Identification and Quantitation of Microcystins by Targeted Full-Scan LC-MS/MS

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Key Words

Velos Pro, UltiMate, Water Analysis, Cyanobacteria, Microcystin

Goal

Develop a simple and sensitive LC-MS method for definitive identification and quantitation of microcystins in water.

Introduction

Cyanobacteria, commonly referred to as blue-green algae, are photosynthetic prokaryotes that occur naturally in surface waters. They contribute significantly to primary production and nutrient cycling. Eutrophic, warm and low turbulent conditions in freshwater bodies typically promote the dominance of cyanobacteria within phytoplankton communities. Excessive proliferation of cyanobacteria leads to blooms that disrupt ecosystems, adversely affect the taste and odor of water, and increase water treatment costs. Blooms of toxic cyanobacteria species in surface drinking water sources and recreational waters threaten human health. Gastrointestinal illness, skin irritation, and death following renal dialysis have been attributed to acute cyanotoxin exposure. Chronic exposure can cause liver damage and may be associated with primary liver cancer.[1] The incidence and severity of cyanobacterial blooms are increasing globally, underscoring the importance of cyanotoxin monitoring.

The most commonly encountered cyanotoxins are the microcystins, a group of hepatotoxic cyclic heptapeptides produced by various genera of cyanobacteria, including Microcystis, Planktothrix, and Anabaena. The chemical structure of a microcystin, depicted in Figure 1, is characterized by the presence of the amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid (Adda), which modulates the biological activity of these toxins, and N-methyldehydroalanine (Mdha). Microcystin nomenclature is based on the L-amino acids present at two positions (X and Y in Figure 1) in the molecule. Over 80 structural variants are known, differentiated by the two variable L-amino acids as well as by chain modifications. The inhibition of serine/threonine protein phosphatases type 1 and 2A is considered the major mechanism of microcystin toxicity. Microcystin-LR, one of the most prevalent and potent microcystins, is designated as possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC).[2] The potential risk of chronic exposure to microcystins in drinking water supplies prompted the World Health Organization (WHO) to issue a provisional guideline of 1 µg/L as the maximum concentration of total microcystin-LR (free plus cell-bound) in drinking water.[3] Many national and regional governments have since adopted this guideline value directly or have established slightly modified variants.
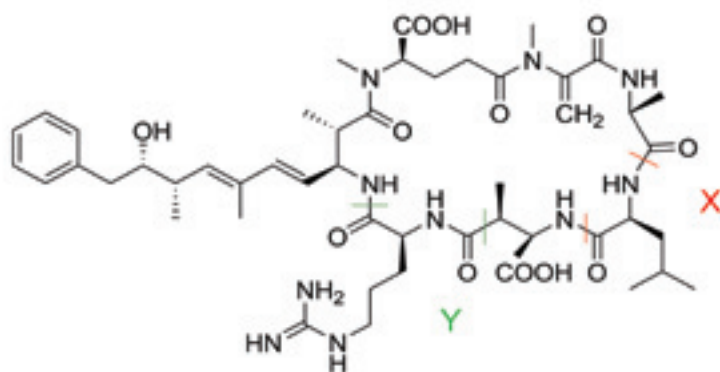

Figure 1. The chemical structure of MC-LR contains leucine (L) and arginine (R) at positions X and Y, respectively. Microcystin nomenclature is based on the L-amino acids present at these two positions.

A toxic cyanobacterial bloom usually consists of multiple microcystin congeners in varying concentrations. Several techniques for the analysis of microcystins have been developed. Mouse bioassays, protein phosphatase inhibition assays, and enzyme-linked immunosorbent assays (ELISA) are effective for rapid screening but lack specificity. Reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is the most common approach used for the separation, detection and quantitation of microcystins. An ISO method for microcystin analysis by HPLC-UV has been validated for MC-RR, MC-YR and MC-LR.[4] However, UV detection is susceptible to interferences from water matrices and requires sample cleanup and concentration to achieve desirable detection limits. Furthermore, UV-based methods do not provide unequivocal identification of known microcystins nor enable identification of unexpected variants. Liquid chromatography in combination with multi-stage mass spectrometry (LC-MS^n) enables structural characterization and unambiguous identification of trace levels of microcystins. LC-MS/MS in multiple reaction monitoring (MRM) acquisition mode allows highly selective and sensitive quantitation and confirmation of target microcystins, but this approach requires extensive compound-dependent parameter optimization and cannot be used to detect unexpected toxins. Full-scan MS/MS approaches obviate the need for compound optimization and enable determination of all microcystins present in a sample.

The Thermo Scientific Velos Pro dual-pressure linear ion trap mass spectrometer delivers sensitivity and speed for qualitative and quantitative applications. High-quality full-scan MS^n spectra enable confident structural elucidation and identification. Rapid scanning and fast cycle times generate more scans across chromatographic peaks for robust quantitation and allow the acquisition of more MS^n spectra in shorter chromatographic runs. A wide dynamic range of up to six orders of magnitude facilitates identification and quantitation of low-abundance compounds in complex matrices. Complementary fragmentation techniques may be performed in parallel to enable more MS^n information to be obtained from a single sample. In this application note, we describe a simple and sensitive targeted full-scan LC-MS/MS method for the identification and quantitation of the microcystins MC-RR, MC-YR, and MC-LR using the Velos Pro™ ion trap mass spectrometer coupled to a Thermo Scientific Dionex UltiMate 3000 x2 Dual RSLC system.

Experimental

Sample Preparation
MC-RR, MC-YR and MC-LR standards were purchased from Sigma-Aldrich®. A stock solution of a mixture of these three microcystins was prepared at a concentration of 5 µg/mL. Calibration solutions, with concentrations of 0.025 µg/L to 50 µg/L, were prepared by serial dilution of the stock solution.

LC-MS/MS Analysis
A 50 µL sample was injected on a Thermo Scientific Acclaim 120 guard cartridge with 150 L/min, washed for two minutes to waste and then eluted onto a Thermo Scientific PepMap100 analytical column for separation. LC-MS/MS analysis was performed on an UltiMate™ 3000 x2 Dual RSLC system coupled to an Velos Pro mass spectrometer.

LC Parameters

Guard cartridge:	Acclaim™ 120 C18 (10 x 3.0 mm i.d., 5.0 µm particle size, 120 Å pore size)
Analytical column:	Acclaim PepMap100 C18 (150 x 1.0 mm i.d., 3.0 µm particle size, 100 Å pore size)
Mobile Phase A:	Water containing 0.1% formic acid
Mobile Phase B:	Acetonitrile containing 0.1% formic acid
Column temperature:	40 °C
Sample injection volume:	50 µL
Flow rate:	150 µL/min
Gradient:	Table 1

Table 1: LC Gradient

Time	% A	% B
0.1	98	2
1.5	98	2
2.0	80	20
3.0	60	40
7.4	40	60
7.5	2	98
7.9	2	98

MS Parameters

Ionization mode:	Positive electrospray ionization (ESI)
Collision energy:	35%
Isolation window:	2
Targeted full-scan MS/MS:	MC-RR [M+2H]$^{2+}$ at m/z 520 [m/z 150-1100] MC-YR [M+H]$^+$ at m/z 1045 [m/z 285-1100] MC-LR [M+H]$^+$ at m/z 995 [m/z 285-1100]

Results and Discussion

Structural Identification and Confirmation

Figure 2 shows extracted ion chromatograms and MS/MS spectra obtained from full-scan LC-MS/MS analysis of a mixture containing MC-RR, MC-YR and MC-LR at concentrations of 0.5 µg/L. MC-RR, MC-YR and MC-LR eluted at 5.62, 6.85, and 6.93 minutes, respectively. The MS/MS spectrum of MC-RR was generated by collision-induced dissociation (CID) of the $[M+2H]^{2+}$ ion and is characterized by major fragment ions at *m/z* 505, 452 and 887, which correspond to $[M+2H-CO]^{2+}$, $[M+2H-C_9H_{10}O]^{2+}$ and $[M+H-C_9H_{10}O-NH_3]^+$, respectively ($C_9H_{10}O$ is a fragment of the Adda residue). The closely eluting compounds MC-YR and MC-LR are easily distinguished by their

MS/MS spectra. The MS/MS spectrum of MC-YR, generated by CID of the $[M+H]^+$ ion, contains major fragment ions at *m/z* 1017, 599, and 916, which correspond to $[M+H-CO]^+$, $[Arg+Adda+Glu + H]^+$, and $[Arg+Adda+Glu+Mdha+Ala+Tyr + H]^+$, respectively. The CID MS/MS spectrum of the $[M+H]^+$ ion of MC-LR is characterized by major fragment ions at *m/z* 967, corresponding to $[M+H-CO]^+$; *m/z* 599, corresponding to $[Arg+Adda+(Glu \text{ or } MeAsp) + H]^+$; *m/z* 866, corresponding to $[Ala+Adda+Arg+(Glu \text{ or } MeAsp) +Leu+Mdha + H]^+$; and *m/z* 553, corresponding to $[Ala+Arg+(Glu \text{ or } MeAsp) +Leu+Mdha+ H]^+$.
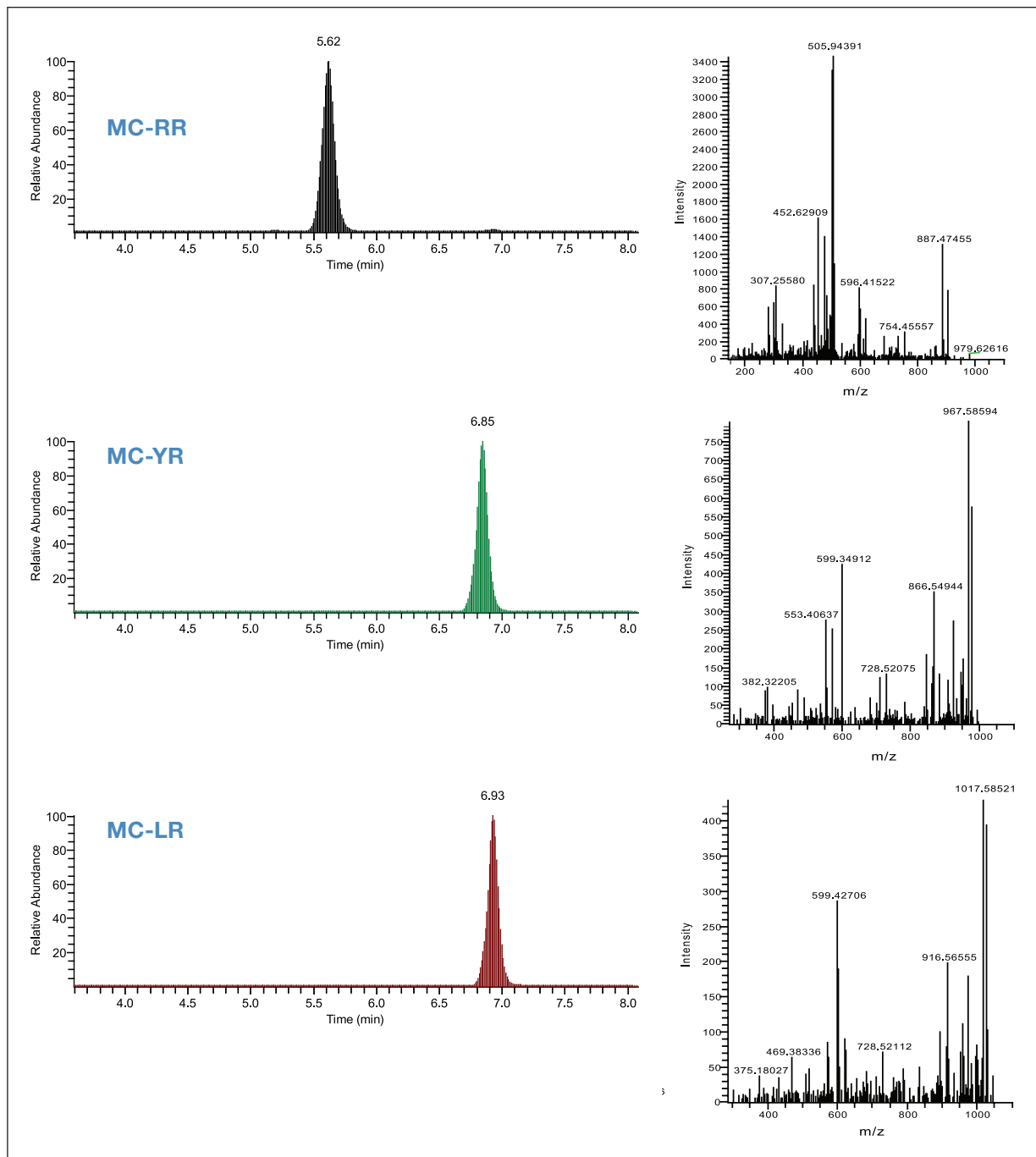

Figure 2. Extracted ion chromatograms and MS/MS spectra for MC-RR, MC-YR and MC-LR at concentrations of 0.5 µg/L

Quantitative Analysis

The high scan speeds and fast analytical cycle time of the Velos Pro mass spectrometer enabled higher numbers of analytical scans across chromatographic peaks for optimal quantitative reliability (Figure 3). Excellent linearity in detector response was observed over the range of 0.05-50 µg/L for all three microcystins. Calibration curves for MC-RR, MC-YR and MC-LR are shown in Figure 4, with coefficients of determination of 0.9986, 0.9994, and 0.9994, respectively. The lowest detectable amount (LOD) of 0.025 µg/L and quantifiable amount (LOQ) of 0.05 µg/L were achieved for each microcystin. Both QC samples, at levels of 0.5 and 5 µg/L, achieved quantitation accuracy better than 94% for all three microcystins. Signal-to-noise ratios of >25 with automatic ICIS algorithm integration in Thermo Scientific Xcalibur software were obtained for MC-LR at the LOQ (Figure 5), demonstrating that this LC-MS/MS method can be used to determine MC-LR at concentrations well below the WHO's recommended guideline level of 1 µg/L.

Method reproducibility was investigated by analyzing five replicate injections of each analyte. Peak area RSDs for MC-LR and MC-YR were less than 7% and 11%, respectively, over the entire linear dynamic range (Table 2). For MC-RR, peak area RSDs over the range 0.10-50 µg/L were under 6%; at the LOQ, the peak area RSD was 16% (Table 2). Retention time precisions were 0.3% RSD or less over the entire dynamic range (Figure 6) for all three microcystins. Tap water, filtered water and surface pond water were analyzed using this method. No microcystins were in any of the three water sources.
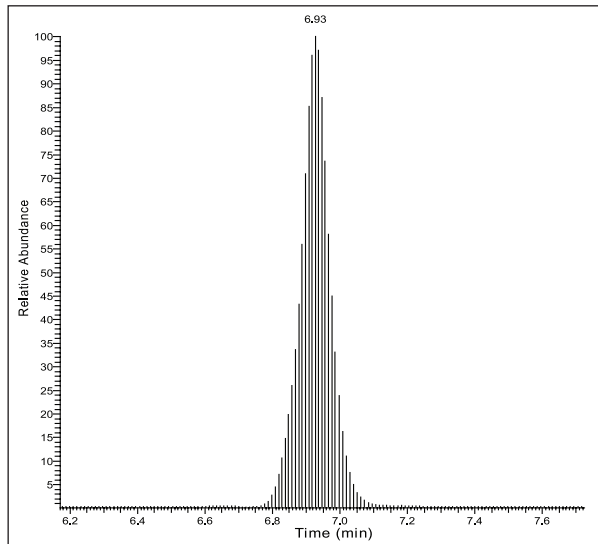

Figure 3. High scan speeds and fast cycle times enable more than 20 data points to be acquired across the MC-LR chromatographic peak.
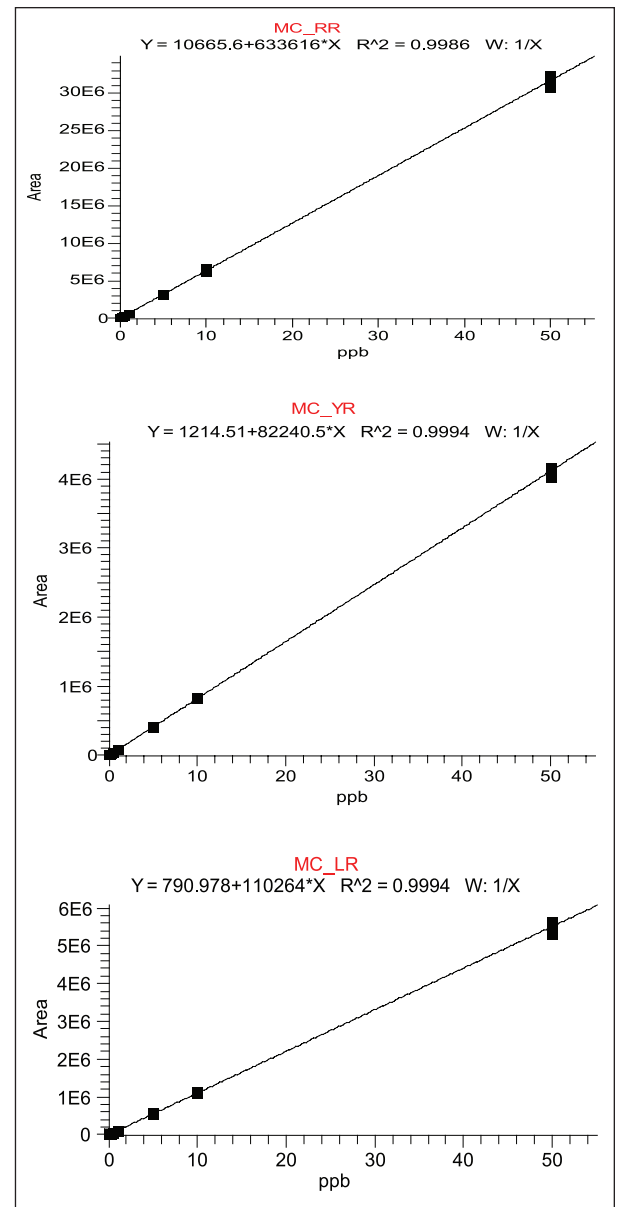

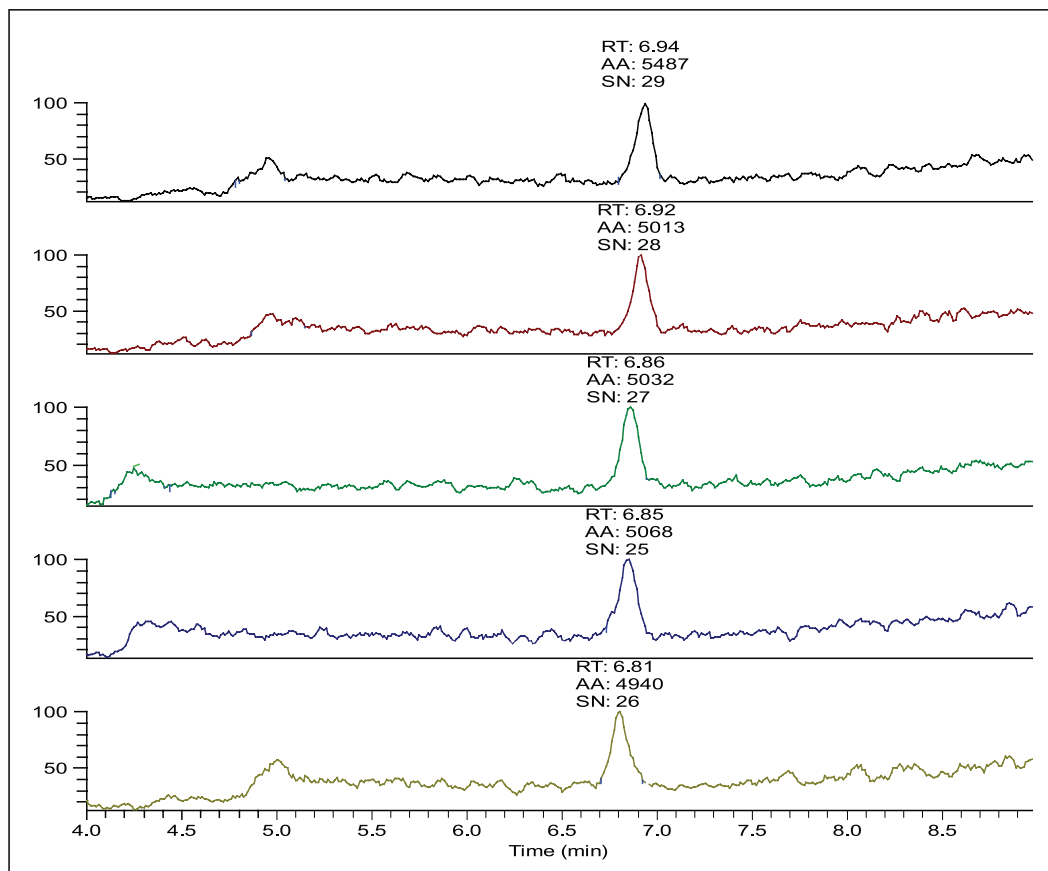Figure 4. Calibration curves for quantitation of MC-RR, MC-YR and MC-LR

Figure 5. For MC-LR at the LOQ (0.05 µg/L), S/N > 25 and peak area RSD = 6.91%
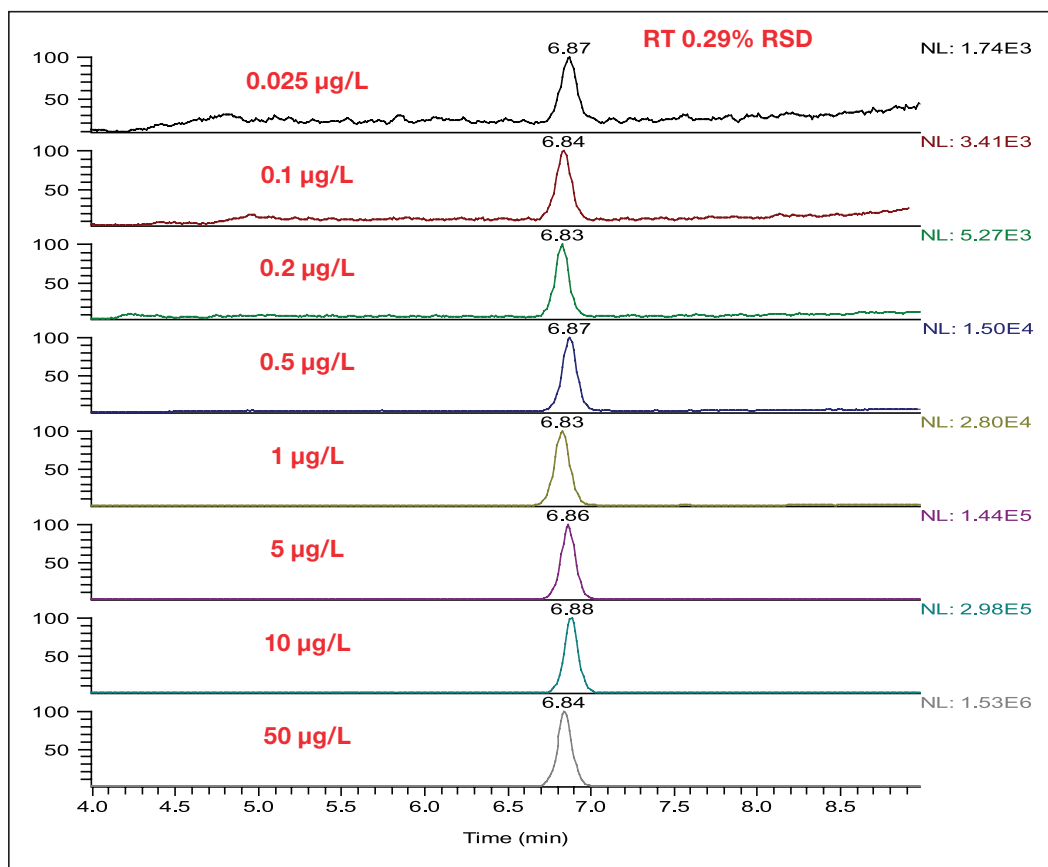

Figure 6. High retention-time precision (< 0.3% RSD) over a wide linear dynamic range

Table 2. Peak area precision (from five replicate injections) for LC-MS/MS assay of MC-RR, MC-YR and MC-LR

Levels µg/L	MC-RR	MC-YR	MC-LR
0.05	16.01	10.5	6.91
0.10	2.82	5.88	3.97
0.20	3.54	5.25	4.89
0.50	4.86	8.54	3.03
1.00	5.84	1.76	4.25
5.00	2.28	2.13	2.47
10.00	4.54	1.30	1.31
50.00	2.40	1.76	2.66

Conclusion

A simple, sensitive and robust LC-MS method for quantitative determination of microcystins was developed. Targeted full-scan MS/MS analysis using the LTQ Velos Pro linear ion trap mass spectrometer provided excellent selectivity and sensitivity for the identification and quantitation of MC-RR, MC-YR and MC-LR across a wide linear dynamic range. The LOD and LOQ were 0.025 µg/L and 0.05 µg/L, respectively. The LOQ was significantly lower than the provisional guideline value established by the WHO for MC-LR concentrations in drinking water. Assays performed in full-scan MS/MS mode enable compound confirmation and quantitation without the need for compound-dependent parameter optimization. The method was used to analyze tap, filtered and surface pond water. No microcystins were detected from these three water sources.

References

1. *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management;* Chorus I, Bartram J, eds. Published by E & FN Spon, London, on behalf of the World Health Organization, Geneva. 1999

2. Ingested Nitrate and Nitrite and Cyanobacterial Peptide Toxins. *IARC Monogr Eval Carcinog Risks Hum,* **2010**, 94:1–477.

3. *WHO Guidelines for Drinking-water Quality*, 3rd Ed., Vol. 1, Recommendations; WHO, Geneva. 2004

4. *ISO Water Quality: Determination of microcystins - method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection.* ISO, Geneva, Switzerland (ISO 20179:2005).

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