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Detection and confirmation of saxitoxin analogues in freshwater benthic *Lyngbya wollei* algae collected in the St. Lawrence River (Canada) by liquid chromatography–tandem mass spectrometry

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ABSTRACT

The presence of cyanotoxins in benthic *Lyngbya wollei* algae samples collected in a fluvial lake along the St. Lawrence River, Canada, was investigated using a multi-toxins method. Hydrophilic interaction liquid chromatography (HILIC) and reverse phased liquid chromatography (RPLC) were coupled to triple quadrupole mass spectrometry (LC-QqQMS) for quantification and to quadrupole-time of flight mass spectrometry (LC-QqTOFMS) for screening and confirmation. The presence of two saxitoxin analogues, LWTX-1 and LWTX-6, was confirmed in benthic *Lyngbya wollei* algae samples. Concentration of LWTX-1 was between 209 ± 5 and $279 \pm 9\ \mu g\ g^{-1}$. No other targeted cyanotoxin (such as anatoxin-a, nodularin, microcystin-LR, microcystins-RR and saxitoxin) was found in the samples. The presence of LWTX-6 was observed by using a screening approach based on an in-house database of cyanotoxins, an algorithm of identification and high resolution mass spectrometry measurements on the precursor and product ions. This work demonstrates the need for more research on the fate of benthic cyanotoxins in aquatic ecosystems such the St. Lawrence River.

1. Introduction

Cyanotoxins are among the oldest environmental organic contaminants known as they were first reported in an Australian lake in 1878 [1]. They are metabolic by-products released by cyanobacteria, also called blue-green algae. These species are ubiquitous inhabitants of surface water, but when several physical, chemical and biological mechanisms combine and interact with the ecosystem, they can form dense blooms [2,3]. Harmful algal blooms (HABs) develop when these increased populations release cyanotoxins into the aquatic environment during cell growth or cell lysis. Consequently, surrounding water becomes toxic to animals and humans.

In the last few decades, reports on HABs around the world have increased [2]. Moreover, recent studies suggest that global warming could contribute to a further increase in the formation of HABs [4]. Therefore a growing interest in HABs has arisen in the last few years since these blooms deteriorate water quality thus affecting agricultural and recreational activities and also potentially contaminating drinking water supplies [5,6]. Among the cyanotoxins, two classes of substances have attracted more attention because of their toxicity: the hepatotoxins and the neurotoxins. The hepatotoxins are comprised of three main groups: microcystins, nodularins and cylindrospermopsins. The neurotoxins are comprised of anatoxins and saxitoxins (also called paralytic shellfish poisons or PSP).

For instance in Canada, cyanobacterial research in freshwaters has focused primarily on microcystins and anatoxin-a [7]. However, recent studies on the distribution of benthic macroalgae in fluvial lakes of the St. Lawrence River observed the presence of *Lyngbya wollei,* a filamentous benthic cyanobacterium, at the bottom of the river ecosystem [8]. These cyanobacteria are responsible for the production of several analogues of saxitoxin, such as decarbamoylsaxitoxin and decarbamoylgonyautoxin-2 and -3, as well as six other compounds known as *L. wollei* toxins (LWTXs) [9]. According to mouse bioassays, LWTXs do not appear to be as toxic as other saxitoxins [9]. However given their structural similarity to saxitoxin, abiotic or biotic transformation of LWTXs to other more toxic analogues cannot be ruled out without additional bioassays. This type of transformation has been observed for N-sulfocarbamoyl saxitoxins (C1 and C2) which can be converted to the more toxic decarbamoylgonyautoxins [10].

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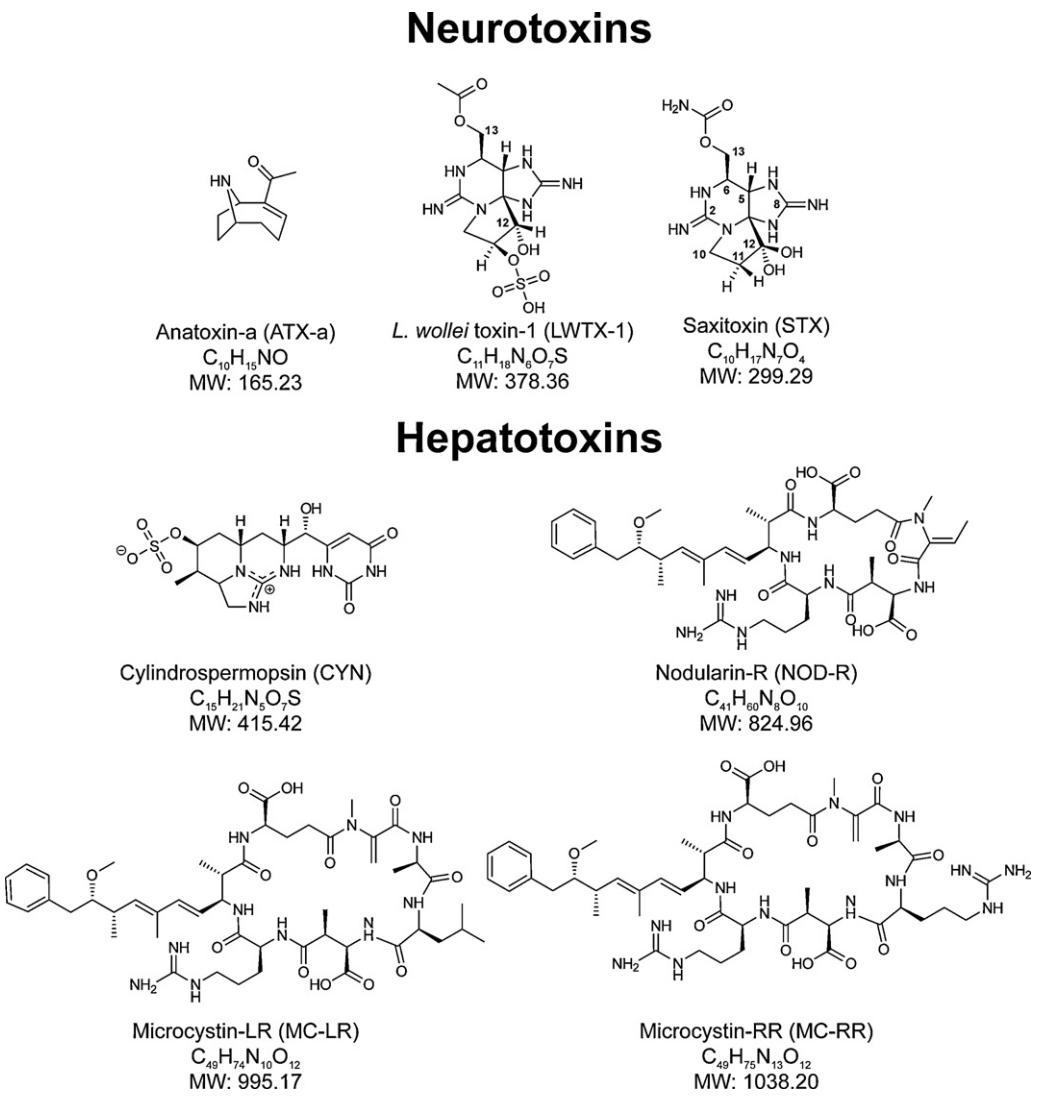


Fig. 1. Chemical structures of studied cyanotoxins along with their respective molecular formula and molecular weight.

Cyanotoxins present several challenges that have hindered the development of analytical methods. The scarcity of certified calibration standards makes quantitative analysis difficult since scientists must also extract and purify target cyanotoxins from HABs samples. Also, the chemical diversity of these substances further complicates the development of multi-toxin methods of analysis, which is now required for complex samples containing a variety of toxins.

Since HABs can release mixtures of cyanotoxins rather than just single toxins [11], therefore methods of analysis capable of identifying different analogues concurrently are necessary. Since cyanotoxins have been studied for decades, numerous methods have been developed for their detection [12,13] but most lack the specificity required to unambiguously identify and quantify cyanotoxins in complex matrices such as algae. In the last few years, specific and quantitative multi-toxin methods of analysis based on liquid chromatography combined with tandem mass spectrometry (LC–MS/MS), have been published [14–17]. However, these methods often require a number of time-consuming sample preparation steps, thus further complicating their analysis.

The goal of our research was threefold: (a) to develop and validate a common and simple multi-toxins method for LC–MS/MS analysis of six representative hepatotoxins and neurotoxins (Fig. 1) in different types of algae (e.g., *L. wollei* field samples and *Anabaena flos-aquae* and *Microcystis aeruginosa* culture samples); (b) to investigate and confirm the presence of these cyanotoxins in *L. wollei* field samples; and (c) to screen for the presence of other cyanotoxins using high resolution-mass spectrometry and a database of cyanotoxins.

2. Material and methods

2.1. Reagents and materials

Standard (≥95% purity grade) anatoxin-a (ATX-a), nodularin (NOD), microcystin-RR (MC-RR), and microcystin-LR (MC-LR) were purchased from Enzo Life Sciences Inc. (Plymouth Meeting, PA, USA). Ampoules of certified standard solutions of cylindrospermopsin (CYN, 30 ± 2 μM in purified water), saxitoxin dihydrochloride (STX, 65 ± 3 μM in 2 mM hydrochloric acid), and an in-house reference materials containing *L. wollei* toxin-1 (LWTX-1, 39.9 μM in 0.01 M acetic acid) and 7-desmethylmicrocystin-LR (7dmMC-LR, 10.0 μM in 50% methanol/water) were obtained from the Certified Reference Materials Program (NRC, Halifax, NS, Canada). The internal standards *p*-aminobenzoic acid-d_4 (PABA-d_4) and clarithromycin-d_3 (CLA-d_3) were provided from Toronto

Research Chemicals (North York, ON, Canada) and Sigma–Aldrich Co. (St. Louis, MO, USA), respectively. The HPLC-grade solvents methanol (MeOH), acetonitrile (MeCN) and water (H_2O) were purchased from Tekniscience (Terrebonne, QC, Canada), while hydrochloric acid (HCl) and acetic acid (CH_3COOH) were bought from American Chemicals Ltd. (Montreal, QC, Canada). Ammonium formate (NH_4OOCH_3, purity ≥99.0%), formic acid (HOOCH, purity ≥98.0%), and DL-phenylalanine (purity 99%) were manufactured by Fluka and purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Stock solutions of ATX-a, CYN, LWTX-1 and STX (1.0 mg L^{-1}) were prepared in HCl (3 mM), and MC-RR, NOD, MC-LR, and 7dmMC-LR in MeOH/H_2O (1:1, v/v) prior their storage at −20 °C in amber glass bottles. Internal standard stock solutions (1–5 mL) of PABA-d_4 (10 mg L^{-1}) and CLA-d_3 (5 mg L^{-1}) were prepared respectively in MeOH and MeCN in amber glass bottles and stored at 4 °C. Bottles and PTFE-lined caps were previously cleaned at Environment Canada following a rigorous validated protocol approved by the Canadian Association for Laboratory Accreditation (CALA) under ISO/IEC/17025:2005 guidelines. All corrosive and pure standard chemicals were handled carefully under a ventilated fume hood wearing appropriate protection.

2.2. Sample collection

2.2.1. Lyngbya wollei samples

Algal samples (*L. wollei*) were collected in August 2010 from a scum-forming bloom at the bottom of Lake St. Louis along of the shallow (2–3 m depth) shore of the St. Lawrence River. Three sampling sites (stations) were chosen close to the north shore of the lake (coordinate: X utm 590287, Y utm 5031155). Approximately 30 g of material was harvested at each site. After collection, the wet algae were rinsed twice manually using purified water and wring out to diminish the water content. Around 3 g wet wt of each frozen algal samples were then freeze-dried (yielding approx. 0.3 g dry wt) in a FreeZone Triad model 7400040 lyophilizer (Labconco, Kansas City, MO, USA) and stored at −80 °C until use.

2.2.2. Anabaena flos-aquae culture

Strains of *A. flos-aquae* (Lybg.) (CPCC64) were raised in batch cultures using BG-11 media. The *Anabaena* samples were used to test the capacity of the proposed multi-toxin method to detect ATX-a in real (non spiked) samples to demonstrate the satisfactory chromatographic resolution of ATX-a and its natural isobaric interference phenylalanine. All batch cultures were grown in an incubator (New Brunswick Scientific, Edison, NJ, USA) under a light:dark cycle of 16:8 h at 56 μmol m^{-2} s^{-1} PAR and a temperature of 24 ± 2 °C. All batch cultures were renewed regularly to maintain exponential growth. For each batch, cells were collected by centrifugation at 4 °C for 5 min at 5000 rpm using a Sorvall Legend RT+, centrifuge (Thermo Scientific, Waltham, MA, USA). Pellets were collected, frozen at −80 °C and then lyophilized before being pooled prior to extraction. *A. flos-aquae* samples were extracted following the same procedure reported in Section 2.3.

2.2.3. Microcystis aeruginosa culture

As with *Anabaena*, *M. aeruginosa* samples were used to verify the efficiency of the multi-toxin method developed to identify microcystins. A strain of *M. aeruginosa* (CPCC299) was raised in batch cultures using Bold Basal Medium. The batch was grown in an incubator (New Brunswick Scientific, Edison, NJ, USA) under a light: dark cycle of 14:10 h at 50 μmol m^{-2} s^{-1} PAR and at a temperature of 21 ± 2 °C. The culture was renewed regularly to maintain exponential growth. Cells were collected by filtration through a 0.7 μm pore size glass microfiber filters (Whatman grade GF/F, 47 mm diameter), frozen at −80 °C and then lyophilized before being pooled prior to extraction. *M. aeruginosa* samples were extracted following the same procedure as described in Section 2.3.

2.3. Extraction procedure

Lyophilized algae (20–50 mg, dry mass) were ground and weighed accurately into some 1.5 mL safe-lock Eppendorf tubes. Extraction solvent (1.0 mL of 50% MeOH/H_2O with 0.1 M CH_3COOH) was added to each and the mixtures were shaken by vortex for 10 s. Tubes were then placed in a sonication bath for 5 min, followed by an ultrasonication for 2 min with a UIS250v ultrasonic processor and a VialTweeter sonotrode (amplitude: 75, cycle: 1) manufactured by Hielsher (Teltow, Germany). After centrifugation (3500 rpm, 4 °C, 5 min), supernatants were filtered using syringes equipped with a 25 mm PTFE syringe filter (Fisherbrand, pore size 0.45 μm) and collected in 16 mm × 150 mm borosilicate glass screw-top conical tubes. The previous steps were repeated twice on weighed samples, and all aliquots were combined resulting in an approximate final volume of 3 mL. Then 100 μL of each internal standard solution (PABA-d_4 10 mg L^{-1}, CLA-d_3 5 mg L^{-1}) was added to each individual tube, and the resulting solutions were evaporated to dryness under a gentle stream of N_2. The dried aliquots were suspended in 0.5 mL of MeCN/H_2O (9:1) with 5 mM NH_4OOCH_3 and 3.6 mM HCOOH (pH 3.5). One portion of each extract was aliquotted for immediate analysis using the HILIC–MS/MS (hydrophilic interaction liquid chromatography tandem mass spectrometry) method. Another portion was diluted 1:1 with H_2O containing NH_4OOCH_3 and 3.6 mM HCOOH (pH 3.5) prior to RPLC–MS/MS (reversed-phase liquid chromatography tandem mass spectrometry) analysis.

Since the observed LWTX-1 concentrations were found to be high in *L. wollei* samples, it was decided that only 20 mg of sample would be extracted. The combined extraction solutions were diluted by dispersing four equal aliquots of 25 μL in four 16 mm × 150 mm borosilicate glass screw-top conical tubes and adding to each tube 0.5 mL of MeOH and 100 μL of each internal standard solution. The extracts were dried and then reconstituted (dilution factor of 120) with MeCN/H_2O (9:1) with 5 mM NH_4OOCH_3 and 3.6 mM HCOOH (pH 3.5) and injected for HILIC separation.

2.4. Liquid chromatography–mass spectrometry (LC-QqQMS, LC-QToFMS)

Liquid chromatography was performed using an Agilent 1200 Series LC system equipped with binary pumps, degasser, and thermostated autosampler maintained at 4 °C. For polarity consideration, the chromatographic separation of all studied cyanotoxins was performed using two different LC methods (HILIC and RPLC commonly named C18). The first group of cyanotoxins (ATX-a, CYN, LWTX-1, and STX) was separated on a TSKgel Amide-80 HILIC column (size: 150 mm × 2 mm, particle diameter: 3 μm, pore size: 80 Å) manufactured by TOSOH Bioscience (San Francisco, CA, USA). The mobile phase consisted of solvent A: H_2O and solvent B: MeCN/H20 (95:5) both with 5 mM NH_4OOCH and 3.6 mM HCOOH (pH 3.5). Flow rate was 0.5 mL min^{-1}. The following gradient was used (%B): 0 min (90%), 1 min (90%), 2.25 min (75%), 6.5 min (75%), 7.5 min (45%), and 15 min (45%). An equilibration time of 15 min was required to return the column to its initial condition, resulting in a total run time of 30 min. The second group of cyanotoxins (MC-RR, NOD and MC-LR) was separated using RPLC with a Gemini C18 column (150 mm × 2 mm, 3 μm, 110 Å) manufactured by Phenomenex (Torrance, CA, USA). With the same solvents and flow rate used previously for HILIC column, the gradient used was (%B): 0 min (1%), 1 min (1%), 1.25 min (35%), 6 min (35%), 8 min (97%), and 10 min (97%). An equilibration time of 10 min was used

Table 1
Optimized LC-(ESI+) QqQ conditions for the analysis of cyanotoxins.

Methods	Substances	t_R (min)	SRM transitions (m/z)	Fragmentor (V)	Collision energy (V)
HILIC	ATX-a	4.51	166.1 → 131.0, 91.0	89	13, 25
	CYN	5.38	416.1 → 194.0, 176.0	140	40, 50
	LWTX-1	8.50	379.1 → 299.1, 138.2	115	13, 30
	STX	11.87	300.2 → 203.9, 138.0	90	20, 28
	PABA-d_4 (ISTD)	1.03	142.2 → 124.1	80	14
RPLC	MC-RR		519.9 → 135.0, 127.0	130	30, 50
	NOD	7.04	825.5 → 227.1, 135.1	255	60, 65
	MC-LR	8.49	995.6 → 212.9, 134.9	260	70, 75
	CLA-d_3 (ISTD)	7.44	751.2 → 161.0	150	30

Table 2
Cyanotoxins identified by the non-target screening method with score > 80.

Sample	Name	t_R (min)	$[M+H]^+$ Theoretical mass (u)	$[M+H]^+$ Experimental Mass (u)	Δ Error (mDa)	Formula	Score
Site 3	*Lyngbya wollei* toxin-6	10.27	282.1437	282.1440	−0.38	C11H18N6O3	99.15
Site 3	Dihydroanatoxin-a	3.42	167.1309	167.1310	−0.09	C10H17NO	97.72
Site 3	*Lyngbya wollei* toxin-1	7.91	378.0974	378.0958	1.67	C11H18N6O7S	90.02
Site 3	*Lyngbya wollei* toxin-5	10.42	298.1387	298.1390	−0.29	C11H18N6O4	86.21
Site 4	*Lyngbya wollei* toxin-6	10.29	282.1438	282.1440	−0.27	C11H18N6O3	99.06
Site 4	*Lyngbya wollei* toxin-1	7.88	378.0951	378.0958	−0.63	C11H18N6O7S	98.17
Site 4	Dihydroanatoxin-a	3.42	167.1310	167.1310	−0.02	C10H17NO	87.31
Site 4	*Lyngbya wollei* toxin-5	10.45	298.1390	298.1390	0.02	C11H18N6O4	85.90
Site 4	Hydroxyhomoanatoxin-a (S/R)	4.00	209.1413	209.1416	−0.30	C12H19NO2	83.80
Site 4	Dihydroanatoxin-a	3.88	167.1308	167.1310	−0.18	C10H17NO	83.63
Anabena	Dihydroanatoxin-a	2.88	167.1307	167.1310	−0.26	C10H17NO	93.80
Anabena	Epoxyhomoanatoxin-a	4.98	195.1261	195.1259	0.18	C11H17NO2	82.61
Anabena	Epoxyanatoxin-a/Dihydrohomoanatoxin-a	4.37	181.1097	181.1103	−0.53	C10H15NO2	82.36

resulting in a total run time of 20 min. For both methods, the column temperature was maintained at 45 °C. Injection volumes of 15 μL and 20 μL were used for the HILIC and RPLC methods, respectively.

The LC systems were coupled to two different mass spectrometers. For quantitative analysis a 6410 triple quadrupole mass spectrometer (QqQMS) manufactured by Agilent Technologies (Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source was used. The capillary was maintained at 2500 V, and the cone voltage was optimized for each cyanotoxin in the positive-ion mode (ESI+). Additional parameters were held constant for all cyanotoxins: gas temperature 325 °C; gas flow 10 L min^{-1}; nebulizer 35 psi. MassHunter software from Agilent Technologies was used for data acquisition and processing. All optimized parameters are listed in Table 1. For qualitative analyses a 6530 quadrupole-time of flight mass spectrometer (QqTOFMS) also manufactured by Agilent Technologies, was employed. The QqTOFMS was equipped with a thermal gradient focusing (Jet Stream technology) ESI source. This type of source uses super heated N_2 sheath gas to improve desolvation and ion focus into the mass spectrometer, thus ameliorating sensitivity [18]. Source parameters consisted of the following: gas temperature 350 °C; sheath gas temperature 350 °C, sheath gas flow 11 L min^{-1}, drying gas flow 11.5 L min^{-1}, nebulizer 30 psig, fragmentor 100 V and capillary voltage 4000 V. The QqTOFMS was operated in the 4 GHz High Resolution mode with a low mass range (1700 m/z). Purine (121.050873 m/z) and Hexakis (922.009798 m/z) were used as internal reference masses to improve mass accuracy. Two different MS acquisition modes were used. First, full scan was used to measure exact masses of the precursor ions using an acquisition range m/z 50–1250 at a rate of 1 spectra s^{-1}. Identified compounds with a score > 80 were selected for tandem MS analysis using a mass range of m/z 50–1250 with an acquisition rate of 1 spectra s^{-1}, quadrupole isolation width 4 m/z, collision energies of 5, 15 and 30 V, with an acquisition time of 1000 ms $spectrum^{-1}$.

2.5. Method validation

Although the multi-toxin LC–MS method was developed using limited available certified standards, all validation tests (e.g., recovery, matrix effects, and linearity) were completed for the extracted sample matrices. The limits of detection (LOD) of the method (signal-to-noise ratio (S/N) > 3) were also estimated in the spiked extracted matrix.

Recovery tests were performed on spiked samples to determine the precision and accuracy of the method. Because the target cyanotoxins can be found in algae, recovery of each toxin were calculated as follow: the peak area ratio obtained by LC-QqQMS (i.e. analyte area/ISTD area) of the spiked sample before extraction minus the peak area ratio of the unspiked sample divided by the peak area ratio of the extracted sample spiked after extraction. Matrix effects were also investigated on algae samples. Hence, the area of the spiked standard (100 ng of toxin in 50 mg of lyophilized algae) minus the area of the extracted unspiked matrix was compared to the area of a 100 ng standard prepared directly in the LC mobile phase. The signal suppression or enhancement was calculated in percentage. Values <100% indicate matrix suppression whereas values >100% indicate matrix enhancement. Finally, the linearity of standard addition calibration curves of all studied toxins were evaluated in real matrix for a concentration range of 10–100 ng per 50 mg of spiked algae sample.

3. Results and discussion

3.1. Sample preparation and method validation

In this study, lyophilized ground algae were extracted according to Hiller et al. [15] with some modifications such as solvent composition. The solvents used in the purification of cyanotoxins remained the key parameter of the whole sample extraction process. For example, extractions of *L. wollei* algae using MeOH/H_2O

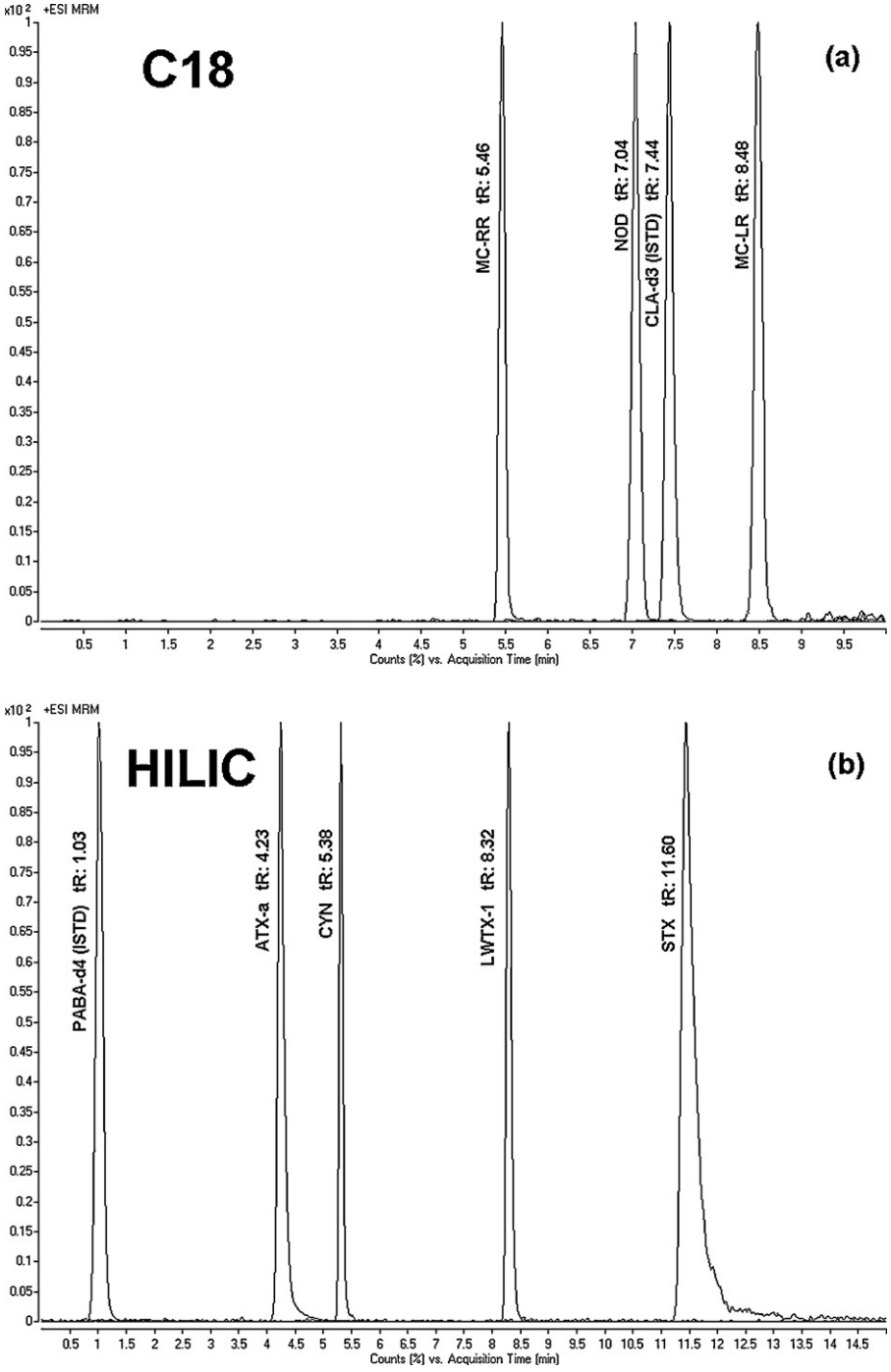


Fig. 2. LC–MS/MS chromatograms of standard solutions of studied cyanotoxins separated by RPLC column (a) and HILIC column (b).

(1:1) [19] resulted in dark-green solutions that were deemed unsuitable for the LC–MS/MS systems. A solvent mixture made of MeOH/H_2O (1:1) with 0.1 M CH_3COOH gave the best percentage of recovery (80–105%) for *L. wollei* samples (detailed results are listed in supplementary material, Table S1). This solvent mixture was selected with regard to the polarity of toxins to be extracted and their respective solubility in the tested media. It also facilitated the purification of the matrix, since the diluted CH_3COOH solution limits the co-extraction of color pigments such as chlorophyll-a [20], which could cause additional matrix effects. This way, the capacity of the multi-toxin method was maximized to efficiently extract and detect the total toxin content of different algae species.

Major precursor ions for all toxins were observed as singly charged pseudo-molecular ions $[M+H]^+$ except MC-RR for which the doubly charged ion $[M+2H]^{2+}$ was more intense. In the case of STX and LWTX-1 in spite of being doubly charged cations at the mobile phase pH, their precursor ions were also observed as singly charged ions. This was previously observed by Quilliam et al. [21].

Method linearity was tested across a range of concentrations that was previously reported for cyanotoxins in algae [14,15]. Hence, the linearity of calibration curves in extracted matrix yielded a correlation coefficient $r^2 > 0.99$ for a concentration range of 10–100 ng per 50 mg of spiked algae. Due to limited availability of toxins, upper limits of linearity could not be test during our validation process. Method sensitivity for the different toxins was found

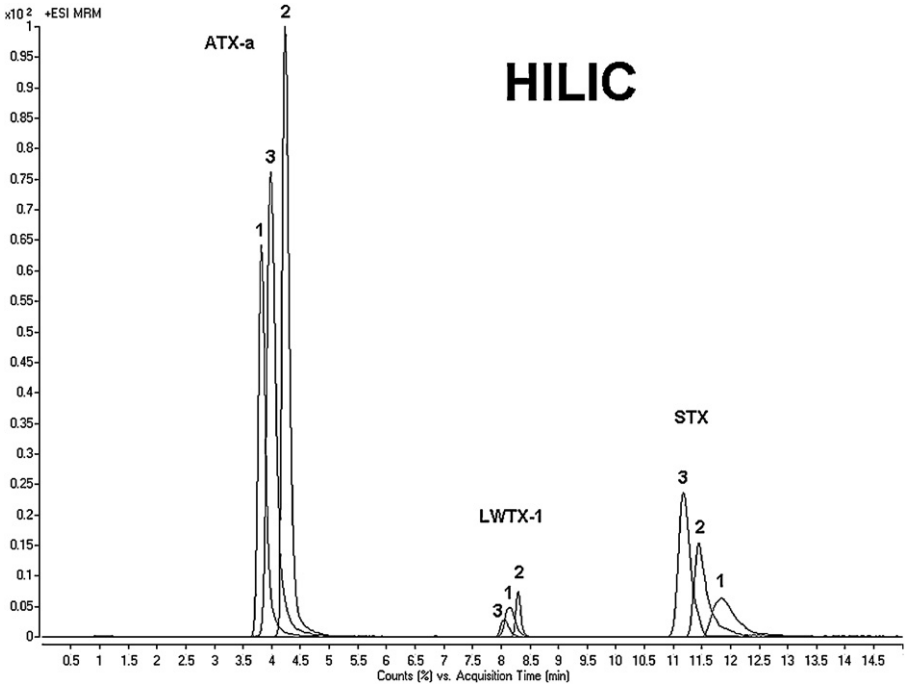


Fig. 3. LC–MS/MS reconstituted ion chromatograms of a standard solution injected on HILIC column using different mobile phase buffer composition: NH_4OOCH 2 mM, HCOOH 3.6 mM (1), NH_4OOCH 5 mM, HCOOH 3.6 mM (2), and NH_4OOCH 10 mM, HCOOH 3.6 mM (3).

to be acceptable with an estimated LOD ranging from 2 to 537 pg. The mean limit of quantification (LOQ, $S/N > 10$) estimated for all toxins was roughly 50 fmol injected on column (injection volume: 15–20 μL, solvent of reconstitution: 0.5 mL). Precision was also investigated to ensure good reproducibility after multiple injections in LC-QqQMS. Hence, five replicate injections of a *L. wollei* extract solution (50 mg algae spiked with 100 ng of each toxin) gave satisfactory results for the HILIC and RPLC methods with mean RSD values of 7.5% and 6.9%, respectively.

An issue inherent to electrospray MS is that the ionization source is highly susceptible to co-extracted matrix components [22,23]. Therefore, sample extracts were used to assess the matrix effect on the quantification of the target toxins. Signal suppression (<100%) in *L. wollei* extracts ranged from 86% (CYN) to 16.9% (MC-RR) with a mean value of 36%. In order to overcome matrix effect interferences within algae extracts, standard addition experiments were then used for quantitation. Detailed results of LOD, LOQ, RSD%, and matrix effects values are reported in supplementary material, Table S1.

3.2. Chromatographic development

The monitoring of algae in surface water for the presence of deleterious toxins is of critical importance for environmental and health risk assessment. Since it has been reported that benthic cyanobacteria have the capacity to produce hepatotoxins, such as MC-LR [24] and CYN [25], the development of an analytical method that could provide simultaneous detection and reliable identification of different cyanotoxins is of great interest. As pointed out by Dell'Aversano et al. [14], the wide range of structures and water solubility of some cyanobacterial toxins make it difficult to resolve all the toxins in one analysis. To avoid potential chromatographic issues, it was decided to analyze the hydrophobic hepatotoxins (MC-LR, MC-RR, and NOD) on a C18 column in the RPLC mode and the hydrophilic neurotoxins (ATX-a, CYN, LW-1, and STX) on an Amide-80 column in the HILIC mode. Since all analyses can be performed with a single extract using the same mobile phase constituents, the developed method proved to be suitable for a preliminary screening of cyanotoxins. The choice of two stationary phases of different sorbent polarities gave satisfactory chromatographic separation as well as good peak shapes for all studied toxins (Fig. 2). The use of two different selected reaction monitoring (SRM) transitions for each toxin ensured excellent specificity during the analysis, thus decreasing the possibility of false negatives.

While the chromatography of the hepatotoxins was excellent on the C18 column, poor results were obtained for neurotoxins analyzed under the same conditions. Indeed, low retention times (t_R) were observed for ATX-a and CYN in this column. Moreover, the peak shapes of ATX-a and LWTX-1 were not acceptable, showing increased broadening and tailing compared to other toxins. Therefore, to address these chromatographic problems, an Amide-80 column was tested in the HILIC mode using modified version of the method proposed by Dell'Aversano et al. [14]. In the HILIC mode, analytes are eluted following a partition (hydrophilic) process between a highly organic mobile phase and a water layer formed on the polar surface of the stationary phase. Retention of polar organic molecules also results from electrostatic and hydrophobic interactions between the analytes and the two phases [26]. Hence, the behavior of this column can be compared to normal phase liquid chromatography with the advantages of LC–MS solvent compatibility. In this way, all neurotoxins were eluted faster when a lower proportion of the organic solvent (MeCN) was used in a gradient table (results not shown). In order to improve the chromatographic separation between all targeted toxins, an initial mobile phase composition of 90% solvent B (MeCN/NH_4OOCH (95:5) with 5 mM and 3.6 mM HCOOH) was used for the gradient. To obtain optimal analytical conditions with the Amide-80 column, further adjustments related to the mobile phase composition were necessary. This column was highly sensitive to subtle mobile phase modifications. The column behavior changed completely when the salt composition of the mobile phase was slightly modified. As depicted in Fig. 3, the order of elution for toxins is affected by the NH_4OOCH concentrations. Indeed, it was very difficult to predict the eluted order of the toxins, as well as their peak shapes for a given salt concentration.

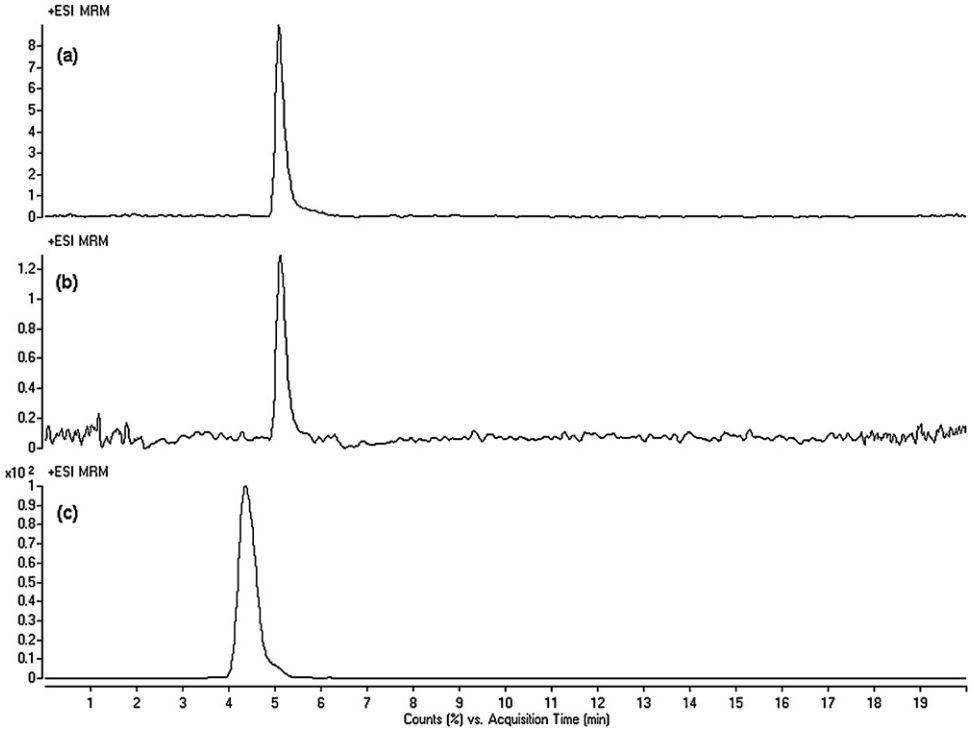


Fig. 4. LC–MS/MS chromatographic separation of ATX-a and phenylalanine on HILIC column: STD solution of phenylalanine (a), *Anabaena flos-aquae* culture extract (b), and *Anabaena flos-aquae* culture extract spiked with 100 ng ATX-a (c).

However, the optimal analytical results were obtained using the 5 mM buffer solution. At higher salt concentration (e.g., 10 mM), much more ion suppression was observed during the ESI process, resulting in smaller peak areas.

In biological and environmental matrices, investigations of suspected ATX-a are often troubled by interferences. As previously reported [27], a possible confusion or false-positive can occur in routine LC-MS analysis due to the presence of the natural amino acid phenylalanine (Phe) which is isobaric with ATX-a (m/z 166.1) and has a similar LC retention time with C18 column. In their forensic analysis, Furey et al. [27] proposed some interesting strategies to avoid the misidentification of Phe as ATX-a. Instead of using chemical derivatization or multiple-stage MS confirmation, the developed HILIC method provides enough resolution to separate ATX-a from Phe in algae matrices. An example of the satisfactory chromatographic resolution is represented in Fig. 4 for *A. flos-aquae* cultures extract that clearly showed some amounts of Phe.

3.3. Method application to samples analysis

Samples of *L. wollei* algae collected in Lake St. Louis at stations 2, 3, and 4 were screened for hepatotoxins and neurotoxins. Hence, the extraction procedure was successfully applied to the analysis of the combined extracellular and intracellular content in cyanotoxins. The quantitative part of the analytical work was achieved by LC-QqQMS using the available reference material, while the qualitative part involving some structure characterizations and confirmations was done by LC-QqTOFMS. Additional results are also reported in the following section about an extracted cultured sample of *Microscystis* using RPLC.

3.3.1. LC-QqQMS analysis

Among the seven target cyanotoxins, only LWTX-1 was detected by LC-QqQMS in *L. wollei* algae (Fig. 5a and b). Quantitative results revealed the presence of LWTX-1 at $215 \pm 4\ \mu g\ g^{-1}$ ($n = 2$) for station 2 samples, $209 \pm 5\ \mu g\ g^{-1}$ ($n = 2$) for samples collected at station 3, and $279 \pm 9\ \mu g\ g^{-1}$ ($n = 2$) for station 4 samples. Since there are very few environmental studies available on *L. wollei* algae in the literature, no comparison could be made on LWTX-1 concentrations. Historically, this saxitoxin analogue was isolated and characterized for the first time in 1997 from an algal bloom of *L. wollei* collected from Guntersville Reservoir on the Tennessee River (AL, USA) [9]. Preliminary analysis made by our research team at Halifax (National Research Council of Canada, NS) showed the presence of saxitoxin analogues in *L. wollei* growing in the St. Lawrence River (unpublished results), however to our best knowledge the present study is the first to report quantitative data of LWTX-1 in *L. Wollei* algae. Contrary to other existing *L. wollei* studies around the world [15,25,28] no CYN or other STX analogues were detected in final algal extracts of the present samples.

In addition to *L. wollei* analysis, method robustness was tested by analyzing a cultured cyanobacterial sample of *M. aeruginosa*. As expected, quantitative LC-QqQMS results confirmed the high abundance of MC-LR using the RPLC method (Fig. 5c and d) at a mean concentration of $646.1 \pm 86.0\ \mu g\ g^{-1}$ ($n = 2$). Further investigations enabled the positive detection of the 7-desmethyl analogue of MC-LR to a lesser proportion. Also, the cultured *A. flos-aquae* were screened for the possible presence of ATX-a. Our results showed that the algae did not produce any toxin in detectable concentrations. Optimal parameters such as light, temperature, PO_4^{3-} and NO_3^- concentration required for the production of toxins from cultured algae are difficult to obtain [29]. Nevertheless, the *A. flos-aquae* extracts were used to show the satisfactory chromatographic separation between ATX-a and Phe (see Section 3.2). Following these findings, the adapted multi-toxin method proved its usefulness for the analysis of various algae samples using either the HILIC or the RPLC column.

3.3.2. LC-QqTOFMS analysis

A previously described method based on high-resolution mass spectrometry was used to screen the presence of other non-targeted cyanotoxins in the samples [30] and to confirm the

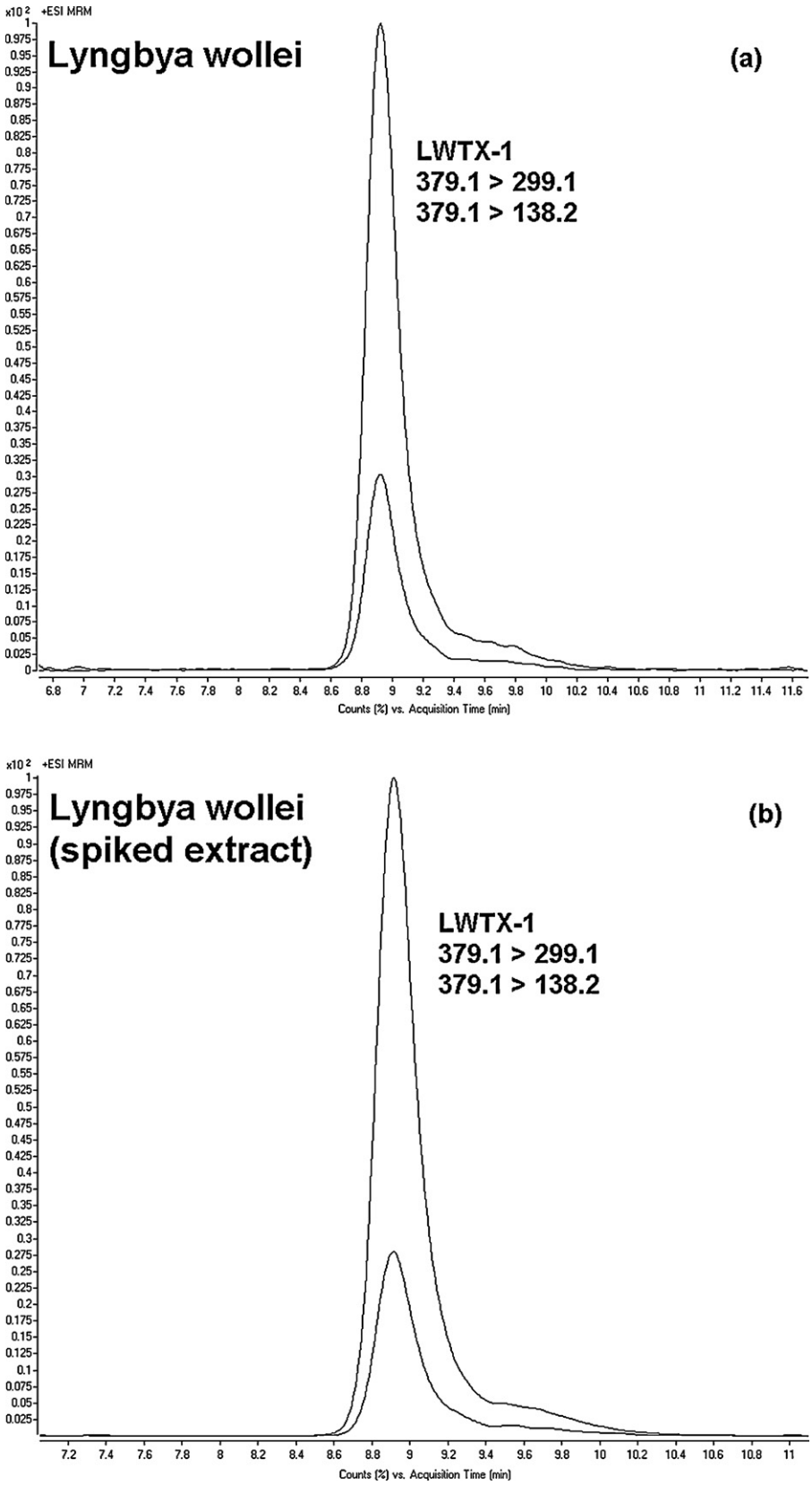
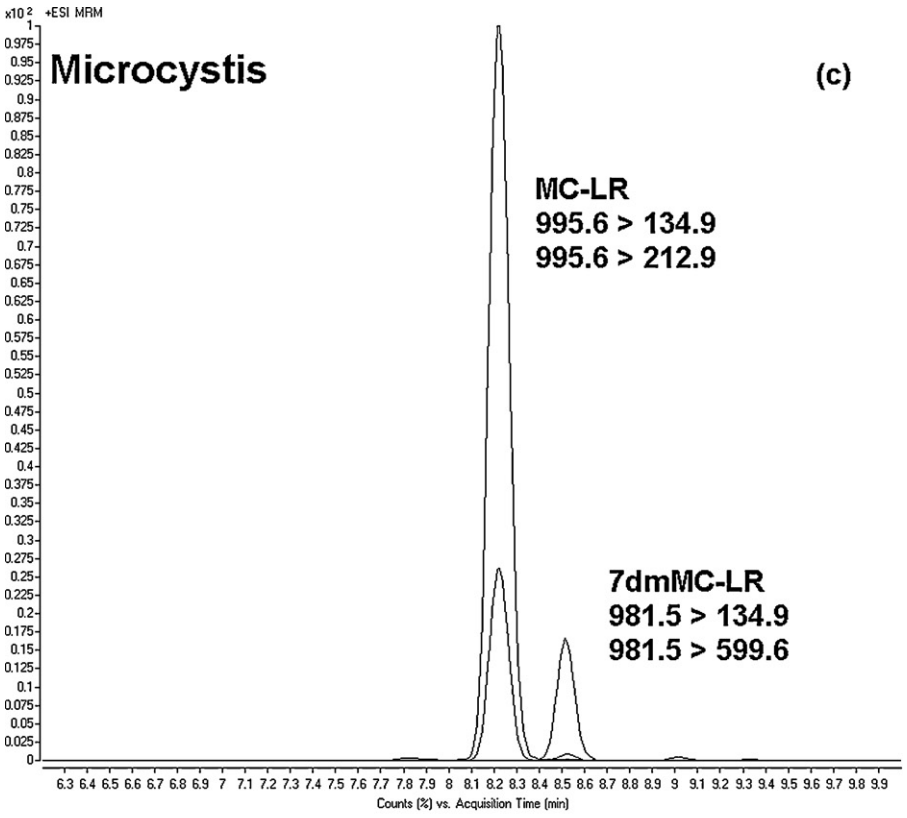


Fig. 5. LC–MS/MS reconstituted ion chromatograms of extracted *Lyngbya wollei* algae collected in St. Lawrence River, station 4 (a), extracted *Lyngbya wollei* algae collected in St. Lawrence River spiked with 25 ng of LWTX-1 (b), *Microcystis* culture algae extract (c), and *Microcystis* culture algae extract spiked with 25 ng MC-LR, 7dmMC-LR (d).



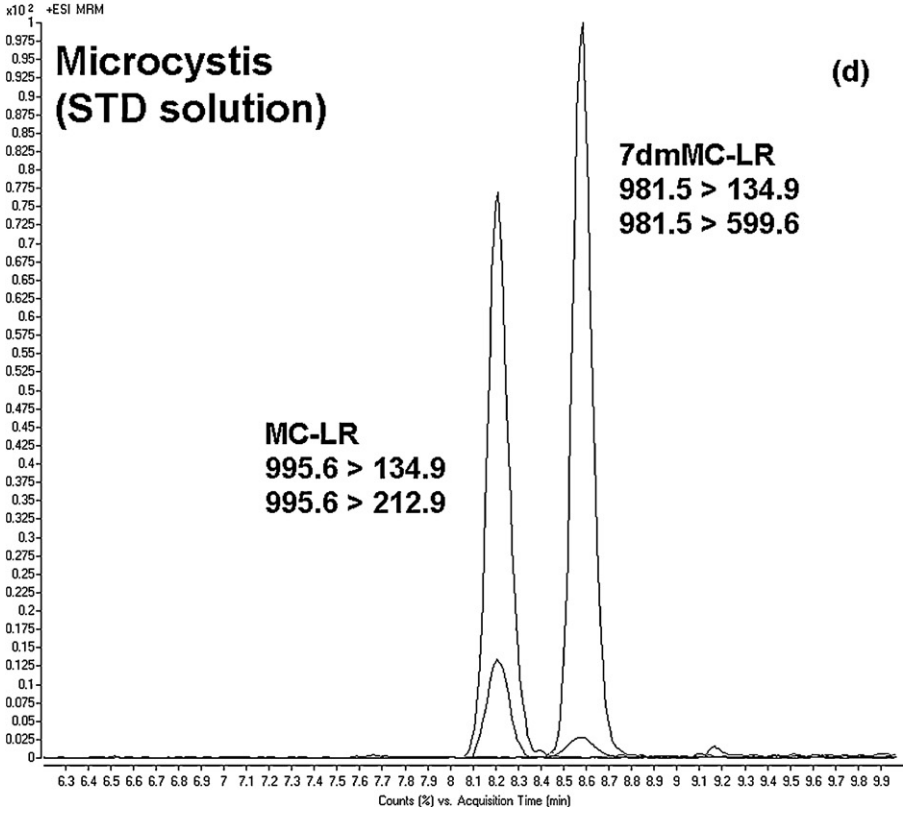


Fig. 5. (*Continued*).

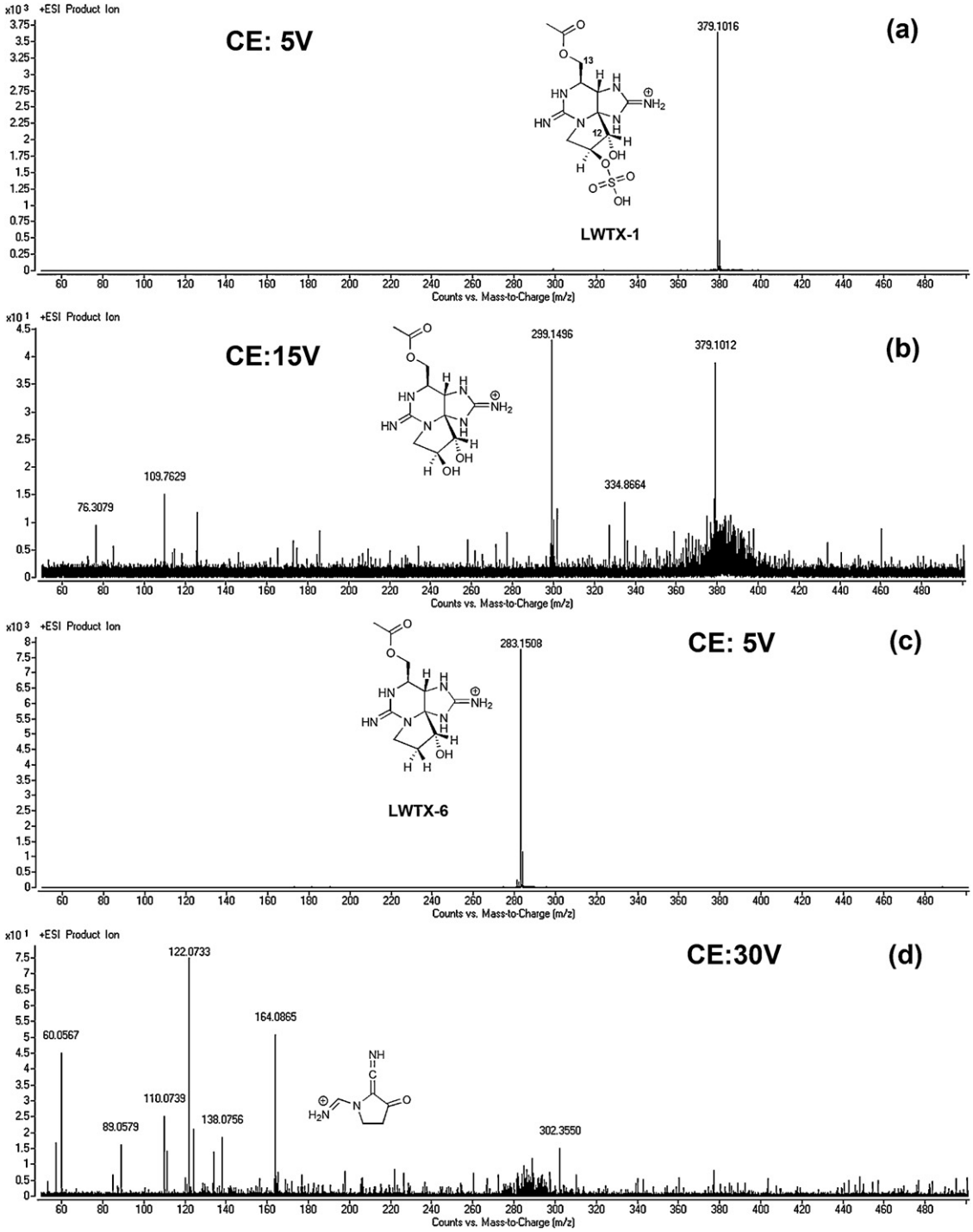


Fig. 6. Tandem mass spectra acquired in the LC-QqTOFMS system for: LWTX-1 at low collision energy (a) and its product ion (b), LWTX-6 at low energy (c) and its product ion (d).

presence of LWTX-1 in the *L. wollei* samples. As in the quantitative analysis, samples were separated with both the RPLC and HILIC methods. To identify the presence of suspect cyanotoxins, an in-house database of 46 cyanotoxins (see supplementary material, Table S2) was used in combination with the "Find by Molecular Feature" function of the MassHunter Qualitative Analysis software (version B.03.01). This function uses chromatography parameters (relative height, retention time) and a scoring algorithm that compares experimental accurate mass measurements, abundance of the isotope cluster and isotope peak spacing to expected values in order to assign a matching score to the identified compounds. The major advantage of using such function is the rapid screening of hundreds substances of interest in large data files within a couple of hours only, thus avoiding tedious manual data extraction and searching.

This approach identified with a high score (>80) the presence of 5 cyanotoxins (dihydroanatoxin-a, hydroxyhomoanatoxin-a (S/R), LWTX-1, LWTX-5 and LXTX-6) in samples from sites 3 and 4 (Table 2) analyzed using the HILIC method. No cyanotoxins were found with the RPLC method. Following these results, MS/MS was

used to confirm the presence of those toxins. Thus, precursor ions were isolated in the quadrupole of the QqTOF and then fragmented in the collision cell at various energies (5, 15, 30 V) in order to perform accurate mass measurements on the fragment ions. These experiments showed only two compounds that could be confirmed in *L. wollei* samples: LWTX-1 and LWTX-6. Isolation and fragmentation of the precursor ion of LWTX-1 (m/z 379.1018) generated an abundant product ion at m/z 299.1496 (Fig. 6a and b). This ion corresponds to $[M+SO_3H]^+$, due to elimination of SO_3 from the sulfate ester at C-12 [9]. Accurate mass error from theoretical values for this fragment was −3.3 mDa. As for LWTX-6 when its precursor ion at m/z 283.1508 (Fig. 6c and d) was isolated and fragmented in the collision cell of the QqTOF, an ion at m/z 138.0756 was observed that could be interpreted as $C_6H_8N_3O^+$ (−9.4 mDa). This same product ion was observed in collision induced dissociation (CID) spectrum of saxitoxin (STX) [31]. Cyanotoxins STX and LWTX-6 differ only by the functional group attached to C-13, a primary amide in the case of STX and acetate in the case of LWTX-6 and thus must share CID fragmentation pathways.

Since the isolation width of the quadrupole during MS/MS experiments was large (4 m/z), MS/MS mass spectra of LWTX-1 and LWTX-6 likely contained product ions of other molecules as well, which made interpretation difficult. In the case of LWTX-6, characteristic ions (using STX fragmentation as a model) such as m/z 204, 281, 221 and 179 are absent from the experimental MS/MS spectrum. However, according to European Commission Decision 2002/657/EC [32], at least 4 identification points are required in order to confirm the presence of a contaminant. Since we have one high-resolution precursor ion and a high-resolution product ion, the number of identification points earned is 4.5. Therefore, the results obtained are sufficient to confirm the presence of LWTX-1 and LWTX-6 in the samples.

4. Conclusion

A useful adapted multi-toxin method has been validated for the monitoring of two important classes of toxins e.g., hepatotoxins, neurotoxins present in algae. The application of the developed method enabled the detection by LC–MS/MS of two saxitoxin analogues (LWTX-1, LWTX-6) in *L. wollei* extracts collected in the St. Lawrence River (Canada). The method was also robust to extract specifically other cyanotoxins that can be found in different common algae such as *A. flos-aquae* and *M. aeruginosa*. The combination of LC-QqQMS and LC-QqTOFMS proved to be a powerful tool for the detection and the confirmation of various cyanotoxins, especially when no all standards are available. Since abiotic or biotic transformation of LWTXs to other more toxic analogues toxins cannot be ruled out, this work highlights the need to develop new sample preparation protocols for the detection and/or quantification of toxins in surface water, wastewaters, and aquatic organisms. The reported results suggest the need for further investigation into the ecotoxicological effects of these saxitoxin analogues to better determine the environmental risk they pose to aquatic organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.10.092.

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