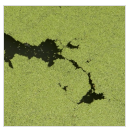

Jun 09, 2020

LCMS-SOP Determination of Microcystins in Water Samples by High Performance Liquid Chromatography (HPLC) with Single Quadrupole Mass Spectrometry (MS)

Gregory L Boyer[1]

[1]Department of Chemistry, State University of New York (SUNY), College of Environmental Science and Forestry, Syracuse, NY

1 Works for me dx.doi.org/10.17504/protocols.io.bck2iuye

Greg Boyer

ABSTRACT

This standard operating procedure is used to determine and quantify microcystins and nodularin (intracellular and/or extracellular) in whole water samples or filter samples using HPLC-coupled with single quadrupole mass spectrometry (LC-MS). The method described here is a modification of the methods described in Boyer (2007) for particulate microcystins in water samples and does not use solid phase extraction. It was designed to use a 50% acidified methanolic extraction solvent, but is compatible with protocols for direct water analysis (US-EPA, 2016) and higher concentration methanolic extracts (Cerasino et al., 2017). It is not suitable for use with tissues samples or other samples with complex matrix interferences, such as soil or sediments.

The method is designed for high throughput (1000-1500 injections per month) using a low-resolution (unit mass) mass spectrometer. It gives results of similar accuracy to the commonly used Abraxis ADDA ELISA with additional information on the microcystin congener profiles.

ATTACHMENTS

Microcystins_LCMS_SOP_for_DEC_(20January2020)_provisional_(1).pdf

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Formic Acid 99.0 % Optima™ LC/MS Grade	A117-50	Fisher Scientific

EQUIPMENT

NAME	CATALOG #	VENDOR
Ace 3 C18, 3 micron particle size, 150 x 3.0 mm column with an inline solvent filter	50-900-01131	

WARNING: The method requires use of volatile organic solvents and handling microcystin hepatotoxins. This should be done by persons familiar with safe laboratory practice.

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

1. Scope

This standard operating procedure is used to determine and quantify microcystins and nodularin (intracellular and/or extracellular) in whole water samples or filter samples using HPLC-coupled with single quadrupole mass spectrometry (LC-MS). The method described here is a modification of the methods described in Boyer (2007) for particulate microcystins in water samples and does not use solid phase extraction. It was designed to use a 50% acidified methanolic extraction solvent, but is compatible with protocols for direct water analysis (US-EPA, 2016) and higher concentration methanolic extracts (Cerasino et al., 2017). It is not suitable for use with tissues samples or other samples with complex matrix interferences, such as soil or sediments.

The method is designed for high throughput (1000-1500 injections per month) using a low-resolution (unit mass) mass spectrometer. It gives results of similar accuracy to the commonly used Abraxis ADDA ELISA with additional information on the microcystin congener profiles.

2. Abbreviations

- ELISA: Enzyme-linked immunosorbent assay
- HPLC-MS: High performance liquid chromatography-mass spectrometry
- HPLC-MS/MS:High performance liquid chromatography with tandem mass spectrometer
- PDA: Photodiode array detector
- SPE: Solid phase extraction
- Filter Sample: A filter containing the biomass of a whole water sample, which is extracted directly. The biomass is collected by filtering a known volume of whole water sample through the filter. Filter water samples are used to evaluate particulate microcystins.
- Whole water sample: Sample collected directly from a water body containing water and any biotic or abiotic particulate matter. Whole water samples are used to evaluate dissolved and particulate microcystins.
- Sample Set: A series of samples to be analyzed including the initial column wash, matrix blank, microcystin and nodularin standards, chromatographic standards (LRRF), and the field samples to be analyzed.
- Method Set: Waters EmpowerTM chromatography term that includes the procedure to obtain and display extraction ion

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chromatograms, single wavelength chromatograms, total ion trace and the pump pressures. Method sets may also contain individual integration reporting parameters for the different channels and protocols for deriving and updating calibration curves.

- IDL: Instrument detection limit as determined by injection of standard of microcystin in water through the LC-MS system. Expressed in micrograms (µg) on column.
- MDL: Method detection limit as determined used in IDL and correcting for the injection volume, volume filter or lyophilized, extraction volume and molecular weight of the individual congeners. Expressed in micrograms (µg) LR equiv. per liter.
- Congener: Refers to an specific individual member of the more than 250 compounds that make up the class of toxins called "microcystin". Common microcystin congener abbreviations are given below (see Appendix for additional information).
- MC-AR: Microcystin containing Ala and Arg in the variable sites X & Y.
- MC-dLA: Generic demethyl microcystin containing Leu and Ala in variable sites X & Y. The specific amino acid lacking the methyl group is not specified.
- MC-dLR: Demethyl microcystin containing Leu and Arg in variable sites X & Y.
- MC-dRR: Demethyl microcystin containing Arg and Arg in variable sites X & Y.
- MC-FR: Microcystin containing Phe and Arg in variable sites X & Y.
- MC-hIR (aka MC-HilR) Microcystin containing homo-Ile and Arg in variable sites X & Y. MC-h4YR Microcystin containing tetramethyl homoTyr and Arg in variable sites. MC-hYR Microcystin containing homoTyr and Arg in variable sites X & Y.
- MC-LA Microcystin containing Leu and Ala in variable sites X & Y.
- MC-LF Microcystin containing Leu and Phe in variable sites X & Y.
- MC-LL Microcystin containing Leu and Leu in variable sites X & Y.
- MC-LR Microcystin containing Leu and Arg in variable sites X & Y.
- MC-LW Microcystin containing Leu and Trp in variable sites X & Y.
- MC-LY Microcystin containing Leu and Tyr in variable sites X & Y.
- MC-MeLR: Microcystin containing Leu and Arg in variable site X & Y and the 0- methyl ester of isoGlu (isoGLU methyl esters are a possible artifact of sample or standard storage in acidified methanol).
- MC-mLA: Methylated microcystin containing Leu and Ala in variable site X & Y. The position of the methyl group in the methylated microcystins is not specified.
- MC-mLR: Methylated microcystin containing Leu and Arg in variable site X & Y. MC-mRR Methylated microcystin containing Arg and Arg in variable site X & Y. MC-RR Microcystin containing Arg and Arg in variable site X & Y.
- MC-WR: Microcystin containing Trp and Arg in variable site X & Y.
- MC-YR: Microcystin containing Tyr and Arg in variable site X & Y.
- MC-zLR: Microcystin containing Leu and Arg in variable site X & Y with the [(4E,6Z)] or [4(Z), 6(E)] ADDA group.
- NOD: General abbreviation for Nodularin-R, a common congener in the nodularin class of cyanobacteria toxins. NOD-R containing Arg in the single variable site.
- LRRF: Chromatography standard that consists of a mixture of equal amounts of microcystins LR, RR, and LF.

3. Principle and Background for the Method

Microcystins and the related nodularins are toxins produced by cyanobacteria that may be found in recreational and potable water supply (Carmichael and Boyer, 2016). There are currently 250 reported microcystins and 10 nodularin derivatives (Spoof and Catherine, 2017). This creates an analytical challenge for monitoring water sources for these toxins. Analytical methods for microcystins fall into two general categories. (1) Assays. These can be structure or bio-activity based but give a single integrated value for all the toxins in the water body. Common microcystin assays include the structural- based ELISA (Carmichael and An, 1999; US-EPA, 2016) or bioactivity-based protein phosphatase inhibition assay (Carmichael and An, 1999; Kaloudis et al., 2017). Assays give a single value for all microcystin structures present in the sample, but give little information on the actual toxins present in the water body. Assays are generally run using a single microcystin congener as a standard (usually MC-LR) and the results are expressed in terms of MC-LR equivalents assuming the response of the different microcystin congeners in the assay is similar to MC-LR[1]. (2) Analysis. In contrast to an assay, an analysis such as HPLC-MS (Harada, 1996), HPLC-PDA (ISO 20179, 2003) or HPLC-MS/MS (Shoemaker et al., 2015; Triantis et al., 2017) separate the toxins using HPLC, then measures the individual congener using electrospray ionization coupled with mass spectrometry or photodiode array spectroscopy. These analyses can be done with (Shoemaker et al., 2015) or without (Triantis et al., 2017) solid phase extraction depending on the sensitivity desired and the nature of the starting matrices. Highly targeted methods such as HPLC-MS/MS are the most selective, but require individual microcystin congeners to tune the instrument for the individual fragmentation patterns and voltages. These individual response factors can vary dramatically between the different congeners and this limits the LC-MS/MS approach only to those congeners where sufficiently analytical standards are available. In contrast, the initial ionization voltage (e.g. capillary voltage) of the microcystin ring system is very similar for the different microcystin congeners. With careful tuning, an generic ionization response can be obtained for multiple different microcystins, allowing the use of a single microcystin congener to generate the standard curve. This approach is similar to the ELISA assay for microcystins with the added advantage that you obtain information on the congener profiles. Individual microcystin congeners are identified based on their relative retention time, the molecular weight of the parent compound, and, if in sufficient concentration, their diagnostic PDA spectrum. This allows for the identification and quantification of microcystin congeners where analytical standards are not readily available, and provides for enhanced selectivity and sensitivity over the AOAC-validated microcystin method (ISO 20179, 2003) based on the PDA alone. The approach can be tailored to individual water bodies that contain high levels of "unique" microcystins (e.g. MC-H4YR) as, once the retention time and molecular weight of those microcystins have been identified, they can be added to the extracted ion database and quantified using the same standard curve.

4. Reagents, Apparatus, and HPLC Conditions

4.1 Reagents:

- Water: H_2O, HPLC grade, 0.22 micron filtered; PHARMCO-AAPER 23200HPLC[2]
- Methanol: CH_3OH, UV/HPLC grade; PHARMCO-AAPER 33900HPLC
- Acetonitrile: CH3CN, UV/HPLC grade; PHARMCO-AAPER 30000HPLC[3]
- Formic acid: HCOOH, Fisher Optima LC/MS grade A117-50; 50 ml bottle or less

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Formic Acid 99.0 % Optima™ LC/MS Grade
by Fisher Scientific
Catalog #: A117-50
CAS Number: 64-18-6

- Trifluoroacetic acid: TFA, $C_2HF_3O_2$, >99.0% for UV spectroscopy; Fluka 91703, 50 ml bottle or less

4.2 Apparatus

- **HPLC Pump:** Waters Alliance 2695 Separations Module
- **HPLC Column:** (Mac Mod Analytic Cat. No. ACE-111-1503)

Ace 3 C18, 3 micron particle size, 150 x 3.0 mm column with an inline solvent filter
HPLC Column
Mac-Mod Analytic 50-900-01131

- Alternate column: ACE 5 C18 5 micron particle size, 150 x 3.0 mm (Catalog # ACE-121-1503)
- Use of a 5 micron over 3 micron particle size will give a slightly prolonged column life with only minor loss of resolution

- **UV / Photo Diode Array (PDA) Detector:** Waters 996 or 2996 Photodiode Array Detector scanning between 210 and 400 nm at 1.2 nm resolution.

This is used as a confirmatory method for microcystin identification at high concentrations and for quality control of the standards and mass spectrometer.

- **MS Detector:** Waters Micromass ZQ 4000[4]
- **HPLC-MS Computer Software:** Empower™-2 or Empower™-3 Data and Control Software

4.3 HPLC Conditions

- Trifluoroacetic acid-based:[5]

HPLC mobile phase solution (A): Water containing **0.02 % (v/v) TFA** .

HPLC mobile phase solution (B): Acetonitrile containing **0.02 % (v/v) TFA** .

- Formic Acid-based:

HPLC mobile phase solution (A): Water containing **0.1 % (v/v) TFA** .

HPLC mobile phase solution (B): Acetonitrile containing **0.1 % (v/v) TFA** .

Time	Flow (mL/min)	%A	%B
0	0.3	70	30
3	0.3	65	35
18	0.3	15	85
20	0.3	0	100
22	0.3	0	100
23	0.3	70	30
30	0.3	70	30

Table 1: HPLC mobile phase gradient[6]

Typical injection volume is **10 µl** - **20 µl** , **not to exceed 30 µl**, and the sample chamber is held at **7 °C** (±2°). Column temperature is unregulated but should be between **20 °C** - **24 °C** (**Room temperature**). PDA data is collected over the entire **00:30:00** run to monitor for column bleed and equilibration.

4.4 LC-MS Conditions (specific for the Waters MicroMass ZQ4000):

LC-MS conditions are instrument specific and will need to be determined individually for your specific instrument. For illustrative purposes, here are the specific parameters used with a Waters Micromass ZQ4000 mass spectrometer.

Source temperature	150°C
Desolvation gas temperature	350°C
Desolvation gas flow	600 L/hour
Con gas flow	40 L/hour
Capillary voltage	3.25 kV

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Con voltage	65 V
MS Scan range	m/z 650-1250 (see section 7.2)
Scan time	0.3 sec
Interscan Delay	0.1 sec
MS Data Acquisition	0-20 min

Lens and Extractor voltages are tuning dependent and are not specified in this method. They will need to be determined individually on site.

4.5 HPLC Standards

4.5.1 Microcystin standards:

- Primary Standard LR: Microcystin LR, Enzo Life Sciences catalog number ALX-350-012-C100 or equivalent. (100 µg vial); CAS 101043-37-2
- Primary Standard LF: Microcystin LF, Enzo Life Sciences catalog number ALX-350-081-C100 or equivalent. (100 µg vial); CAS 154037-70-4
- Primary Standard RR: Microcystin RR, Enzo Life Sciences catalog number ALX-350-043-C100 or equivalent. (100 µg vial); CAS 111755-37-4
- Primary Standard NOD: Nodularin-R. Enzo Life Sciences catalog number ALX-350-061-C100 or equivalent. (100 µg vial); CAS 118399-22-7
- Quality Control Std: While a quality control standard can be prepared from Enzo Life Sciences catalog number ALX-350-431 (10 µg vial MC-LR) per their instructions, it should ideally be obtained from a second source. Microcystin LR is available from Novakits catalog NRC-CRM-MC (certified 10 µg/mL) as a certified standard or as a 10 µg/ml analytical standard under part number STDMICROLRD.
- Certified reference materials (CRM) are also available directly from NRC Canada. The following CRM have been used since 2019. CRM come with their exact concentrations and should be used without modification.
    1. Microcystin-LR: available from NRC Canada as CRM-MCLR (~10 µg/ml)
    2. Microcystin-RR: available from NRC Canada as CRM-MCRR (~10 µg/ml)

4.5.2 Preparation of the Microcystin-LR stock solution:

The MC-LR primary standard is used to generate MS response factors. It is therefore essential to check the purity of the MC-LR by HPLC-MS and the concentration by UV spectroscopy prior to preparation of any working standards. **1 ml water** is added to the **100 µg primary standard ALX-350-012-C100** to give an approximately **100 Parts per Million (PPM)** solution. A subsample of this solution was diluted **1:10** with **100 % methanol** and the absorbance determined at 239 nm by UV-Vis spectroscopy. The concentration of MC-LR is obtained from Beer's Law using the molar extinction coefficient of 39,800 (Harada et al., 1990); Appendix 2.

$$C = \frac{A}{Eb}$$

where C = concentration in moles/liter, A= absorbance at 239 nm, E= Molar extinction coefficient, and b = path length in cm. The primary standard is labeled with its measured concentration stored at **-20 °C** .

4.5.3 Preparation of Microcystin LR Working Standards:

The stock solution from 4.5.2 above is diluted in **50 % methanol** [7] to give low and high working solutions of 0.2 and 2 ppm. These solutions are stored at **-20 °C** in glass auto-sampler vials equipped with a Teflon™ liner.

- **2 Parts per Million (PPM) standard** is prepared by adding **20 µl MC-LR stock solution** to **0.98 ml solvent**
- **0.2 Parts per Million (PPM) standard** is prepared by diluting the **2 Parts per Million (PPM) standard 1:10** with solvent

4.5.4 Preparation of the Microcystin-LRRF and NOD-R stock and working solution:

The LRRF microcystin standard is used to check the chromatographic performance of the system. MC-RR is one of the first microcystins to elute using the conditions specified in section 4.3, MC-LF is one of the last microcystins to elute, and MC-LR falls near the middle of the chromatographic retention times. Compounds eluting significantly outside of this chromatographic window are unlikely to be microcystins[8].

1 ml water is added to the primary standard **ALX-350-043-C100** and **1 ml methanol** added to **ALX-350-081-C100** to give an approximately **100 µg/ml stock solution** of MC-RR and MC-LF respectively. If desired, the absolute concentrations of these stock solutions can be determined by Beer's Law (see section 4.5.2) using the extinction coefficients in Appendix 2. To prepare the final **2 Parts per Million (PPM) working solution** , **20 µl** of the **100 ppm MC-RR, MC-LR and MC-LF primary standards** are added to **0.94 ml 50% methanol** or **water**.

Nodularin-R is used as an independent check on the chromatographic performance and ionization efficiency of the system. A nominal **100 Parts per Million (PPM) primary standard** is prepared by adding **1 ml water** to **100 µg ALX-350-061-C100** . A nominal **2 Parts per Million (PPM) working standard** is prepared by adding **20 µl** of this **primary standard** to **0.98 ml water** . The primary and working standards are stored at **-20 °C** .

5. General Procedure

5.1 Sample Set construction

A typical sample setup is shown below. Each sample set starts with column rinse to condition the column to the existing mobile phase, followed by an instrument blank using the extraction solvent, the high MC-LR standard, the NOD-R standard, the low MC-LR standard, LRRF standard, and another sample blank.

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Vial	Name	Purpose
1	Rinse	Condition the column to solvents used that day
2	Blank	Determination of column background
3	2 ppm MC-LR	Calibration standard used to generate response factors and ionization efficiency
4	0.2 ppm NOD	Secondary check on mass spectrometer ionization efficiency
5	0.2 ppm MC-LR	Secondary check on minimum detection limit
6	2 ppm LRRF	Determination of the chromatographic retention time window
7	Blank	Ensures that the column is not contaminated and monitors for injector carryover.
10-29	Unknowns	
2	Blank	Determination of column background
3	2 ppm MC-LR	Calibration standard used to generate response factors, IDL and ionization efficiency
4	0.2 ppm NOD	Secondary check on mass spectrometer ionization efficiency and IDL
30-49	Unknowns	
2	Blank	Determination of column background
3	2 ppm MC-LR	Calibration standard used to generate response factors and ionization efficiency
4	0.2 ppm NOD	Secondary check on mass spectrometer ionization efficiency and IDL
50-69	Unknowns	
Continue as needed. The last six runs of all samplet sets are given below		
2	Blank	Determination of column background
3	2 ppm MC-LR	Calibration standard used to generate response factors and ionization efficiency
4	0.2 ppm NOD	Secondary check on mass spectrometer ionization efficiency
5	0.2 ppm MC-LR	Secondary check on minimum detection limit
6	2 ppm LRRF	Determination of the chromatographic retention time window
7	Blank	Ensures that the column is not contaminated and monitors for injector carryover.
Shut down protocol		

Table 2. Representative sample set layout

5.2 Method Set construction

The LCMS system provides three continuous data channels; the cumulative PDA absorbance channel, the total ion trace from the mass spectrometer, and the pump pressure at the column head. Pump pressure is used for system troubleshooting and will not be discussed further.

The PDA data channel is collected continuously between **0-30 min** (including the column equilibration time) and provides information on column contamination and the microcystins present in the sample. The derived microcystin chromatograph is obtained by extracting the channel at 239 nm. No correction is made for those microcystins with a 232 absorbance maximum. (Appendix 2)

The derived ion channels (EIC) are obtained by extracting the ion current at the appropriate mass with a peak separation of **0.5 amu**. These masses correspond to the protonated molecular ion $[MH^+]$ or sodium adduct $[MNa^+]$ of the microcystin (Appendix 1) and are used for quantitation of the individual toxins. For hydrophobic microcystins (LA and greater), the sodium adduct may become increasing abundant in these samples. For those microcystins, the EIC consists of the sum of the parent protonated molecular ion $[MH]^{+1}$ and the sodium adduct of the parent compound $[M+Na]^{+1}$ (Appendix 1).

A typical LC-MS trace is shown below:

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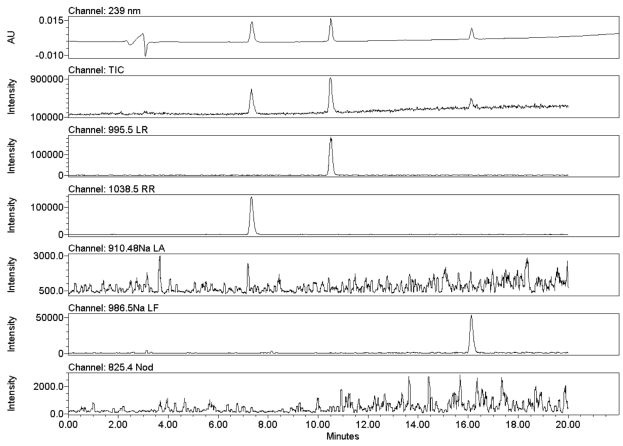

Figure 1. Representative LCMS method chromatogram for an LRRF standard containing ~1 ppm MC-LR and MC-RR, and 0.5 ppm MC-LF. The top line is the PDA trace at 239 nm, second trace is the total ion current (TIC), third trace is the *m/z* 995.5 EIC (MC-LR), fourth trace is the *m/z* 1038.5 EIC (MC-RR), fifth trace is the *m/z* 910.4 EIC (MC-LA plus sodium adduct), six trace is the *m/z* 986.5 EIC (MC-LF plus sodium adduct), and the bottom trace is the *m/z* 825.4 EIC (NOD-R). NOD and MC-LA were not present in this sample. The method set auto-scales all traces so that the largest peak is full scale. The NOD and MC-LA channel are expanded approximately 50-100-fold relative the MC-LR EIC and an example of channel noise.

5.3 Calculation of Results

5.3.1 Determination of the sample set response factors:

The response factor is expressed in terms of µg MC-LR_{equiv} per peak area. It is calculated by taking the concentration of the standard (usually **100 µg MC-LR**) in parts per million (ppm), multiplying by the milliliters of standard injected on column, and dividing by the area of the standard peak:

$$\frac{concentration\ of\ standard\ in\ \frac{\mu g}{mL} \cdot \frac{\mu L\ of\ sample\ injected}{1000}}{area\ of\ standard\ peak} = response\ factor$$

MC-LR response factors are determined for all MC-LR standards in a given sample set. An average sample set MC-LR response factor is obtained by averaging the individual response factors. Coefficient of variation across the sample set for the MC-LR response factor should be less than 5% and must be less than 15%. Variations of greater than 10% are indicative that either the standard has gone bad, or that the ionization efficiency and dropped over the course of the sample set (see section 5.3.2).

To determine the response factor in MC-LR_{equiv} using congeners other than MC-LR, the individual toxin response factor is determined as described above and then corrected to MC-LR_{equiv} by multiplying by the ratio of the molecular weight of MC-LR divided by the individual congener molecular weight (see section 5.3.4). Coefficient of variation across the sample set for the MC-LR_{equiv} response factor when including all microcystin congeners in the average should be less 15%.

Nodularin represents a special case. It is generally run at a lower concentration closer to the instrument detection limit. Its response factor is calculated separately and not included in the average MC-LR_{equiv} response factor. It provides an independent measure of the sample ionization efficiency, IDL, and stability of the analytical standards over the course of the analytical run (section 6.3).

5.3.2 Calculation of the MC-LR ionization efficiency:

The MC-LR ionization efficiency is calculated using the area of EIC channel at *m/z* 995.5 divided by the area of the PDA channel at 239 nm. It provides a repeated measurement of sample cone occlusion, a leading cause of decreased sensitivity in samples without SPE clean-up. The calculated MC-LR ionization efficiency is instrument dependent and must be individually determined for a given mass spectrometer and PDA detector. It is not dependent on the standard concentration or microcystin congener (±10%) provided you can measure the area in the PDA trace and EIC trace with similar precision.

MC-LR ionization efficiency is determined for all MC-LR standards in the sample set and compared to the maximum ionization efficiency obtained using a clean and freshly calibrated source (e.g. ZQ4000, the observed ratio is ~60). When the ionization efficiency of the standard drops to 25% of the maximum value (e.g. 15), the run should be aborted, the sample cone cleaned and the any run after the last satisfactory standard rerun.

The nodularin ionization efficiency is calculated separately and provides an independent check on the sample cone cleanliness and performance of the mass spectrometer.

5.3.3 Calculation of instrument detection limits (IDL):

Method 1: The system (HPLC+PDA+MS) IDL is calculated by multiplying the MC-LR_{equiv} response factor by 3 times the standard deviation of 7 peak areas from 7 replicate injections of the microcystin-LR standard at a concentration near the detection limit (Wisconsin Department of Natural Resources, 1996). It is expressed in terms of micrograms on-column.

$$Response\ factor\ \left(\frac{\mu g}{area}\right) \cdot 3 \cdot Standard\ deviation\ (area) = Detection\ limit\ in\ \mu g$$

Method 2: IDL's can also be calculated using an empirical approach rather than a statistical approach. This approach is often more suitable when dealing with multiple channels of data and samples that have differing degrees of background interference and noise.

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Here, the analyst constantly monitors the output of the instrument and empirically decides on a minimum peak area they would be confident in identifying as a peak. The individual congener IDL is then calculated from the minimum peak area and MC-LR_{equiv} response factor using the following equation.

$$Response\ factor\ \left(\frac{\mu g}{area}\right) \cdot minimum\ observable\ peak\ area = Detection\ limit\ in\ \mu g$$

Instrument detection limits are instrument specific and should be re-measured any time there is a significant change in the instrument performance or background sample matrix. IDL's calculated using methods 1 and 2 should be in agreement with each other. If method 2 is used to determine the IDL, then the calculated MDL becomes the practical limit of quantitation (LOQ) for the sample.

5.3.4 Calculation of individual sample method detection limits (MDL):

While IDLs are instrument specific and generally consistent for an instrument in good operating condition, method detection limits (MDL) are very sample specific and must be determined and reported for each individual sample. MDL are expressed in terms of µg per liter of starting sample, e.g. liter of lake water.

The sample MDL are determined using the IDL determined above and correcting for the injection volume, sample volume filtered or lyophilized and the extraction volume. The basic calculation is illustrated below.

$$\frac{IDL\ (\mu g\ on\ column)}{injection\ volume\ (ml)} \cdot \frac{extraction\ volume\ (ml)}{volume\ filtered\ (ml)} \cdot 1000 = sample\ MDL\ (\mu g/l)$$

5.3.5 Requirements for positive identification of microcystin in a sample:

The identification of a peak in the corresponding EIC requires the following.

(1) Presence of a peak with a retention time within 5% expected retention time based on Appendix or its updated modification. Care must be taken that many of the molecular weights corresponding to a molecular ion are present as other compounds. It is essential that the retention time of the peak be at the appropriate time.

(2) Presence of a molecular ion at the predicted mass to charge ratio if it has been previously found in NYS samples (Appendix 1) or in Spoof and Catherine (2017), Appendix 3 for novel microcystins. The requirements to add a novel microcystin to Appendix 1 requires confirmation of the retention time with either a known standard or a sample of sufficient concentration where a clean PDA spectrum can be obtained (see 3 below).

(3) For samples with greater than **10 ng microcystin** injected on column, the presence of an appropriate 232 nm or 239 nm signature in the PDA trace must accompany the presence of the molecular ion. This becomes more significant with on column amounts greater than **50 ng** , and becomes more unreliable for samples under **10 ng** as the amount of interference due to the matrix increases.

(4) If available, additional information such as high resolution exact mass measurements or MS/MS fragmentation may be used to help determine the specific congeners. These should be used with caution as exact mass measurements only provide the empirical formula and microcystins with the same empirical formula may elute with similar retention times. Tandem mass spectrometer fragmentation patterns (MS/MS) may or may not distinguish between closely related congeners. When in doubt, the operator should label the congener with the known information (microcystin methyl ester) and not indicate the specific amino acid modified without confirming information.

5.3.6 Calculation of individual sample results

Total concentration of microcystins in the sample is obtained by summing across the individual congener concentrations after conversion to MC-LR_{equiv}. This is usually done is a two-step process.

(A)

$$\frac{Area\ Congener\ 1}{injection\ volume\ (ml)} \cdot MCLR\ response\ factor\ \left(\frac{ug}{area}\right) \cdot \frac{MW\ MC{-}LR}{MW\ Congener} = congener\ conc.\ in\ voucher\ \left(\frac{as\ ug\ LRequiv}{ml}\right)$$

(B)

$$Sum\ of\ all\ congeners\ \left(\frac{\mu g}{ml}\right) \cdot \frac{extraction\ volume\ (ml)}{volume\ filtered\ (ml)} \cdot 1000 = total\ MCs\ (\mu g/l)$$

5.3.7 Expression of results

Individual sample results should be expressed in terms of µg MC-LR equivalents per liter, and reported along with the number of analytic runs used to determine the concentrations and the individual sample MDL calculated using the MC-LR response factor for that sample set.

6. Quality Control Considerations

6.1 Column equilibration

The first run of each day is the column rinse. It is used to condition the column after the system has been shut down or to recondition the column after running solvents have been changed or replenished. Each HPLC run includes a **00:02:00 flush** with **100% B** (time 20-22 min) followed by a **00:07:00 re-equilibration** (time 23-30 min) in the starting solvent. This corresponds to 4 column volumes and has been shown to be sufficient for the columns specified in section 4.2. Changing to a larger column or lower flow rate will require an increase in the re-equilibration time[9]. Changing the starting mobile phase percentage (increasing %A) may also affect the column re-equilibration and require additional time.

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6.2 Instrument Blanks

The second run of each day is the extraction blank. It confirms that there are no residual microcystins absorbed to the column. Extraction blanks are run every 20 runs and after every series of standards to ensure that there is no carryover from the injector. For unknown samples that are weakly positive and run immediately after an extremely positive sample, it may be necessary to rerun those samples following an instrument blank to ensure there is no sample carryover. Instrument blanks must be 100% negative in this method. Any positive response in the instrument blank requires that the entire sample set be discarded and rerun.

True matrix blanks are not part of this method since the matrix changes for every sample. However, given that a large fraction of the samples will be blank, the analyst should be alert to contamination when there are a continuing series of weakly positive samples. When in doubt, the sample order should be scrambled and rerun. Nascent voucher samples are always retained at **-80 °C** as part of the extraction sample. It may be necessary to discard the current HPLC vial and retrieve a new vial if sample contamination is probable.

6.3 Determination of ionization efficiency

At the start of each season, preferably after the mass spectrometer has been serviced by factory authorized personnel, an MC-LR high standard in water is run using the conditions described in section 4.3. The area of the peak in the extracted ion channel at m/z 995.5 is measured along with the corresponding peak in the 239 nm channel from the PDA spectrum (section 5.3.2). The ratio of MS peak area to PDA peak area is a very important diagnostic as these samples are run without SPE cleanup. Cleanliness of the sample cone is critical and will dramatically affect the method detection limit. Once the MC-LR ionization efficiency has dropped to 25% of the maximum value obtained with a cleaned source, the run should be aborted and the sample cone cleaned. Individual operators may decide it is time-efficient to pre-emptively clean the cone at the start of a sample set. However, LR ionization efficiency should always be measured at the start and end of each run and after every 20 unknown samples to ensure that conditions have not changed during the sample set.

Determination of ionization efficiency also provides a mechanism to matrix effects on the ionization of MC-LR in natural samples without standard addition. Many of the samples will have natural levels of MC-LR at sufficient concentration to get measurable peak areas in the 239 nm and 995.5 EIC trace. In these cases, the unknown sample MC- LR ionization efficiency can be used to estimate the effect of the natural matrix on microcystin ionization. The ionization efficiency in the unknown should be very similar to the ionization efficiency in the standards. There are two reasons for decreased ionization efficiency in unknown samples. (1) There is a very high concentration of microcystin in the voucher sample and you have started to saturate the MS detector. The PDA detector is linear over a much greater concentration range than the MS detector. (2) There may be other microcystins present that are contributing to the PDA absorbance, but not the *m/z* 996.5 EIC trace. A common example of this is the presence of demethyl MC-LR, which has virtually identical PDA signature and retention time to MC-LR, but whose molecular ion is *m/z* 981.5, not *m/z* 996.5.

We recommend including a secondary standard (NOD-R) at the start and end of each sample set as an independent determination of ionization efficiency. If questions arise, NOD-R can be added directly to the sample matrix as an independent determination of ionization efficiency.

6.4 Identification of microcystin in a sample

The identification of a microcystin in an unknown sample always requires at least two independent pieces of evidence. These include:

(1) The presence of a peak in the extracted ion chromatogram corresponding to the molecular ion of the suspect microcystins.

(2) The peak must have a retention time within 5% expected retention time of known microcystins in New York State based on Appendix 1 or its updated modification.

(3) For peak whose molecular ion corresponds to microcystins in Spoof and Catherine, (2017), but which have not been previously found in NYS samples (Appendix 1) so that the retention time is known, the retention time must be determined using a either a known standard or a sample of sufficient concentration where a clean PDA spectrum, and/or LC-MS/MS fragmentation pattern can be obtained (see section 5.3.5).

(4) For samples with greater than **10 ng** - **50 ng microcystin** injected on column, the presence of an appropriate 232 nm or 239 nm signature in the PDA trace must accompany the peak in the EIC trace. Peaks without the appropriate PDA spectrum should not be considered as microcystins. This becomes more and more unreliable as the concentration of microcystin injected on column decreases and amount of interference due to the matrix increases.

6.5 Quality control standards to ensure accurate quantitation

Certified reference materials are now available from NRC Canada for MC-LR and MC-RR. For samples with low sample through-put, or who only run samples occasionally, it may be appropriate to use the certified standards directly. However, higher through-put analytical labs will want to prepare their own standards per section 4.2.

Certified microcystin standards should be run at least once per month to check the concentrations of the working standard. These certified standards are sufficient high in concentration (**10 µg/ml**) that the PDA 238 nm trace can be used to calculate a PDA response factor to recalibrate the working standards if necessary. Once that PDA response factor is known, it can be compared to the daily MC-LR standards to rapidly identify when the secondary standard concentration is suspect.

The concentration of working standards should always be checked after an extended period in the auto-sampler even at **4 °C** , if the sample compartment is allowed to warm up due to instrument failure or unintended shutdown, or there have been changes to the PDA detector such as replacement of the deuterium lamp.

6.6 Linearity

This method uses a two-point calibration curve updated every 20 samples rather than a more detailed calibration curve run less frequently. Response factors calculated from this calibration are equal to the slope of a linear calibration curve. Linearity of the calibration curve is a function of the individual mass spectrometer used for the analysis. To ensure that you are operating in the

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linear range of the mass spectrometer, it is required that you run a linearity curve after any major changes to the instrument and after the instrument has sat idle for an extended period of time. This includes after factory required servicing and preventative maintenance. Linearity curves for the ZQ-4000 for MC-LR are given in Appendix 3. Injections > **150 ng MC-LR** on-column will show 10 deviations from linearity approaching 10% and should be diluted and rerun[10].

6.7 System Calibration and preventative maintenance

The mass spectrometer should be calibrated by factory authorized personnel on at the start of each sampling season. This calibration needs to include a calibration at the appropriate scan speeds e.g. **2000 amu/sec** as specified in section 4.4. The molecular weight of the microcystin standards should be recorded and any deviations greater than **0.25 amu** (0.025%) noted as this may affect the unit mass window and require adjustment of the ion window in the method set. In these cases, the sample should be reintegrated using the new parameters. Deviations <10% can be ignored.
For systems used for high through-put samples (e.g. >5,000 injections per season), it is recommended that the mass spectrometer undergo yearly preventative maintenance including but not limited to the cleaning of the ion source and quadrupole.

7. Other considerations

7.1 Appropriate sample matrices

The method here is designed to give both untargeted (PDA and TIC) and targeted (EIC) analysis of microcystins in water samples, cyanobacteria biomass and filtrates. This system has been validated with cyanobacteria biomass, biomass on glass fiber filters, and raw water samples. Extraction of microcystins using from these matrices using 50% acidified methanol and ultrasound gives 100% recovery of added MC-RR, LR, LA, LF and NOD-R (Boyer, 2007). Use of solid phase extraction is not required for clean-up to reduce matrix suppression in recreational water samples. Laboratory fortified matrix spike samples are therefor not required as the same information can be obtained using a combination of the measured ionization efficiency (section 6.3) of the MC-LR standards and any naturally occurring MC-LR in unknown samples.

This approach is not appropriate for use with fish tissues, soils, and sediment samples. These sample matrices require SPE clean-up to reduce matrix interference when microcystin concentrations are low and use of an internal standard to determine sample recovery and potential matrix suppression (Schmidt et al., 2013; Smith and Boyer, 2009). The use of single quadrupole LC-MS as described here leads to unacceptable high sample MDL and the overestimation of microcystins at low toxin concentrations. Better techniques using LC-MS/MS exist for these matrices.

7.2 Tuning for Microcystin RR

MC-RR and other Arg-Arg containing microcystins such MC-dRR tend to ionize as the diprotonated molecular ion $[M+2H]^{+2}$. This causes the molecular ion to appear at a m/z corresponding to half of the molecular weight; *m/z* 519.8 versus *m/z* 1038.5. The MS conditions in section 4.4 do not scan below *m/z* 650 to minimize background ions in the TIC due to lower molecular weight compounds. To ensure that MC-RR is not underestimated in the *m/z* 1038 EIC, it is necessary to detune the MS slightly to maximize the 1038 signal relative to the 519 signal. This results in a slight decrease in sensitivity for MC-RR, but gives a response factor nearly identical to that obtained for MC-LR and MC-LF, and allows the use of a single response curve for all the different congeners.

As a quality control consideration, the operator should occasionally scale down to **500 amu** to ensure that the MC-RR is not ionizing as the di protonated molecular ion. If this problem consistently occurs and you need higher accuracy for the RR-containing congeners, then you will need to run a separate standard curve for these compounds.
A certified CRM is available for microcystin-RR from NRC Canada (section 4.5.1).

7.3 Choice of Mobile Phase

The method was developed using [M]**0.2 % TFA** as the acidic modifier in the mobile phase. In subsequent studies, we recognized that a 2-fold increase in sensitivity could be achieved by the elimination of TFA suppression (Eshraghi and Chowdhury, 1993) and switching to [M]**0.1 % formic acidic** as the acidic modifier. This change had little to no impact on the resolution and consistency of the retention times. With dedicated columns and careful HPLC maintenance, we observe less than 1% variation in the microcystin retention times over a 4-6 month period.

We also investigated the use of ammonium salt (acetate or formate) mobile phases. The slight increase in sensitive observed using these mobile phases for MC-LR, was counteracted by the difficulty in preparing the mobile phase reproducibly, the inherent instability of these mobile phase during multiday sample sets, and variable ionization efficiency of ammonium-based mobile phases with the different congeners. For these reason, these ammonium based mobile phases are not recommended for this application.

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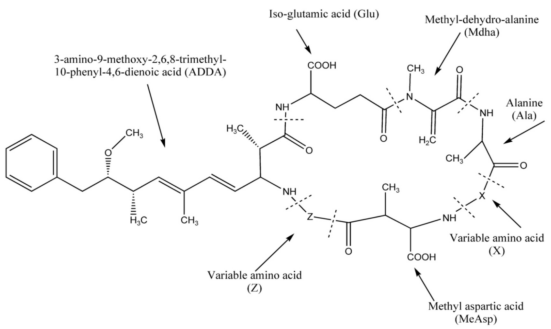
Appendix 1: Name, Retention Time, Molecular Ions and PDA Pattern for Microcystins Currently Measured Using this Method.



All microcystins consist of the generalized structure cyclo(-D-Ala[1]-X[2]-D-MeAsp[3]-Y[4]-Adda[5]-D- Glu[6]-Mdha[7]-). X and Y are variable L-

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amino acids. Demethyl derivatives occur at Mdha, MeAsp and ADDA residues. These cannot be differentiated by LCMS and require LC-MS/MS or knowledge of their retention time.

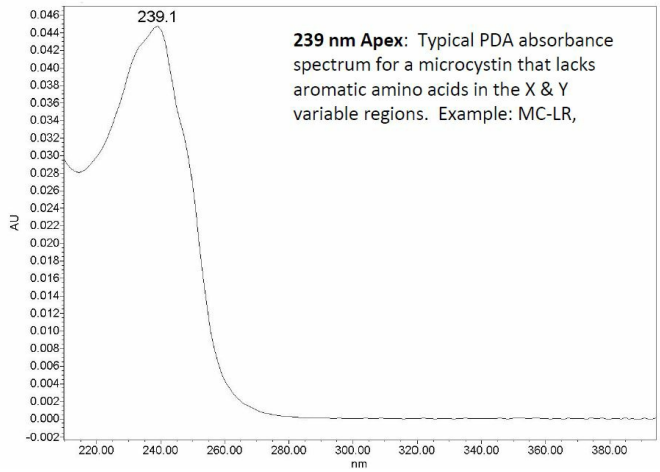
Microcystin	Retention Time (Minutes)	Observed Mass to Charge Ratio (m/z)	Calculated Exact mass (MH+)	PDA Absorbance pattern
MC-dRR*	8.2	1024.5	1024.5574	239 nm
MC-RR	8.3	1038.5	1038.5731	239 nm
MC-mRR	8.3	1052.4	1052.5524	239 nm
MC-h4YR	9.6	1049.5	1049.5666	239 nm
MC-hYR	10.4	1059.5	1059.5510	239 nm
MC-YR	10.6	1045.5	1045.5353	232 nm
MC-dLR	10.9	981.5	981.5404	239 nm
MC-LR	11.0	995.5	995.5561	239 nm
MC-AR	11.3	953.5	953.5091	239 nm
MC-mLR	11.5	1009.5	1009.5717	239 nm
MC-WR	11.7	1068.5	1068.5513	224 nm
MC-FR	12.2	1029.5	1029.5404	232nm
MC-meLR	12.5	1009.5	1009.5717	239 nm
MC-dLA + Na	13.9	896.5 + 918.5	896.4764	239 nm
MC-LA + Na	13.9	910.5 + 932.5	910.4921	239 nm
MC-LY + Na	16.4	1002.5 + 1024.5	1002.5182	232 nm
MC-mLA + Na	14.8	924.6 + 946.6	925.5077	239 nm
MC-LL+ Na	16.0	952.5 + 974.5	952.5390	239 nm
MC-LF + Na	16.5	986.5 + 1008.5	986.5234	232 nm
MC-LW + Na		1025.5 + 1047.5	1025.5343	224 nm
MC-zLR	11.0	995.5	995.5561	-
nodularin-R	9.4	825.4	825.4511	232/239 nm (11)

Appendix 2: Typical PDA Absorbance Pattern for Microcystins with (232 pattern) or without (239 pattern) Aromatic Residues in the Variable Regions.

Microcystins Extinction Coefficients available in the literature:

Microcystin	Molar Extinction Coefficient (E)	Reference
MC-LR	39800	(Harada et al., 1990)
MC-LR	36500 in MeOH	(Honkanen et al., 1990)
MC-LR	30900	(Sano et al., 2008)
MC-YR	38100 and 41100	(Blom et al., 2001)
MC-RR	39800	(Harada et al., 1990)
MC-LA	36500	Unpublished Carmichael*
MC-LA	36500	(Backer et al., 2010)
D-Asp3-LR	31600	(Harada et al., 1990)
D-Asp3, E-Dhb7 MC-RR	50400	(Blom et al., 2001)
[Dha 7] MC-LR	46800	(Harada et al., 1990)
NOD	35500	Carmichael and Brittain unpubl*

*as cited in Determination and Significance of Emerging Algal Toxins (Nicholson et al., 2007)



239 nm Apex: Typical PDA absorbance spectrum for a microcystin that lacks aromatic amino acids in the X & Y variable regions. Example: MC-LR,

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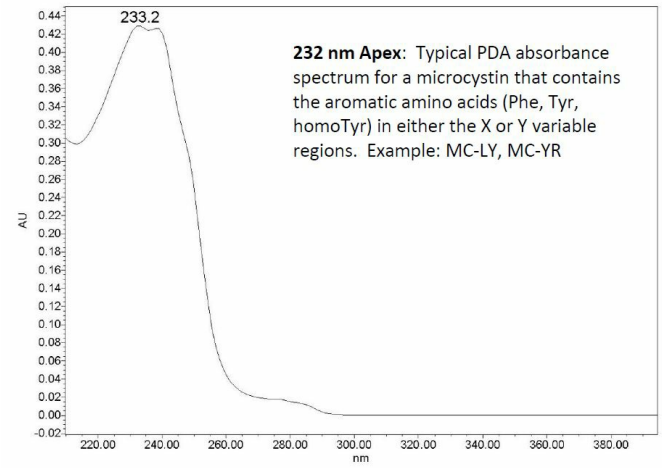
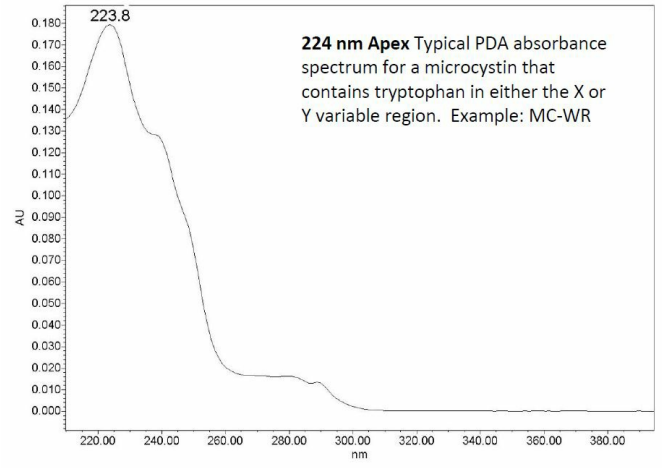
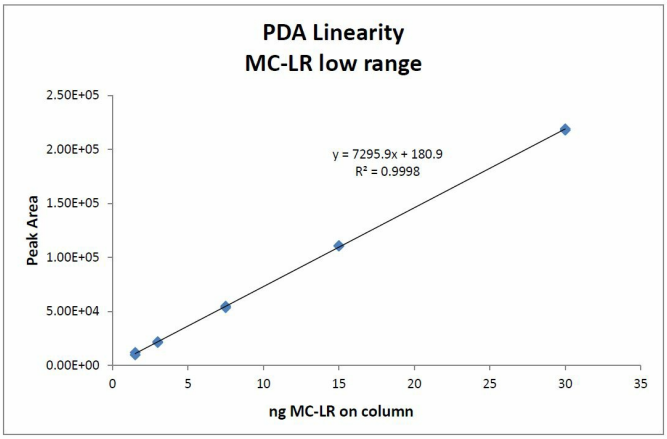




Appendix 3: Linearity Curves and Deviations from Linearity for the ZQ4000



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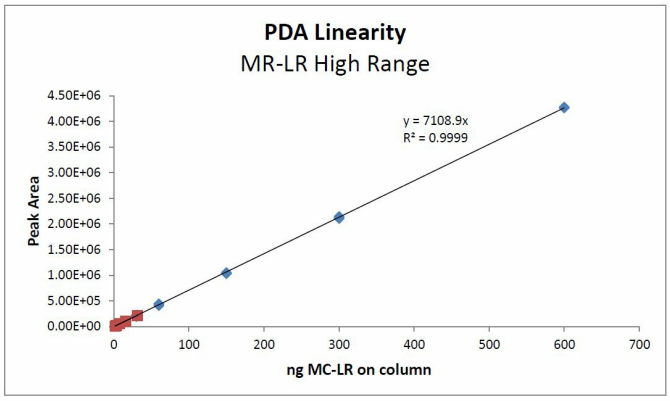

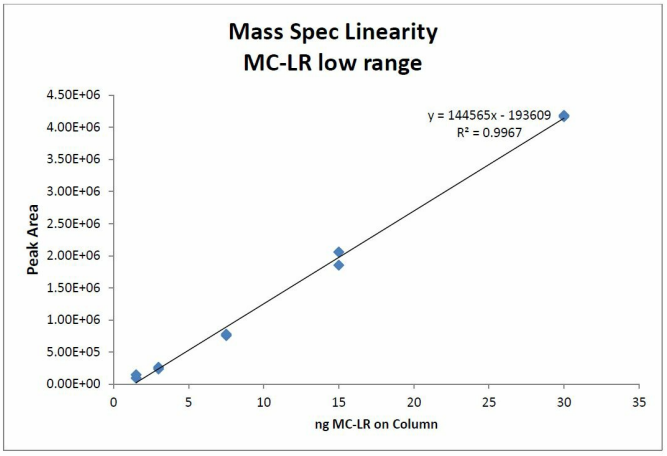

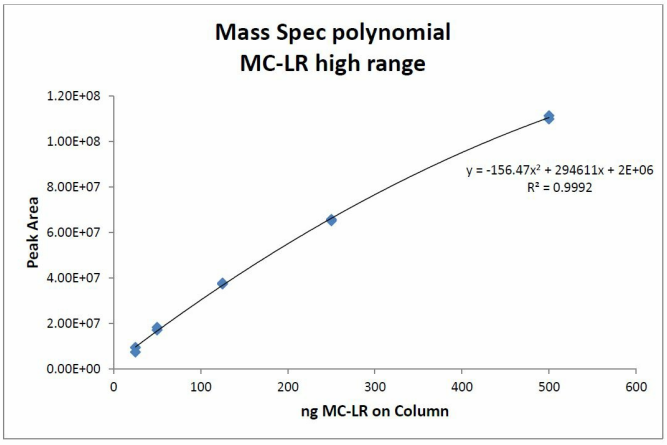

Appendix 4: Commercial sources of microcystin congeners

Certified analytical standards are currently only available for microcystin LR, RR and NOD-R. Other microcystin congeners are available in sufficient purity to confirm their retention time and molecular ion. Users should use care and check the quality of the analytical standard by LC-MS, LC-MS/MS and NMR if applicable as several commercial suppliers have provided mislabeled or mixtures of microcystins.

Congener	Source	Catalog number	Available sizes	Form
MC-dRR [Asp3]	Enzo Life Sciences	ALX-350-168	25, 100 µg	Dry residue on vial
MC-dRR	Novakits	STDDMMCRR5	5 µg	1 ml liquid
MC-dLR [Asp3]	Enzo Life Sciences	ALX-350-173	25, 100 µg	Dry residue on vial
MC-dLR [Asp3]	Novakits	STDDMMCLR5	5 µg	1 ml liquid
MC-dLR [Dha7]	Novakits	NRC-CRM-DMC	5 µg	0.5 ml liquid
MC-RR	Enzo Life Sciences	ALX-350-043	50,100, 500, 1000 µg	Dry residue on vial
MC-RR	Novakits	STDMICRORRD	10 µg	1 ml liquid
MC-RR*	Cyano Biotech	MCRR-a	10 µg	1 ml liquid
MC-RR std	Enzo Life Sciences	ALX-350-432	10 µg	Dry residue on vial
MC-RR std*	Novakits	NRC-CRM- MCRR	10 µg/ml	0.5 ml liquid
MC-hIR	Enzo Life Sciences	ALX-350-177	25 µg	Dry residue on vial

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MC-hYR	Enzo Life Sciences	ALX-350-174	25, 100 µg	Dry residue on vial
MC-LA	Enzo Life Sciences	ALX-350-096	25, 100, 1000 µg	Dry residue on vial
MC-LA	Novakits	STDMICROLAD	10 µg	1 ml liquid
MC-LF	Enzo Life Sciences	ALX-350-081	25, 100, 1000 µg	Dry residue on vial
MC-LF	Novakits	STDMICROLFD	5 µg	1 ml liquid
MC-LF	Cyano Biotech	MCLF-a	7.5 µg	1 ml liquid
MC-LR	Enzo Life Sciences	ALX-350-012	100, 500, 1000 µg	Dry residue on vial
MC-LR	Novakits	STDMICROLRD	10 µg	1 ml liquid
MC-LR	Cyano Biotech	MCLR-a	10 µg	1 ml liquid
MC-LR std	Enzo Life Sciences	ALX-350-431	10 µg	Dry residue on vial
MC-LR std*	Novakits	NRC-CRM- MCLR	10 µg/mL	0.5 ml liquid
MC-LY	Enzo Life Sciences	ALX-350-148	25, 100, 1000 µg	Dry residue on vial
MC-LY	Novakits	STDMICROLYD	5 µg	1 ml liquid
MC-LY	Cyano Biotech	MCLY-a	7.5 µg	1 ml liquid
MC-LW	Enzo Life Sciences	ALX-350-080	25, 100, 1000 µg	Dry residue on vial
MC-LW	Novakits	STDMICROLWD	5 µg	1 ml liquid
MC-LW	Cyano Biotech	MCLW-a	7.5 µg	1 ml liquid
MC-YR	Enzo Life Sciences	ALX-350-044	25, 100 µg	Dry residue on vial
MC-YR	Novakits	STDMICROLWD	10 µg	1 ml liquid
MC-YR	Cyano Biotech	MCYR-a	10 µg	1 ml liquid
MC-WR	Enzo Life Sciences	ALX-350-167	25, 100 µg	Dry residue on vial
Nod-R	Enzo Life Sciences	ALX-350-061	100, 250, 1000 µg	Dry residue on vial
NOD-R	Novakits	STDNODULD	10 µg	1 ml liquid
NOD-R std*	Novakits	NRC-CRM- NODR	12 µg/ml	0.5 ml liquid
Nodularin-linear	Enzo Life Sciences	ALX-350-182	100 µg	Dry residue on vial

*Certified reference material provided by NRC Canada
Updated August 2019

Footnotes

[1] Approximately 20% of the known microcystins and 40% of the nodularins contain modifications to the ADDA group. Even ELISA assays directed against this"constant" aminoacid can be highly variable in their response to different congeners.

[2] This method is routinely run using building distilled water that was further polished using aBarnstead Ultrapure 4 cartridge system to give true R18 grade water (Ω>17.8). Laboratorieswithout access to high quality analytical grade water will need to purchase HPLC grade water.

[3] Use of a suitable grade Acetonitrile is essential for this method. After switching to PHARMCO-AAPER from another major supplier, our retention times have varied by less than 0.2% over a two-year period.

[4] This method has also been validated using a Water's Micromass ZQ2000 and in MS1 and MS2 mode using a Water's TQD.

[5] This method was initially developed and validated using 0.02% TFA as the solvent modifier. A suitable replacement is 0.1% formic acid in water and in acetonitrile. The use of ammonium formate as the solvent modifier may or may not give better sensitivity but should be avoided due to difficulties in (a) reproducible making up the solvent systems leading to more variable retention times and (b) difficulties with the formation of ammonium adducts to the molecular ion that made tuning the instrument to give equivalent ionization efficiencies for the different congeners more difficult.

[6] These conditions were developed for use with a Waters Alliance 2695 HPLC system. Slight modifications are needed for use with other systems such as a UPLC or Agilent 1100/1200 system.

[7] Hyenstrand et al (2001) has shown that low concentrations of microcystins in water readily absorbed to plastic labware including disposable polypropylene pipette tips. This absorption can be blocked by preparing standards as methanol solutions.

[8] Even highly modified microcystins such as the ML-LR glutathione derivative (RT ~ 8 min) fall within this window generated by MC-RR and MC-LF.

[9] Adequate column re-equilibration times are essential to achieve retention time consistency of less than 1%. Use of a 3 mm column diameter will decrease your re-equilibration time. The required re-equilibration time may need to be determined experimentally on you particular HPLC system.

[10] A concentration of 150 ng microcystin on column would correspond to 750 ug/L lake concentrations using standard extraction and run conditions. These levels would clearly be above any safe guideline values and the minor deviations from linearity may be inconsequential to the final conclusions.

[11] The UV spectrum of nodularin-R is similar to the 232 nm pattern but distinctive with 238 nm apex slightly greater than the 232 nm apex.

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