Identification of Unknown Microcystins in Alberta Lake Water

Application Note

Environmental

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Abstract

Detection, characterization, and tentative identification of very low levels of unknown microcystins in lake water are possible in the absence of analytical standards using a combination of triple quadrupole LC/MS and LC/Q-TOF analysis and a Personal Compound Database (PCD) compiled from the Word Health Organization (WHO) list of microcystins.

Agilent Technologies

Introduction

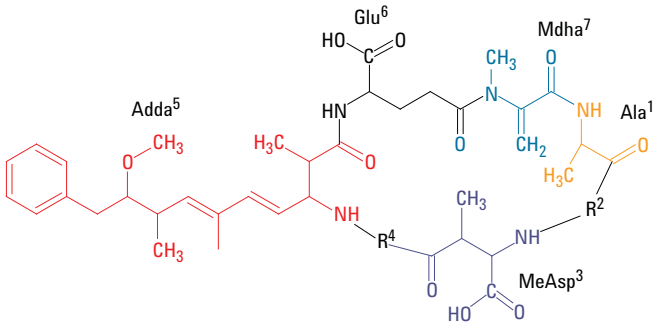
The occurrence of cyanobacterial toxins in Canadian fresh waters is a serious environmental and public health concern [1]. Microcystins (MCs) are a class of common cyanobacterial toxins present in Canadian lakes, and they are potent inhibitors of eukaryotic protein phosphatases [2]. Microcystins are also powerful hepatotoxins, and may promote tumor development in mammals, presenting a serious threat to livestock and human drinking water sources. These cyanobacterial toxins have been detected in every province in Canada, often at levels above maximum guidelines for recreational water quality [2].

The MCs are cyclic peptides containing seven amino acids. They have the general structure shown in Figure 1. Table 1 gives the chemical structures for eight microcystins surveyed in Alberta lakes. The most frequently reported MC is LR, containing leucine and arginine at positions R^2 and R^4, respectively.

The Alberta Centre for Toxicology (ACFT) conducted a comprehensive study of microcystins in Alberta lakes, and it continues to monitor fresh water sources using liquid chromatography and triple quadrupole mass spectrometry. While this method tests for eight microcystins, a nontargeted compound was also detected in a sample with the same transition qualifier ion as MC YR (*m/z* 1045), but it had an incorrect retention time (RT) and qualifier ion ratio.

This application note describes two methods developed through a collaboration between Alberta Centre for Toxicology (ACFT) and Vogon Laboratory Services to provide sensitive detection of a wide range of microcystins and establish the identity of this newly observed compound. A confirmation method was first developed using an Agilent 1290 Infinity LC System and an Agilent 6460 Triple Quadrupole LC/MS, with a different retention time pattern from the reference ACFT method. Accurate mass determination on an Agilent 6540 Q-TOF LC/MS system was then used to provide tentative identification of the unknown peak as desmethylated microcystin HtyR.

All microcystins were detected at 0.1 ng/mL, which is well below the 2007 Canadian Drinking Water Guideline of 1.5 µg/L. Using a Personal Compound Database (PCD) of 52 compounds created in the Agilent MassHunter software suite from the World Health Organization (WHO) list of microcystins, the Q-TOF data enabled the tentative identification of an additional seven microcystins.



General microcystin structure

Position	Abbreviation	Amino acid
R^1	Ala^1	Alanine
R^2	Leu^2 (L)	Leucine
R^3	$MeAsp^3$	Methylaspartic acid
R^4	Arg^4 (R)	Arginine
R^5	$Adda^5$	3-amino-9-methoxy-2,6,6-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid
R^6	Glu^6	Glutamic acid
R^7	$Mdha^7$	N-methyldehydroalanine

Figure 1. General microcystin (MC) structure.

*Table 1. Eight Microcystins Surveyed in Alberta Lakes**

Microcystin	R^2	R^4	Formula	Neutral mass
LR	Leucine	Arginine	$C_{49}H_{74}N_{10}O_{12}$	994.5488
Desmethyl LR	Leucine	Arginine	$C_{48}H_{72}N_{10}O_{12}$	980.5331
RR	Arginine	Arginine	$C_{49}H_{75}N_{13}O_{12}$	1037.5658
YR	Tyrosine	Arginine	$C_{52}H_{72}N_{10}O_{13}$	1044.5280
LA	Leucine	Alanine	$C_{46}H_{67}N_7O_{12}$	909.4848
LW	Leucine	Phenylalanine	$C_{54}H_{72}N_8O_{12}$	1024.5270
LF	Leucine	Tryptophan	$C_{52}H_{71}N_7O_{12}$	985.5161
HtyR	Homotyrosine	Arginine	$C_{53}H_{74}N_{10}O_{13}$	1058.5437

*See Figure 1 for the general microcystin structure

Experimental

Instruments

The confirmation method was developed on an Agilent 1290 Infinity LC System equipped with an Agilent G4226A Autosampler and coupled to an Agilent 6460 Triple Quadrupole LC/MS System. The instrument conditions are listed in Table 2.

The method for tentative identification of unknown microcystins was developed on a 1290 Infinity LC system equipped with a G4226A Autosampler and coupled to an Agilent 6540B Q-TOF LC/MS System with Jet Stream electrospray source. The instrument conditions are listed in Table 2.

Table 2. LC and Triple Quadrupole MS Run Conditions

LC conditions		
Column	Agilent Poroshell SB-C18, 3.0 × 100 mm, 2.7 µm (p/n 685975-306)	
Column temperature	50 °C	
Injection volume	20 µL	
Mobile phase	A) 1 mM ammonium fluoride in water (HPLC grade) B) 20% isopropanol in acetonitrile (LC/MS grade)	
Autosampler temperature	5 °C	
Flow rate	0.6 mL/min	
Gradient	Time (min)	% B
	0	20
	3.0	30
	5.0	50
	6.0	100
Stop	7 minutes	
Post time	2 minutes	
Triple quadrupole MS conditions		
Ionization mode	ESI with Agilent Jet Stream Technology	
Drying gas temperature	350 °C	
Drying gas flow	12 L/min	
Nebulizer pressure	40 psig	
Sheath gas temperature	400 °C	
Sheath gas flow	11 L/min	
Capillary voltage	4,000 V	
Nozzle voltage	1,000 V	
EMV	400 V	
Q-TOF MS conditions		
Mode	Targeted MS/MS	
Acquisition	Profile and centroid; 2 GHz	
Range	100–1,700 amu	
Acquisition rate (MS)	3 scans/s	
Acquisition rate (MS/MS)	1 scan/s	
Reference masses	121.0509 and 922.0098	

Analysis parameters

The 6460 Triple Quadrupole LC/MS multiple reaction monitoring (MRM) analysis parameters are shown in Table 3.

Table 3. MRM Analysis Parameters[a] for the Target Compounds Using a Triple Quadrupole LC/MS

Microcystin	Precursor[b] (*m/z*)	Product ion (*m/z*)	Collision energy (V)
LR	995.6	135.2	80
		213.2	80
Desmethyl-LR	981.5	135.2	80
		213.2	80
RR	520.0	135.2	30
		213.2	40
YR	1045.5	135.2	80
		213.2	70
LA	910.5	135.2	70
		213.2	70
LY	1002.5	135.2	80
		213.2	70
LW	1025.5	135.2	80
		213.2	60
LF	986.5	135.2	70
		213.2	50
HtyR	1059.5	135.2	80
		213.2	70

[a] The fragmentor and cell acceleration voltages were 150 V and 2 V, respectively, for all transitions.

[b] All precursors are singly charged, except RR which is doubly charged.

Sample preparation

Water samples (10 mL) were taken through three freeze/thaw cycles, then sonicated for 5 minutes, followed by filtration through 0.2-µm cellulose filters directly into autosampler vials.

Results and Discussion

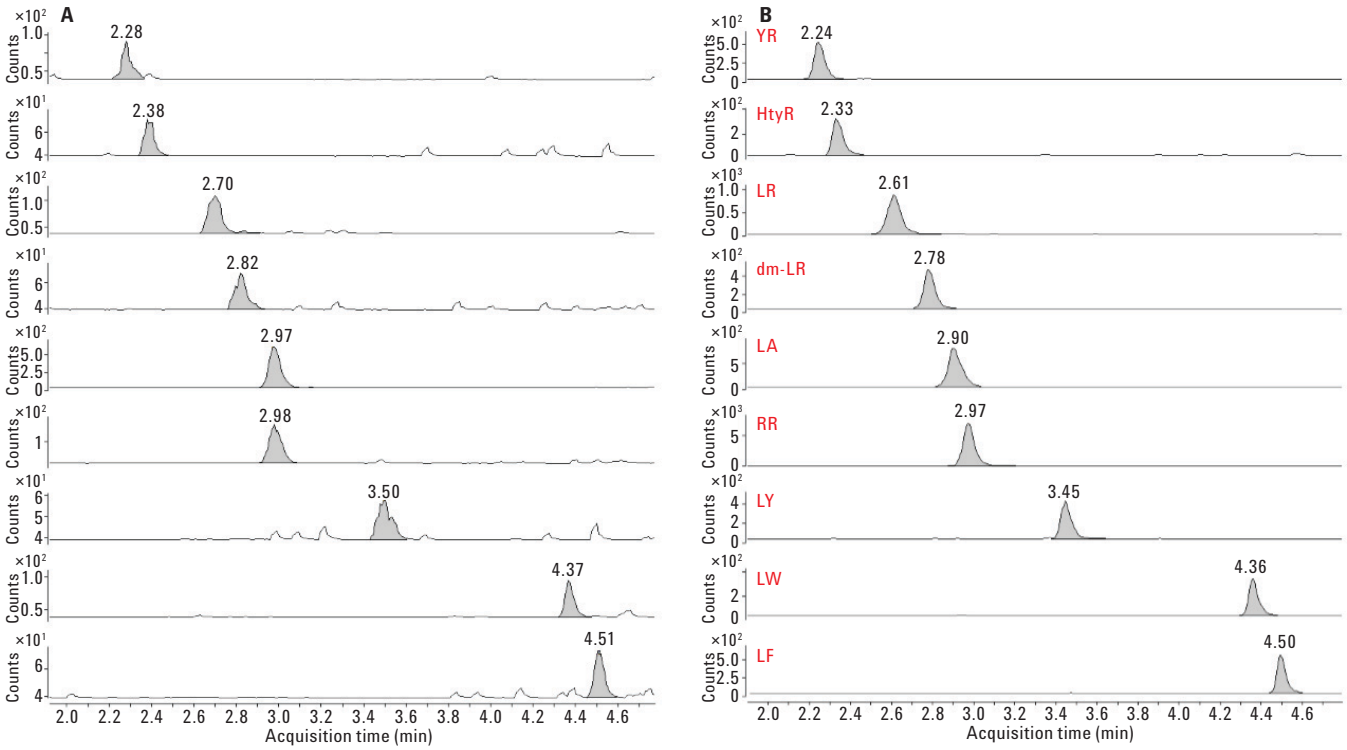
Identifying an unknown microcystin

One sample analyzed using the ACFT method revealed a compound with the same transition and qualifier ion as YR (*m/z* 1045), but at the wrong retention time (RT) and qualifier ion ratio. It was tentatively identified as desmethyl-HtyR, based on the loss of 14 amu from HtyR (*m/z* 1059).

To confirm the identification of this unknown MC, a triple quadrupole LC/MS confirmation method was developed, which provided a different retention time pattern from the reference ACFT method. Additional microcystin analytes were added to the method to help characterize compounds. Finally, samples were also analyzed using an LC/Q-TOF method to confirm the identity of the unknown compound.

Confirmation method

The triple quadrupole LC/MS confirmation method was optimized to provide a minimum detection level at approximately 10% of the Canadian Drinking Water Guidelines (CDWG), which is 1.5 µg/L, based on MC LR. The lowest level calibrators used for the method were 0.1 µg/L for MCs YR, LR, and RR, and 0.2 µg/L for all others. Figure 2 illustrates the complete separation achieved for nine MCs with the high performance liquid chromatography (HPLC) chromatographic method at both the lowest calibrator levels and calibrator levels near the CDWG.



Lowest level calibrator

0.1 µg/L for YR, LR, and RR
0.2 µg/L for all others

Calibrator near DW guidelines

0.9 µg/L for YR, LR, and RR
1.9 µg/L for all others

Figure 2. Example total ions chromatograms (TICs) for nine microcystins at concentrations approximately 10% (A) and 100% (B) of the CDWG of 2007.

Quantitation calibration coefficients (R^2) were excellent, ranging from 0.9978 to 0.9995 for the six MCs using a minimum calibrator level of 0.2 µg/L and a maximum calibrator level of 50 µg/L (Table 4). The R^2 values ranged from 0.9992 to 0.9997 for the three MCs using a minimum calibrator level of 0.1 µg/L and a maximum calibrator level of 25 µg/L. A representative calibration curve for MC LR is shown in Figure 3, illustrating excellent linearity even at extremely low concentrations. Accuracy ranged from 84% to 121% for the six MCs with a minimum calibrator level of 0.2 µg/L, and 110% to 119% for the three MCs with a minimum calibrator level of 0.1 µg/L (Table 4).

Analyzing the unknown

Using the confirmation method to analyze the unknown MC from an Alberta lake water sample showed a peak with the same transition and qualifier ions as YR, but with an RT of 1.91 minutes, rather than 2.24 minutes. The qualifier ion ratio (*m/z* 213.2/135.2) was also too low to be YR (2% versus 24%). The low abundance of the *m/z* 213.2 peak indicates possible desmethylation of the N-methyldehydroalanine (Mdha) moiety in the YR structure. Unfortunately, desmethyl HtyR is not commercially available. Therefore, Q-TOF for accurate mass analysis was used to further confirm the desmethylation hypothesis.

Table 4. Calibration Coefficients and Accuracy of Recovery

Calibration range	MC	R^2	Accuracy[a] (%)
0.2 to 50 µg/L	Hty R	0.9995	95
	Desmethyl-LR	0.9993	84
	LA	0.9988	121
	LY	0.9998	92
	LW	0.9989	118
	LF	0.9978	119
0.1 to 25 µg/L	YR	0.9993	118
	LR	0.9997	110
	RR	0.9992	119

[a] Quantitative accuracy is listed for calibrator 1 (0.1 or 0.2 µg/L)

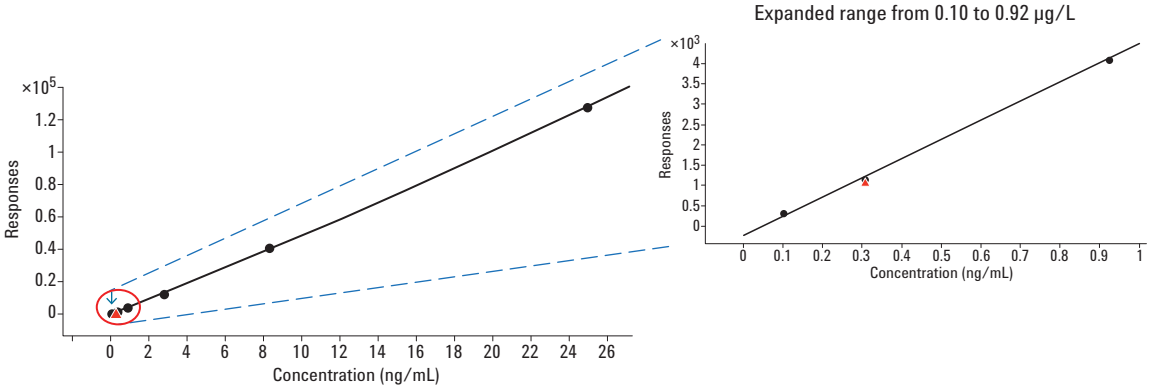


Figure 3. A calibration curve for MC LR using three-fold serial dilutions from 0.1 to 25 µg/L, including the expanded range from 0.10 to 0.92 µg/L, to illustrate excellent linearity.

LC/Q-TOF analysis

The accurate mass determined for the precursor ion for the unknown MC using liquid chromatography/Q-TOF MS (LC/Q-TOF) was 1059.5500, which matches the actual mass for desmethylated HtyR with a mass error of 5.0 ppm. This further supports the hypothesis that the unknown is formed by the desmethylation of HtyR. Examination of the transition ions reveals that both the unknown and YR contain the two *m/z* 135 nominal mass ions characteristic for microcystins (Figure 4). The actual masses of the two ions are 135.0804 and 135.1168, formed by different fragmentation patterns of the Adda group. Both ions are well separated in both microcystins at mass resolution 14,000.

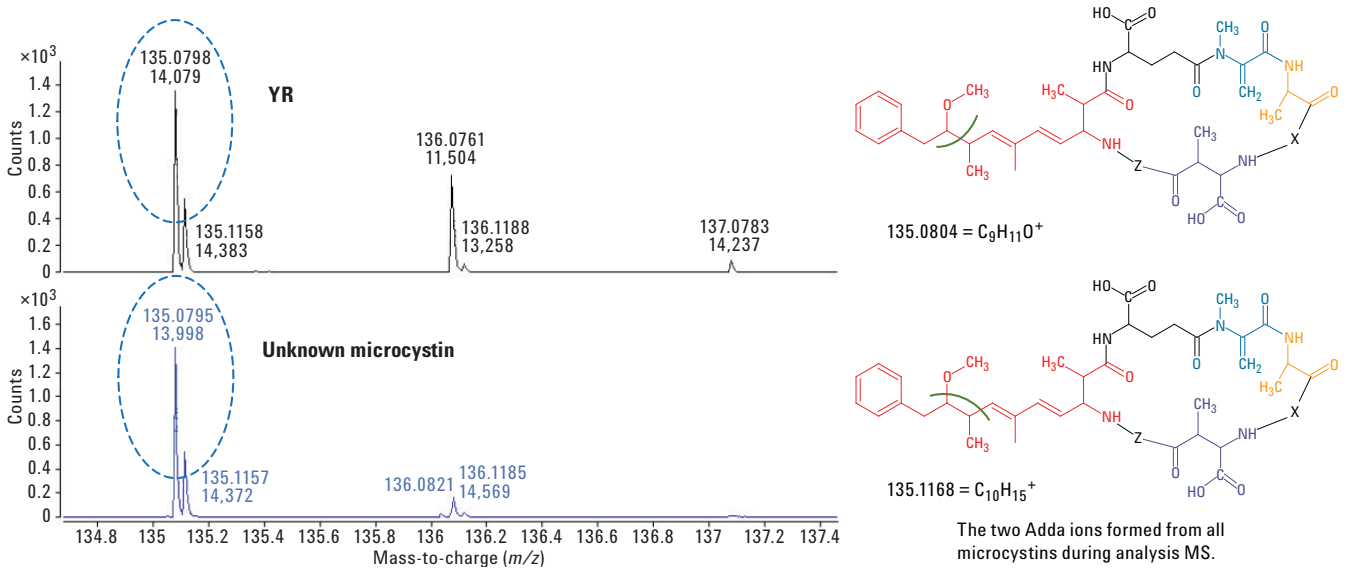


Figure 4. The two ions derived from the Adda group and characteristic of all microcystins are present and well separated in both YR and the unknown microcystin, using LC/Q-TOF.

However, the *m/z* 213 ion formed from cleavage of the Glu + Mdha groups from the microcystins is present as expected in MC YR, but present at only trace levels in the unknown MC (Figure 5). This indicates that the unknown MC is not YR. Desmethylation of the Glu + Mdha group would be expected to produce an ion with an accurate mass of 199.0713 (Figure 6). This ion is observed in the spectra from the unknown MC, but not in the spectra of MC YR. These results also support the hypothesis that the unknown MC is dm-HtyR.

A previous structural LC/MS/MS characterization of microcystins had identified eight major ions designated a-h [3]. Two of these, ions f and h, can be used to confirm the identity of the unknown MC. Ion f contains the R^7 and R^2 moieties (Figure 1), which should be dm-Mdha (Dha) and Hty, respectively for the microcystin dm-HtyR. Ion h contains the R^2 moiety, as well as a second possible desmethylation site, MeAsp[3]. Depending on the identities of R^7 and R^2 and the presence or absence of desmethylation at position R^3, the accurate masses of these two ions will vary and be diagnostic of the microcystin structure.

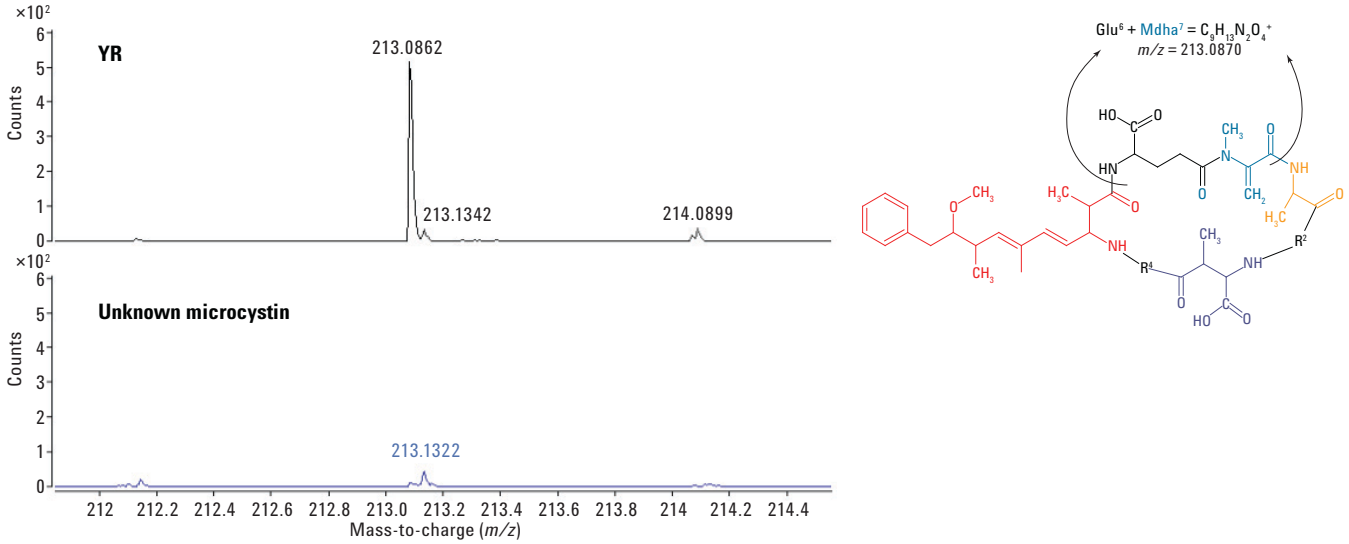


Figure 5. Q-TOF spectra of YR and the unknown microcystin in the m/z range from 212 to 214.4. The m/z 213 ion characteristic of the Glu + Mdha group is present in microcystin YR but at low abundance in the unknown MC.

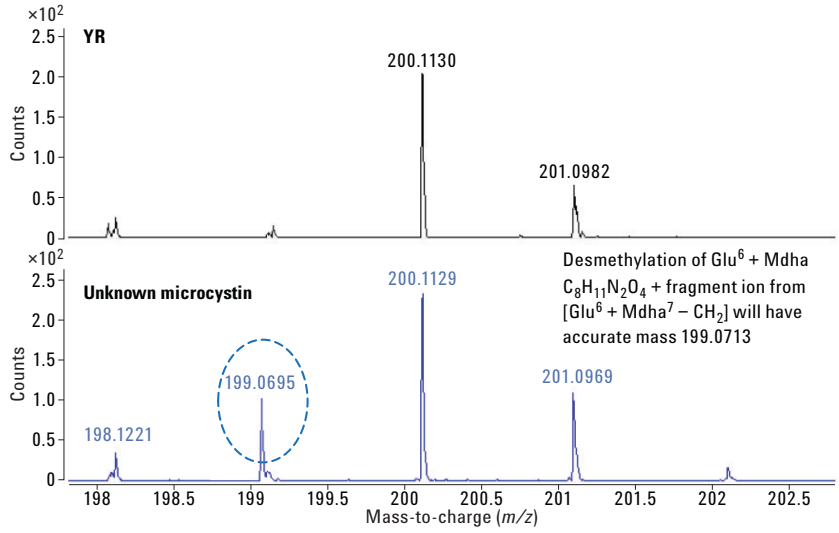


Figure 6. Q-TOF spectra of YR and the unknown microcystin in the m/z range from 198 to 202. The m/z 199 peak is present in the unknown microcystin, indicating desmethylation of the Glu + Mdha group. This peak is not present in YR.

Analysis by Q-TOF of ion f in the unknown microcystin revealed accurate masses that confirmed the presence of Dha at position 7 and Hty at position 2, consistent with the identification of the unknown as dm-HtyR. Analysis of the HtyR standard also gave the correct accurate mass for ion f (Figure 7).

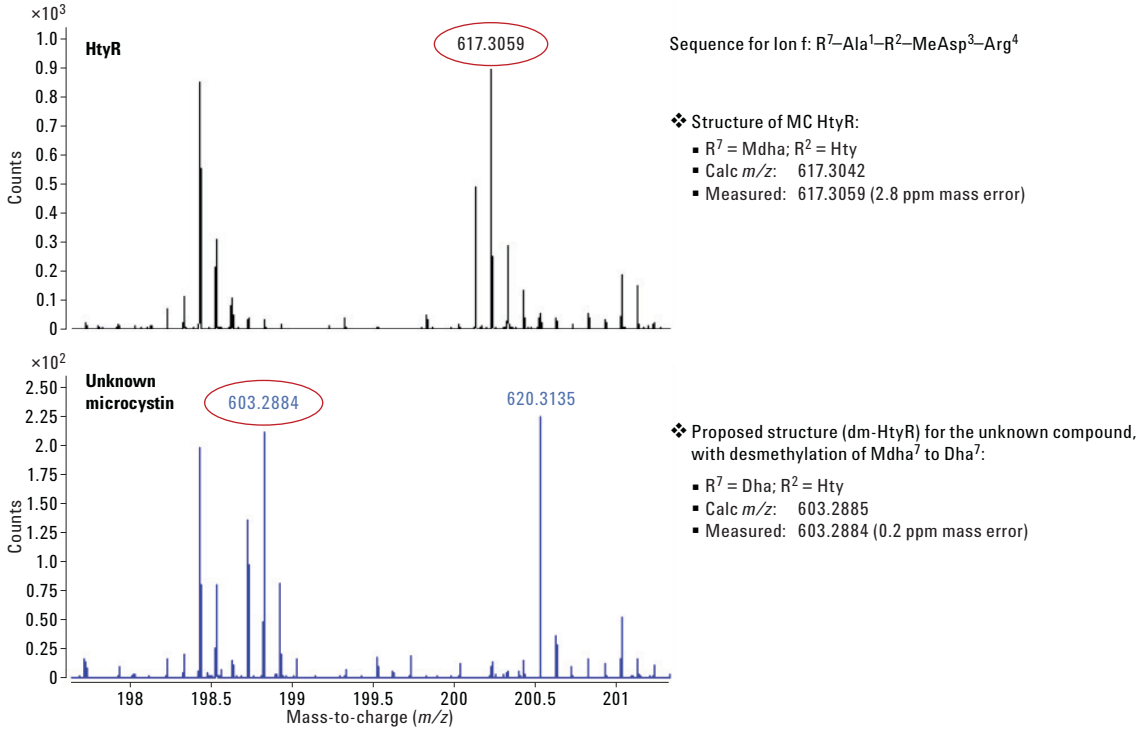
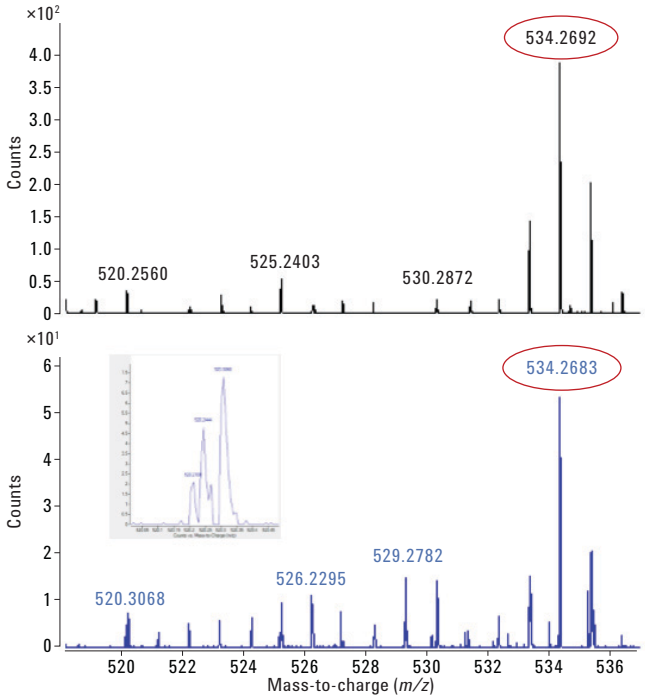


Figure 7. Q-TOF spectra of ion f for HtyR and the unknown microcystin. The accurate mass for the f ion observed in HtyR (upper) matched the calculated mass for the ion containing Hty at position 7 and Mdha at position R^3. In contrast, the mass observed for ion f in the unknown MC matched the calculated mass for the ion containing Hty at position 7 and Dha at position R^3.

The spectra of the h ion confirmed that minimal desmethylation is occurring in the R^3 position in the unknown MC, as its accurate mass matched the calculated mass for methylated aspartic acid in position 3, in both the unknown and the HtyR standard (Figure 8).



Sequence for Ion h: Ala^1–R^2–$MeAsp^3$–Arg^4

❖ Structure of MC HtyR:

- R^2 = Hty
- Calc *m/z*: 534.2671
- Measured: 534.2692 (2.3 ppm mass error)

❖ Proposed structure (dm-HtyR) for the unknown compound, with no desmethylation at position 3:

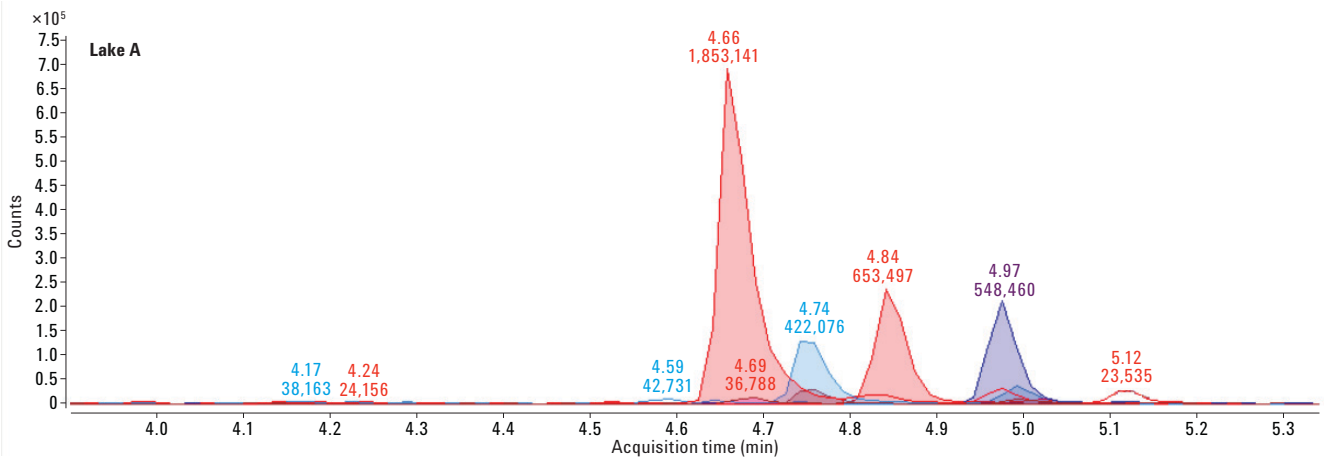
- R^7 = Hty
- Calc *m/z*: 534.2671
- Measured: 534.2683 (4.0 ppm mass error)

Figure 8. Q-TOF spectra of ion h for HtyR and the unknown microcystin. The accurate mass for the h ion observed in both HtyR (upper) and the unknown MC matched the calculated mass for the ion containing Hty at position R^7 and Mdha at position R^3. In contrast, only a very small peak was observed at nominal mass m/z 520 in both microcystins, indicating that little or no desmethylation was occurring at position R^3.

Using a Personal Compound Database to identify multiple unknown microcystins

The accurate mass capabilities of the Q-TOF can be used to tentatively identify other microcystins that may be present in Alberta lakes, based only on the mass of their H+ adducts. The first step in the process is to build a Personal Compound Database (PCD) using the Agilent MassHunter PCDL Manager Software, which enables the user to create and edit a customizable PCD, including compounds, accurate-mass, and retention time information. One of the advantages of accurate mass scan data is the ability to retrospectively search acquired data for new compounds using such a database.

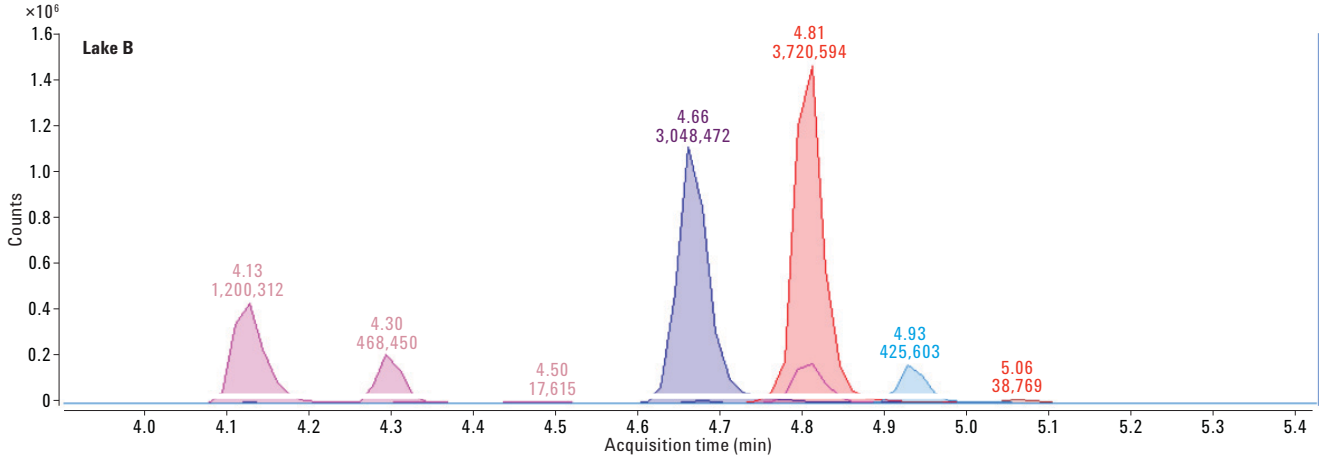
In this study, the WHO list of microcystins [4] was used to enter the formulas for 52 microcystins into a PCD, which generates accurate masses for the H+ adducts. The Find by Formula tool in MassHunter was then used to search for these accurate masses in the sample total ions chromatogram (TIC) data file. Using this process with the Q-TOF, seven unknowns were tentatively identified in samples from Lake A and Lake B, in addition to the desmethyl HtyR (Figures 9 and 10). Additional analysis by Q-TOF MS/MS and comparison to analytical standards is needed to confirm the presence and identity of these MCs. Work is on-going to develop an exact-mass calculator model to help identify nontargeted MCs and their variants.



RT	Compound name	Formula	*m/z*	Area	Score	Mass diff (ppm)*
4.66	MC-YR	C52 H72 N10 O13	1045.5346	1,853,141	98.5	-0.7
4.69	MC-LR	C49 H74 N10 O12	995.5549	36,788	90.4	-0.1
4.74	MC-HtyR-DAsp3-Dha7	C51 H70 N10 O13	1031.5185	422,076	96.8	-0.7
4.76	MC-HtyR	C53 H74 N10 013	1059.5501	94,974	98.4	-0.3
4.84	MC-DesMe-LR	C48 H72 N10 O12	981.5405	653,497	99.1	-0.1
4.97	MC-HphR-Dha7	C52 H72 N10 O12	1029.5400	548,460	99.6	-0.2
4.99	MC-LR-DAsp3-Dha7	C47 H70 N10 O12	967.5236	116,668	94.3	-1.5
4.99	MC-LR	C49 H74 N10 O12	995.5559	32,185	92.7	-0.6

*Mass difference determined by subtracting the theoretical mass of the compound from the calculated mass derived from the Q-TOF analysis, expressed in ppm.

Figure 9. Extracted ion chromatograms of a water sample from Lake A, and a table including retention time (RT), compound name, calculated m/z, and difference of the calculated mass from the theoretical mass for each unknown tentatively identified using the Find by Formula tool in MassHunter and the personal compound database (PCD) created for the microcystins in the WHO list. A mass tolerance of 5 ppm and a minimum match score of 70 were used to make the identification of H+ adducts.



RT	Compound name	Formula	*m/z*	Area	Score	Mass diff (ppm)*
4.13	MC-HtyR-DAsp3-Dha7	C51 H70 N10 O13	1031.5193	1,200,312	99.5	-0.4
4.30	MC-LR-DAsp3-Dha7	C47 H70 N10 O12	967.5246	468,450	99.7	-0.3
4.50	MC-LR-DAsp3-Dha7	C47 H70 N10 O12	967.5256	17,615	86.4	0.7
4.66	MC-HtyR-DAsp3-ADMAdda5-Dhb7	C53 H72 N10 O14	1073.5305	3,048,472	99.4	0.4
4.68	MC-LR	C49 H74 N10 O12	995.5549	18,836	97.7	-1.1
4.81	MC-LR-DAsp3-ADMAdda5-Dhb7	C49 H72 N10 O13	1009.5359	3,720,594	99.6	0.5
4.93	MC-LY	C52 H71 N7 O13	1002.5181	425,603	99.4	-0.3
5.06	MC-LR-ADMAdda5	C50 H74 N10 O13	1023.5505	38,769	95.9	-0.6

*Mass difference determined by subtracting the theoretical mass of the compound from the calculated mass derived from the Q-TOF analysis, expressed in ppm.

Figure 10. Extracted ion chromatograms of a water sample from Lake B, and a table including retention time (RT), compound name, calculated m/z, and difference of the calculated mass from the theoretical mass for each unknown tentatively identified using the Find by Formula tool in MassHunter and the personal compound database (PCD) created for the microcystins in the WHO list. A mass tolerance of 5 ppm and a minimum match score of 70 were used to make the identification of H+ adducts.

Conclusion

When analytical standards are available, the Agilent 1290 Infinity LC System and an Agilent 6460 Triple Quadrupole LC/MS provides an excellent platform for the targeted analysis of microcystins. Analysis by triple quadrupole LC/MS using the Agilent 6540 Q-TOF LC/MS System can confirm suspect compounds using the accurate mass of the molecular ion adducts, as well as MS/MS fragments. The combination of these two technologies supported the hypothesis that dm-HtyR was present in an Alberta lake water sample. Databases can be compiled using the Agilent MassHunter PCDL Manager Software to include the chemical formula for additional microcystins based on reported analogues in the literature. Using MassHunter Find by Formula, previously acquired data files can be retrospectively searched against PCDL databases and libraries for these additional compounds as they become known.

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