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RESEARCH PAPER

Determination of BMAA and three alkaloid cyanotoxins in lake water using dansyl chloride derivatization and high-resolution mass spectrometry

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Abstract A new analytical method was developed for the detection of alkaloid cyanotoxins in harmful algal blooms. The detection of the nonproteinogenic amino acid β-*N*-methylamino-L-alanine (BMAA) and two of its conformation isomers, 2,4-diaminobutyric acid (DAB) and *N*-(2-aminoethyl) glycine (AEG), as well as three alkaloid cyanotoxins, anatoxin-a (ANA-a), cylindrospermopsin (CYN), and saxitoxin (STX), is presented. The use of a chemical derivatization with dansyl chloride (DNS) allows easier separation with reversed phase liquid chromatography. Detection with high-resolution mass spectrometry (HRMS) with the Q-Exactive enables high selectivity with specific fragmentation as well as exact mass detection, reducing considerably the possibilities of isobaric interferences. Previous to analysis, a solid phase extraction (SPE) step is used for purification and preconcentration. After DNS derivatization, samples are submitted to ultra high-performance liquid chromatography coupled with heated electrospray ionisation and the Q-Exactive mass spectrometer (UHPLC-HESI-HRMS). With an internal calibration using isotopically-labeled DAB-D_3, the method was validated with good linearity (R^2>0.998), and method limits of detection and quantification (MLD and MLQ) for target compounds ranged from 0.007 to 0.01 μg L^{-1} and from 0.02 to 0.04 μg L^{-1}, respectively. Accuracy and within-day/between-days variation coefficients were below 15 %. SPE recovery values ranged between 86 and 103 %, and matrix effects recovery values ranged between 75 and 96 %. The developed analytical method was successfully validated with 12 different lakes samples, and concentrations were found ranging between 0.009 and 0.3 μg L^{-1} except for STX which was not found in any sample.

Keywords Water · Organic compounds/trace organic compounds · Mass spectrometry · Blue-green algae

Introduction

The nonproteinogenic amino acid BMAA is an excitotoxic neurotoxin produced by harmful cyanobacterial blooms. The first identification of BMAA was in 1967 with a major incidence of amyotrophic lateral sclerosis/Parkinson's disease complex (ALS/PDC) on the island of Guam [1, 2]. The neurotoxin was reported to be produced by the cyanobacteria genus *Nostoc* sp. symbiot, which was found in the seeds of cycad tree (*Cycas circinalis*), used to make flour by the Chamorro people from Guam [3]. Furthermore, the discovery of the biomagnification of BMAA through the food chain suggested that concentrations could accumulate to levels sufficient to cause neurodegenerative damages [4]. In summary, BMAA causes the hyperexcitation of the neuronal activity by elevating intracellular calcium levels, and it was found that concentrations as low as 10 and 30 μM, administrated to cortical cell cultures, could induce damages and the death of the motor neurons [5, 6]. Recent studies reported that more than 95 % of cyanobacterial genera can produce BMAA, suggesting its presence in aquatic environments [7]. BMAA is a small hydrophilic molecule, which makes it challenging to analyze, and due to its controversial link to neurodegenerative diseases, it becomes crucial to use highly selective and robust analytical

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methods for its detection [2, 8, 9]. Furthermore, the presence of constitutional isomers, such as 2,4-diaminobutyric acid (DAB), *N*-(2-aminoethyl) glycine (AEG), and β-amino-*N*-methyl-alanine (BAMA) can induce false positives if the analytical method has difficulty to discriminate the different forms [10].

Other alkaloid cyanotoxins have been routinely identified in water bodies including anatoxin-a (ANA-a), cylindrospermopsin (CYN), and saxitoxin (STX). ANA-a is a neurotoxin produced by at least ten genera of cyanobacteria. With a high toxicity (LD_{50}) of 200–250 μg kg^{-1} for mice, this neurotoxin can cause permanent stimulation of respiratory muscles leading to asphyxiation [11–13]. To date, Canada and New Zealand tolerate concentrations below 3.7 and 6 μg L^{-1}, respectively, in drinking water, and for three US states, (California, Oregon, and Washington) the threshold is 1 μg L^{-1} [14]. CYN is an alkaloid toxin with cytotoxic, neurotoxic, and hepatotoxic effects [15–17]. With at least six cyanobacterial genera responsible for its presence, this toxin is linked to tumor promotion and carcinogenic effects in the digestive system due to the inhibition of protein synthesis. The LD_{50} values range from 200 to 2100 μg kg^{-1} for mice, and based on its toxicity, a guideline of 1 μg L^{-1} in drinking water was proposed [18]. Finally, STX is also a potent neurotoxin belonging to a group of paralytic shellfish poisoning (PSP) toxins and known for its severe food poisoning [19]. With a high level of toxicity (LD_{50} value of 10 μg kg^{-1}), STX causes numbness and respiratory failure by disrupting the nervous system; it inhibits the sodium transport by blocking the sodium channels [20, 21]. A guideline of 3 μg L^{-1} in drinking water is used in Australia; however, no guidelines are available in Canada [22].

Several analytical methods have been published for the detection of BMAA, but few consensuses have been made on the reported concentrations. Many separation and detection methods were used such as capillary electrophoresis (EC) [23], gas chromatography (GC) [24, 25] and liquid chromatography (LC) in combination with fluorescence detection [8, 26–31], UV spectroscopy [26, 28], and mass spectrometry [8, 10, 26, 28–30, 32–40]. Precolumn derivatization was routinely used with 6-amino-quinolyl-*N*-hydrosuccinimidyl (6-AQC) [8, 26, 28–30, 34, 36, 37], 9-fluorenylmethyl chloroformate (FMOC) [27, 33], and propyl chloroformate (EzFaast™) [24, 28]. The most commonly used derivatization technique involves a derivatization with 6-AQC, which is widely used for the analysis of amino acids [41]. Derivatization enables easier liquid chromatography separation with reverse phase columns, and the mostly used detectors involve fluorescence detection (HPLC-FD) and mass spectrometry (HPLC-MS) [41]. For the most known commonly used analytical method, HPLC-FD, BMAA concentrations were overestimated, due to the derivatization of other amino acids or small molecules present in complex matrices causing false positives and unspecific detection. However, the use of tandem mass spectrometry (MS/MS) detection showed different results, due to higher selectivity, with significantly lower detected concentrations of BMAA [29, 32, 33, 42]. Several studies presented the possibility of eliminating the derivatization step using hydrophilic separation with HILIC columns coupled with mass spectrometric detection [29, 31–33, 35, 37, 39, 40]. The advantage of the HILIC technique is the simplicity of the sample preparation since the compounds are directly injected and analyzed. However, the major drawback comes from the high dependency on the chromatographic and MS/MS separation abilities [41, 43]. Furthermore, the presence of numerous low mass isobaric compounds and isomers can compromise the selectivity of the analytical methods. More specifically, DAB and AEG have been previously studied and known to interfere with the analysis of BMAA due to problems of coelution [41]. Many studies using derivatization (6-AQC) and HILIC have been able to distinguish BMAA from DAB, but few have been able to differentiate the three isomers [10, 28, 39, 40]. In a recent study, a new approach was used for the analysis of BMAA using DNS derivatization and ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-ESI-MS/MS) [38]. This derivatization was previously reported for the analysis of amines by fluorescence detection [44, 45]. It was also documented for its use on the improvement of chromatographic separation and enhancement of ionization efficiency in mass spectrometry detection [46–49]. With easier preparation steps and faster reaction time (4 min at 60 °C) as well as a specific fragmentation patterns for BMAA and DAB, DNS derivatization was shown to be a usable alternative to 6-AQC method [38].

Considering the challenge toward the analysis of BMAA, there is a need for reliable analytical methods usable routinely for clinical reasons. As described by Cohen [41], BMAA is present at low concentrations in complex matrices in the presence of possible isobaric interferences; therefore, effective sample clean-up is essential prior to analysis to avoid those compounds. Moreover, a selective method is primordial with good chromatographic separation and mass spectrometric detection with specific product ions. High-resolution mass spectrometry (HRMS) detection is proposed in this study with the use of the hybrid mass spectrometer, the Q-Exactive. In summary, it is a benchtop Orbitrap detector, which is combined to a quadrupole precursor selection and a high-energy collisional dissociation cell (HCD). The advantage of this hybrid mass spectrometer resides in the combination of a quadrupole *m*/*z* value filtration prior to an HCD cell, thus offering the possibility of fragmenting selected precursor ions. With resolving power up to 140,000 full width half mass (FWHM) at *m*/*z* 200, the mass accuracy obtained with the Q-Exactive is between 1 and 3 ppm [50]. These features allow high sensitivity and selectivity detection and quantification. It was previously used

for its capability of high selectivity in peptides sequencing and metabolomics, and a few methods were developed for small molecules quantification in environmental matrices [51–55]. The objective of this study was to develop an analytical method using ultra high-performance liquid chromatography coupled with heated electrospray ionization and the Q-Exactive (UHPLC-HESI-HRMS) for the detection and quantification of BMAA and two of its constitutional isomers, DAB and AEG, as well as three other alkaloid cyanotoxins, ANA-a, CYN, and STX (Fig. 1). A solid phase extraction (SPE) step was used for the clean-up and preconcentration of environmental water samples, then the extract was submitted to a derivatization step with DNS. The use of HRMS in a fragmentation mode (t-MS^2) allows us to determine the fragmentation pattern of the different derivative compounds and thereafter suggest the structures of the principal product ions detected with high mass accuracy. The method was validated with the use of deuterated 2,4-diaminobutyric acid (DAB-D_3) as internal standard. The extraction recovery, the method detection and quantification limits (MDL and MQL), the linear dynamic range, the accuracy, the precision, and matrix effects were evaluated with spiked real bloom water samples. The method was finally applied to real field-collected cyanobacterial bloom water samples to assess the quantity of each of the studied cyanotoxins.

Materials and methods

Chemicals, reagents, and stock solutions

L-BMAA hydrochloride (BMAA, purity≥97 %), DL-2,4-diaminobutyric acid dihydrochloride (DAB, purity≥97 %), and DL-phenylalanine (PHE, purity≥99 %) were purchased from Sigma-Aldrich Chemical Co. (Oakville, ON, Canada). *N*-(2-Aminoethyl) glycine (AEG, purity≥95 %) was purchased from TCI America (Portland, OR, USA), (±)-anatoxin-a/furamate salt (ANA-a, purity≥99 %) was purchased from Abcam Biochemicals (Cambridge, MA, USA), cylindrospermopsin (CYN, purity≥97 %) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA), and 2,4-diaminobutyric acid-2,4,4-D_3 dihydrochloride (DAB-D_3, 99 at.% D) was purchased from CDN isotopes (Pointe-Claire, QC, Canada). Ampoules of certified standard solutions of saxitoxin dihydrochloride (STX, 66.3 μM in 3 mM hydrochloric acid) were obtained from the Certified Reference Materials Program (NRC, Halifax, NS, Canada). Sodium tetraborate (Borax, purity≥99 %), dansyl chloride (DNS, purity≥99 %), citric acid (purity≥99.5 %), and formic acid (HCOOH, purity≥95.0 %) were purchased from Sigma-Aldrich (Oakville, ON, Canada). All solvents used were of high-performance liquid chromatography (HPLC) grade purity from Fisher Scientific (Whitby, ON, Canada). Individual stock solutions of BMAA, DAB, AEG, CYN, and DAB-D_3 were prepared in HPLC grade water, and ANA-a was prepared with acidified water (0.1 M formic acid) all at a concentration of 100 mg L^{-1} prior their storage at −20 °C. STX solutions from ampoules were transferred to amber glass bottles prior their storage at −20 °C. A 100-mg L^{-1} stock solution of PHE was prepared daily in HPLC grade water prior to analysis. All BMAA, DAB, and AEG solutions were prepared and stored in polypropylene bottles and vials knowing that BMAA can strongly adhere on glass surfaces [41]. As for ANA-a, CYN, and STX solutions, they were prepared and stored in glass bottles and vials. According to compound stability, new stock solutions were prepared every 4 months [17,

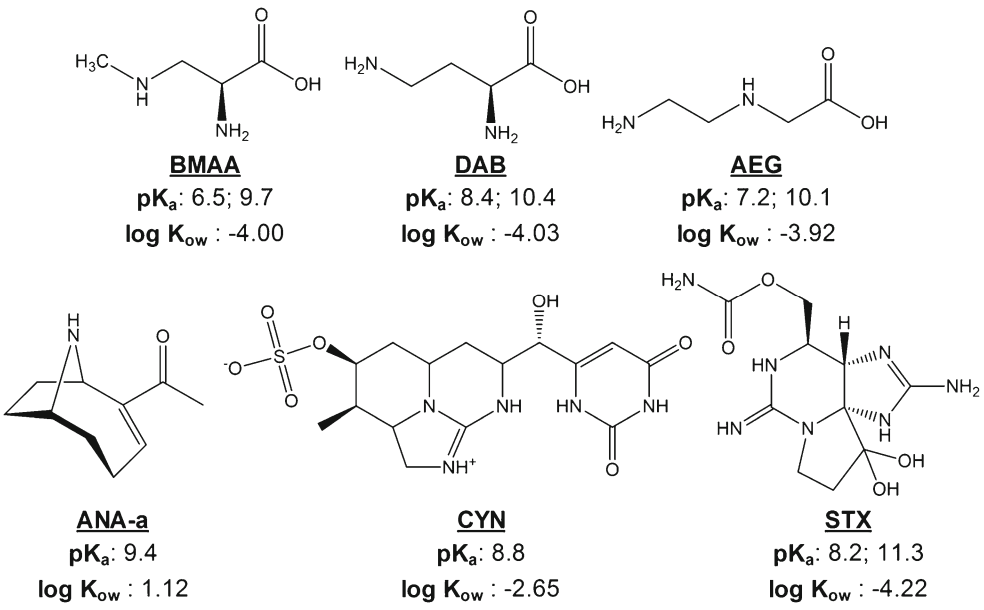

Fig. 1 Chemical structures, pK_a, and partition coefficients (log K_{ow}) of the studied cyanotoxins: β-*N*-methylamino-L-alanine (*BMAA*), 2,4-diaminobutyric acid (*DAB*), and *N*-(2-aminoethyl) glycine (*AEG*), anatoxin-a (*ANA-a*), cylindrospermopsin (*CYN*), and saxitoxin (*STX*)

38, 55]. All working solutions were prepared by dilution with HPLC-grade water from individual stock solutions. The solvents for the chromatographic mobile phases were prepared daily.

Cyanobacterial bloom samples

Environmental samples were provided by the monitoring program realized by the *Ministère du Développement Durable, de l'Environnement, et de lutte aux changements climatiques,* (MDDELCC—The Ministry of the Environment of the province of Québec, Québec, Canada). The lakes were sampled from 2009 to 2013 as part of a project to monitor cyanobacteria genera and their toxins around the province of Québec, Canada, and they were chosen for their high occurrence of cyanobacterial blooms. The samples have been stored at −20 °C until analysis to reduce degradation. Before each analysis, the samples were submitted three times to a freeze–thaw lysis followed by filtration using 0.22-μm nitrocellulose membrane obtained from Millipore (Billerica, MA, USA) [41, 56–59]. All recovery data and validation parameters were acquired using spiked relevant environmental matrix, which consisted of lake water bloom samples containing nonharmful cyanobacterial cells. This matrix assures the method development to take account of matrix effects without cyanotoxins contamination.

Solid-phase extraction procedures

A strong cation-exchange polymeric sorbent Strata-X-C cartridge (Phenomenex, Torrance, CA, USA) with 200 mg bed mass and a volume of 6 mL was used for sample clean-up and preconcentration. Other strong cation-exchange sorbents were previously used for sample pretreatment for the analysis of BMAA by LC-MS/MS, and the SPE conditions were inspired from these studies [31, 34, 37, 60]. The procedure was done using a 12-position manifold manufactured by Phenomenex (Torrance, CA, USA). The SPE was performed with 100-mL aliquots of samples with pH adjusted to 4 with citric acid. The conditioning step was done with 5 mL of methanol (MeOH) for cartridge activation followed by 5 mL of acidified water with citric acid (pH 4). The acidified samples were then loaded on the cartridge columns at a rate of 2 mL min^{-1} using a mechanical pump. The cartridges were washed with 5 mL of acidified water (pH 4) containing 15 % MeOH (*v*/*v*). Elution was performed with 5 mL of MeOH containing 3 % NH_4OH into conical-bottom polypropylene centrifuge tubes. The eluates were completely dried under a gentle stream of nitrogen at room temperature with a nine-port Reacti-Vap unit from Pierce (Rockford, IL, USA). The dried fractions were then reconstituted with the DNS reactive solution.

Dansyl chloride derivatization

The derivative procedures with DNS were previously described and optimised by Salomonsson et al. [38] for the derivatization of BMAA. A direct derivatization was done with the dried fractions obtained after the SPE procedures by adding 250 μL of a Borax buffer (0.2 M, pH 9.5) and 250 μL of DNS in acetone (1 mg mL^{-1}). The tubes were vortexed and placed in an Innova 4230 refrigerated incubator shaker from New Brunswick Scientific (Edison, NJ, USA) at 60 °C for 10 min with agitation at 150 rpm. A slightly longer derivatization time was used compared to Salomonsson et al. [38] (4 min) due to the temperature equilibration of the incubator and the solutions. The efficiency of the reaction could not be directly evaluated; however, the reaction completion was evaluated over time (1–30 min) by spiking the analytes in solution (100 μg L^{-1}) and using the plateau of signal intensity. The samples were finally cooled at room temperature and then directly submitted to the UHPLC-HESI-HRMS analysis for the target compounds: BMAA-DNS, DAB-DNS, AEG-DNS, ANA-a-DNS, CYN-DNS, STX-DNS, and DAB-D_3-DNS. The complete workflow is illustrated in Fig. 2 with the reaction scheme of DNS derivatization presented in Fig. 3.

UHPLC-HESI parameters

The chromatographic separation was performed with a Thermo Scientific Dionex Ultimate 3000 Series RS pump coupled with a Thermo Scientific Dionex Ultimate 3000 Series TCC-3000RS column compartments and a Thermo Fisher Scientific Ultimate 3000 Series WPS-3000RS autosampler controlled by Chromeleon 7.2 Software (Thermo Fisher Scientific, Waltham, MA, USA and Dionex Softron GMbH Part of Thermo Fisher Scientific, Germany). The chromatographic column was a Hypersil GOLD™ C18 column (100 mm, 2.1 mm, 1.9 μm particles) preceded by a guard column (5 mm, 2.1 mm, 3 μm particles) (Thermo Fisher Scientific, Waltham, MA, USA), both at 40 °C. The mobile phase for the analysis of DNS derivatives consisted of H_2O with 0.1 % formic acid as mobile phase A and acetonitrile (ACN) with 0.1 % formic acid as mobile phase B. A solvent gradient was used starting from 30 % of B, increasing to 90 % from 0 to 2 min, then increasing to 100 % from 2 to 4 min, and it was held constant for 2 min. Finally, the mobile phase was brought back to initial conditions and maintained 4 min for equilibration resulting in a total run time of 10 min. The flow rate was 0.5 mL min^{-1}, and the injection volume of sample was chosen to be 25 μL. The ionization was performed by a heated electrospray ionization source (HESI-II) configured in positive mode. The

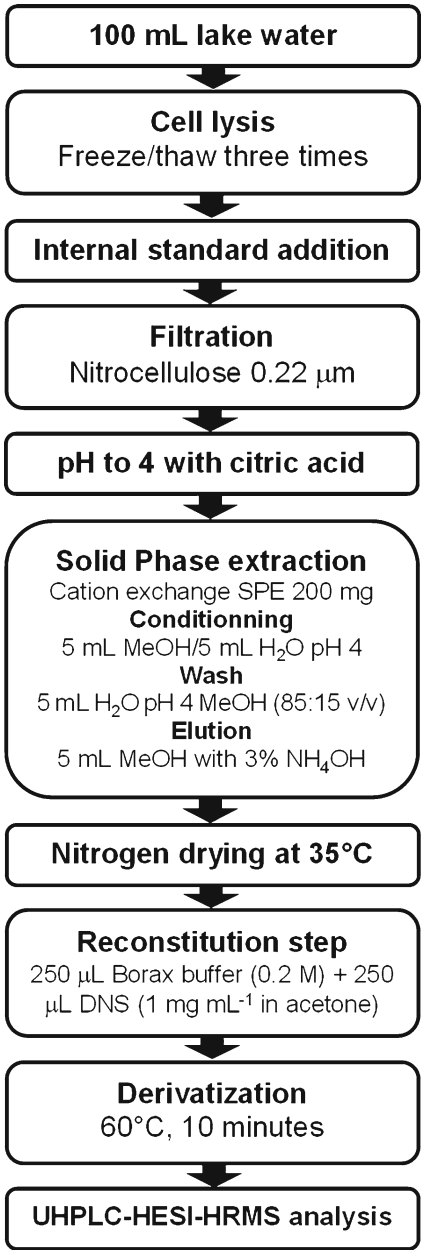

Fig. 2 Analytical method workflow including sample preparation, clean-up procedure, and derivatization

voltage was optimized at +3000 V, the capillary and vaporizer temperatures were set at 400 °C and 350 °C, respectively, and the sheath gas and auxiliary gas flow were set at 30 and 60 arbitrary units, respectively.

High-resolution mass spectrometry detection

Detection was performed using a Q-Exactive mass spectrometer controlled by the Excalibur 2.3 Software (Thermo Fisher Scientific, Waltham, MA, USA), and exact masses were calculated using Qualbrowser in Xcalibur 2.3. Instrument calibration in positive mode was done every 5 days with a direct infusion of a LTQ Velos ESI positive ion calibration solution (Pierce Biotechnology Inc., Rockford, IL, USA). Targeted ion fragmentation (t-MS^2) mode was used for compound quantification and was optimized using individual standards solutions at a concentration of 100 μg L^{-1}. The solutions were directly infused with a syringe at a flow rate of 0.01 mL min^{-1} through a T-union connected to the UHPLC system with a mobile phase flow rate of 0.5 mL min^{-1}. The product ions and their collision energy were chosen by increasing the normalized collision energy (NCE) using the Q-Exactive Tune 2.3 software (Thermo Fisher Scientific, Waltham, MA, USA). All optimized collision energies, precursor and fragment ions, are shown in Table 1. The theoretical exact *m*/*z* values of the precursor and product ions are presented in supplementary materials Table S1 with their respective chemical formulas. In the t-MS^2 mode, the data were acquired at a resolving power of 17,500 FWHM at *m*/*z* 200. The automatic gain control (AGC) target, for a maximum capacity in C-trap, was set at 2×10^5 ions for a maximum injection time of 100 ms. A mass inclusion list was used including the precursor ion *m*/*z* values, their expected retention time with a 1-min window, and their specific fragmentation energy (HCD). The precursor ions are filtered by the quadrupole, which operates at an isolation width of 0.4 amu.

Data analysis and method validation

The data treatment was performed using the Excalibur 2.3 Software (Thermo Fisher Scientific, Waltham, MA, USA). The method validation was done according to the recommendation of validation protocol for environmental chemistry analysis from the Québec's MDDELCC ministry guidelines.

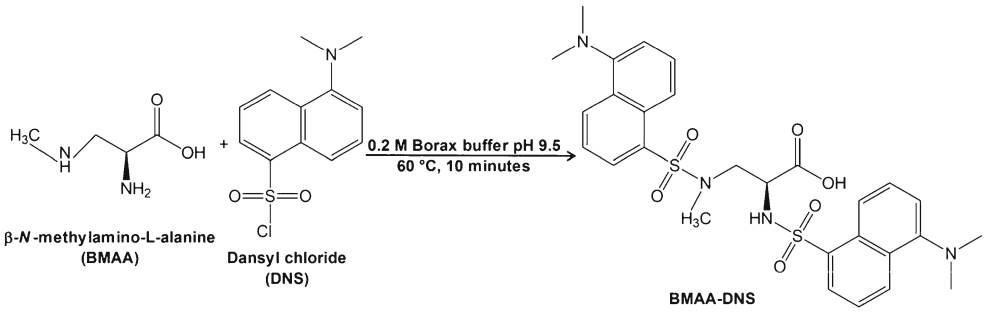

Fig. 3 Reaction scheme of DNS derivatization procedure with BMAA

Table 1 Parameters of HRMS detection of compounds as DNS derivatives

Derivative compounds	RT (min)	Experimental precursor ion (*m*/*z*)	Quantification product ion (*m*/*z*)	Confirmation product ion (*m*/*z*)	Relative intensity ratio[a]	NCE[b] (%)	Average mass accuracy[c] (ppm)	Confirmation ion[d] (*m*/*z*)
BMAA	3.78	585.1836	277.1007	71.0131	5.4±0.6	30	1.26	278.1039
DAB	3.60	585.1836	277.1006	88.0395	2.8±0.2	30	1.80	278.1037
AEG	3.78	585.1835	289.1005	88.0394	3.3±0.3	30	1.95	290.1032
ANA-a	3.77	399.1737	149.0964	131.0856	1.2±0.2	35	1.47	150.0989
CYN	1.27	649.1744	194.1291	176.1184	1.6±0.2	35	1.03	195.1230
STX	2.99	533.1925	204.0877	138.0665	2.3±0.2	35	1.46	205.0899
DAB-D_3	3.60	588.2024	279.1130	88.0396	2.3±0.3	25	1.42	280.1159

[a] Ratio (quantification product ion/confirmation product ion)

[b] Fragmentation energy for precursor ion in HCD cell

[c] Accuracy of average measured *m*/*z* values for quantification product ion

[d] Second abundant ion in isotopic pattern from quantification product ion

For DAB-DNS, ANA-a-DNS, CYN-DNS, and STX-DNS, two product ions with the highest signal intensity were selected as the quantification and the confirmation ions, the first being used to establish the method limits of detection and quantification. The relative intensities of their ratio were used for compound confirmation to avoid false positives. The second most abundant ion from isotopic pattern was used as confirmation ion for the target compounds, and the isotopic ratio was confirmed with <10 % of intensity variations. The structural identification of the product ions from derivative compounds was done using Mass Frontier™ 7.0 Software (HighChem, Bratislava, Slovakia). For a good selectivity in data analysis, a mass tolerance window was set to 5 ppm (±2.5 ppm) for the extracted *m*/*z* values from acquisition [53]. The recovery values for the SPE method were evaluated using dd-H_2O water and nonharmful cyanobacterial bloom (or bloom water blank), at three concentrations of 0.05, 0.25, and 1.25 μg L^{-1}. Extraction recoveries and matrix effects were determined with the mean peak areas of the targeted compounds spiked prior to extraction in matrix-free (dd-H_2O) and matrix-containing (bloom water blank) samples compared to spiked postextraction matrix-free samples, all in triplicate, and results are reported as percentages. A 7-point internal calibration curve was obtained by passing through the SPE method and was prepared with bloom water blanks with concentration levels ranging from 0.025 to 2.5 μg L^{-1} and analyzed in triplicate. The concentration of internal standard (IS) DAB-D_3, 0.750 μg L^{-1}, was selected for its capacity of signal correction (data not shown). Method detection limit (MDL) and method quantification limit (MQL) were established by calculating three and ten times, respectively, the standard deviation of the mean value of five spiked blank matrix samples (n=5) containing approximately five times the estimated concentration for detection limit (0.05 μg L^{-1}). Accuracy, expressed as relative error (%), and within-day/between-days variations, expressed as the relative standard deviation (%), were determined with three different concentrations on the linearity range (0.05, 0.25, and 1.25 μg L^{-1}, n=5) in blank matrix samples. Between-days reproducibility was estimated over 5 weeks. Statistical comparison was used when needed with the Statistical Package for Social Science (SPSS 21.0, Chicago, IL, USA) for Windows. ANOVA test and Tukey's post hoc tests were used with statistical significance defined as $P<0.05$.

Results and discussion

Sample treatment

After the sample lysis, which promotes the breaking of the cyanobacterial cells and leaches the cell-bound toxins, a filtration step is needed to remove the suspended particles. Filtration conditions were studied for all compounds of interest to ensure sample integrity and accuracy. This step can have an impact on the loss of analytes due to undesirable interactions between the target compounds and the filter membrane material. Seven different filters were tested to isolate the unbound extracellular fraction: glass fiber (GF), cellulose acetate (CA), polycarbonate (PC), nitrocellulose (NC), mixed cellulose ester (MCE), nylon (NY), and polypropylene (PP). All compounds were spiked at a concentration of 100 μg L^{-1} in bloom water blanks before filtration, and their mean peak areas were compared to blank samples spiked after filtration. Mean recovery values for each filter and compounds are presented in Fig. 4. Three of the seven filter membranes gave significantly higher recovery values with 92–102 % for NC, 78–118 % for PC, and 65–107 % for NY ($P>0.05$). However, the four other filter membranes showed much lower recovery values with 20–71 % for GF, 11–31 % for CA, 26–110 % for MCE, and 0–22 % for PP. Taking into account these results, NC was deemed the most reliable membrane material for the

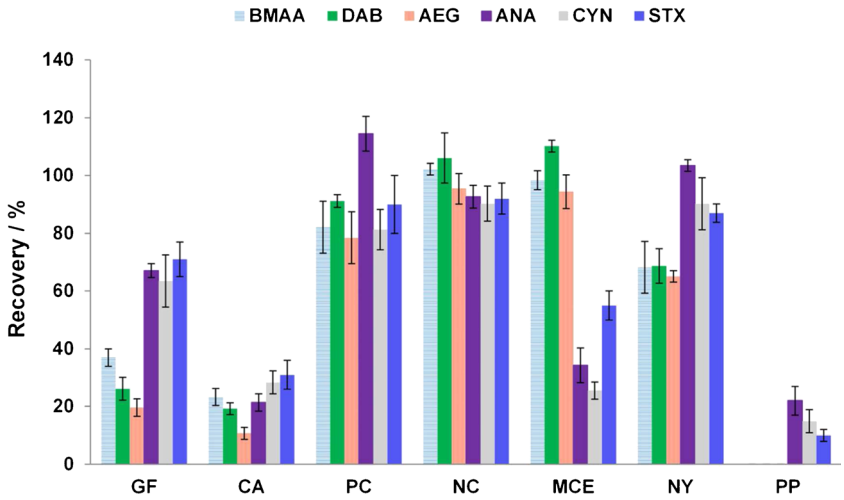

Fig. 4 Comparison of recovery values for the target cyanotoxins on different filter materials, glass fiber (*GF*), cellulose acetate (*CA*), polycarbonate (*PC*), nicrocellulose (*NC*), mixed cellulose ester (*MCE*), nylon (*NY*), and polypropylene (*PP*). *Vertical error bars* represent standard deviations from the mean (n=3)

filtration of the suspended particles in samples while minimizing the loss of the molecules of interest onto the filter.

The bloom water samples are complex matrices with a high presence of organic matter. A solid-phase extraction step is then used to clean-up the samples and also to preconcentrate the analytes of interest and consequently enhance sensitivity by decreasing their method detection and quantification limits (MDL and MQL). A Strata X-C cartridge was used, which contain a strong cation exchange sorbent, and has the advantage of allowing the use of organic solvents during the washing step, thus removing a large portion of interfering organic matter from the matrix. Given that ionic bonds are stronger then van der Waals interactions, this permits us to use a high percentage of organic solvent during the washing step. In order to positively charge the compounds of interest, citric acid was added to set the pH of the solutions to 4, below the pK_as of the target compounds (Fig. 1). For the washing step of the SPE sorbent, 5 mL of water acidified with citric acid (pH 4) containing 15 % MeOH was used to eliminate a maximum of interfering compounds from the matrix. The elution step was done with 5 mL of 3 % NH_4OH in MeOH in order to change the charge of the compounds and then release them from the sorbent. The use of basified water was suggested by Li et al. since it has higher eluent strength, but with the use of 5 mL of MeOH instead, the elution can be completed, and subsequently, the evaporation step is still faster [37]. The recovery values for the SPE procedure were calculated using the mean peak area of targeted compounds spiked in pure water before the extraction compared to pure water spiked after extraction. The results for the different recoveries are shown in Table 2 and gave good results for the three concentration levels (0.05, 0.25, and 1.25 μg L^{-1}) with values ranging from 86 to 103 %. The matrix effects were also determined by comparing the mean peak area of targeted compounds in bloom water blank samples spiked before the extraction compared to pure water spiked after extraction. The signal recoveries for the same three concentration levels are shown in Table 2 and ranged from 75 to 96 %. This small drop of signal could be caused by ion suppression during the ionization due to the presence of interfering molecules from the matrix, as explained by Cohen [41].

Derivatization

The use of DNS for the derivatization of BMAA and DAB was previously described and optimized by Salomonsson et al. [38]. As explained, this derivatization is a good choice for its ease, rapidity, and low cost. It was demonstrated by Guo and Li [47] as a simple method, which produces little to no side-reaction products. In this study, the procedure was adapted from the derivatization proposed by Salomonsson et al. [38]. Due to the use of a different source of heat, which was an incubator instead of a heating block, the reaction time was evaluated using the variation of the mean peak areas of the studied compounds between 1 and 30 min. Results are shown in Fig. S1 (see supplementary materials), and a reaction time of 10 min was necessary to maximize the signal, and after 10 min, no significant increase occurred but higher signal variability was observed (P<0.05).

High-resolution mass spectrometric detection

The Q-Exactive can operate in multiple acquisition modes depending on the analytical needs. In this study, the fragmentation of precursor ions was necessary in order to differentiate the BMAA isomers and give a higher selectivity in the detection. The t-MS^2 mode, given by the mass spectrometer, was then chosen for our purposes due to its selectivity and reliability in small molecule quantification [54]. Some parameters need to be optimized to enhance sensitivity: the AGC target, the maximum injection time (IT) of the ions in the C-trap, and the resolving power (RP). These parameters will affect the

Table 2 Recoveries from SPE procedures and matrix effects of target analytes evaluated at three different concentrations (ng L^{-1}) with standard deviation (SD) (n=3)

Compounds	SPE recovery±SD (%)			Signal recovery from matrix effects±SD (%)		
	25	250	1250	25	250	1250
BMAA	97±11	99±10	102±6	86±10	85±8	90±9
DAB	95±13	99±9	99±5	79±12	78±12	82±9
AEG	93±12	97±7	102±6	86±10	85±10	90±7
ANA-a	99±10	103±12	101±9	90±9	95±8	96±9
CYN	92±9	91±8	94±9	84±10	83±11	87±10
STX	87±11	86±9	90±10	75±12	80±10	81±11

duty cycle of the Orbitrap detection, which affect the number of data acquisitions for each chromatographic peak, essential for a precise quantification. For more details about the operation and technical details, see previous studies of analytical methods using the Q-Exactive [54, 55]. Since the t-MS^2 scan mode produces much fewer ions transferred to the Orbitrap analyzer than a full-scan mode, a smaller AGC target and injection time can be used. This scan mode is quite useful for enhancing sensitivity when highly charged matrices are analyzed. For our purpose, 1×10^5 ions were transferred to the C-trap for a maximum injection time of 50 ms. As for the selected RP, the main criterion is the number of acquisition points for each chromatographic peak, which as to be 7 or higher for a quantitative analysis with an acceptable relative standard variation (RSD) [61]. In our case, our chromatographic peaks are narrow (<10 s), thus giving approximately 12 acquisition data points per peak using a RP of 17,500 m/z 200 (Fig. S2). This RP was then selected given that, together with precursor fragmentation, the selectivity of an exact m/z value is deemed acceptable [55]. Moreover, the mass accuracies were measured below 2 ppm, which is considered acceptable according to the manufacturer recommendations (between 1 and 3 ppm). Finally, selectivity is highly dependent on the mass tolerance (MT), which enables the selection of extracted m/z values from acquisition data. By narrowing the mass tolerance of a monitored ion in a mass extraction window, the presence of false positives is reduced. An adequate mass tolerance was chosen to be 5 ppm (±2.5 ppm), which is in accordance with many studies [53, 62].

The DNS derivatives were ionized in positive mode $[M+H]^+$ before being selected by the quadrupole, and then, the fragmentation took place in the HCD cell. The fragmentation energies were carried out to obtain the optimal normalized collision energy (NCE) for complete fragmentation of precursor ions, and results are shown in Table 1. Fragmentation spectra for BMAA-DNS, DAB-DNS, and AEG-DNS (Fig. 5) showed similar pattern of fragmentation. Considering these, the selected product ions were m/z 585.1836>277.1007; 71.0131 for BMAA-DNS, m/z 585.1836>277.1006; 88.0395 for DAB-DNS and m/z 585.1835>289.1005; and 88.0394 for AEG-DNS (exact m/z values and mass accuracies are presented in Table 1). Specific product ions were selected for each compound in order to avoid signal enhancement caused by mutual contributions. Both m/z 277 and 289 are found in their own fragmentation pattern; however, their intensities are significantly different, with a difference of two orders of magnitude for both, which can be assumed by a fragmentation pattern promoting fragment m/z 277 for BMAA-DNS and m/z 289 for AEG-DNS. In this case, a specific confirmation ion is essential in order to avoid a cross selectivity of the two isomers. In this case, m/z 71 for BMAA-DNS and m/z 88 for AEG-DNS were found to be unique product ions, and their mean peak area ratios (Table 1) were closely studied for every sample in order to confirm the presence of BMAA-DNS without any signal contribution of AEG-DNS, and vice versa. Since the product ions were selected from the derivatives and not from the compounds alone, a structural search using the software Mass Frontier™ was done to confirm their specificity, and results are shown in Figs. 5 and 6. In the case of ANA-a-DNS, CYN-DNS, and STX-DNS, the fragmentation patterns were very similar to those without derivatization. The selected ions were m/z 399.1737>149.0964; 131.0856 for ANA-a-DNS, m/z 649.1744>194.1291; 176.1184 for CYN-DNS and m/z 533.1925>204.0877; and 138.0665 for STX-DNS. The selected product ions of these three compounds were associated to the toxins molecules without the DNS. For all the fragmentation spectra, specific product ions coming from the DNS reactive were present confirming the derivatization step, including these m/z values: m/z 170, 172, 235, 236, and 237. These ions were rejected during the product ions selection of derivative compounds, as they are only specific to DNS and not to target compounds. Finally, for all the compounds, the second most abundant observed ion was used as confirmation, and the isotopic ratio was confirmed with <10 % of intensity variations (Table 1).

Chromatographic separation

A C18 chromatography column was used for the derivative compounds, and the mobile phase included 0.1 %

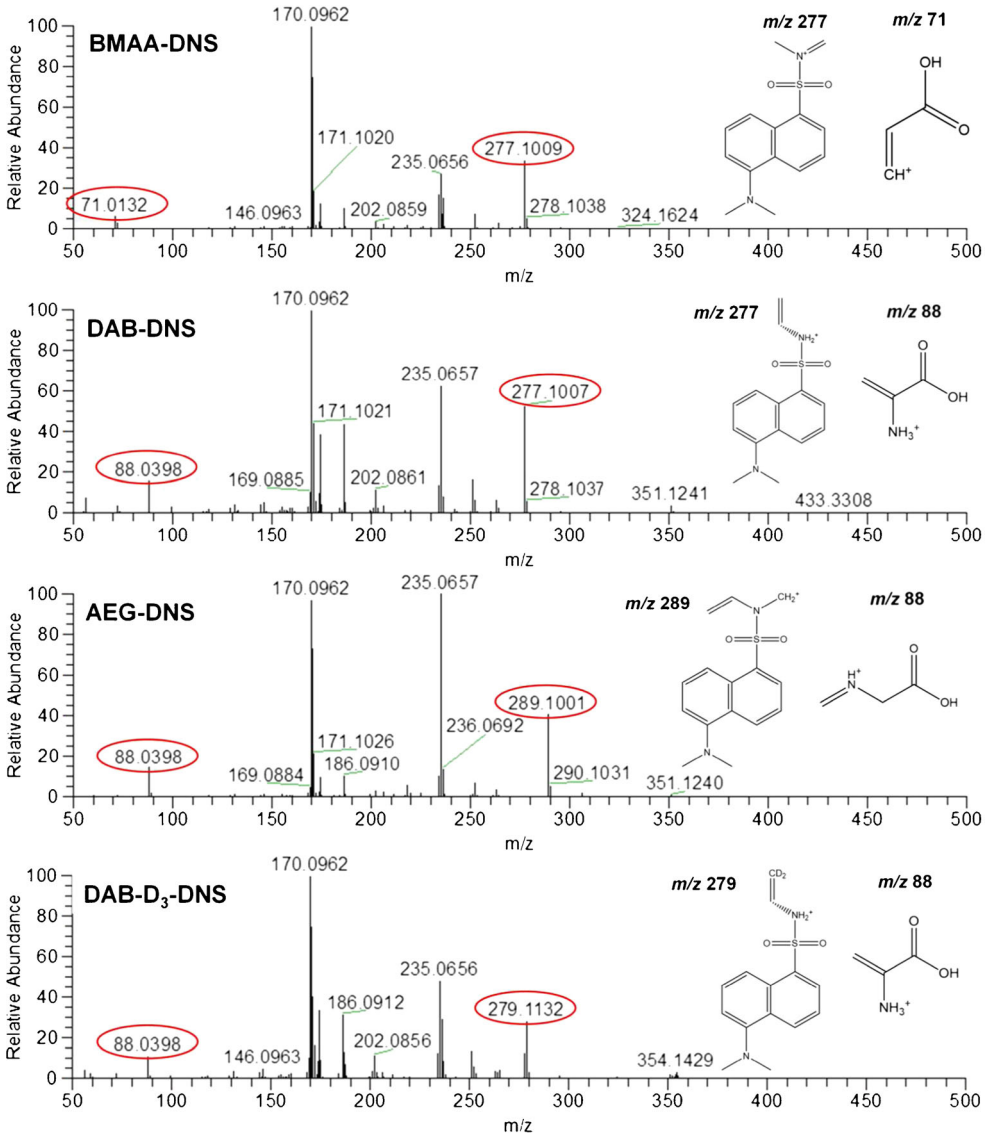

Fig. 5 Fragmentation mass spectra of BMAA-DNS, DAB-DNS, AEG-DNS, and DAB-D_3-DNS with the structures of their quantification and confirmation product ions

formic acid in water and 0.1 % formic acid in acetonitrile (ACN). A minimum of 30 % of ACN in the beginning of elution was necessary to enable a proper elution of the compounds within the gradient ramp, which was divided in two phases for the same reasons. The gradient was adjusted to achieve the separation of BMAA-DNS and its isomers DAB-DNS and AEG-DNS. Solvent flow rate, gradients, and elution time were tested, and the parameters were chosen to be optimal for elution time, compounds separation, and compounds peak shape. BMAA-DNS and DAB-DNS were completely resolved; however, chromatographic separation was laborious between BMAA-DNS and AEG-DNS. Therefore, different chromatographic columns were tested including C18, C8, and phenyl as well as different organic solvents including ACN, MeOH, ethanol, and 2-propanol. Finally, a slow gradient was tested for over 40 min to assess chromatographic separation, without success. Ultimately, to overcome this issue, the use of t-MS^2 mode from the Q-Exactive was necessary, and as explained in the previous section, the choice of specific product ions from both derivative compounds enabled selective quantification. The chromatographic separation is illustrated in Fig. S3 for all derivative compounds, and their retention times sustained no significant variation (approximately ±0.02 min) for 4 months of experiments including approximately 1000 injections on the same column. In the mass inclusion list of the precursor ions, the acquisition time window was set at 1-min center on each retention time of target analytes. Retention time variation was below 0.01 min for 1 day of analysis. The chromatographic run was short with <4 min for compounds elution and a total of 10 min including the column equilibration. This elution time is similar to the previous UPLC methods

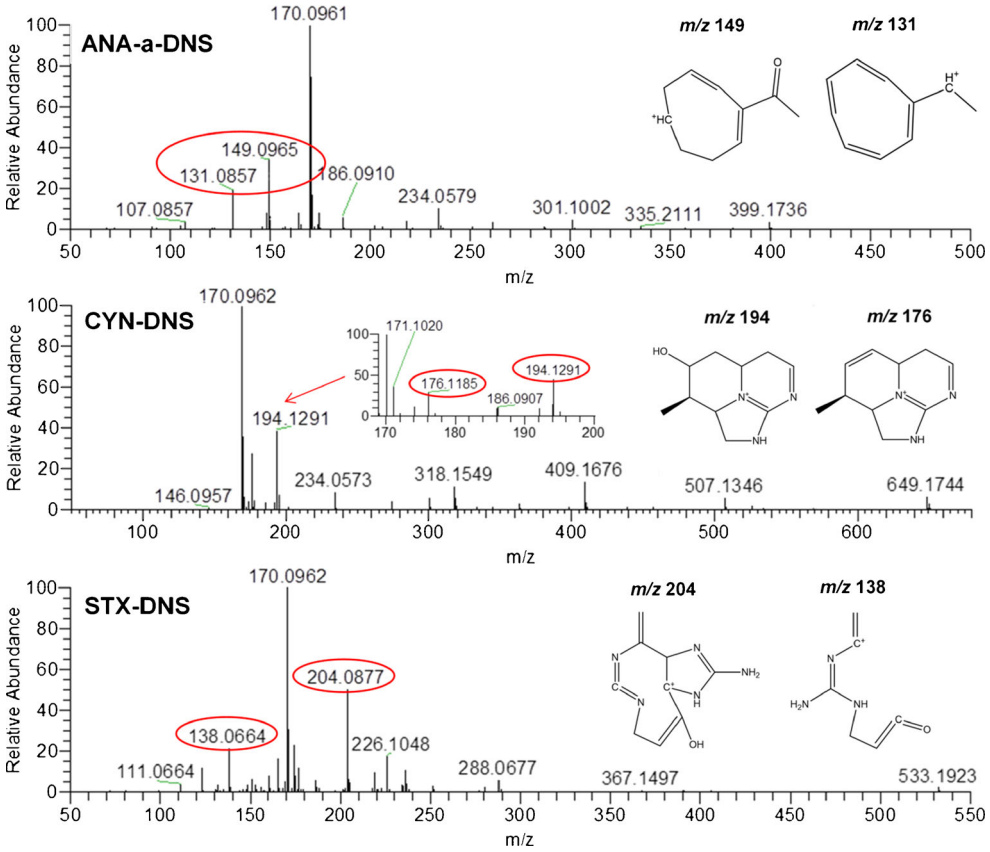

Fig. 6 Fragmentation mass spectra of ANA-a-DNS, CYN-DNS, and STX-DNS with the structures of their quantification and confirmation product ions

with HILIC and reverse phase columns [38, 40]. Finally, the amino acid phenylalanine (PHE), which is considered as an isobaric interference of ANA-a, was derivatized and analyzed to confirm that it would not contribute as a false positive for the detection of ANA-a. Their retention times as DNS derivatives are 3.29 min for PHE-DNS and 3.77 min for ANA-a-DNS making them fully separated, and ultimately, PHE will not interfere during the analysis. If a coelution would have occurred, ANA-a-DNS and PHE-DNS product ions *m/z* values would have been distinguished from each other, given the high resolving power of the Q-Exactive, as explained in a previous study [55].

Method validation

The performances of the UHPLC-HESI-HRMS method were evaluated based on these parameters: linearity, sensitivity, precision, accuracy, matrix effects, and selectivity. The matrix effects were previously discussed in the sample treatment section with the evaluation of the SPE treatment. The use of an isotopically labeled internal standard is highly recommended for the quantitative detection of BMAA; the DAB-D_3 was then selected according to this criterion. As explained previously, all validation parameters were evaluated using bloom water blanks to take account of matrix effects. A 7-point standard addition

Table 3 Method validation parameters with accuracy and precision determined at three different concentrations (μg L^{-1})

Compounds	Accuracy (RE %)			Within-day (RSD %)			Between-days (RSD %)			R^2	Linearity range (μg L^{-1})	MDL (μg L^{-1})	MQL (μg L^{-1})
	50	250	1250	50	250	1250	50	250	1250				
BMAA	8	4	3	5	2	2	9	5	6	0.9992	0.02–2.5	0.008	0.02
DAB	8	5	2	6	2	3	9	7	7	0.9991	0.03–2.5	0.009	0.03
AEG	9	3	2	5	4	2	12	8	6	0.9994	0.02–2.5	0.007	0.02
ANA-a	5	7	3	8	7	4	11	12	8	0.9995	0.02–2.5	0.007	0.02
CYN	10	7	5	8	9	7	13	11	12	0.9990	0.03–2.5	0.01	0.03
STX	11	8	6	10	8	6	15	14	11	0.9989	0.04–2.5	0.01	0.04

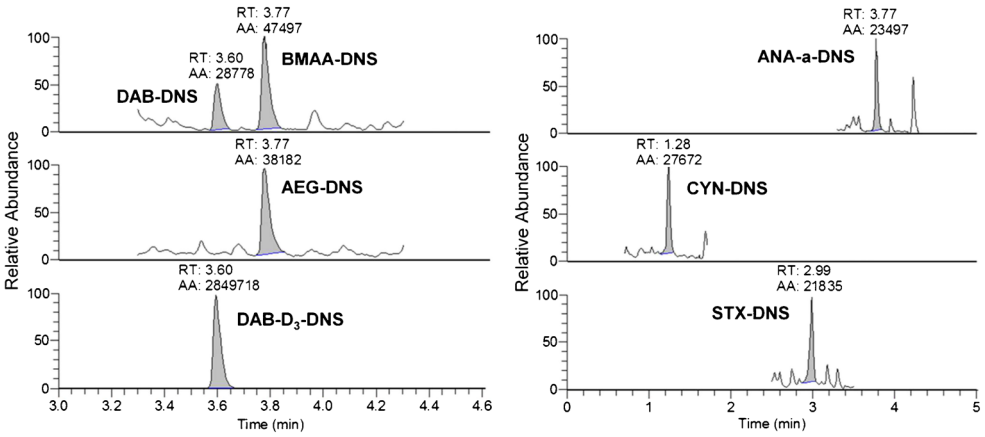

Fig. 7 Chromatograms of DNS derivatives using UHPLC-HESI-HRMS method. Standards were spiked at their detection limit and internal standard (*DAB-D_3*) was spiked at 100 μg L^{-1} in bloom water blank samples

calibration curve spiked prior to the SPE procedure was used with a linearity dynamic range between 0.025 and 2.5 μg L^{-1} analyzed in triplicates. The concentration of DAB-D_3 used in every measure was optimized to be 0.75 μg L^{-1} depending on the lowest variability of signal ratios throughout the linearity range of the calibration curve (data not shown). Table 3 summarizes the validation parameters for all the derivative compounds. The calibration curves showed good linearity, with correlation coefficients close to unity (R^2>0.998). The good linearity throughout the dynamic range confirms the efficiency of the derivatization step for low to high concentrations. The MDL and MQL of the compounds were between 0.007 and 0.01 μg L^{-1} and 0.02 and 0.04 μg L^{-1}, respectively, which is a significant improvement compared with previous studies using analytical methods for the analysis of BMAA in water bodies, which ranged from 0.2 μg L^{-1} and higher [27, 30, 40]. Chromatograms of the different derivative compounds spiked at their respective MDL are shown in Fig. 7. The accuracy and within-day/between-days precisions are presented in Table 3 and were evaluated using three different concentrations to be representative of the linearity range (0.05, 0.25, and 1.25 μg L^{-1}). The accuracy, expressed as the relative errors (RE %), ranged between 2 and 11 %, within-day repeatability and between-days reproducibility, expressed as relative standard deviations (RSD %) ranged between 2 and 10 % and 5 and 15 %, respectively.

The analytical method was tested on cyanobacterial bloom samples, which contained harmful algal blooms assessed by the MDDELCC. The samples were from 12 different lakes around the province of Québec during the algal proliferation season, and results are shown in Table 4. STX was absent in all the samples, which is not unusual since this toxin is produced by very specific genera of cyanobacteria and its presence is knowingly less frequent than other cyanotoxins. CYN was found in two samples, with 0.1 and 0.2 μg L^{-1}. As for ANA-a, it was

Table 4 Cyanotoxins detection in lake samples (μg L^{-1}) with relative standard deviation (RSD %)

No. sample	Location	Date	BMAA	DAB	AEG	ANA-a	CYN	STX
1	Lanaudière	2009–09–04	ND	ND	ND	ND	ND	ND
2	Montérégie	2009–09–24	ND	0.01 (7)	0.08 (8)	0.1 (8)	ND	ND
3	Montérégie	2009–09–24	0.2 (9)	ND	ND	ND	ND	ND
4	Montérégie	2009–09–24	ND	ND	0.05 (10)	ND	ND	ND
5	Estrie	2013–06–14	ND	ND	ND	0.08 (9)	ND	ND
6	Estrie	2013–06–14	0.03 (8)	0.04 (8)	0.05 (9)	0.02 (7)	ND	ND
7	Saguenay	2013–06–20	ND	0.009 (10)	0.06 (8)	ND	ND	ND
8	Saguenay	2013–06–20	0.3 (10)	0.008 (11)	0.009 (11)	ND	0.2 (9)	ND
9	Abitibi-Témiscamingue	2013–06–24	ND	ND	ND	0.2 (6)	ND	ND
10	Abitibi-Témiscamingue	2013–07–31	0.01 (8)	ND	ND	ND	ND	ND
11	Abitibi-Témiscamingue	2013–07–31	ND	0.03 (9)	ND	0.03 (8)	0.1 (11)	ND
12	Montérégie	2013–08–01	ND	ND	0.01 (5)	0.01 (6)	ND	ND

ND not detected

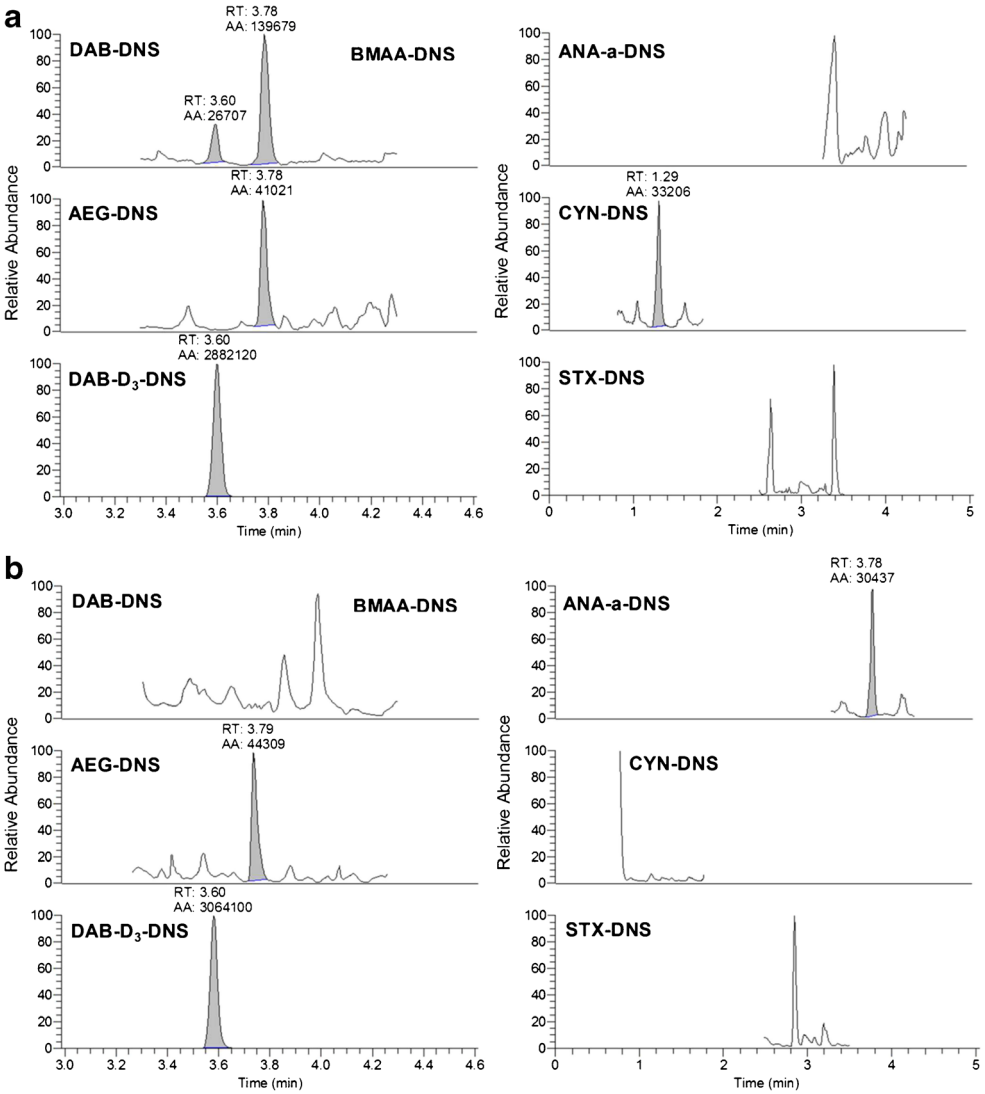

Fig. 8 Example of results for the analysis of **a** sample 8 and **b** sample 12 analyzed according to the validated method using DNS derivatization and UHPLC-HESI-HRMS analysis

found at low concentrations ranging from 0.02 to 0.2 μg L^{-1} in six samples. Finally, our main target, the nonproteinogenic amino acid BMAA, was found in four samples at low concentrations ranging from 0.01 to 0.3 μg L^{-1}. On the other hand, DAB and AEG were found in other samples, at relatively lower concentrations ranging from 0.008 to 0.04 μg L^{-1} for DAB and from 0.009 to 0.08 μg L^{-1} for AEG. Chromatograms of the real samples 8 and 12 are presented in Fig. 8 as examples of signals for all the target derivative compounds (except for STX, which was not present in any samples). It was observed that some samples contained BMAA and not AEG, and vice versa. Moreover, using mean peak area ratios of their selected product ions, we can then assume that there were no contribution of signals for each of these two compounds, and ultimately, the developed analytical method can quantitate BMAA with high selectivity. With the use of DNS derivatization, it was possible to develop a selective analytical method for alkaloid cyanotoxins, and the use of HRMS detection gave a selective detection of targeted compounds and a better understanding of their fragmentation.

Conclusion

A new method for the analysis of the nonproteinogenic amino acid BMAA and two of its conformation isomers DAB and AEG, as well as three alkaloid toxins, ANA-a, CYN, and STX, is presented. The use of DNS derivatization permitted easier liquid chromatography with the help of a reverse phase column. With high-resolution detection using the Q-Exactive mass spectrometer in a fragmentation mode (t-MS^2), a highly sensitive and selective detection of the toxins was possible, and the structures for the quantification and confirmation product ions of the derivative compounds were proposed

using their exact *m*/*z* values. The chromatographic separation was successfully used with the derivative toxins except for BMAA-DNS and AEG-DNS. However, the use of favored product ions confirmed by their signal ratios permitted selective detection of the two compounds without significant signal contribution. An internal calibration was used with isotopically labeled DAB-D_3, and the validated method gave linear correlation coefficients (R^2) above 0.998. MDL and MQL for the target compounds ranged between 0.007 and 0.01 μg L^{-1} and 0.02 and 0.04 μg L^{-1}, respectively, which is an improvement of one order of magnitude compared to similar analytical methods sensitivity. Accuracy and within-day/between-days variation coefficients for target compounds were below 15 %. SPE recovery values ranged between 86 and 103 %, and matrix effects recovery values ranged between 75 and 96 % showing small signal suppression due to ionisation. The high-resolution detection allowed high mass accuracy, which was below 2 ppm. The developed method was successfully validated for the toxins with concentration found to be between 0.009 and 0.3 μg L^{-1} in 12 tested field-collected samples from lakes where cyanobacterial blooms frequently occur. Only STX was not found in any sample, its presence being knowingly uncommon in algal blooms. Finally, this new analytical method using DNS derivatization as well as HRMS detection shows great potential for alkaloid cyanotoxins, and could be applied to more complex matrices such as shellfish and sediments, for sensitive and selective detection.

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