A single direct injection method for the quantitation of cyanotoxins in water

Achieving sub-ng/mL (parts-per-trillion) sensitivity using the SCIEX 7500 system

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

This technical note presents a direct injection method to analyze multiple classes of cyanotoxins in water and achieve sub-ng/mL detection limits. The sensitivity of the SCIEX 7500 system allowed for the omission of time-consuming sample preparation, while attaining limit of quantitation (LOQ) values between 0.0075 and 0.075 ng/mL (Figure 1). The diverse group of cyanotoxins, including microcystins, nodularin-R, anatoxin-a and cylindrospermopsin, were analyzed using a 14-minute LC runtime. Matrix spikes into reverse-osmosis (RO) lab water and drinking water yielded accuracies of ±30% and %CV values <11% (n=6) for all analytes.

Analytical methods for cyanotoxin analysis commonly use multiple sample preparation and instrument methods, such as EPA Methods 544 and 545.[1,2] Here, the conventional solid-phase extraction (SPE) sample preparation method was avoided by using a simple method that consisted of multiple freeze-thaw cycles and dilution with acetonitrile. A similar approach has been reported in the literature with good accuracy and precision.[3] Analysis time was significantly reduced by using a single method instead of analyzing the classes of cyanotoxins separately.

Cyanotoxins are produced by cyanobacteria and are known to be harmful to both humans and animals. These toxins are therefore regulated by government agencies around the world. For example, the US EPA specified health advisory levels (HALs) of ≤0.3 ng/mL for microcystins and 0.7 ng/mL for cylindrospermopsin in drinking water for children less than 6 years old.[4] These low HALs require sensitive analytical methods to ensure community safety.

Key benefits of the SCIEX 7500 system and simplified method for the analysis of cyanotoxins

- The sensitivity of the SCIEX 7500 system enabled LOQs of 0.0075–0.075 ng/mL
- The method benefits from a simple, fast and cost-effective sample preparation and does not rely on costly internal standards for microcystins
- Rapid LC method capable of analyzing microcystins, nodularin-R, anatoxin-a and cylindrospermopsin in a single injection
- The method covers various cyanotoxins, including those often analyzed using EPA Methods 544 and 545
- Accuracies of ±30% and %CV values <11% (n=6) were achieved for matrix spikes in RO lab water and drinking water.

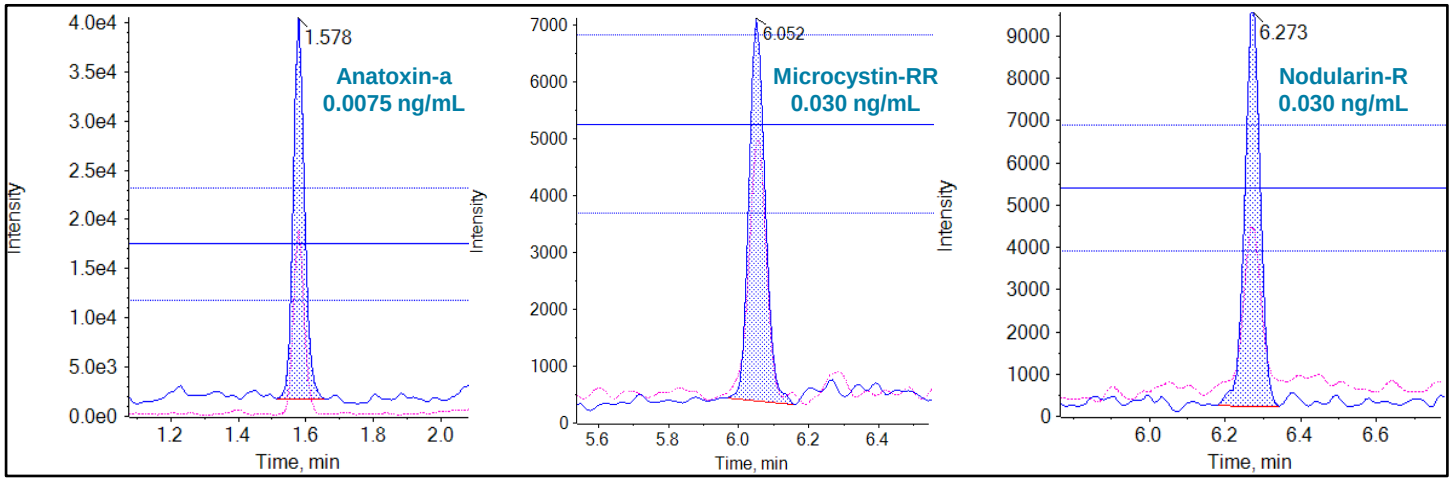

Figure 1. Representative XIC chromatograms of anatoxin-a, microcystin-RR and nodularin-R at the LOQ level with ion ratio tolerance lines overlaying the quantifier and qualifier ions. Compound identification was based on ion ratio calculation with tolerance levels of ±30% for the quantifier and qualifier ions at the LOQ. The LOQs for anatoxin-a, microcystin-RR and nodularin-R were 0.0075 ng/mL, 0.030 ng/mL and 0.030 ng/mL, respectively.

Methods

Standard preparation: Individual stock solutions of anatoxin-a, cylindrospermopsin, nodularin-R and microcystins-RR, -LF, -LR, -LY and -YR were purchased from Cifga (Luga, Spain). Microcystin-LW was obtained from Supelco (Sigma-Aldrich). The individual stock solutions were used to prepare a 200 ng/mL mixed solution in 1:1 (v/v), acetonitrile/water. The resulting solution was further diluted to cover concentrations ranging from 0.0075 to 2.40 ng/mL. The L-phenylalanine-d5 and uracil-d4 stock solutions were purchased from Clearsynth (Mumbai, India).

Extraction spike sample preparation: Pre-extraction matrix spikes were prepared by aliquoting 950 µL of the water sample into culture tubes and adding 50 µL of the spiking solution (variable concentration) to yield concentrations of 0.15 and 0.30 ng/mL. Uracil-d4 and L-phenylalanine-d5 were spiked at final concentrations of 100 ng/mL and 0.6 ng/mL, respectively. The solution was vortexed for 1 minute, stored at -20°C for an hour and then thawed in a water bath for 15 minutes. The freeze-thaw cycle was repeated 2 more times to ensure cell lysis. After 3 cycles, the sample was filtered through a CLARIFY-PVDF 13 mm syringe filter (hydrophilic, 0.22 µm). Finally, the filtered sample was diluted 1:1 by volume with acetonitrile. Blank samples were prepared in a similar manner, without spiking in the analytes. The processed samples were transferred to autosampler vials immediately prior to LC-MS/MS analysis.

Chromatography: An ExionLC AD system was used with a Phenomenex Synergi Polar-RP column (100 Å, 100 × 3.0 mm, 2.5 µm) for chromatographic separation. Table 1 shows the gradient conditions used and the compositions of the mobile phases and rinsing solution. The injection volume was 10 µL, the flow rate was 0.400 mL/min, and the column oven temperature was set to 40°C. The autosampler temperature was set to 8°C and 1 mL of rinsing solution was used for needle washing.

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 2) were monitored for the quantitation and confirmation of the targeted analytes based on the ion ratio calculation. Source and compound-specific parameters are shown in Tables 2 and 3, respectively. Individual Q0D values were optimized (Table 2) to reduce the background noise and improve sensitivity.

Data processing: All data were processed using SCIEX OS software, version 2.1.6. The anatoxin-a response was normalized to the L-phenylalanine-d5 response, cylindrospermopsin was normalized to uracil-d4 response (matrix spikes only).

Table 1. Gradient program used for the analysis of cyanotoxins.

Time (min)	% A	% B
0.0	*100*	*0*
1.5	*100*	*0*
10.0	*2*	*98*
12.0	*2*	*98*
12.1	*100*	*0*
14.0	*100*	*0*

Mobile phase A: 90:10 (v/v), water/acetonitrile with 0.1% formic acid and 5mM ammonium formate
Mobile phase B: acetonitrile
Rinsing solution: 60% 2-propanol, 20% methanol and 20% acetonitrile by volume

Table 2. MRM conditions and compound-specific parameters for the analysis of microcystins, nodularin-R, anatoxin-a and cylindrospermopsin.

Compound	Q1 (m/z)	Q3 (m/z)	EP (V)	CE (V)	CXP (V)	Q0D (V)
Microcystin-RR _1	*519.9*	*135.1*	*8*	*37*	*4*	*50*
Microcystin-RR_2	*519.9*	*103.4*	*8*	*98*	*6*	*50*
Microcystin-LF_1	*986.5*	*852.6*	*6*	*30*	*10*	*25*
Microcystin-LF_2	*986.5*	*135.1*	*6*	*95*	*10*	*25*
Microcystin-LR_1	*995.6*	*103.2*	*10*	*160*	*12*	*5*
Microcystin-LR_2	*995.6*	*135.1*	*10*	*120*	*4*	*5*
Microcystin-LY_1	*1002.5*	*868.4*	*10*	*30*	*14*	*40*
Microcystin-LY_2	*1002.5*	*134.9*	*10*	*100*	*20*	*40*
Microcystin-YR_1	*1045.6*	*103.3*	*6*	*175*	*12*	*110*
Microcystin-YR_2	*1045.6*	*135.1*	*6*	*120*	*6*	*110*
Nodularin-R_1	*825.4*	*103.3*	*10*	*155*	*12*	*20*
Nodularin-R_2	*825.4*	*135*	*10*	*90*	*8*	*20*
Microcystin-LW_1	*1025.6*	*135.2*	*6*	*100*	*4*	*25*
Microcystin-LW_2	*1025.6*	*107.2*	*6*	*140*	*16*	*25*
Anatoxin-a_1	*166.1*	*149.1*	*10*	*20*	*8*	*40*
Anatoxin-a_2	*166.1*	*131.1*	*10*	*25*	*8*	*40*
Cylindrospermopsin_1	*416.2*	*194.1*	*8*	*50*	*6*	*25*
Cylindrospermopsin_2	*416.2*	*336.2*	*8*	*35*	*10*	*25*
L-phenylalanine-d5	*171.1*	*125.1*	*10*	*20*	*12*	*25*
Uracil-d4	*115*	*98*	*8*	*25*	*12*	*40*

Note: Quantifier transitions are designated by "_1", qualifier transitions are designated by "_2".

Table 3. Optimized source parameters for the analysis of microcystins, nodularin-R, anatoxin-a and cylindrospermopsin.

Source and gas parameters	
Curtain gas	*40 psi*
CAD gas	*12 psi*
Ion spray voltage	*3000 V*
Temperature	*400°C*
Ion source gas 1	*45 psi*
Ion source gas 2	*80 psi*

Novel LC method for wide coverage of cyanotoxin compounds

Developing LC methods for analytes with wide-ranging polarities is challenging. During method development, various mobile phases and columns were tested. Good retention and peak shape were achieved using the Phenomenex Polar-RP column using water modified with formic acid and ammonium formate and acetonitrile as mobile phases (Table 2).

A linear 14-minute gradient was developed to retain and chromatographically separate the diverse group of cyanotoxins. The most polar analytes (anatoxin-a and cylindrospermopsin) eluted after the column void volume as shown by the retention factor (k') of 0.56 for cylindrospermopsin. This demonstrates good retention and minimal impact from unretained interferences (Figure 2). Good separation was also obtained between microcystins and nodularin-R, which eluted later.

Method performance in calibration standards

Calibration standards were prepared in solvent and the curve was plotted using the weighing factor 1/x for all 9 compounds. Excellent linearity was achieved with an r value >0.99 and average accuracies (n=3) ranged between 90% and 113% (Table 4). For example, the calibration curves based on the quantifier transitions for anatoxin-a and microcystin-RR covered a linear range of 0.0075–2.4 ng/mL and 0.030–2.4 ng/mL, respectively (Figure 3).

Excellent sensitivity was achieved on the SCIEX 7500 system and LOQs ranged from 0.0075 ng/mL to 0.075 ng/mL in the solvent-based standards. The LOQ was determined based on 2 selective MRM transitions, S/N ratio >10 for both the quantifier and qualifier transitions, accuracy within 10%, %CV <10% and ion ratio tolerance within 30%. Method robustness and reproducibility were confirmed by processing 3 replicate LOQ samples and injecting each in duplicate (n=6). The observed results met the acceptance criteria with an accuracy of ±10% and %CV <10% for all the analytes (Table 4). Example LOQ chromatograms with ion ratio tolerance lines overlaid with the quantifier and qualifier ions are shown in Figure 1. Excellent peak shapes were observed for anatoxin-a, microcystin-RR and nodularin-R. These results indicate that the method was sensitive enough to quantify cyanotoxins at levels considerably lower than the current HALs for microcystins (0.3 ng/mL) and cylindrospermopsin (0.7 mg/mL) in drinking water for children <6 years old.

Table 4. Correlation coefficients (r value) and accuracy ranges for calibration curves and average accuracy and %CV for the quantifier ion at the LOQ.

Compound	Linear range (ng/mL)[1]	LOQ[2]	Correlation coefficient (r)	Accuracy range for calibration standards (%)[3] (n=3)	Average % accuracy at LOQ (n=6)	% CV at LOQ (n=6)
Microcystin-RR	*0.030–2.40*	*0.0300*	*0.999*	*96.4–102*	*102*	*6.0*
Microcystin-LF	*0.030–2.40*	*0.0300*	*0.999*	*92.5–113*	*115*	*6.4*
Microcystin-LR	*0.075–2.40*	*0.0750*	*0.999*	*98.2–101*	*107*	*3.9*
Microcystin-LY	*0.030–2.40*	*0.0300*	*0.999*	*90.2–111*	*124*	*6.3*
Microcystin-YR	*0.030–2.40*	*0.0300*	*1.000*	*96.3–102*	*107*	*7.3*
Nodularin-R	*0.030–2.40*	*0.0300*	*1.000*	*96.4–109*	*100*	*3.8*
Microcystin-LW	*0.075–2.40*	*0.0750*	*0.999*	*97.5–103*	*105*	*7.7*
Anatoxin-a	*0.0075–2.40*	*0.0075*	*0.999*	*94.1–104*	*104*	*7.5*
Cylindrospermopsin	*0.075–2.40*	*0.0750*	*0.999*	*95.7–106*	*103*	*4.1*

[1] The calibration curve and LOQ samples were prepared in 1:1 (v/v), acetonitrile/water
[2] LOQ values were selected based on 2 selective MRM transitions, S/N ratio >10 for the quantifier and qualifier ions of the calibration standard, accuracy within ±10%, %CV <10% and ion ratio tolerance within ±30%
[3] Calibration curve accuracy range was calculated based on the mean accuracy of each standard in 3 replicate injections (n=3) of the single sample

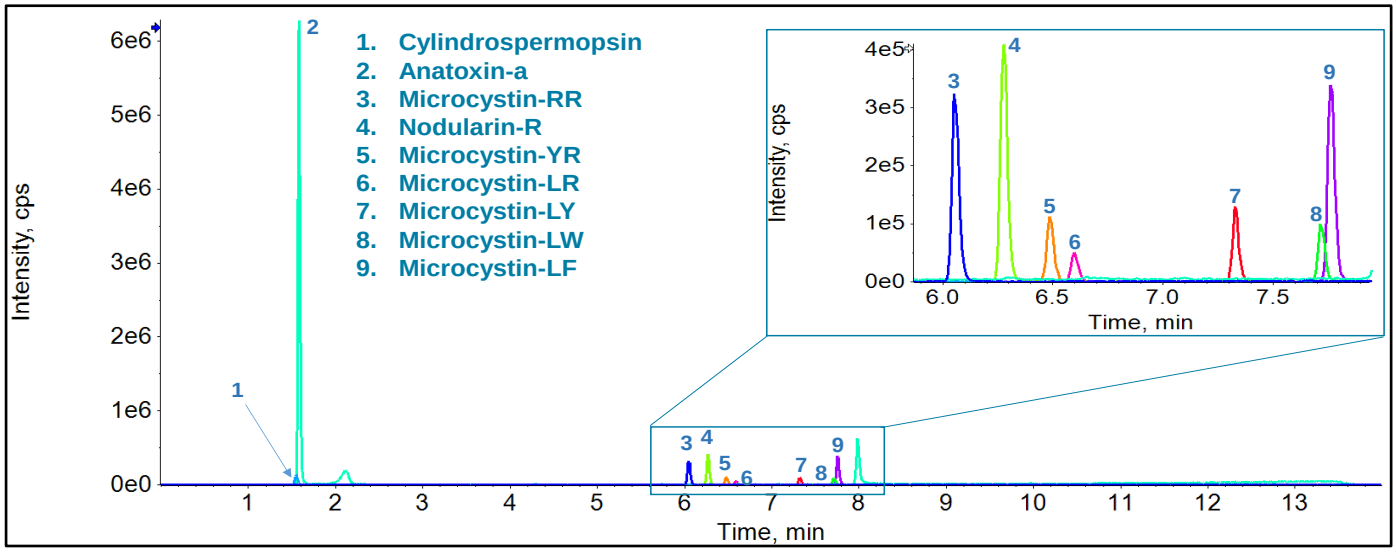

Figure 2. XIC of the 1.2 ng/mL standard for anatoxin-a, cylindrospermopsin, nodularin-R and 6 microcystins. Traces show the quantifier transitions. The Phenomenex Synergi Polar-RP column achieved good chromatographic separation and retention for the analytes, which have different polarities.

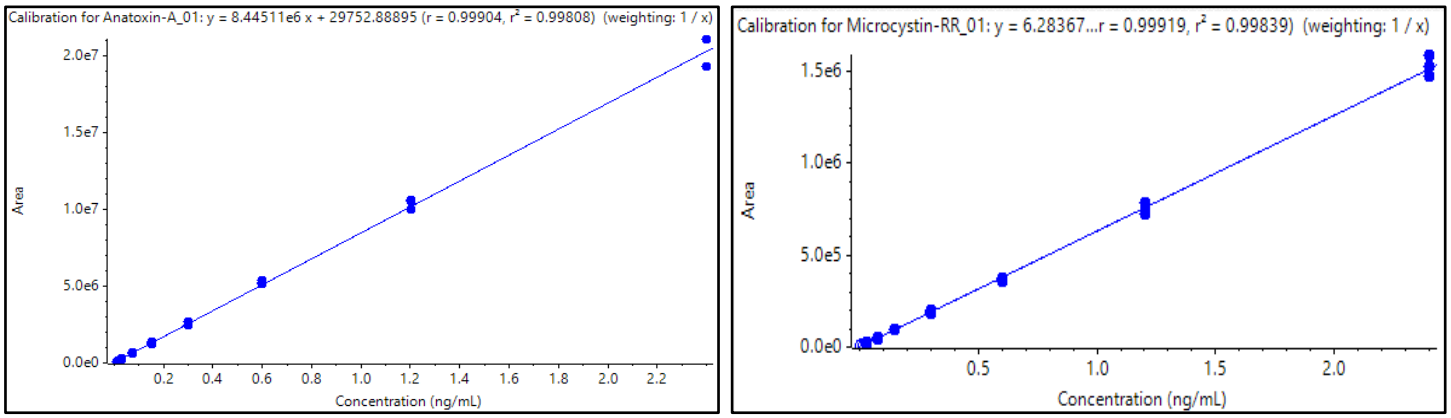

Figure 3. Representative calibration curves from the quantifier ions for anatoxin-a (m/z: 166.1/149.1) and microcystin-RR (m/z: 519.9 / 135.1). Linear ranges of 0.0075–2.4 ng/mL and 0.030–2.4 ng/mL were observed for anatoxin-a and microcystin-RR, respectively. The calibration curves for both analytes had r values >0.99. A weighting factor of 1/x was applied.

Accuracy and precision in water sample spikes

Water samples were collected from the RO lab water supply and a commercial drinking water. These samples were processed, as described, to demonstrate the applicability of the method. Unspiked samples were processed and analyzed against the external solvent calibration curve. None of the analytes showed significant peaks in the unspiked sample.

The water samples were spiked at 0.15 ng/mL and 0.30 ng/mL. Similar to the LOQ experiment, each sample was prepared in triplicate and injected in duplicate (n=6) and compared against an external solvent calibration curve. Accuracies ±30% and %CV values <11% were observed for all compounds in the water samples at both spike levels. These results met the acceptance criteria for accuracy (±30%) and precision (%CV<30%).[1,2] Accuracy and precision data are shown in Table 5.

Table 5. Average accuracy and %CV (n=6) for anatoxin-a, cylindrospermopsin, microcystins and nodularin-R for RO lab water and drinking water samples[1]. All recovery measurements were performed at pre-spiked 0.15 and 0.30 ng/mL concentration levels and compared against the single external solvent calibration curve. The average accuracy and %CV values shown are based on the quantifier transition.

Compound	Lab water				Drinking water			
	0.15 ng/mL		0.30 ng/mL		0.15 ng/mL		0.30 ng/mL	
	Average accuracy (%)	%CV	Average accuracy (%)	%CV	Average accuracy (%)	%CV	Average accuracy (%)	%CV
Microcystin-RR	89.7	3.7	90.8	2.7	93.2	3.8	97.6	2.7
Microcystin-LF	80.4	7.3	80.8	2.0	83.5	5.6	84.0	3.7
Microcystin-LR	87.1	6.0	85.4	3.8	87.1	10	93.0	4.2
Microcystin-LY	83.7	4.6	83.9	1.8	88.0	3.7	88.2	5.2
Microcystin-YR	94.3	7.8	99.1	3.4	97.9	7.4	101.2	2.8
Nodularin-R	99.9	1.7	98.9	2.7	102	6.9	107.3	2.9
Microcystin-LW	86.3	9.4	82.1	5.1	81.1	8.2	86.2	3.6
Anatoxin-a	70.8	11	76.6	11	86.1	5.9	88.7	6.4
Cylindrospermopsin	107	8.4	112	8.3	115	7.6	102	4.5

[1] *All samples were prepared in triplicate and analyzed in duplicate*

Conclusions

- A comprehensive method streamlined the quantification of many cyanotoxins in water in a single injection, instead of using both EPA Methods 544 and 545
- LOQs less than the US EPA HALs specified for drinking water consumption by young children were achieved due to the sensitivity of the SCIEX 7500 system. LOQs ranged from 0.0075–0.075 ng/mL for all analytes using the direct injection method
- Accuracies between 90-110% and %CV values <10% were achieved for all analytes in solvent at the LOQ
- Method applicability was demonstrated in matrix-spikes from various water samples. Pre-extraction spikes performed at 0.15 and 0.30 ng/mL yielded accuracies of ±30% and %CV values <11% for all analytes

References

1. Shoemaker J, Tettenhorst D, Delacruz, A. Method 544. Determination of microcystins and nodularin in drinking water by solid phase extraction and liquid chromatography/tandem mass spectrometry (LC/MS/MS), EPA/600/R-13/474. United States Environmental Protection Agency, Washington, February 2015. https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NERL&direntryid=306953.

2. 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/esam/method-545-determination-cylindrospermopsin-and-anatoxin-drinking-water-liquid-chromatography.

3. Wang C.C., Petty E.E., Smith C.M. (2016) Rapid and efficient analysis of microcystins, nodularin, cylindrospermopsin, and anatoxin-a in drinking water by LC tandem MS. J. AOAC. Int. **99(6)**, 1565-1571.

4. Cyanobacteria and cyanotoxins: Information for drinking water systems. EPA-810F11001. United States Environmental Protection Agency, Office of Water, Washington, September 2014. https://www.epa.gov/sites/default/files/2014-08/documents/cyanobacteria_factsheet.pdf.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices