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Natural product discovery with LC-MS/MS diagnostic fragmentation filtering: application for microcystin analysis

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Corresponding Author:	Justin B Renaud Agriculture and Agri-Food Canada London, Ontario CANADA
Corresponding Author's Institution:	Agriculture and Agri-Food Canada
Corresponding Author E-Mail:	justin.renaud@canada.ca
Order of Authors:	David R McMullin
	Shawn Hoogstra
	Kimberlynn P McDonald
	Mark W Sumarah
	Justin B Renaud
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TITLE:

Natural Product Discovery with LC-MS/MS Diagnostic Fragmentation Filtering: Application for Microcystin Analysis

AUTHORS & AFFILIATIONS:

David R. McMullin[1], Shawn Hoogstra[2], Kimberlynn P. McDonald[1], Mark W. Sumarah[1,2], Justin B Renaud[2]

[1]Department of Chemistry, Carleton University Ottawa, Ontario, Canada

[2]London Research and Development Center, Agriculture and Agri-Food Canada, London, Canada

Corresponding Author:

Justin B. Renaud

Email Address: Justin.renaud@canada.ca

Email Addresses of Co-authors:

David R. McMullin (david.mcmullin@carleton.ca)

Shawn Hoogstra (shoogstr@uwo.ca)

Kimberlynn P. McDonald (kimberlynnMcDonald@cmail.carleton.ca)

Mark W. Sumarah (mark.sumarah@canada.ca)

KEYWORDS:

MZmine, LC-MS/MS, semi-targeted analysis, data-dependent acquisition, natural product discovery, Orbitrap, microcystins

SHORT ABSTRACT:

Diagnostic fragmentation filtering, implemented into MZmine, is an elegant, post-acquisition approach to screen LC-MS/MS datasets for entire classes of both known and unknown natural products. This tool searches MS/MS spectra for product ions and/or neutral losses that the analyst has defined as being diagnostic for the entire class of compounds.

LONG ABSTRACT:

Natural products are often biosynthesized as mixtures of structurally similar compounds, rather than a single compound. Due to their common structural features, many compounds within the same class undergo similar MS/MS fragmentation and have several identical product ions and/or neutral losses. The purpose of diagnostic fragmentation filtering (DFF) is to efficiently detect all compounds of a given class in a complex extract by screening non-targeted LC-MS/MS datasets for MS/MS spectra that contain class specific product ions and/or neutral losses. This method is based on a DFF module implemented within the open-source MZmine platform that requires sample extracts be analyzed by data-dependent acquisition on a high-resolution mass spectrometer such as quadrupole Orbitrap or quadrupole time-of-flight mass analyzers. The main limitation of this approach is the analyst must first define which product ions and/or neutral losses are specific for the targeted class of natural products. DFF allows for the subsequent discovery of all related natural products within a complex sample, including new compounds. In this work, we demonstrate the effectiveness of DFF by screening extracts of *Microcystis*

aeruginosa, a prominent harmful algal bloom causing cyanobacteria, for the production of microcystins.

INTRODUCTION:

Tandem mass spectrometry (MS/MS) is a widely used mass spectrometry method that involves isolating a precursor ion and inducing fragmentation *via* application of activation energy such as collision induced dissociation (CID)[1]. The manner in which an ion fragments is intimately linked to its molecular structure. Natural products are often biosynthesized as mixtures of structurally similar compounds rather than as a single unique chemical[2]. As such, structurally related compounds that are part of the same biosynthetic class often share key MS/MS fragmentation characteristics, including shared product ions and/or neutral losses. The ability to screen complex samples for compounds that possess class-specific product ions and/or neutral losses is a powerful strategy to detect entire classes of compounds, potentially leading to the discovery of new natural products[3-6]. For decades, mass spectrometry methods such as neutral loss scanning and precursor ion scanning performed on low resolution instruments have allowed ions with the same neutral loss or product ions to be detected. However, the specific ions or transitions needed to be defined prior to performing the experiments. As high-resolution mass spectrometers have become more popular in research laboratories, complex samples are now commonly screened using non-targeted, data-dependent acquisition (DDA) methods. In contrast to traditional neutral loss and precursor ion scanning, structurally related compounds can be identified by post-acquisition analysis[7]. In this work, we demonstrate a strategy we have developed termed diagnostic fragmentation filtering (DFF)[5,6], a straight-forward and user-friendly approach to detect entire classes of compounds within complex matrices. This DFF module has been implemented into the open-source, MZmine 2 platform and available by downloading MZmine 2.38 or newer releases. DFF allows users to efficiently screen DDA datasets for MS/MS spectra which contain product ion(s) and/or neutral loss(es) that are diagnostic for entire classes of compounds. A limitation of DFF is characteristic product ions and/or neutral losses for a class of compounds must be defined by the analyst.

For example, each of the more than 60 different fumonisin mycotoxins identified[8,9] possess a tricarballylic side chain, that generates a *m/z* 157.0142 ($C_6H_5O_5^-$) product ion upon fragmentation of the $[M-H]^-$ ion[4]. Therefore, all putative fumonisins in a sample can be detected using DFF by screening all MS/MS spectra within a DDA dataset that contain the prominent *m/z* 157.0142 product ion. Similarly, sulfated compounds can be detected by screening DDA datasets for MS/MS spectra that contain a diagnostic neutral loss of 79.9574 Da (SO_3)[3]. This approach has also been successfully applied for detecting new cyclic peptides[5] and natural products that contain tryptophan or phenylalanine residues[6].

To demonstrate the effectiveness of DFF and its ease of use within the MZmine platform[10], we have applied this approach to the analysis of microcystins (MCs); a class of over 240 structurally related toxins produced by freshwater cyanobacteria[11-13].

The most commonly reported cyanotoxins are MCs, with the MC-LR (leucine [L]/arginine [R]) congener most frequently studied (**Figure 1**). MCs are monocyclic non-ribosomal heptapeptides,

biosynthesized by multiple cyanobacteria genera including *Microcystis, Anabaena, Nostoc,* and *Planktothrix*[12,13]. MCs are composed of five common residues and two variable positions occupied by L-amino acids. Nearly all MCs possess a characteristic β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) residue at position 5[11]. The MS/MS fragmentation pathways of MCs are well described[14,15]; the Adda residue is responsible for the prominent MS/MS product ion, *m/z* 135.0803$^+$ ($C_9H_{11}O^+$) as well as other product ions including *m/z* 163.1114$^+$ ($C_{11}H_{15}O^+$) (**Figure 2**). Non-targeted DDA datasets of *Microcystis aeruginosa* cellular extracts can be screened for all microcystins present using these diagnostic ions, granted that the microcystins have an Adda residue.

PROTOCOL:

1. Preparation of non-targeted liquid chromatography (LC)-MS/MS datasets

NOTE: DFF can be performed using any high-resolution mass spectrometer and analytical method optimized for a target class of analytes. MC optimized LC-MS/MS conditions on Orbitrap mass spectrometer are listed in the **Table of Materials**.

1.1 **Downloading MZmine 2** (http://mzmine.github.io/)

NOTE: Example data CPCC300.raw can be found at https://drive.google.com/open?id=1HHbLdvxCMycSasyNXPRqIe5pkaSqQoS0.

1.1.1 Under the **Raw data methods** drop down menu, select the **Raw data import** option.

1.1.2 Choose the data file(s) to be analyzed. Single or multiple files may be imported.

1.2 (Optional) If vendor data format is not supported by MZmine, use Proteowizard[16] to generate centroided .mzml data files.

1.2.1 Choose the **Peak Picking** filter to apply vendor-supplied centroiding algorithm.

2. Diagnostic fragmentation filtering of imported DDA files

2.1 Using the cursor, select and highlight the data file(s) in the **Raw data files** column of the main MZmine screen.

2.2 Under the **Visualization** drop down menu, select the **Diagnostic fragmentation filtering** option.

2.3 In the DFF dialogue box that appears (**Figure 3**), input the following options:

2.3.1 Retention time – use **Auto range** or define the range of retention times in minutes when the targeted class of analytes will elute.

2.3.2 Precursor *m/z* - use **Auto range** or define the *m/z* range of the targeted class of analytes, including the possibility for multiple charged compounds when appropriate.

2.3.3 *m/z* tolerance – Input the achievable MS/MS mass accuracy of the MSinstrument; 0.01 m/z or 3.0 ppm is appropriate for an Orbitrap platform. If only diagnostic product ions will be investigated, input **0.0** into the Diagnostic neutral loss value (Da) option. Conversely, if only diagnostic neutral losses will be investigated, input **0.0** into the Diagnostic product ions (*m/z*) option.

2.3.4 Diagnostic product ions (*m/z*) – Input the class specific product ion(s) *m/z*. Separate multiple product ions with a comma.

NOTE: Inputting multiple product ions will visualize spectra that contain all listed product ions.

2.3.5 Diagnostic neutral loss value (Da) – Input the class specific neutral loss(es). Separate multiple neutral losses with a comma.

NOTE: Inputting multiple neutral losses will visualize spectra that contain all listed neutral losses. Inputting both diagnostic product ions and neutrals losses will visualize spectra that satisfy all the criteria.

2.3.6 Minimum diagnostic ion intensity (% base peak) – As a % of the base peak of the MS/MS spectra, define the minimum intensity for diagnostic product ions and/or neutral losses to be considered.

2.3.7 Peaklist output file – Select a path and filename to output the results.

2.3.8 Click the **OK** button to start the DFF analysis. A DFF plot will appear upon successfully completing the above steps

NOTE: Two .csv data files will be generated. *{Peaklist output file}.csv* contains the precursor *m/z*, scan numbers, and retention times of the scans. This can be used in existing MZmine modules including ***Raw data methods > Peak detection > Targeted peak detection*** to generate extracted ion chromatograms of precursors that met the defined DFF criteria. *{Peaklist output file}_data.csv* contains the precursor *m/z*, product ion *m/z* and retention times to allow generation of DFF plots outside of MZmine.

3. Example use of DFF for microcystin analysis

3.1 Sample preparation

3.1.1 Sterilize 250 mL Erlenmeyer flasks containing 30 mL of sterile MA media[17] or other cyanobacteria growth media (BG-11) fitted with a foam stopper.

3.1.2 Inoculate sterilized growth media with a cyanobacteria culture to approximately 5×10^5 cells mL^{-1} under aseptic conditions. Monitor cell density with a hemocytometer. In this example, grow *M. aeruginosa* strain CPCC300 photoautotrophically at 27 °C, illuminated with cool white fluorescent light (30 µE m^{-2} s^{-1}) using a 12 h light: 12 h dark regime. Swirl the cells once per day.

3.1.3 Separate the cells from the culture medium after 26 days by vacuum filtration using 47 mm diameter GF/C glass microfiber filter papers.

3.1.4 Add 3 mL of 80% methanol $_{(aq)}$ to harvested cells in 14 mL test tube(s).

3.1.5 Vortex and subsequently sonicate the test tube(s) containing cyanobacteria cells for 30 s each. Store the test tube(s) at -20 °C for 1 h. Return the test tube to room temperature and allow the sample(s) to thaw for 15 min.

3.1.6 Repeat step 3.1.5 two additional times to effectively lyse the cells.

3.1.7 Filter the resulting cyanobacteria cell extract(s) through a 0.22 µm PTFE syringe filter(s).

3.1.8 Dry extract(s) with an evaporator at a temperature of 30 °C using a gentle stream of nitrogen gas. Store the extract dry at -20 °C until LC-MS/MS analysis.

3.1.9 Reconstitute the dried residue with 500 µL of 90% methanol $_{(aq)}$ and vortex for 30 s in an amber HPLC vial prior to analysis.

3.2 Analyze the cyanobacteria extract using a DDA acquisition method on a high-resolution mass spectrometer.

NOTE: The optimized LC-MS conditions for MC analysis used here are listed in **Table of Materials**.

3.3 Prepare the DDA datafile(s) and import into MZmine following steps 1.1 and 1.2.

3.4 Select the datafiles and start the DFF modules following steps 2.1-2.2.

3.5 For MC analysis, use the following settings within the DFF module (**Figure 3**).

3.5.1 Retention time – Input the range of **2.00** to **6.00** min.

3.5.2 Precursor *m/z* – Input *m/z* range of **430.00** to **1200.00**.

3.5.3 *m/z* tolerance – Apply *m/z* tolerance of **0.01** m/z or **3.0** ppm.

3.5.4 Diagnostic product ions (m/z) – Input *m/z* of **135.0803, 163.1114** as the diagnostic product ions

3.5.5 Diagnostic neutral loss value (Da) – Input **0.0** to define that no diagnostic neutral losses are being used.

3.5.6 Minimum diagnostic ion intensity (% base peak) – Use **15.00** as the minimum intensity threshold

3.5.7 Peaklist output file – Define the output file as **putative_MCs.csv**.

3.6 Click the **OK** button to start the DFF analysis. A DFF plot (**Figure 4**) will appear upon successfully completing the above steps

REPRESENTATIVE RESULTS:

The DFF plot generated following the analysis of *M. aeruginosa* CPCC300 is shown in **Figure 4**. The *x*-axis of this plot is the *m/z* of the precursor ions that satisfied the defined DFF criteria while the *y*-axis shows the *m/z* of all product ions within the MCs MS/MS spectra. For this analysis, the criteria for MC detection included precursor ions within the *m/z* range of 440-1200, retention times between 2.00–6.00 min. Most importantly, these MS/MS spectra contain both *m/z* 135.0803 and 163.1114 (± 3 ppm) above the defined 15% basepeak intensity threshold. Under these conditions, a total of 4116 MS/MS spectra were acquired during the LC-MS/MS DDA analysis. Of those, 26 spectra satisfied the DFF criteria were detected in the *M. aeruginosa* CPCC300 extract. However, multiple MS/MS spectra can be acquired on the same compound, particularly for higher intensity ions. In this extract, only 18 unique precursor *m/z* were found. The smallest ion (*m/z* 497.2746, $[M+2H]^{2+}$) is the doubly charged complement of the $[M+H]^{+}$ precursor *m/z* 993.5389, which was also detected by DFF. Based on previously published studies on this *M. aeruginosa* strain[18], the major MCs detected can be confidently assigned as MC-LR and [D-Asp^3]MC-LR. Investigating the mass spectra of the remaining putative MCs revealed that two were ^{13}C isotopes of other detected MCs (*m/z* 993.5389, 1025.5343) and another was an adduct of and MC of *m/z* 993.5389. Of the 12 remaining putative MCs, four corresponded to the masses of known MCs, and eight were previously unreported compounds (**Supplementary File. Table S1**).

FIGURE AND TABLE LEGENDS:

Figure 1: Chemical structure of MC-LR. The Adda residue is common in a large proportion of known MCs and produces diagnostic product ions at *m/z* 135.0803 and 163.1114. Other MC variants that contain a dimethyl-Adda and acetyldemethyl-Adda residue at position 5 are known and would not produce the same product ions.

Figure 2: MS/MS spectra of MC-LR. MS/MS spectra acquired on a Orbitrap mass spectrometer showing the prominent product ion at *m/z* 135.0803 derived from the Adda residue. An additional product ion at *m/z* 163.1114 is also derived from the Adda residue and increases the selectivity of the DFF analysis.

Figure 3: DFF dialogue box within MZmine. The product ions and/or neutral losses that are

diagnostic for the targeted class of compounds are inputted. Retention time and precursor ion filters can be used to increase selectivity of the analysis. The minimum diagnostic ion intensity refers the threshold intensity of the diagnostic product ions and neutral losses that must be achieved in order for the spectra to satisfy the DFF criteria. Lowering this value may result in false positive hits.

Figure 4: DFF plot for MC analysis of *M. aeruginosa cellular extract*. DFF analysis of the *M. aeruginosa* CPCC300 extract found 26 spectra that met the defined DFF criteria, comprising 18 unique *m/z* values. Right clicking the plot allows the user to "Zoom Out" the domain and/or range axes. A doubly charged precursor ion was detected at *m/z* 497.2746 and corresponded to an unknown MC at $[M+H]^+$ 993.5389. The two known MCs produced by strain CPCC300 are [D-Asp^3]MC-LR and MC-LR [18]. In total, eight putative MCs did not correspond to the *m/z* of known MCs, four MCs corresponded to the *m/z* of multiple congeners and three were found to be isotopes/adducts of other MCs (**Supplementary File. Table S1**). The DFF plot shown here was generated manually in Excel from the *"putative_MCs_data.csv"* that was automatically made upon executing the DFF module.

Supplementary File. Optimized conditions for LC-MS/MS analysis of M. aeruginosa extracts.

DISCUSSION:

DFF is a straight-forward and rapid strategy for detecting entire classes of compounds, especially relevant for natural product compound discovery. The most important aspect of DFF is defining the specific MS/MS fragmentation criteria for the targeted class of compounds. In this representative example, DFF was used to detect all Adda residue containing MCs present in an *M. aeruginosa* cellular extract. Although the vast majority of MCs contain an Adda residue, other residues at this position have been known, notably demethyl- and acetyldemethyl-Adda variants[19]. Any MCs with these residues would not be detected using the defined criteria. However, as DFF is a post-acquisition approach, additional diagnostic fragments can easily be investigated on the same dataset using the simple step-by-step protocol outlined here. This also allows the analyst to detect compounds with hypothetical modifications that would alter the diagnostic product ions and/or neutral loss.

Adducts and in-source fragments may also meet DFF criteria and be incorrectly interpreted as unique analytes. False positives may arise when other compounds present in the extract exhibit the same product ions and/or neutral losses. In both cases, this can be alleviated by using additional product ions and neutral losses that increase method selectivity.

Although precursor ions may meet all of the DFF criteria defined by the analyst and represent compounds within the targeted class, their absolute identity will still be putative. Using the identification confidence levels, proposed by Schymanski (2014), MCs detected using this MS/MS approach have a 'level 3' identification confidence when unequivocal molecular formula of the precursor ion can be assigned by accurate mass and the isotope profile[20]. In this example, eight putative MCs had masses that corresponded to multiple, isobaric MCs[11]. Absolute identity would have been achieved by either comparison of retention time and MS/MS spectra with an authentic

standard or confirmed by NMR and other spectroscopic methods after purification. Putative compounds that do not correspond to masses of any known members of the targeted class, such as the eight putative MCs detected here, represent tangible targets for discovering new natural products.

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DISCLOSURES:

The authors have nothing to disclose

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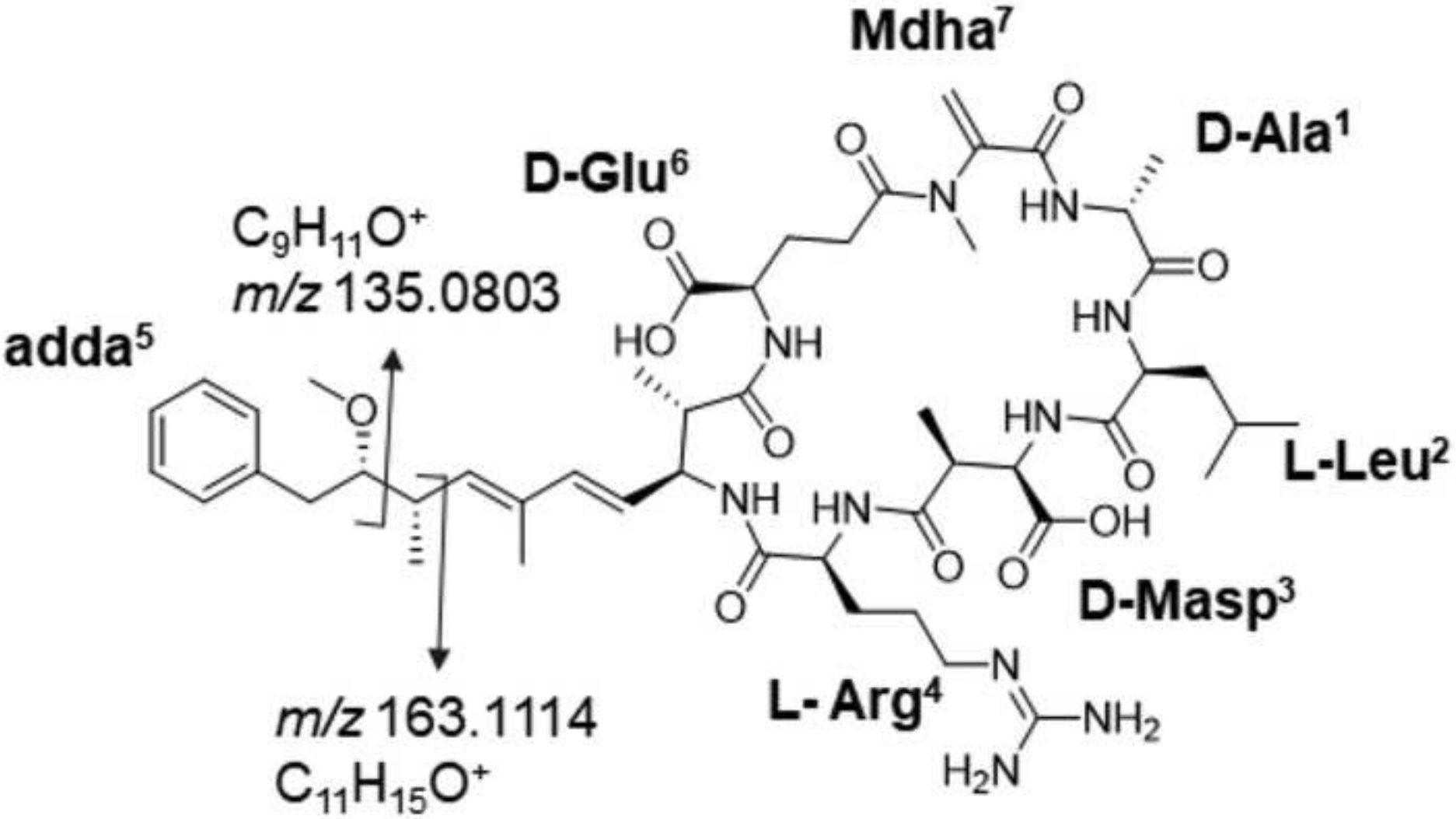
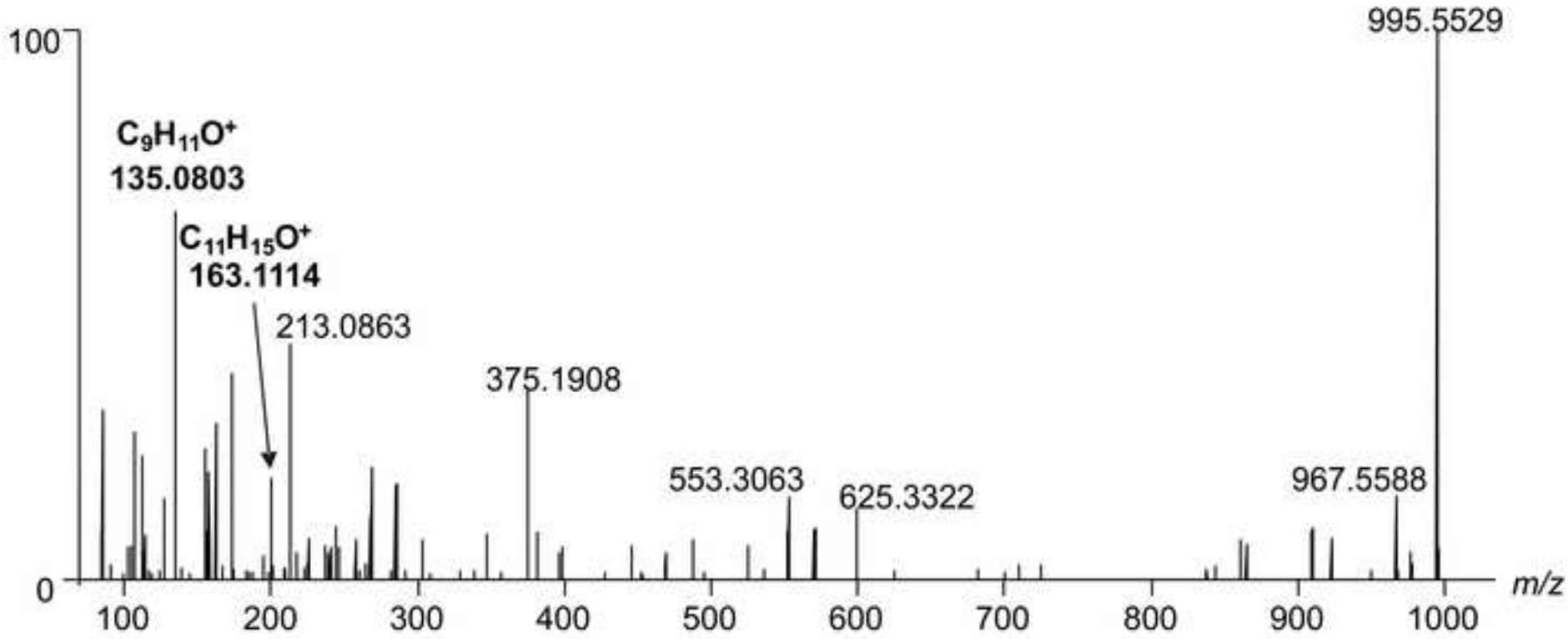
Figure



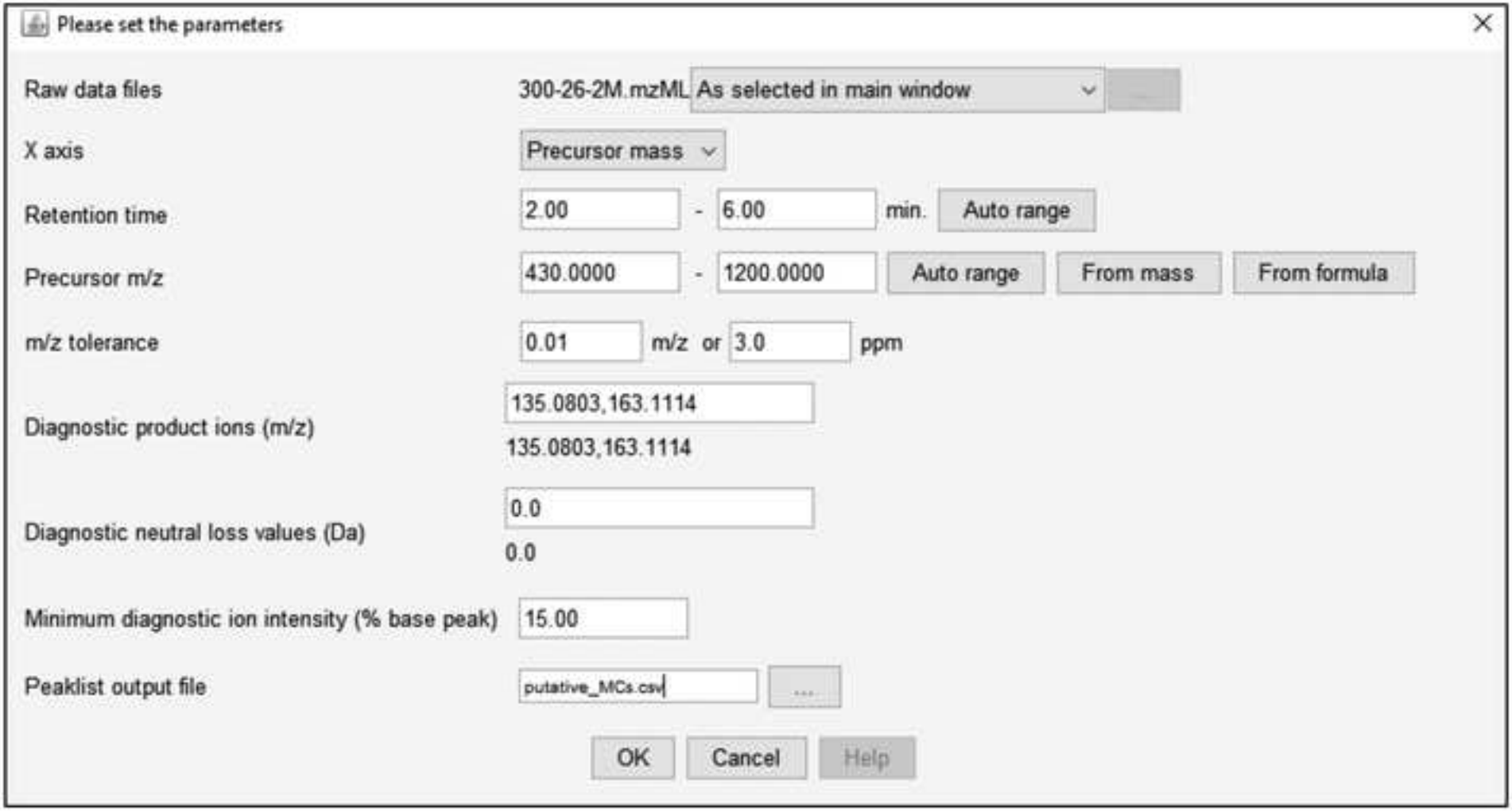
Figure

Click here to access/download;Figure;Figure 2.tif



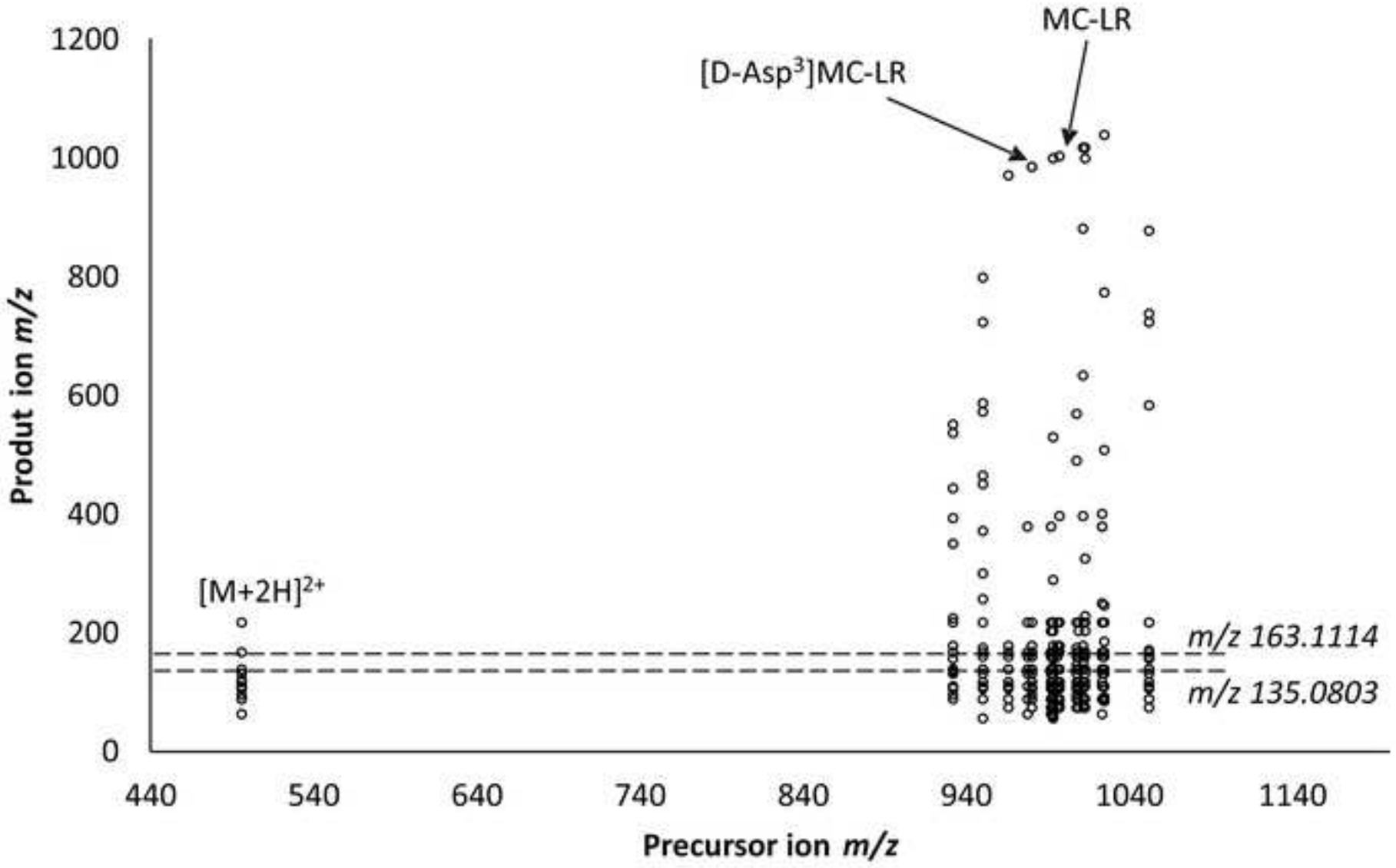
Figure

Click here to access/download;Figure;Figure 3.tif



Figure

Click here to access/download;Figure;Figure 4.tif



Name of Material/ Equipment	Company	Catalog Number
Cyanobacteria		
Microcystis aeruginosa CPCC300	CANADIAN PHYCOLOGICAL CULTURE CENTRE	CPCC300
Software		
Proteowizard (software)		software
Mzmine 2		software
LC-MS		
Q-Exactive Orbitrap	Thermo	-
1290 UHPLC	Agilent	
C18 column	Agilent	959757-902
Solvents		
Optima LC-MS grade Methanol	Fisher	A456-4
OptimaLC-MS grade Acetonitrile	Fisher	A955-4
OptimaLC-MS grade Water	Fisher	W6-4
LC-MS grade Formic Acid	Fisher	A11710X1-AMP
Vortex-Genie 2	Scientific Industries	SI-0236
Centrifuge Sorvall Micro 21	Thermo Scientific	75-772-436
Other		
Amber HPLC vials 2 mL/caps	Agilent	5182-0716/5182-0717
0.2-µm PTFE syringe filters	Pall Corp.	4521
Whatman 47mm GF/A glass microfiber filters	Sigma-Aldrich	WHA1820047
Media		
MA media (pH 8.6) (quantity / L)		
$Ca(NO_3)\cdot 4H_2O$, 50 mg	Sigma-Aldrich	C2786
KNO_3, 100 mg	Sigma-Aldrich	P8291
$NaNO_3$, 50 mg	Sigma-Aldrich	S5022
Na_2SO_4, 40 mg	Sigma-Aldrich	S5640
$MgCl_2.6H_20$, 50 mg	Sigma-Aldrich	M2393

Sodium glycerophosphate, 100 mg	Sigma-Aldrich	G9422
H_3BO_3, 20 mg	Sigma-Aldrich	B6768
Bicine, 500 mg	Sigma-Aldrich	RES1151B-B7
	P(IV) metal solution, 5 mL	
Bring the following to 1 L with ddH_2O		
NaEDTA·2HO	Sigma-Aldrich	E6635
$FeCl_3$ ·6H2O	Sigma-Aldrich	236489
$MnCl_2·4H_2O$	Baker	2540
$ZnCl_2$	Sigma-Aldrich	Z0152
$CoCl_2·6H2O$	Sigma-Aldrich	C8661
$Na_2MoO_4·2H_2O$	Baker	3764

Cyanobacteria BG-11 50X Freshwater Solution	Sigma-Aldrich	C3061-500mL

Comments/Description

ɔo.ca/canadian-phycological-culture-centre/

http://proteowizard.sourceforge.net/
http://mzmine.github.io/

Equipped with HESI ionization source
Equipped with binary pump, autosampler, column compartment
Eclipse Plus C18 RRHD column (2.1 × 100 mm, 1.8 µm)

Watanabe, M. F. & Oishi, S. Effects of environmental factors on toxicity of a cyanobacterium (Microcystis aeruginosa) under culture condit

ions. Applied and Environmental microbiology. 49 (5), 1342-1344 (1985).

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CORRESPONDING AUTHOR

Name:	Justin Renaud
Department:	AGRICULTURE & AGRI-FOOD CANADA
Institution:	Her majesty the Queen in right of Canada as represented by the minister of Agriculture and Agri-food Canada.
Title:	Researcher
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AUTHORS & AFFILIATIONS:

David R. McMullin[1], Shawn Hoogstra[2], Kimberlynn P. McDonald[1], Mark W. Sumarah[1,2], Justin B Renaud[2]

[1]*Department of Chemistry, Carleton University Ottawa, Ontario K1S 5B6 Canada*

[2]*London Research and Development Center, Agriculture and Agri-Food Canada, London, Canada*

Corresponding Author:

Justin B. Renaud, PhD

Email Address: Justin.renaud@canada.ca

Email Addresses of Co-authors:

David R. McMullin (david.mcmullin@carleton.ca)
Shawn Hoogstra (shoogstr@uwo.ca)
Kimberlynn P. McDonald (kimberlynnMcDonald@cmail.carleton.ca)
Mark W. Sumarah (mark.sumarah@canada.ca)

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