinathan[1], Sashank Pillai[2], Craig M. Butt[2], Simon Roberts[2]
[1]SCIEX, India; [2]SCIEX, USA

This technical note demonstrates the trace level quantification of anatoxin-a and cylindrospermopsin, following EPA Method 545[1], to achieve detection limits of 0.005 ng/mL. The detection limits were 60x and 140x below the microcystin and cylindrospermopsin health advisory levels (HALs) for young children. The sensitivity of the SCIEX 7500 system allowed for a simple, direct injection of 5 µL. In addition, an LC run time of 10 minutes minimized co-eluting interferences while maintaining throughput. Matrix applicability was shown by spiking four individual water samples at two levels, and all water samples showed excellent accuracy (<±20%) and precision (<10%).

Cyanobacteria naturally occur in surface water and can form harmful algal blooms (HABs) under eutrophic conditions of warm temperatures and excess nutrients [2]. HABs produce toxic compounds such as anatoxin-a and cylindrospermopsin, also known as cyanotoxins, which can be detrimental to humans and animals. Global regulations have established the minimum reporting levels (MRLs) for both compounds to be quantified to ensure water quality. For example, the US EPA (EPA-810F11001) has specified HALs equal to or below 0.3 ng/mL for microcystins and 0.7 ng/mL for cylindrospermopsin in drinking water for children less than six years old. The recommended HALs for adults and school-age children (above six years) are equal to or below 1.6 ng/mL for microcystins and 3.0 ng/mL for cylindrospermopsin in drinking water.

Key features of the SCIEX 7500 system for the analysis of cyanotoxins

- Simple, robust, reproducible and rapid sample preparation to meet the requirements of the EPA 545 method.
- Method sensitivity that exceeds the global regulatory requirements for drinking water
- Excellent chromatographic retention and retention time reproducibility for the analyte and internal standards
- Matrix spike samples (n=6) showed accuracy within ±20% and %CV <10% at 0.010 and 0.025 ng/mL for anatoxin-a and 0.020 and 0.050 ng/mL for cylindrospermopsin

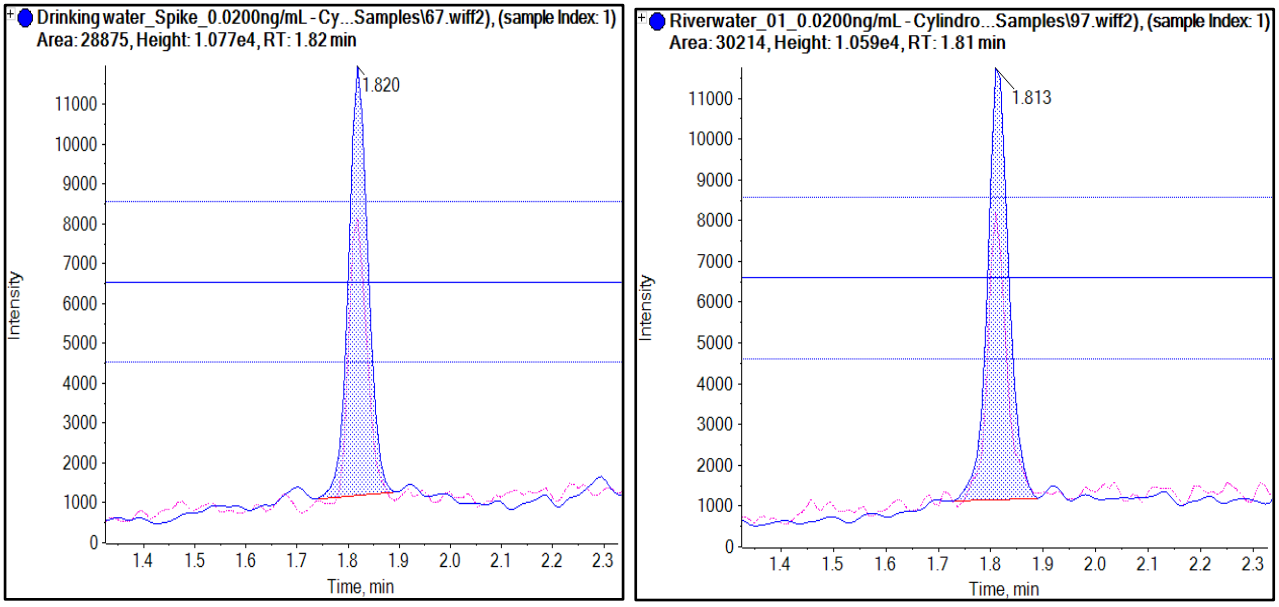

Figure 1. XIC chromatogram of cylindrospermopsin spiked and prepared into drinking water (left) and river water #1 (right) at 0.020 ng/mL. Chromatograms show overlaid ion ratio tolerance lines of the quantifier and qualifier ions and demonstrate detection confirmation based on ion ratios within ±30% tolerance.

Methods

Internal standard (IS) preparation. L-Phenylalanine-d5 and uracil-d4 stock solutions were manufactured by Clearsynth (Mumbai, India). Intermediate and spiking stocks were prepared using methanol: water (50:50 v/v). Internal standard concentrations in the samples were 0.050 ng/mL of L-phenylalanine-d5 and 1.00 ng/mL of uracil-d4.

Standard preparation. Individual stock solutions of anatoxin-a (5 µg/mL) and cylindrospermopsin (10 µg/mL) were manufactured by Cifga (Luga, Spain). Intermediate and spiking stocks were prepared in methanol: water (50:50 v/v). The calibration standards were prepared in LC-MS grade water and ranged from 0.005-1.00 ng/mL for anatoxin-a and 0.005-2.00 ng/mL for cylindrospermopsin. Calibration standards also were spiked with the internal standards.

Pre-spiked water sample preparation. Matrix spikes were prepared by aliquoting 850 µL of the water sample (lab water, drinking water, two separate river water samples), 50 µL of the analyte spiking solution, 50 µL of L-phenylalanine-d5, and 50 µL of uracil-d4 spiking solutions to yield a final volume of 1 mL. The solution was vortexed for 1 minute and filtered through a CLARIFY-PVDF 13mm syringe filter (hydrophilic, 0.22u, non-sterile, luer/slip). Next, blank samples were prepared using 1 mL of each water sample and filtered directly using the syringe filters without spiking. After filtration, samples were transferred to autosampler vials for the LC-MS/MS analysis.

Chromatography. An ExionLC AD was used with a Phenomenex Luna Omega 3 µm Polar C18 column (100Å 100 × 2.1 mm) for the chromatographic separation; see Table 1 for the gradient conditions used with the flow rate of 0.350 mL/min. A 5 µL injection volume was used, and the column oven temperature was set to 40°C.

Mass spectrometry. The SCIEX 7500 system was operated in multiple reaction monitoring (MRM) mode with electrospray ionization in positive ion mode. Two selective MRM transitions (Table 3) were monitored for the quantitation and confirmation of the targeted analytes based on the ion ratio calculation. The number of data points was calculated across the chromatographic peak for both analytes. Source and compound-specific parameters are presented in Tables 2 and 3, respectively. In addition, individual Q0D values are optimized for all the compounds (Table 3) to reduce the background noise and improve sensitivity and selectivity.

Data processing: All data was processed using SCIEX OS software (2.1.6).

Table 1. Gradient program for the analysis of anatoxin-a and cylindrospermopsin

Time (min)	% A	% B
0.00	*98*	*2*
1.00	*80*	*20*
6.00	*40*	*60*
8.00	*40*	*60*
8.20	*98*	*2*
10.00	*98*	*2*

Mobile phase A: 0.2 % (v/v) acetic acid in water
Mobile phase B: methanol

Table 2. Optimized source parameters for the analysis of anatoxin-a and cylindrospermopsin.

Source and gas parameters	
Curtain gas	*40 psi*
CAD gas	*8 psi*
Ion spray voltage	*1500 V*
Temperature	*350°C*
Ion source gas 1	*55 psi*
Ion source gas 2	*80 psi*

Table 3. MRM transitions and compound-dependent parameters for cyanotoxins and the internal standards

Compound	Q1 (m/z)	Q3 (m/z)	EP	CE	CXP	Q0D	Internal standard
Anatoxin-A	*166.1*	*149.1*	*10*	*20*	*8*	*40*	*L-Phenylalanine-d5*
Cylindrospermopsin	*416.2*	*194.1*	*8*	*50*	*6*	*25*	*Uracil-d4*
L-Phenylalanine-d5	*171.1*	*125.1*	*10*	*20*	*12*	*25*	*N/A*
Uracil-d4	*115.0*	*98.0*	*8*	*25*	*12*	*40*	*N/A*

Chromatographic separation using a 10 min LC gradient

The chromatographic conditions for compound analysis were optimized to provide a fast run time of 10 minutes and an injection volume of 5 µL. Previous work has shown that the analytes and internal standards are well retained by the Phenomenex Luna Omega C18 column[3]. The 10 min gradient was developed to achieve good analyte separation while also reducing co-elution with matrix interferences and ensuring column equilibration. As a result, Figure 2 shows an excellent chromatographic separation of the analyzed compounds.

Linear dynamic range and method sensitivity

The calibration curve was plotted using triplicate injections for each standard level. Both anatoxin-a and cylindrospermopsin showed r > 0.99 with an accuracy range between 74-118% (Table 4) using the weighting factor 1/x. The internal standard normalized calibration curves for the anatoxin-a and cylindrospermopsin quantifier transitions are shown in Figure 3. Excellent linear dynamic range was observed between 0.005-1.00 ng/mL for anatoxin-a and 0.005-2.00 ng/mL for cylindrospermopsin.

The method LOQs were established by spiking anatoxin-a and cylindrospermopsin into LC-MS grade water and determined as 0.005 ng/mL for both compounds. Statistical robustness was ensured by processing 3 replicate LOQ samples and injecting each sample in duplicate (n=6). Figure 4 shows chromatograms of anatoxin-a and cylindrospermopsin at LOQ level. The LOQ value was selected based on two selective MRM transitions, S/N ratio >10 for quantifier and qualifier of calibration standard, accuracy within ± 30%, % CV <15% and ion ratio tolerance within ± 30%. As a further determination of method reproducibility, the LOQ sample was analyzed against the solvent calibration curve. The observed results met the acceptance criteria with an accuracy of ±10% and precision <10% for both the analytes (Table 4). The achieved sensitivity of the SCIEX 7500 system was 60x and 140x lower than the US EPA HALs for microcystins and cylindrospermopsin, respectively, considering exposure for children <6 years old.

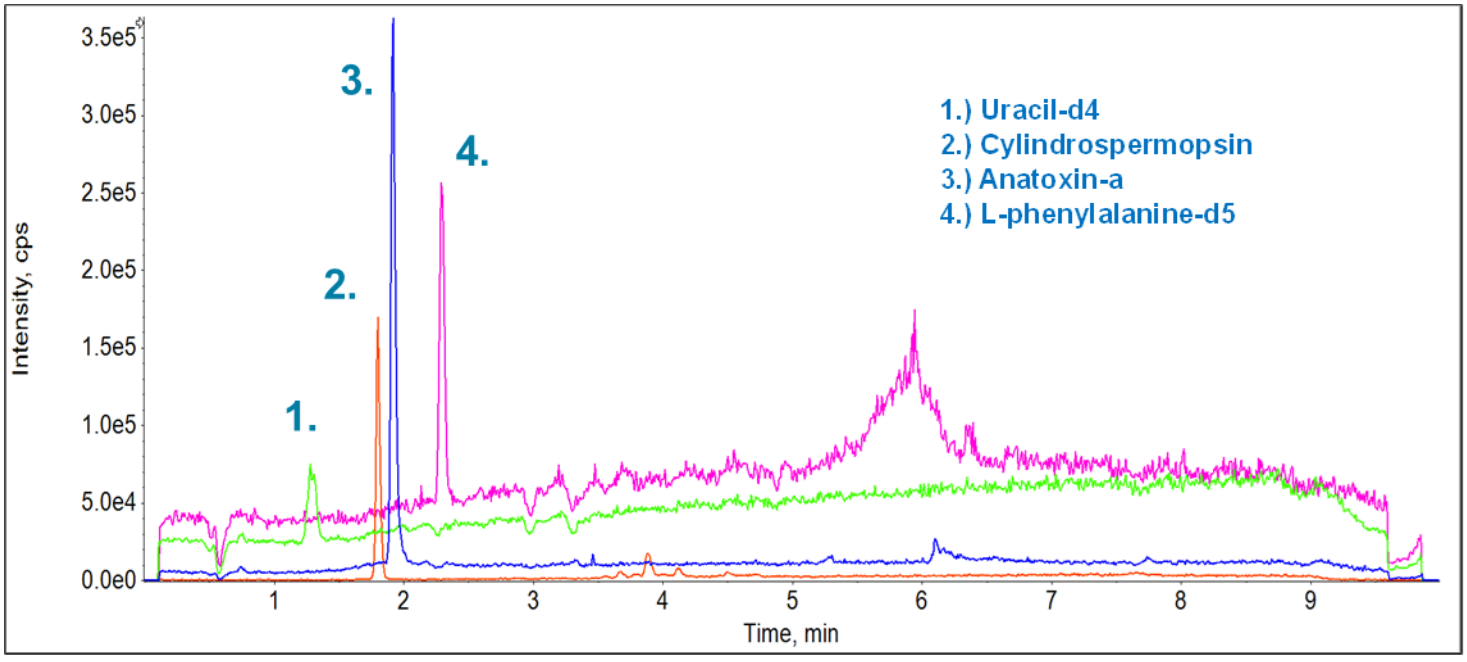

Figure 2. XIC of anatoxin-a (0.100 ng/mL), cylindrospermopsin (0.200 ng/mL) from the quantifier transitions, and XIC of L-phenylalanine-d5 and uracil-d4 at 0.050 and 1.00 ng/mL respectively. The Phenomenex Luna Polar C18 column achieved good chromatographic separation.

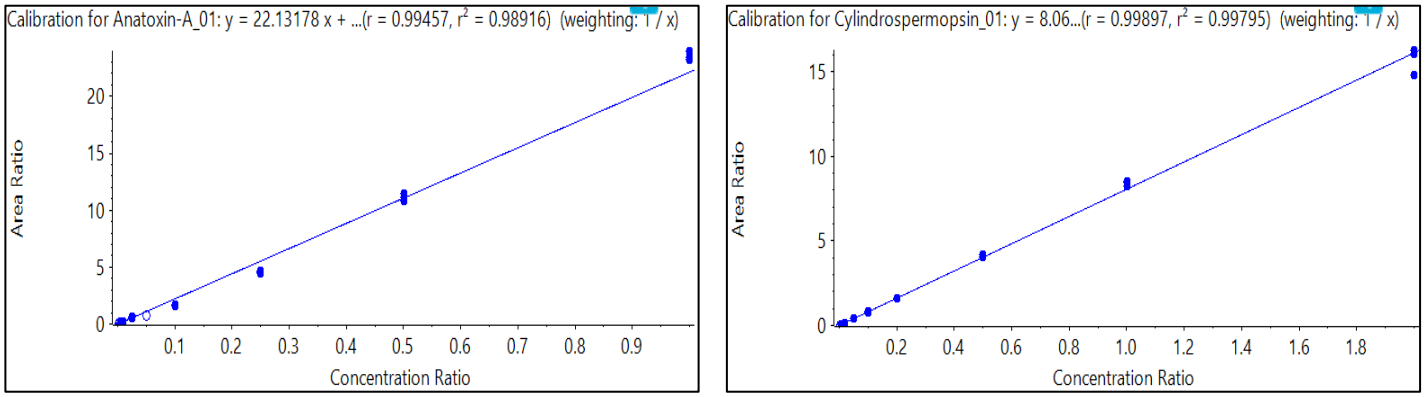

Figure 3. Representative calibration curve for anatoxin-a (left) and cylindrospermopsin (right) using the weighting factor 1/x. The excellent linear dynamic range of 0.005-1.00 ng/mL for anatoxin-a and 0.005-2.00 ng/mL for cylindrospermopsin was achieved with an r-value of >0.99.

Table 4. Calibration curve correlation coefficient (r) and accuracy range across the calibration curve[1] for quantifier ion.

Compound	Linear range (ng/mL)	LOQ [2]	Correlation coefficient (r)	Accuracy (%) range of calibration standards [3]
Anatoxin-a	*0.005-1.00*	*0.005*	*0.995*	*74.0-118*
Cylindrospermopsin	*0.005-1.00*	*0.005*	*0.999*	*93.0-104*

[1] Calibration curve and LOQ samples prepared by using the diluent-LC MS grade water.
[2] LOQ value selected based on two selective MRM transitions, S/N ratio >10 for quantifier and qualifier of calibration standard, accuracy within ± 30%, % CV <15%, and ion ratio tolerance within ± 30%.
[3] The accuracy range for the calibration curve is calculated based on the mean accuracy of each calibration standard in 3 replicate injections (n=3) of the single sample.

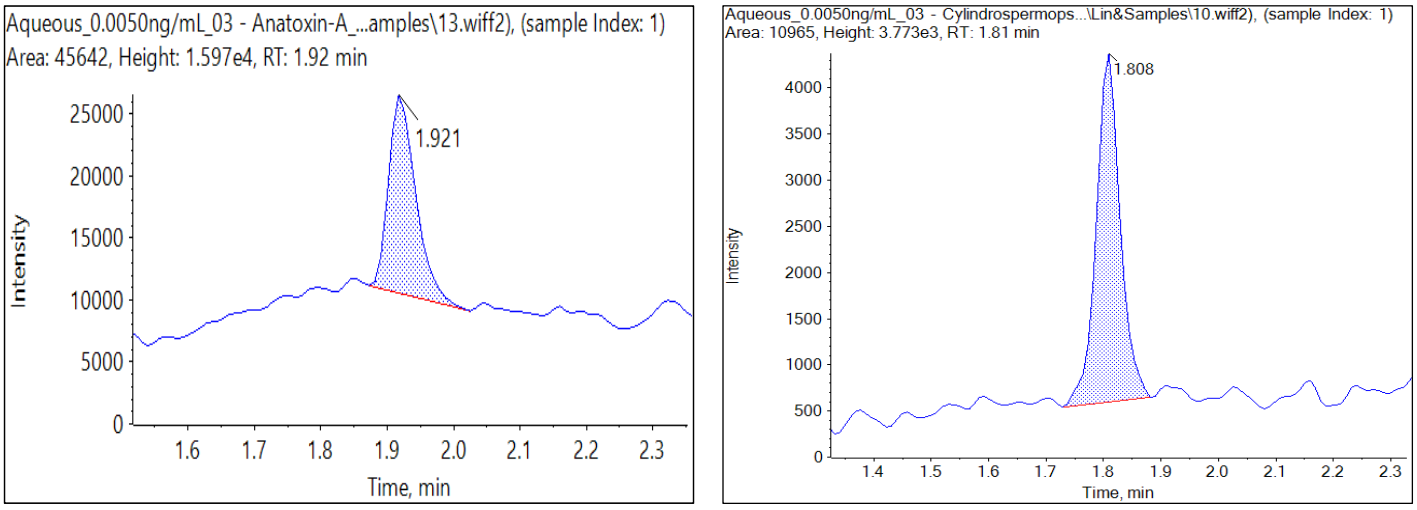

Figure 4. Chromatogram of anatoxin-a (left) and cylindrospermopsin (right) at the LOQ concentration of 0.005 ng/mL. XICs show the quantifier transitions for both anatoxin-A (m/z: 166.1 / 149.1) and cylindrospermopsin (m/z: 416.2 / 194.1). Excellent peak shape and sensitivity were achieved for both analytes.

Method applicability in 4 real-world water samples

Four different water samples were collected and processed to demonstrate the method applicability (drinking water, lab water, river water #1 and river water #2). The water samples were spiked at two different levels, which were at least one order of magnitude lower than the US EPA HALs (0.010, 0.025 ng/mL for anatoxin-a and 0.020, 0.050 ng/mL for cylindrospermopsin). Similar to the LOQ experiment, statistical robustness was ensured by preparing each sample in triplicate and analyzed in duplicate (n=6) against a solvent calibration curve.

Tables 5 and 6 show excellent accuracy (± 20%) and precision <10%) for anatoxin-a and cylindrospermopsin in all four water samples at both spike levels. Unspiked, "blank" samples were also processed and analyzed against the solvent calibration curve. No significant peaks were found in the unspiked sample for either analyte indicating these contaminants were not present in the water samples.

Table 5. Average accuracy and %CV (n=6) for anatoxin-a and cylindrospermopsin for drinking and RO water samples. All recovery measurements were performed at prespiked 0.010, 0.025 ng/mL for anatoxin-a and 0.020, 0.050 ng/mL for cylindrospermopsin (n=6) concentration levels against the single solvent calibration curve. The average accuracy and %CV showed here from the quantifier transition.

Compound	Drinking water				Lab water			
Anatoxin-a	***0.010 ng/mL***		***0.025 ng/mL***		***0.010 ng/mL***		***0.025 ng/mL***	
	Average accuracy	*%CV*	*Average accuracy*	*%CV*	*Average accuracy*	*%CV*	*Average accuracy*	*%CV*
	97.0	*5.3*	*101.2*	*3.2*	*80.6*	*4.7*	*93.4*	*4.1*
Cylindrospermopsin	***0.020 ng/mL***		***0.050 ng/mL***		***0.020 ng/mL***		***0.050 ng/mL***	
	Average accuracy	*%CV*	*Average accuracy*	*%CV*	*Average accuracy*	*%CV*	*Average accuracy*	*%CV*
	83.7	*7.1*	*86.6*	*8.3*	*85.7*	*6.7*	*84.4*	*6.9*

Note: *For each spiking level, 3 replicate samples were processed and each sample injected in duplicate.*

Table 6. Average accuracy and %CV (n=6) for anatoxin-a and cylindrospermopsin for River water 01 and River water 02. All recovery measurements were performed at prespiked 0.010, 0.025 ng/mL for anatoxin-a and 0.020, 0.050 ng/mL for cylindrospermopsin levels against the single calibration curve. The average accuracy and %CV showed here from the quantifier transition.

Compound	River water #1				River water #2			
Anatoxin-a	***0.010 ng/mL***		***0.025 ng/mL***		***0.010 ng/mL***		***0.025 ng/mL***	
	Average accuracy	*%CV*	*Average accuracy*	*%CV*	*Average accuracy*	*%CV*	*Average accuracy*	*%CV*
	103.1	*2.1*	*102.2*	*4.5*	*90.7*	*5.3*	*95.4*	*5.1*
Cylindrospermopsin	***0.020 ng/mL***		***0.050 ng/mL***		***0.020 ng/mL***		***0.050 ng/mL***	
	Average accuracy	*%CV*	*Average accuracy*	*%CV*	*Average accuracy*	*%CV*	*Average accuracy*	*%CV*
	83.7	*7.7*	*87.4*	*4.9*	*83.5*	*5.2*	*81.2*	*5.7*

Note: *For each spiking level, 3 replicate samples were processed and each sample injected in duplicate.*

Conclusions

The method showed:

- Simple and fast sample preparation for the analysis of anatoxin-a and cylindrospermin matrix spikes and real-world samples.
- The limit of quantification (LOQ) was 0.0050 ng/mL for both analytes. These LOQs were below the US EPA HALs for young children, and this age class represents the strictest HALs established by the US EPA.
- Method applicability was shown by analyzing unspiked and pre-spiked water samples, demonstrating sensitivity, accuracy, and precision in real-world water samples.
- The recovery of pre-spiked water samples was 80-120% for anatoxin-a and cylindrospermopsin, meeting the acceptance criteria outlined in the EPA 545 method.

References

1. Method 545: Determination of cylindrospermopsin and anatoxin-a in drinking water by liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS), EPA 815-R-15-009. United States Environmental Protection Agency, Washington, April 2015. https://www.epa.gov/sites/default/files/2017-10/documents/epa_815-r-15-009_method_545.pdf.
2. Abbas, F.; Porojan, A. Mowe, M.A.D.; Lehane, M.; Mitrovic, S.M.; Lim, R.P.; Yeo, D.C.J. (2020) Sample extraction and liquid chromatography–tandem mass spectrometry (LC-MS/MS) method development and validation for the quantitative detection of cyanobacterial hepatotoxins and neurotoxins in Singapore's reservoirs. *Marine and Freshwater Research* ***71(5)****: 673-688*.
3. Aqeel, Z.; Lomas, S.; Schiessel, D. (2016) Determination of cylindrospermopsin and anatoxin-a in drinking water by UHPLC/MS/MS using a Luna® Omega 1.6 µm Polar C18 (EPA Method 545). Phenomenex, Torrance, CA. https://www.phenomenex.com/documents/2022/05/20/19/14/determination-of-cylindrospermopsin-and-anatoxina-in-drinking-water-by-uhplcmsms-using-a-luna-omega.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
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