Quantitation of Microcystins and Nodularins in Water Samples Using LC-MS/MS

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Overview

This application note describes the quantitation of 8 individual microcystin (MC) isoforms and Nodularin-R using the SCIEX QTRAP® 4500 system with Turbo V™ source in positive mode electrospray ionization (ESI). Chromatography was performed using a Phenomenex Kinetex® C8 column with an 11.0 min gradient run. Excellent sensitivity, accuracy and precision was shown with LOQ values ranging from 5.5 to 43.8 ng/L, varying by compound. The calculated lowest concentration minimal reporting levels (LCMRL) for the standards ranged from 4.8 ng/L for MC-RR to 91.8 ng/L for MC-YR, suggesting that the direct analysis of ambient water samples is possible. However, EPA Method 544 advises a 500-fold concentration factor which equates to LCMRL values of 0.010 to 0.184 ng/L in the water sample.

Introduction

Microcystins (MC) and nodularins (NOD) are toxins produced by cyanobacteria in saline and freshwaters. MC and NOD are released during cell death and are potential drinking water contaminants. Therefore, accurate and sensitive methods for quantifying MC and NOD in water samples are needed.

MC and NOD both share the common amino acid ADDA, but MC are cyclic heptapeptides whereas NOD are cyclic pentapeptides. Over 130 MC and 10 NOD isoforms have been identified primarily based on variations of two L-amino acids in their cyclic peptide structure [1,2].

MC and NOD are primarily liver toxicants and toxicity varies by isoform with the Microcystin-LR (leucine/arginine variant) thought to be the most harmful. Therefore, the quantification of individual isoforms in necessary. MC and NOD contamination from harmful algal blooms is widespread in surface and drinking water, resulting in occasional consumption advisories [3,4]. The US EPA 10-day drinking water health advisory for microcystins is 0.3 µg/L for infants and children up to 6 years old, and 1.6 µg/L for adults [5]. In addition, Health Canada has set a maximum acceptable concentration (MAC) of MC-LR of 1.5 µg/L [6] and the World Health Organization (WHO) MC-LR provisional guideline is 1 µg/L [7]. Drinking water guidelines for NOD do not exist.

Previous analysis techniques for MC and NOD in water include LC-MS, LC-UV and enzyme linked immunosorbent assay (ELISA). However, liquid chromatography tandem mass spectrometry methods are superior analytical techniques due to the high selectivity, high dynamic linear range and ability to quantify many MC and NOD isoforms in a single analysis run.

Experimental

Standards

Neat standards were obtained from Enzo Life Sciences (Farmingdale, NY) and reconstituted in 1 ml of methanol. An intermediate mixed stock was prepared by diluting the standards in methanol to yield 500 ng/ml for MC-RR and Nodularin-R, and 2000 ng/ml for MC-LA, MC-LF, MC-LR, MC-LY, MC-LW, MC-YR, MC-WR. Calibration standards were prepared with 5% acetonitrile in water to match the initial LC conditions. Standards were prepared in glass vials to reduce sorption to plastic surfaces. All standards were kept at -20 °C until analysis.

HPLC System

A SCIEX ExionLC™ AC was used as the LC system. Chromatographic separation was achieved under gradient conditions using a Phenomenex Kinetex® C8 column (2.6 µm particle size, 100 x 2.1 mm) and flow rate of 0.500 mL/min **(Table 1)**. The mobile phases were water (“A”) and acetonitrile (“B”), both

modified with 0.1% formic acid. The column oven was set to 40ºC and injection volume was 20 µL. To reduce sample carryover the autosampler rinse solvent was 60:20:20 isopropyl alcohol: methanol: acetonitrile using a rinse volume of 2 mL and dip time of 8 s.

MS/MS Detection

Analysis was performed on a SCIEX QTRAP® 4500 system with a Turbo V™ source using an electrospray ionization (ESI) probe in positive mode. Compound-specific and ion source parameters were manually optimized (Tables 2 & 3) and two MRMs per compound were monitored except for MC-LY which showed only 1 product ion. The *Scheduled* MRM™ (sMRM) algorithm was used to maximize dwell times and optimize the number of points across the chromatographic peaks. The MRM detection window was set to 45 s and target scan time was 0.25 s.

Data Analysis and Calculations

The standard batch was run 7 times to generate method performance statistics (i.e. accuracy and precision of LOQ standard) as well as to calculate the LCMRL values. Quantification was performed with MultiQuant™ 3.0.2 using 1.0 Gaussian smoothing and 1/x or $1/x^2$ weighted linear regression. The signal/noise ratio was calculated using the peak-to-peak S/N algorithm in PeakView® 2.2 on unsmoothed chromatograms. The LOD was determined as S/N>3. The LOQ was determined using the following criteria: S/N>8, at least 8 points across the peak and accuracy between 80-120%. LOQ and LOD concentrations were calculated using the first MRM transition, per compound, described in Table 3.

Table 1. LC gradient program at a flow rate of 0.5 mL/min, injection volume = 20 µL.

Step	Time (min)	A (%)	B (%)
0	0.0	95	5
1	0.5	95	5
2	6.0	40	60
3	7.0	5	95
4	9.0	5	95
5	9.1	95	5
End	11.0		

Table 2. Ion source parameters.

Parameter	Value
Curtain Gas (CUR)	30 psi
Collision Gas (CAD)	high
IonSpray voltage (IS)	3500 V
Temperature (TEM)	650°C
Nebulizer Gas (GS1)	50 psi
Heater Gas (GS2)	60 psi

The lowest concentration minimum reporting level (LCMRL) was calculated as described by Winslow et al. [8] using Excel 2016. The LCMRL values were calculated using the LOD standard and subsequent three standard levels. Briefly, the measured versus actual concentrations were plotted and linear regression calculated. The 99% prediction intervals and data quality objective bounds (50% and 150% sample recovery) were calculated and plotted on the original graph. The LCMRL was defined as the intersection of the upper and lower prediction interval lines with the data quality objective (DQO) bounds, using the higher calculated concentration.

Results and Discussion

Using the developed gradient program, baseline separation was achieved for all compounds with excellent peak shape **(Figure 1)**. The gradient is 15 min shorter than the program described in EPA Method 544, resulting in considerable time savings but still maintaining baseline separation.

The LOD concentrations varied by compound and ranged from 2.7 to 21.9 ng/L **(Table 4)**. Specifically, MC-LA, MC-RR and Nodularin showed the lowest LOD values, whereas MC-LR and MC-YR showed the highest. The LOQ concentrations also varied by compound (5.5-43.8 ng/L) and showed similar trends as the LOD values. MRM chromatograms for the LOQ standard (43.8 ng/L) of MC-LR, following 1 Gaussian smooth, are shown in **Figure 2**. The reported LOQ concentrations are significantly below the US EPA drinking water advisory level for children of 300 ng/L.

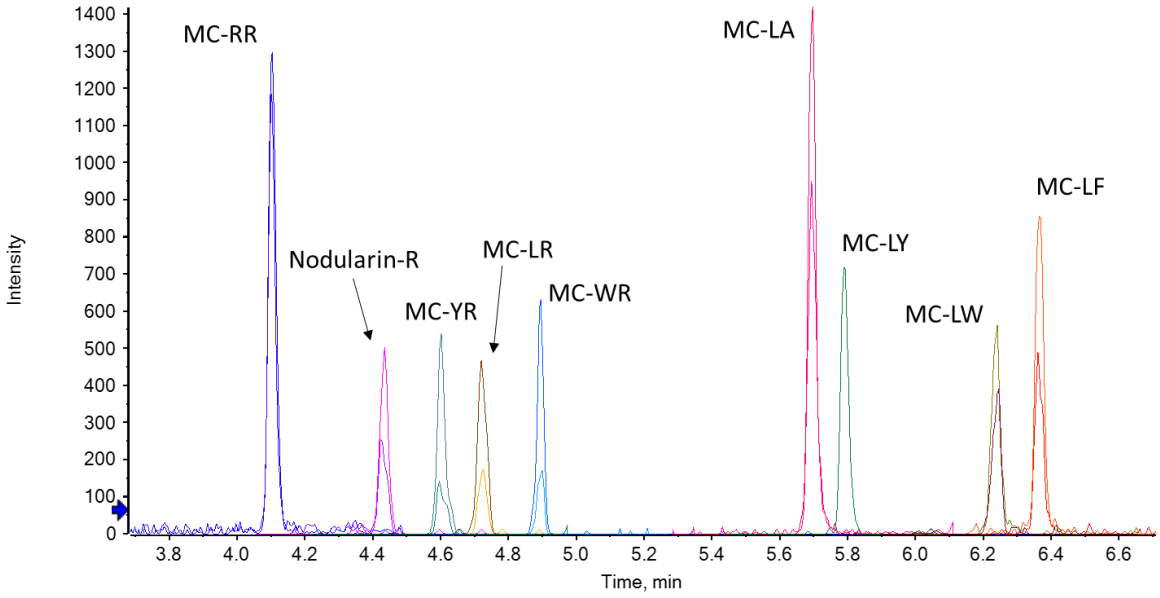

Figure 1. Overlaid chromatogram of 21.9 ng/L standard for MC-RR and Nodularin-R, 87.5 ng/L standard for MC-YR, MC-LR, MC-WR, MC-LA, MC-LY, MC-LW and MC-LF using the SCIEX QTRAP® 4500. 1 Gaussian smooth performed.

Table 3. MRM masses and compound-specific MS parameters for QTRAP® 4500 system.

Compound	Q1	Q3	EP (V)	DP (V)	CE (V)	CXP (V)
MC-LA 1	910.5	776.1	10	70	26	15.0
MC-LA 2	910.5	135.0	10	70	91	8.0
MC-LF 1	986.5	852.5	10	70	30	17.0
MC-LF 2	986.5	134.7	10	70	100	9.0
MC-LR 1	995.6	102.8	10	70	165	6.0
MC-LR 2	995.6	135.2	10	70	139	11.0
MC-LW 1	1025.5	107.1	10	70	146	15.0
MC-LW 2	1025.5	135.2	10	70	106	14.0
MC-LY 1	1002.5	135.3	10	70	119	12.0
MC-RR 1	519.9	135.1	10	70	35	9.3
MC-RR 2	519.9	103.1	10	70	96	9.5
MC-WR 1	1068.6	103.0	10	70	165	9.0
MC-WR 2	1068.6	134.9	10	70	150	11.0
MC-YR 1	1045.5	103.1	10	70	160	6.0
MC-YR 2	1045.5	135.4	10	70	139	10.0
Nodularin-R 1	825.5	103.0	10	70	160	5.8
Nodularin-R 2	825.5	135.3	10	70	110	12.0

Table 4. Method performance parameters (sensitivity, linear range, LOQ accuracy and precision, signal-to-noise). Peak-to-peak S/N was calculated using PeakView® 2.2 with unsmoothed chromatograms.

Analyte	Calibration Range (ng/L)	LOD (ng/L)	LOQ (ng/L)	Linear Correlation (r^2)	Accuracy of LOQ Std. (%)	Precision of LOQ Std. (%)	Peak-to-Peak S/N at LOQ
MC-LA	10.9 – 100,000	5.5	10.9	1.000	113.0	4.6	13.3
MC-LF	21.9 – 100,000	10.9	21.9	0.991	102.0	4.8	10.7
MC-LR	43.8 – 10,000[1]	21.9	43.8	0.998	104.0	18.0	10.9
MC-LW	21.9 – 100,000	10.9	21.9	0.983	99.7	15.2	11.2
MC-LY	21.9 – 100,000	10.9	21.9	0.987	98.9	12.8	18.3
MC-RR	5.47 – 25,000	2.7	5.5	0.996	98.1	8.8	10.0
MC-WR	21.9 – 10,000	10.9	21.9	0.999	105.0	14.1	10.8
MC-YR	87.5 – 10,000[1]	21.9	43.8	0.998	106.0	20.8	10.0
Nodularin-R	10.9 – 25,000	5.5	10.9	0.999	101.0	16.3	10.8

[1] MC-LR and MC-YR have been shown to be linear up to 40,000 ng/L in previous data.

The LOQ standard showed excellent accuracy, with the mean accuracy ranging from 98.1% to 113% (n=7). Further, the precision of the LOQ standard was very good and was generally <20% (n=7). Finally, the LOQ standard signal-to-noise ratio was >10 for all compounds.

The method showed approximately 3 orders of linear dynamic range for all compounds with linearity maintained up to 25,000 ng/L for MC-RR and Nodularin-R, and up to 100,000 ng/L for MC-LA, MC-LF, MC-LW and MC-LY. Previous analysis showed that MC-LR and MC-YR were linear up to 40,000 ng/L.

LCMRL values were calculated using the results of the standards analysis **(Table 5)**. For all compounds, the LCMRL graphs met the required criteria of seven replicate samples at four concentration levels, and at least one standard level below the calculated LCMRL [8]. An example LCMRL graph is shown in **Figure 3** for MC-LR. The LCMRL values – calculated as "in vial" concentrations – ranged from 4.8 ng/L for MC-RR to 91.8 ng/L for MC-YR. However, EPA Method 544 uses solid phase extraction techniques to clean and concentrate the water samples with a suggested concentration factor of 500-fold. Therefore, the LCMRL values – calculated on "sample" basis – range from 0.10 ng/L to 0.184 ng/mL

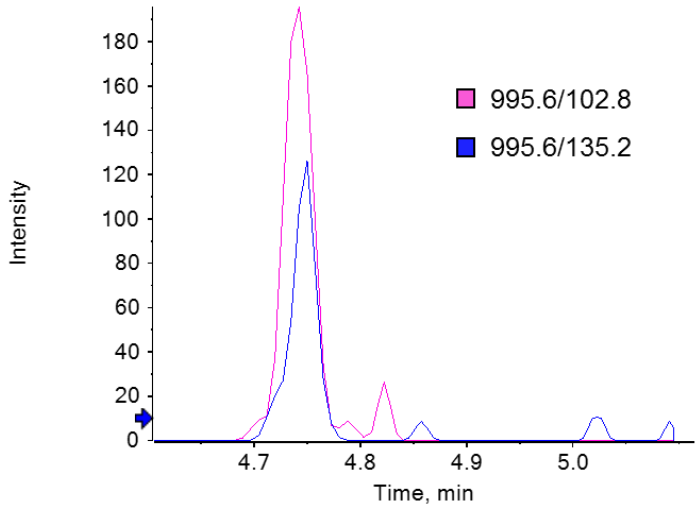

Figure 2. MRM chromatogram for the LOQ standard (43.8 ng/mL) of MC-LR. 1 Gaussian smooth performed.

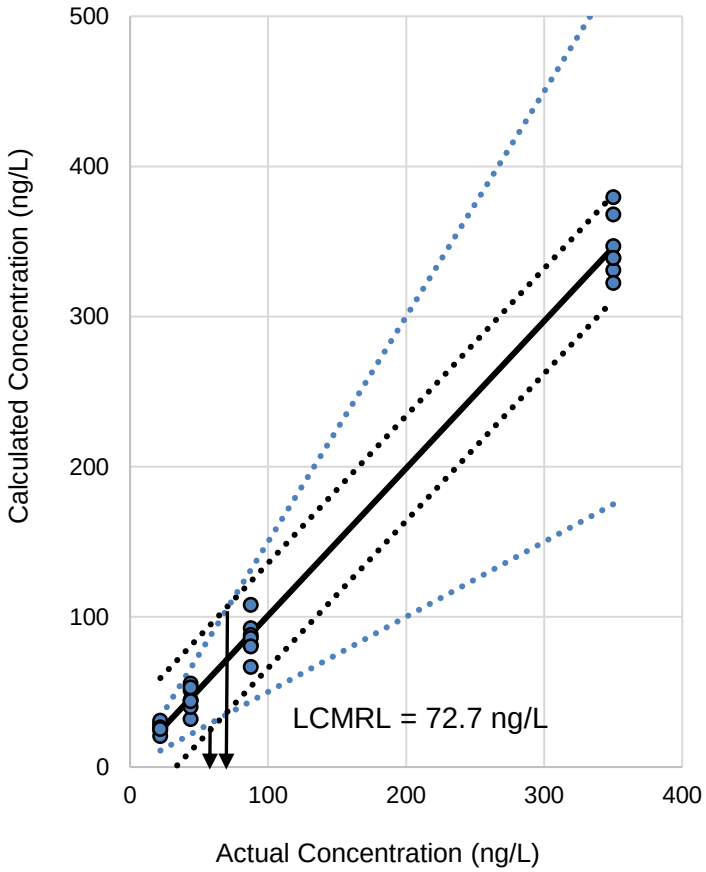

Figure 3. LCMRL graph for MC-LR.

Table 5. Lowest concentration minimum report level (LCMRL) concentrations.

Analyte	LCMRL (ng/L)
MC-LA	13.9
MC-LF	24.7
MC-LR	72.7
MC-LW	31.2
MC-LY	31.4
MC-RR	4.8
MC-WR	49.8
MC-YR	91.8
Nodularin-R	12.9

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