APPLICATION NOTE

Liquid Chromatography/
Mass Spectrometry

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Quantitation of Microcystins and Nodularin in Drinking Water Samples to Meet EPA Method 544 Requirements Using QSight LC/MS/MS

Introduction

Cyanobacteria, also known as blue-green algae, can produce cyanotoxins known as microcystins and nodularins. Cyanobacteria are found in surface waters such as lakes, streams, and ponds and when conditions are favorable, cyanobacteria can bloom and release cyanotoxins into the water. Cyanotoxins often exist intracellularly in cyanobacteria and are released into the water when cells die. Blooms caused by cyanobacteria can be harmful to the human health, animals, and the environment. Microcystin-LR (MC-LR) is one of the most common cyanotoxins and ingestion will cause negative human health effects such as liver inflammation and hemorrhage, vomiting and diarrhea, and abdominal pain.[1] There are both non-toxic and toxic versions of cyanobacteria and it is impossible to tell if a species of cyanobacteria is toxic or non-toxic by its appearance.[1] The US EPA has a health advisory for a maximum level of microcystins as 0.3 µg/L for infants and young children and 1.6 µg/L for school-aged children and adults.[3] The World Health Organization recommends microcystin levels be kept below 1 µg/L in drinking water.[4]

Molecular tests are available to determine if cyanobacteria carry the toxin gene but quantitative analysis, such as liquid chromatography tandem mass spectrometry (LC/MS/MS), is required to determine if cyanobacteria are producing cyanotoxins.[1] EPA Method 544 uses solid phase extraction (SPE) LC/MS/MS for quantitation of six intracellular and extracellular microcystins and nodularin in 500 mL drinking water samples.[2] In this study, we show that the PerkinElmer QSight® LX50 ultra high-performance liquid chromatography (UHPLC) system coupled with the PerkinElmer QSight® 200 series triple quadrupole mass spectrometer can analyze the six microcystins and nodularin (microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LY, microcystin-RR, microcystin-YR, nodularin-R) as outlined in EPA method 544 with good detection limits from 16 ng/L to 80 ng/L.

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Experimental

Hardware/Software

A PerkinElmer QSight LX50 UHPLC system was used for chromatographic separation coupled with a PerkinElmer QSight 200 series triple quadrupole mass spectrometer for analytes detection. Simplicity 3Q™ software was used to perform all instrument control, data acquisition, and analysis.

Method

The LC method and MS source parameters are shown in Table 1. The LC gradient was modified from the recommended conditions in EPA Method 544 to reduce the overall run time.

The MS source parameters, which includes HSID temperatures, source temperatures, drying gas, and nebulizer gas were optimized for maximum sensitivity as shown in Table 1. The solution for each analyte was infused on the QSight MS using a 2-ppm solution to determine optimal collision energies (CE), entrance voltages (EV), and collision cell lens voltage (CCL2) as shown in Table 2.

Table 1: LC Method and MS Source Parameters.

LC Conditions						
Column:	PerkinElmer Brownlee SPP C18, 2.7 µm, 2.1 x 100 mm (Part # N9308404)					
Mobile Phase/ LC Gradient:	Mobile Phase A: 0.1% FA in water Mobile Phase B: 0.1% FA in acetonitrile					
		Time (min)	**Flow (mL/min)**	**%A**	**%B**	**Curve**
	1	Initial	0.6	95	5	
	2	4.0	0.6	95	5	Linear
	3	9.0	0.6	5	95	Linear
	4	10.0	0.6	5	95	Linear
	5	10.1	0.6	95	5	Linear
	6	13.0	0.6	95	5	Linear
Column Oven Temp.:	50 °C					
Injection Volume:	10 µL					
MS Source Conditions						
Ionization Mode:	ESI - positive					
Drying Gas (Nitrogen) Setting:	120					
HSID Temp.:	250 °C					
Electrospray Voltage:	5100 V					
Source Temp.:	315 °C					
Nebulizer Gas (Nitrogen) Setting:	350					
Detection Mode:	MRM Mode					

Table 2: Optimized MS/MS Parameters for cyanotoxins standards and surrogate.

Analyte	Acronym	Precursor Ion	Product Ion	CE	EV	CCL2	Quantifier/Qualifier
Microcystin-LR	MC-LR	498.0 $[M+2H]^{2+}$	135.0	-24	30	-65	Quant
Microcystin-LR	MC-LR	498.0 $[M+2H]^{2+}$	482.3	-12	15	-80	Qual
Microcystin-LA	MC-LA	910.4 $[M+H]^{+}$	135.0	-88	55	-180	Quant
Microcystin-LA	MC-LA	910.4 $[M+H]^{+}$	106.9	-114	30	-185	Qual
Microcystin-YR	MC-YR	523.4 $[M+2H]^{2+}$	135.0	-22	30	-85	Quant
Microcystin-YR	MC-YR	523.4 $[M+2H]^{2+}$	91.1	-116	30	-145	Qual
Microcystin-LF	MC-LF	493.9 $[M+2H]^{2+}$	135.0	-50	15	-90	Quant
Microcystin-LF	MC-LF	493.9 $[M+2H]^{2+}$	102.9	-94	30	-100	Qual
Microcystin-RR	MC-RR	519.9 $[M+2H]^{2+}$	135.0	-38	5	-130	Quant
Microcystin-RR	MC-RR	519.9 $[M+2H]^{2+}$	103.0	-90	15	-140	Qual
Microcystin-LY	MC-LY	1002.4 $[M+H]^{+}$	135.0	-82	20	-175	Quant
Microcystin-LY	MC-LY	1002.4 $[M+H]^{+}$	374.9	-46	20	-185	Qual
Nodularin-R	NOD	825.4 $[M+H]^{+}$	135.0	-74	10	-205	Quant
Nodularin-R	NOD	825.4 $[M+H]^{+}$	103.0	-138	15	-195	Qual
Ethylated MC-RR	C2D5-MC-RR	515.0 $[M+2H]^{2+}$	103.1	-95	12	-110	IS

Calibration Standards Preparation

The analyte primary dilution standard solution (PDS) was prepared in methanol from analyte stock standard solutions according to section 7.2.2.2 in EPA Method 544. Seven calibration standards were prepared in methanol containing 10% water from dilutions of the analyte PDS according to EPA method 544 section 7.2.3. The surrogate primary dilution standard (SUR PDS) was prepared by diluting the stock to 6.49 ng/µL in methanol per section 7.2.1.1 of EPA Method 544. The SUR PDS was added to the calibration curve to yield a concentration of 129.8 µg/L per section 7.2.3 of EPA Method 544. Analyte concentration for the calibration curve ranged from 3.125 – 200 µg/L, except for MC-LY and MC-LA which ranged from 6.25 - 400 µg/L. An example of a calibration curve for one of microcystins (MC-RR) is shown in Figure 1.

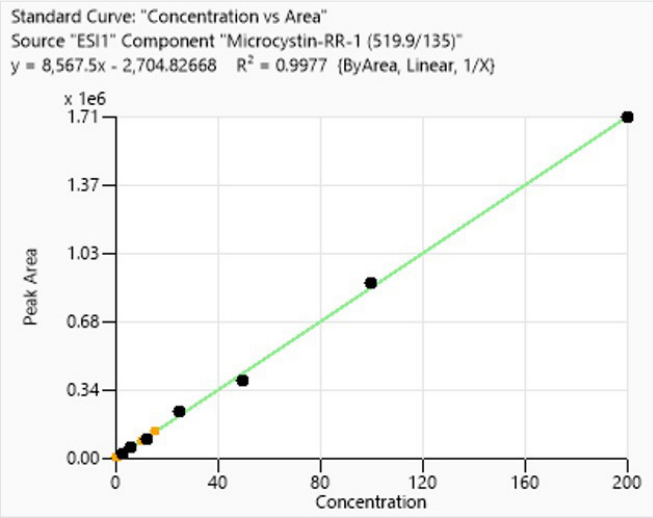

Figure 1: Microcystin-RR calibration curve (in neat solution) ranging from 3.125 µg/L to 200 µg/L

Sample Preparation

All field samples, laboratory fortified blanks (LFB), laboratory reagent blanks (LRB), and continuing calibration checks (CCC) were collected in 500 ml amber glass bottles containing 7.75 g/L of Trizma, 2 g/L of 2-Chloroacetamide, 100 mg/L of ascorbic acid, and 0.35 g/L of ethylenediaminetetraacetic acid trisodium salt per section 8.1.2 of EPA Method 544. The field samples were obtained by opening the cold-water tap, allowing the system to flush for 3-5 minutes, then collecting the 500 mL sample. LRBs, LFBs, and CCCs were prepared by transferring reagent water to 500 mL amber glass bottles that contained the preservatives in section 8.1.2 of EPA Method 544. Samples were held at 10°C until ready for analysis. A constant amount of SUR PDS was added to all field samples, LFBs, and CCCs prior to filtration and SPE extraction.

Intracellular toxins need to be released from the cyanobacteria through filtration and a freezing cycle, according to section 11.3 of EPA Method 544. A manual SPE vacuum manifold system was used for all extractions. Solid phase extractions were performed in accordance with section 11.4 of EPA Method 544 using PerkinElmer Supra-Poly HLB SPE columns (PN N9306662). The SPE cartridges were conditioned with 15 mL of methanol, followed by rinsing with 15 mL of reagent water. 4 to 5 mL of reagent water was added to each cartridge and the sample transfer tubes were attached. The vacuum was turned on and the filtered sample was added to the cartridge. The vacuum was adjusted so the flow rate was 10 - 15 mL/min. Once all the sample was extracted, the sample bottles were rinsed with 10 mL of reagent water and the rinse was drawn through the sample transfer tube. The bottles were then rinsed with 5 mL of reagent water and rinse was drawn through the sample transfer tube. Air was drawn through the cartridge for 10 minutes. Using 5 mL of 10% reagent water in methanol, the sample bottles were rinsed, and the analytes were eluted from the cartridge by pulling the 5 mL of 10% reagent water in methanol though the sample transfer tubes. The 5 mL sample bottle rinse and elution using 10% reagent water in methanol was repeated. The 10 mL extracts were concentrated to dryness using a gentle stream of nitrogen in a heated water bath of 60°C. 1 mL of 10% reagent water in methanol was added to the collection vial and vortexed. The 1 mL aliquot was transferred to an autosampler vial for LC/MS/MS analysis.

Results and Discussion

LC and MS/MS Methods

The LC gradient was optimized to ensure separation of the analytes, minimize run time, and optimize peak shape. Mobile phase compositions used were 0.1% formic acid in water for mobile phase A (MPA) and 0.1% formic acid in acetonitrile for mobile phase B (MPB). Optimizing the mobile phase composition allowed for the reduction of the run time from 26 minutes as outlined in EPA Method 544 to 13 minutes in the method presented here. Figure 2 shows the extracted ion chromatogram (EIC) for 6 microcystins and nodularin at a concentration level of 100 µg/L.

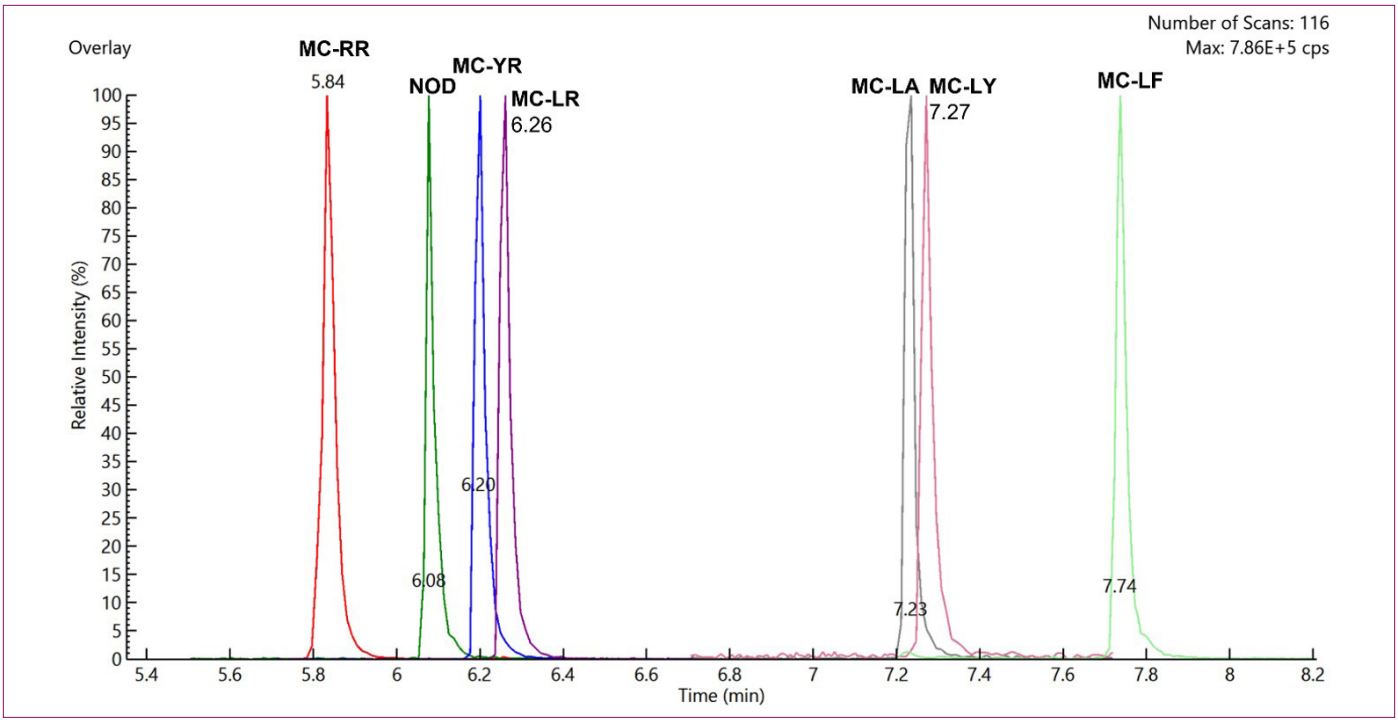

Figure 2: Extracted ion chromatogram of 100 µg/L sample in neat solution.

The QSight MS/MS MRM parameters were optimized for each analyte and surrogate internal standard by direct infusion using positive ion electrospray ionization. Precursor and product masses were selected and then the entrance voltage (EV), collision cell energy (CE), and collision cell lens 2 voltage (CCL2) were optimized for each analyte using the autotune feature in Simplicity 3Q. A timed MRM method containing the quantifier and qualifier ion for each analyte was built using the retention time and acquisition window of each analyte.

Determination of Method Minimum Reporting Level

The minimum reporting level (MRL) was determined according to EPA Method 544 section 9.2.4. Seven laboratory fortified blanks were fortified, extracted, and analyzed using LC/MS/MS. Table 3 shows the established MRL for each analyte.

Table 3: Minimum reporting levels determined for each analyte.

Analyte	Fortified Concentration (ng/L)
MC-RR	24
Nodularin-R	20
MC-YR	16
MC-LR	16
MC-LA	80
MC-LY	80
MC-LF	60

According to section 9.2.4.2 of EPA Method 544, the upper prediction interval of results (PIR) for the results of the MRL experiment must be ≤150% recovery, while the lower PIR must be ≥ 50% recovery. The upper PIR and lower PIR recoveries are shown in Table 4, and all those numbers are within required ranges.

Table 4: Upper PIR and lower PIR results for each analyte after analyzing seven LFBs.

Analyte	Upper PIR	Lower PIR
MC-RR	115	52
Nodularin-R	109	64
MC-YR	137	52
MC-LR	109	67
MC-LA	144	77
MC-LY	148	60
MC-LF	122	92

Determination of Accuracy and Precision

The accuracy and precision were determined according to section 9.2.2 of EPA Method 544. Four laboratory fortified blanks were fortified near the midrange of the calibration curve, extracted, and analyzed using LC/MS/MS. For determination of precision, the %RSD of the results of the four replicates must be less than 30%. For determination of accuracy, the average recovery of replicate values must be within +/- 30% of the true value. Table 5 shows the %RSD and % recovery for each replicate.

Table 5: Fortified concentration of four LFBs fortified near midrange of the calibration curve and %RSD and average recovery for each analyte.

Analyte	Fortified Concentration (ng/L)	Mean Result (ng/L)	%RSD	% Recovery
MC-RR	300	275	2	92
Nodularin-R	300	243	5	81
MC-YR	300	296	5	99
MC-LR	300	272	10	91
MC-LA	300	375	11	125
MC-LY	300	327	14	109
MC-LF	300	283	9	94

Field Sample Analysis

Field samples of tap water were collected from Meriden, CT and Shelton, CT. Both field samples were spiked with an aliquot of the SUR PDS. A laboratory reagent blank (LRB), two continuing calibration checks (CCC), and laboratory fortified blank (LFB) were prepared according to section 9.3 of EPA Method 544. The LRB is to confirm that background contaminants are not interfering with quantitation of the method analytes. The low CCC was fortified at the MRL and midrange CCC was fortified at the midrange of the calibration curve according to section 10.2.7 of EPA Method 544. The LFB was fortified at the low end, 2 times above the MRL.

The results for LRB confirmed that there were no background contaminants from the sample preparation. The LFB's recovery was within the method requirements of 50-150%. The low CCC's recovery was within the method requirement of +/- 50% of the true value and the midrange CCC was within the method requirement of +/-30% of the true value, shown in Table 6.

Table 6: Percent recoveries of the laboratory fortified blank fortified two times above the MRL, the low continuing calibration check, and the midrange continuing calibration check.

Analyte	LFB, Fortified Concentration (ng/L)	LFB Results (ng/L)	% Recovery LFB	Low CCC, Fortified Concentration (ng/L)	CCC Results (ng/L)	% Recovery Low CCC	Midrange CCC, Fortified Concentration (ng/L)	CCC Results (ng/L)	% Recovery Mid CCC
MC-RR	48.0	30.5	64	24.0	18.9	79	80.0	58.4	73
Nodularin-R	40.0	32.4	81	20.0	25.2	126	80.0	82.9	104
MC-YR	32.0	23.6	74	16.0	10.9	68	80.0	79.3	99
MC-LR	32.0	31.0	97	16.0	9.5	59	80.0	88.6	111
MC-LA	160.0	135.0	84	80.0	68.8	86	160.0	154.6	97
MC-LY	160.0	196.3	123	80.0	102.0	127	160.0	142.2	89
MC-LF	120.0	117.8	98	60.0	48.9	81	80.0	85.6	107

The two field sample results were all reported as below the MRL.

Conclusion

This application note outlines an LC/MS/MS method for the analysis of microcystins and nodularin in drinking water samples as outlined in EPA Method 544 using a PerkinElmer QSight LX50 ultra-high performance liquid chromatography (UHPLC) system along with a PerkinElmer QSight 200 series triple quadrupole mass spectrometer. Our LC method allows for a 13-minute reduction in runtime compared to the suggested LC method in EPA Method 544. The determined MRLs, ranging from 16-80 ng/L, for all analytes are well below the US EPA guidelines and WHO guidelines for the maximum levels of microcystins in drinking water. The method showed good accuracy with all recoveries within 70-130% and good precision with all RSD below 30%. The field sample analysis showed the two field drinking water samples analyzed were below the MRL for this method.

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