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A Single Direct Injection Method for the Quantitation of Cyanotoxins in Water

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Introduction

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. These low HALs require sensitive analytical methods to ensure community safety.

Analytical methods for cyanotoxin analysis commonly use multiple sample preparation and instrument methods, such as EPA Methods 544 and 545. 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. Analysis time was significantly reduced by using a single method instead of analyzing the classes of cyanotoxins separately.

This technical note presents a direct injection method to analyze multiple classes of cyanotoxins in water and achieve sub-ng/mL detection limits using a Synergi 2.5 µm Polar-RP column. 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.

Sample Preparation

Standard Preparation

Individual stock solutions of Anatoxin-a, Cylindrospermopsin, Nodularin-R and Microcystins-RR, -LF, -LR, -LY, -LW, and -YR were used to prepare a 200 ng/mL mixed solution in Acetonitrile / Water (1:1, v/v). The resulting solution was further diluted to cover concentrations ranging from 0.0075 to 2.40 ng/mL.

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-D_4 and L-Phenylalanine-D_5 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 13 mm PVDF CLARIFY Syringe Filter™ (hydrophilic, 0.22 µm, nonsterile, luer/slip, Part No.: AF8-7709-12). 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.

LC Conditions

Column: Synergi™ 2.5 µm Polar-RP
Dimensions: 100 x 3.0 mm
Part No.: 00D-4371-Y0
Mobile Phase: A: Water / Acetonitrile (90:10, v/v), with 0.1 % Formic Acid and 5 mM Ammonium Formate
B: Acetonitrile

Gradient:

Time (min)	%B
0	0
1.5	0
10	98
12	98
12.1	0
14	0

Flow Rate: 0.4 mL/min
Injection Volume: 10 µL
Temperature: 40 °C
LC System: SCIEX® ExionLC™
Detection: MRM
Detector: SCIEX 7500

MRM Conditions

Polarity: Positive
Source Temperature: 400 °C
GS1: 45 psi
GS2: 80 psi
CUR: 40 psi
CAD: 12 psi
ISV: 3000 V

Table 1. MRM Parameters.

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-Phenylalanin-D_5	171.1	125.1	10	20	12	25
Uracil-D_4	115	98	8	25	12	40

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

Results and Discussion

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 Synergi 2.5 µm Polar-RP column using water modified with Formic acid and Ammonium Formate, and Acetonitrile as mobile phases.

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.

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 to 2.4 ng/mL and 0.030 to 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, Signal-to-Noise (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.

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 %). Accuracy and precision data are shown in **Table 5**.

Figure 1. Representative Extracted Ion Chromatogram (XIC) of Anatoxin-a, Microcystin-RR, and Nodularin-R at the LOQ Level with Ion Ratio Tolerance Lines Overlaying the Quantifier and Qualifier Ions.

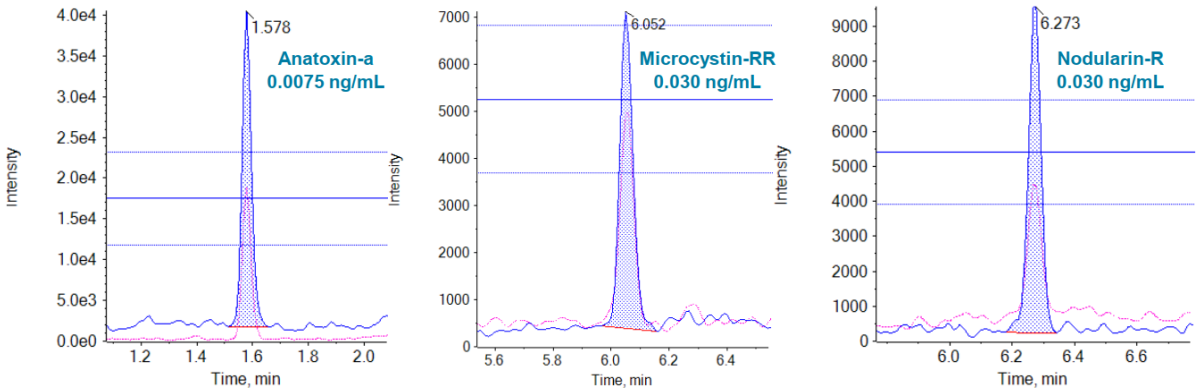

Figure 2. XICs of the 1.2 ng/mL Standard for Anatoxin-a, Cylindrospermopsin, Nodularin-R, and 6 Microcystins.

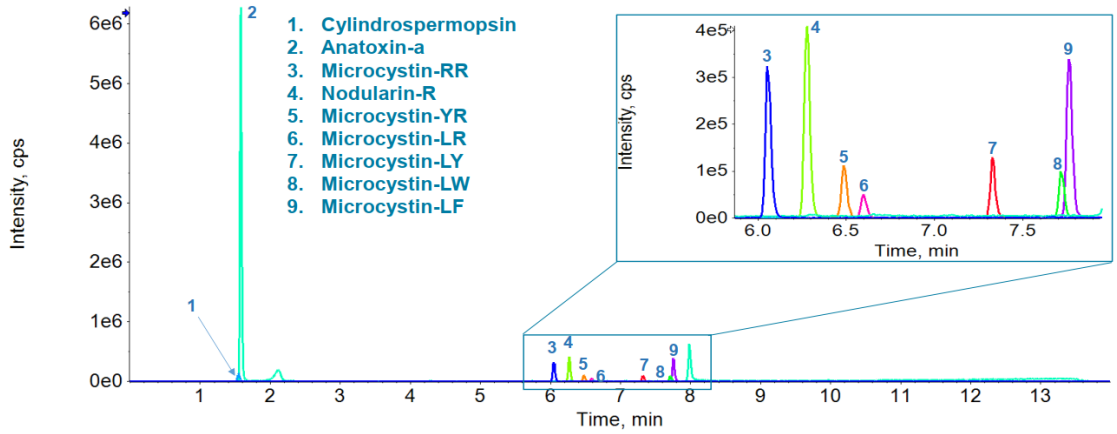

Table 4. Correlation Coefficient (r value) and Accuracy Ranges for Calibration Curves and Average Accuracy and %CV for the Quantifier Ion at the LOQ.

Compound	Linear Range (ng/mL)[1]	LOQ[2]	Correlation Coefficient (r)	Accuracy Range of 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

Notes:
[1]The calibration curve and LOQ samples were prepared in Acetonitrile / Water (1:1, v/v).
[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 3. Representative Calibration Curves from the Quantifier Ions for Anatoxin-a and Microcystin-RR.

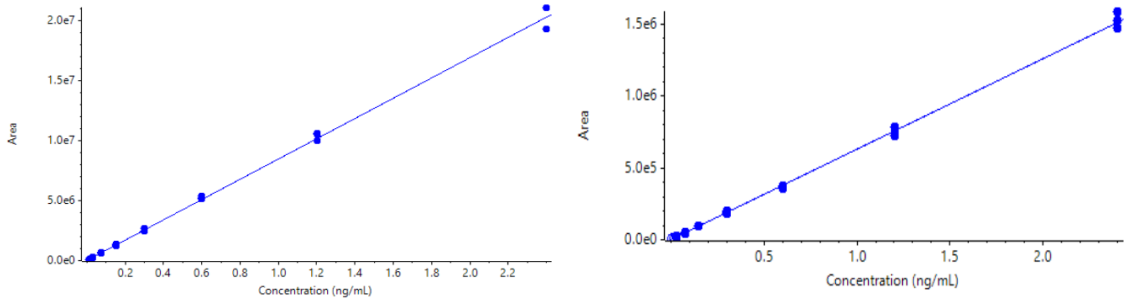

Table 5. Average Accuracy and %CV (n=6) for Anatoxin-a, Cylindrospermopsin, Microcystins, and Nodularin-R for RO Lab Water and Drinking Water Samples.

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

Note: All samples were prepared in triplicate and analyzed in duplicate.

Conclusion

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 to 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.

Synergi™ Ordering Information

2.5 µm High Speed Technology (HST) Columns (mm)						
Phases	30 x 2.0	50 x 2.0	100 x 2.0	50 x 3.0	100 x 3.0	50 x 4.6
Max-RP	00A-4372-B0	00B-4372-B0	00D-4372-B0	00B-4372-Y0	00D-4372-Y0	00B-4372-E0
Hydro-RP	00A-4387-B0	00B-4387-B0	00D-4387-B0	00B-4387-Y0	00D-4387-Y0	00B-4387-E0
Polar-RP	00A-4371-B0	00B-4371-B0	00D-4371-B0	00B-4371-Y0	00D-4371-Y0	00B-4371-E0
Fusion-RP	00A-4423-B0	00B-4423-B0	00D-4423-B0	00B-4423-Y0	00D-4423-Y0	00B-4423-E0

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