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Application News

LCMS-8060NX High Performance Liquid Chromatograph Mass Spectrometer

Highly Sensitive Quantitation of Cylindrospermopsin and Anatoxin-a in Water using the Triple Quad LCMS-8060NX in Accordance with EPA Method 545

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User Benefits

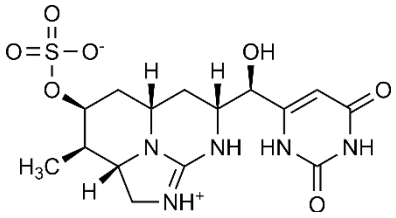
- Shimadzu LCMS-8060NX triple quadrupole mass spectrometer can easily achieve the quantitation requirements of EPA Method 545 on cylindrospermopsin and anatoxin-a analysis
- This method provides lower LOQ and wider dynamic range than those reported in EPA Method 545 so that samples with broader range of concentrations can be analyzed in a single batch without the need of additional sample prep (concentration or dilution).
- Run time of 8 minutes while ensuring good chromatographic separation and peak shape.

■ Background

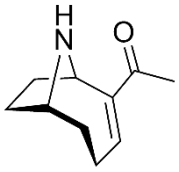
Cyanobacteria, commonly known as blue-green algae, and harmful algae blooms are proliferating in water bodies more frequently because of warmer temperatures and nutrient-rich environments across the world. They possess the potential to significantly impact water quality because they can produce cyanotoxins, such as cylindrospermopsin and anatoxin-a. Exposure to cyanotoxins can lead to a range of adverse health effects in human and animals, varying from mild skin rashes to severe illness.

Cylindrospermopsin can cause damage to the liver, kidneys, and other organs. It is known for its cytotoxic effects.[1] According to the Drinking Water Health Advisory published by US EPA, cylindrospermopsin in drinking water should be less than 0.7 µg/L for children younger than six years old, and less than 3.0 µg/L for school-age children and adults.[2] Anatoxin-a, also known as Very Fast Death Factor (VFDF), is a secondary, bicyclic amine alkaloid and cyanotoxin with acute neurotoxicity. Exposure to anatoxin-a may result in symptoms such as loss of coordination, muscle twitching, convulsions, and swift death, often caused by respiratory paralysis.[3]

Due to the serious threats these cyanotoxins pose to both recreational and drinking waters, various countries, including the United States, actively engage in monitoring and regulatory efforts. These initiatives aim to detect and address the presence of cyanotoxins, ensuring the safety of water sources and protecting public health. Monitoring practices and regulations are crucial components of water management strategies to address the potential hazards associated with harmful algal blooms and their toxins.



Cylindrospermopsin



Anatoxin-a

■ Method

Cylindrospermopsin and anatoxin-a were purchased from Enzo Life Science. Internal standards uracil-d4 and L-phenylalanine-d5 were obtained from Toronto Research Chemicals. LC-MS grade solvents (acetic acid, methanol, and water) were sourced from Honeywell.

Standard preparation: Stock standards of anatoxin-a and cylindrospermopsin were prepared by adding 1 mL of methanol/water (1:1) directly to manufacturers' vials. A standard of 1 mg/mL anatoxin-a fumarate and a 100 µg/mL of cylindrospermopsin stock standards were obtained. The initial standard stock solutions were further diluted to 1 µg/mL by methanol/water (1:1). A series of calibration standards were prepared from 1 µg/mL stock solution using LC-MS grade water containing the sample preservatives (1.0 g/L sodium bisulfate and 0.10 g/L ascorbic acid) as diluent to obtain the final concentrations of 0.02-20 ng/mL for anatoxin-a and 0.005-10 ng/mL for cylindrospermopsin.

Uracil-*d*4 and L-phenylalanine-*d*5 internal standard stock solutions were prepared by weighing 1 mg of the solid material into a HPLC autosampler vial and diluting to volume with hot (50 ℃) LC-MS grade water (uracil-*d*4) and methanol/water (1:1) (L-phenylalanine-*d*5). Calibration standards and samples were spiked with internal standards at final concentrations of 0.1 ng/mL for L-phenylalanine-*d*5 and 1.0 ng/mL for uracil-*d*4.

Sample preparation: The preparation of the samples followed the procedures outlined in EPA Method 545.[4] Five distinct water samples, including LC-MS grade water, tap water 1, filtered tap water 2, one sample from local stream and one sample from a pond (Columbia, MD), were collected and treated to showcase the suitability of the method. The samples were spiked with 1.0 g/L sodium bisulfate and

0.10 g/L ascorbic acid, and were filtered through 0.2 µm pore size PVDF membrane.

Table 1: Gradient time program of mobile phases

Time (min)	%A	%B
0	98	2
1.0	80	20
3.5	60	40
3.51	40	60
4.0	40	60
4.01	98	2
8	98	2

Mobile phase A: 0.2% acetic acid in water
Mobile phase B: methanol

Instrumentation parameters: A Shimadzu LCMS-8060NX triple quadrupole mass spectrometer was used to provide the highly sensitive and robust analysis. The chromatographic separation of the analytes and internal standards was achieved in only 8 minutes using a Shim-pack GIST C18 column (100 x 2.1 mm, 2.0 µm, PN: 227-30001-04). The flow rate of the mobile phase was 0.3 mL/min, with an injection volume of 20 µL. The column oven temperature was maintained at 40 ℃. Details of the gradient conditions are shown in **Table 1**.

The LCMS-8060NX was utilized in positive ion mode with electrospray ionization, operating in multiple reaction monitoring (MRM) mode. Quantitation and confirmation of the targeted analytes were performed through the monitoring of two selective MRM transitions. **Tables 2 and 3** provide details on the parameters used, including both source-specific and compound-specific information. A divert valve was used and sent the eluent from the first minute to waste to avoid mass spectrometer contamination from the preservatives.

Data analysis: Data was acquired using LabSolutions software, analyzed using LabSolutions Insight™ LCMS and reviewed by exception with customizable QA flags. Insight features fast data processing and data review, allowing scientists to analyze data efficiently.

Table 2: MS conditions

Interface	: ESI
Mode	: MRM
Polarity	: Positive
Interface Voltage	: 1 kV
Focus Voltage	: 2 kV
Nebulizing Gas Flow	: 3.0 L/min
Heating Gas Flow	: 10.0 L/min
Interface Temperature	: 300 ℃
DL Temperature	: 250 ℃
Heat Block Temperature	: 400 ℃
Drying Gas Flow	: 10.0 L/min

Table 3: Detailed MRM settings and assigned internal standards for the analysis of cylindrospermopsin and anatoxin-a

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Dwell Time (msec)	Q1 (V)	Collision Energy (V)	Q3 (V)	Internal standards
Cylindrospermopsin (Quantifier)	416.10	194.25	38	-16.0	-35.0	-21.0	Uracil-*d*4
Cylindrospermopsin (Qualifier)	416.10	336.25	38	-16.0	-23.0	-12.0	Uracil-*d*4
Anatoxin-a (Quantifier)	166.00	131.20	38	-17.0	-20.0	-27.0	L-phenylalanine-*d*5
Anatoxin-a (Qualifier)	166.00	43.00	38	-17.0	-24.0	-17.0	L-phenylalanine-*d*5
L-phenylalanine-*d*5	171.10	125.30	59	-18.0	-14.0	-25.0	N/A
Uracil-*d*4	115.10	98.05	59	-21.0	-20.0	-19.0	N/A

■ Results and Discussion

Chromatographic separation: The utilization of the Shim-pack GIST C18 column, along with gradient conditions, led to effective retention of analytes with baseline separation of the target analytes. Full separation of cylindrospermopsin, anatoxin-a and the two internal standards was achieved under an 8-minute gradient condition, as shown in **Figure 1**.

Calibration and LOQ: A linear calibration curve for cylindrospermopsin was achieved in the concentration range of 0.005 – 10 ng/mL, while the calibration range for anatoxin-a was 0.02 – 20 ng/mL. **Figure 2** shows the linearity of the calibration curve. Excellent linearity with R^2 greater than 0.99 for both compounds was achieved over the wide calibration range. The calibration curve was generated by analyzing triplicate injections of each standard concentration, with the accuracy of all injections falling within the range of 80% to 120%. For both cyanotoxins, %RSD of concentration for all calibrator replicates was less than 10%, which indicates the excellent robustness and reproducibility of the system. No carryover was observed in the blank injections after the highest calibrators.

Limits of quantitation (LOQs) of the method were 0.005 ng/mL for cylindrospermopsin and 0.02 ng/mL for anatoxin-a. LOQ was determined based on accuracy, reproducibility and S/N ≥ 10. The accuracy of the data points at LOQ was within 80–120% with the %RSD < 7%, meeting the EPA Method 545 requirements. Representative chromatograms of the quantifier ions in LOQ injections were shown in **Figure 3**.

Spiked water sample analysis: To showcase the applicability of the method, five water samples (LC-MS grade water, tap water 1, filtered tap water 2, stream water and pond water (Columbia, MD)) were prepared and processed to analyze the two cyanotoxins. No detectable peaks were observed in the unspiked samples, suggesting the absence of cyanotoxins in the original water samples. The accuracy and precision was evaluated by spiking two levels of cylindrospermopsin (0.05 and 0.25 µg/L) and anatoxin-a (0.2 and 1 µg/L) into the water samples. Accuracies within a 25% range of the anticipated values, along with %RSD values below 10%, were noted for both cylindrospermopsin and anatoxin-a at the two spiking levels for all water samples (**Table 4**). Results were within the acceptable limits listed in EPA 545.

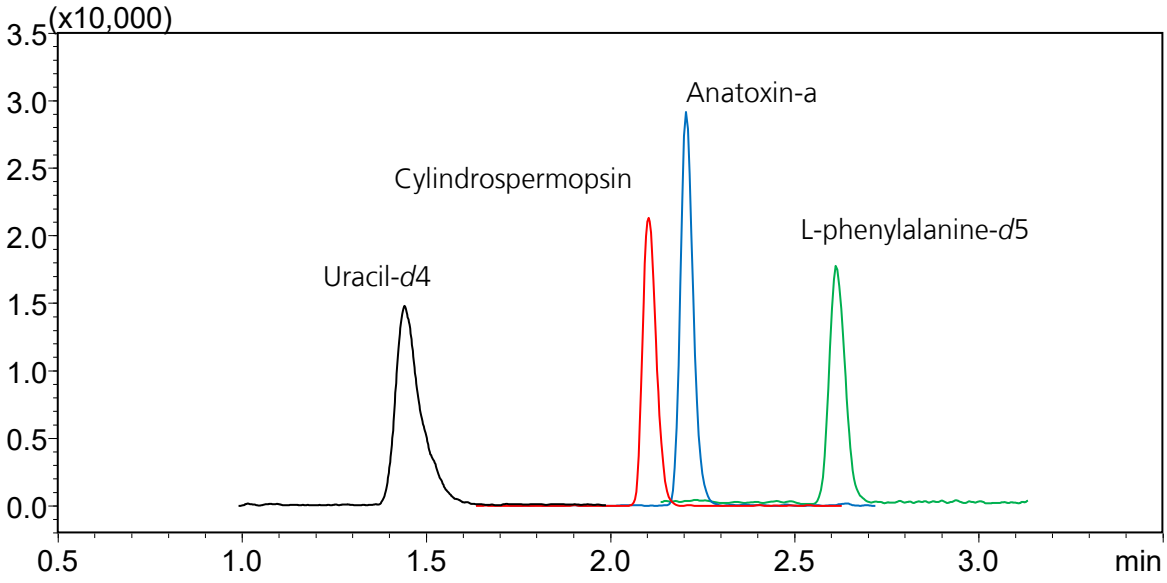


Figure 1: MRM quantifier ion chromatograms of cylindrospermopsin (0.1 ng/mL), anatoxin-a (0.4 ng/mL), L-phenylalanine-*d*5 (0.10 ng/mL) and uracil-*d*4 (1.0 ng/mL). The Shim-pack GIST C18 column successfully retained compounds while demonstrating effective baseline chromatographic separation.

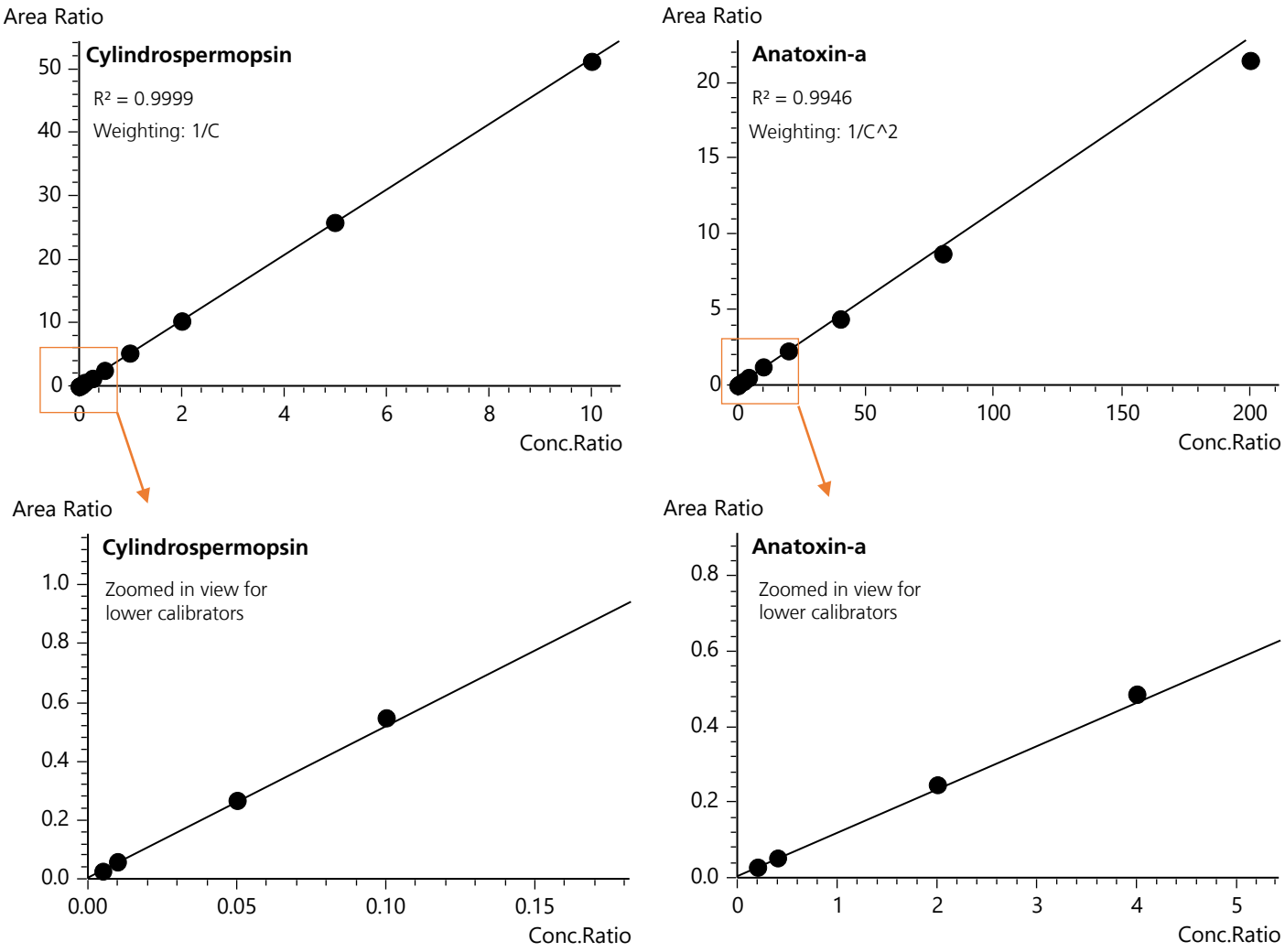


Figure 2: Calibration curves for cylindrospermopsin (0.005 – 10 ng/mL) and anatoxin-a (0.02 – 20 ng/mL), and the zoomed views for lower calibrators.

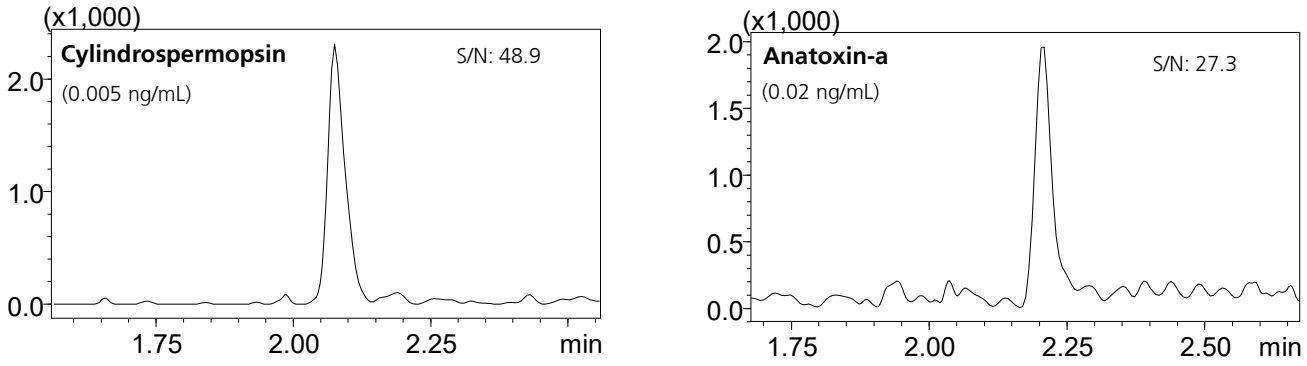


Figure 3: Representative chromatograms of the quantifier ions in LOQ injections.

•**Table 4.** Precision and accuracy for the analysis of cylindrospermopsin and anatoxin-a in water samples

Replicate no.	Cylindrospermopsin (0.05 µg/L)		Anatoxin-a (0.2 µg/L)		Cylindrospermopsin (0.25 µg/L)		Anatoxin-a (1.0 µg/L)	
	Conc. (µg/L)	Accuracy	Conc. (µg/L)	Accuracy	Conc. (µg/L)	Accuracy	Conc. (µg/L)	Accuracy
LC-MS Water								
1	0.0496	99.2%	0.1909	95.4%	0.2505	100.2%	0.9720	97.2%
2	0.0502	100.3%	0.1845	92.3%	0.2495	99.8%	0.9615	96.2%
3	0.0500	99.9%	0.1913	95.7%	0.2477	99.1%	0.9601	96.0%
RSD% (Conc)	0.58		2.02		0.57		0.68	
Tap Water 1								
1	0.0418	83.7%	0.2478	123.9%	0.2023	80.9%	1.2180	121.8%
2	0.0405	81.1%	0.2449	122.4%	0.2018	80.7%	1.2353	123.5%
3	0.0400	80.0%	0.2435	121.8%	0.1999	80.0%	1.2393	123.9%
RSD% (Conc)	2.31		0.88		0.62		0.92	
Filtered Tap Water 2								
1	0.0412	82.3%	0.2349	117.5%	0.2056	82.2%	1.2197	122.0%
2	0.0433	86.7%	0.2310	115.5%	0.2122	84.9%	1.233	123.3%
3	0.0406	81.2%	0.2357	117.9%	0.2100	84.0%	1.2161	121.6%
RSD% (Conc)	3.47		1.09		1.61		0.73	
Stream Water								
1	0.0461	92.1%	0.2405	120.3%	0.2210	88.4%	1.1899	119.0%
2	0.0432	86.5%	0.2411	120.6%	0.2226	89.1%	1.1842	118.4%
3	0.0422	84.5%	0.2259	113.0%	0.2255	90.2%	1.1191	111.9%
RSD% (Conc)	4.54		3.65		1.03		3.38	
Pond Water								
1	0.0486	97.1%	0.2369	118.4%	0.2524	101.0%	1.1334	113.3%
2	0.0491	98.3%	0.2356	117.8%	0.2531	101.2%	1.1557	115.6%
3	0.0551	110.3%	0.2373	118.6%	0.2589	103.6%	1.1261	112.6%
RSD% (Conc)	7.15		0.36		1.41		1.36	

■ Conclusion

In this application, a rapid LCMS method was successfully developed for the analysis of cylindrospermopsin and anatoxin-a in water. Full separation of the two cyanotoxins and internal standards was achieved in only 8 minutes using the Shim-pack GIST C18 column. A linear relationship was obtained in a wide calibration range for cylindrospermopsin (0.005 – 10 ng/mL) and anatoxin-a (0.02 – 20 ng/mL) with R^2 over 0.99.

The applicability of the method was verified by spiking anatoxin-a and cylindrospermopsin into five different water samples at two spiking levels, and the results of accuracy were all within 80 - 125% with %RSD less than 10%. This application demonstrates the sensitivity and reproducibility of Shimadzu LCMS-8060NX in the analysis of cylindrospermopsin and anatoxin-a in water within the QA limits outlined in EPA Method 545.

■ References

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