CEST 2017

15th International Conference on Environmental Science and Technology

Rhodes, Greece, 31 August to 2 September 2017

Analysis Of Saxitoxins In Spirulina Supplements and Cyanobacterial Mass Using SPE and HILIC-LC-MS/MS.

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Abstract Cyanobacteria (green-blue algae) can release hazardous secondary metabolites, called cyanotoxins, with a variety of chemical structures and modes of toxicity. Among them, saxitoxins are neurotoxins, mostly known for causing food poisoning when eating shellfish seafood. Saxitoxin analysis from shellfish has been thoroughly investigated. However, analysis of saxitoxins from spirulina supplements, derived from blue-green algae, is understudied, despite the fact that spirulina is used as part of the human diet and could lead to an increased risk for the consumer due to contamination from cyanotoxins. In this study, an analytical method has been developed using Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) for the analysis of saxitoxins from spirulina supplements and cyanobacterial mass. Target saxitoxins were Saxitoxin, decarbamoylsaxitoxin, Neosaxitoxin, Gonyautoxin 2&3, decarbamoylgonyautoxin 2&3. Separation was achieved using a HILIC chromatographic column with a gradient elution program of acetonitrile and water, both containing ammonium formate 2mM and formic acid 3.6mM. Identification was achieved using MS/MS in positive ESI mode. Extraction of saxitoxins was achieved with acetic acid in acetonitrile/water 50:50 with recoveries 30-40% due to matrix suppression. Solid Phase Extraction (SPE) with different cartridges (C18, Carbon Graphitized) did not improve the obtained recoveries. Nevertheless, column clogging was decreased, as column pressure remained in a steady value. Limits of detection (LODs) were 0.14-0.23μg g^{-1} (dry weight) and mean relative standard deviation (%RSD) was 15-20%.

Keywords: Saxitoxins, Spirulina, Extraction, SPE, HILIC-MS/MS

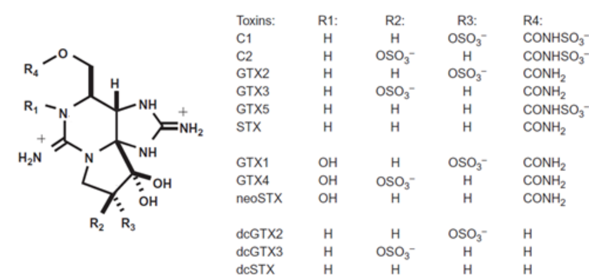
1. Introduction

Cyanobacteria (blue-green algae) are ancient microorganisms, which under favorable conditions can release secondary metabolites, called cyanotoxins [1]. Massive proliferation of cyanobacteria poses a serious concern for human health and animal life [2]. Humans may be exposed to cyanotoxins through several routes: oral (water, food and dietary supplements), dermal and via inhalation [3]. In the past decades, organic dietary supplements consisting of blue-green algae were increasingly consumed by athletes, pregnant or breast-

CEST2017_00534

feeding women, and vegetarians, for their beneficial health effects. Up until now, these products are frequently consumed by humans in larger quantities and over longer periods of times. Consequently, the consumer's risk of a critical exposure to algal toxins is rising [4].

Saxitoxins (STXs), also known as Paralytic Shellfish Poisoning toxins (PSPs), are the most toxic cyanotoxins. They can be produced by marine dinoflagellates and freshwater cyanobacteria [5]. Intraperitoneal lethal dose, LD_{50}, derived from Mouse Bioassay (MBA), was found to be equal to 10μg kg^{-1} [6]. Over the last century, STXs have been associated with numerous human intoxications resulting in numbness, complete paralysis and even death [7]. However, no intoxication through drinking water has been documented so far. While no official guideline has been proposed for STXs in drinking water, Australia is considering a 3 μg STX eq L^{-1} to be used [8, 9]. PSPs are alkaloids tricyclic compounds (figure 1), relatively polar and soluble in water [10].



Toxins:	R1:	R2:	R3:	R4:
C1	H	H	OSO_3^-	$CONHSO_3^-$
C2	H	OSO_3^-	H	$CONHSO_3^-$
GTX2	H	H	OSO_3^-	$CONH_2$
GTX3	H	OSO_3^-	H	$CONH_2$
GTX5	H	H	H	$CONHSO_3^-$
STX	H	H	H	$CONH_2$
GTX1	OH	H	OSO_3^-	$CONH_2$
GTX4	OH	OSO_3^-	H	$CONH_2$
neoSTX	OH	H	H	$CONH_2$
dcGTX2	H	H	OSO_3^-	H
dcGTX3	H	OSO_3^-	H	H
dcSTX	H	H	H	H

Figure 1. Structures of saxitoxin and its analogues [11]

Observed concentrations of STXs in environmental samples are generally quite low with values up to a few μg L^{-1} [12-14]; however, higher concentration ranging from 10-1000 μg L^{-1} have also been reported [15, 16]. Regarding occurrence of saxitoxins in water, biomass and fish tissues from Greece, only few studies have been carried out, mostly with the use of Enzyme-linked Immunosorbent Assay (ELISA) technique [17-20]. However, the possibility of ELISA false-positive results has to be considered and further studies are needed to investigate samples found positive for STXs [17]. Only two studies concerning the presence of STX in Greece

have been reported so far, using advance analytical technique such as liquid chromatography - tandem mass spectrometry (LC-MS/MS) with a Hydrophilic Interaction Liquid Chromatography (HILIC) column [21, 22]. The method used, although not optimized in terms of sample extraction and chromatographic separation, resulted in the detection of saxitoxin and neosaxitoxin in water and biomass samples from lake Vistonis and Karla.

The aim of this study is to develop a sensitive and accurate analytical method for the successful extraction and analysis of saxitoxin and most of its analogues (Saxitoxin, STX; decarbamoylsaxitoxin, dc-STX; Neosaxitoxin, NeoSTX; Gonyautoxin 2&3, GTX2&3; decarbamoylgonyautoxin 2&3, dc-GTX2&3) in cyanobacterial mass including cyanobacterial food supplements.

2. Materials and Methods

2.1. Chemicals and reagents

All substances had purity > 95%. Methanol (MeOH) of HPLC grade (99.99%) was obtained from Fischer Scientific (Leics, UK), acetonitrile (ACN) of gradient grade for HPLC (≥ 99.9%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (FA) (>98%) was purchased by Riedel-de Haën (Seelze, Germany). High purity water (18.2 M) was produced on-site using a Temak TSDW10 system (Temak S.A.). STX, NeoSTX, dc-STX, GTX2&3, dc-GTX2&3 standards were supplied by NRC Canada. All substances had purity >95%.

2.2. Methods

Optimum extraction of saxitoxins from cyanobacterial mass is performed by suspending lyophilized material in acetic acid in acetonitrile/water 50:50, mixing well and sonicated. The mixture is centrifuged and the supernatant is collected, dried and reconstituted in suitable solvent. The supernatant is analyzed directly by LC-MS/MS or first cleaned-up by SPE and subsequently analyzed by LC-MS/MS. Optimum SPE clean-up was performed with C18 cartridges with acidic eluent.

LC-MS/MS was carried out on a Finnigan TSQ Quantum Discovery Max triple-stage quadrupole mass spectrometer (Thermo, USA), equipped with electrospray ionization (ESI) source. Separation of target analytes was achieved with a Finnigan Surveyor LC system, equipped with a Finnigan Surveyor AS autosampler (Thermo, USA). Xcalibur software 2.1 SP 1160 was used to control the mass spectrometric parameters and for data acquisition. Chromatographic separation was achieved with a Sequant zic-HILIC column with mobile phases consisted of (A) water and (B) ACN both containing 2 mM ammonium formate and 3.6mM formic acid. Flow rate was set at 0.2 mL min^{-1}. Ionization of compounds was performed with ESI probe in positive mode. Detection was carried out in multiple reaction monitoring mode (MRM) using the three most intense and characteristic precursor/product ion transitions obtained from the MS/MS optimization procedure.

3. Results and discussion

High performance liquid chromatography (HPLC) is widely used for separation of PSPs, mostly reversed phase liquid chromatography (RPLC) with fluorescence detector (FLD). However, the lack of chromophores in the compounds required the use of pre or post-column derivatization methods with the use of ion-pairing reagents, which is a disadvantage of the RPLC-FLD method [23]. Such instrumentation requires complex set-up with demanding daily maintenance. In the last decade, LC-MS and lately LC-MS/MS, have been successfully adapted for the analysis of STXs, often by using HILIC for separation and the selective reaction monitoring mode to obtain high selectivity and sensitivity [11, 14, 23-28]. Using HILIC there is no need to use ion-pair reagents or derivatization for polar and ionic analytes [23]. Such instrumentation is the one used in this study.

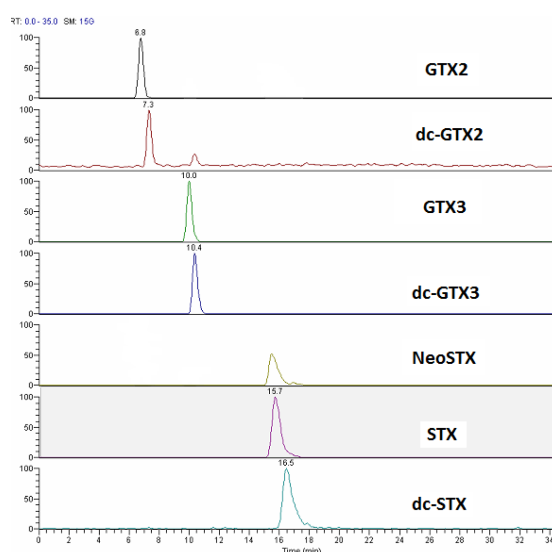


Figure 2. MRM chromatograms of quantification ions of investigated saxitoxins at a concentration of 100 μg L^{-1}.

Different gradient methods and mobile phases were investigated. Buffers of ammonium acetate and ammonium formate were used and formic acid in different concentrations. Optimum chromatographic separation was achieved with a zic-HILIC column with mobile phases consisted of acetonitrile and water, both containing ammonium formate 2mM and formic acid 3.6mM. Identification was achieved using MS/MS in positive Electrospray Ionization (ESI) mode using the optimum ionization parameters of the method development and infusion of the cyanotoxins. Multiple reaction Monitoring (MRM) chromatograms of targeted saxitoxins at a concentration of 100 μg L−1 are presented in figure 2.

Concerning the extraction of saxitoxins, there is a number of studies on toxic algae and the extraction of PSPs. In general lyophilized cyanobacteria are extracted with an acidic solvent. The most common extraction solvent is acetic acid in different mixtures (ex. [14, 29-31]). Extracts can be analyzed directly without any clean-up step (ex. [23, 26, 27]). Only few have used solid phase extraction (SPE) as an additional clean-up step [11, 25].

SPE clean-up, on extracts of shellfish has thoroughly been used with the use of mostly C18 cartridges (ex. [32-35]), but also graphitized [36, 37] and HLB cartridges [24, 37]. In this research, extraction of saxitoxins was achieved with acetic acid in acetonitrile/water 50:50 with recoveries 30-40% due to matrix suppression. SPE experiments were conducted with C18 and Carbon Graphitized cartridges, in order to increase recoveries with. Unfortunately, obtained recoveries were not improved. Nevertheless, solid phase extraction contributed in the minimization of column clogging, as column pressure remained in a steady value. It is a fact that during extraction experiments there was an increase in the column pressure resulting in the need of often regeneration of the column. With this application, limits of detection (LODs) were 0.14-0.23 μg g-1 (dry weight, dw) of cyanobacterial mass, and mean relative standard deviation (%RSD) was 15-20%.

4. Conclusions

Analysis of saxitoxins from cyanobacterial mass and related food supplements has been successfully performed using sample extraction followed by SPE-HILIC-MS/MS analysis. The method developed is sensitive and accurate, with low limits of detection (0.14-0.23 μg g^{-1} dw) becoming an important tool for the monitoring of cyanotoxins in environmental samples.

References

Schindler, D.W., Recent advances in the understanding and management of eutrophication. *Limnology and Oceanography*, 2006. 51(1 II): p. 356-363.

Batoréu, M.C.C., et al., Risk of human exposure to paralytic toxins of algal origin. Environmental *Toxicology and Pharmacology*, 2005. 19(3): p. 401-406.

Metcalf, J.S. and G.A. Codd, Cyanotoxins, in Ecology of Cyanobacteria II: Their Diversity in Space and Time2012. p. 651-675.

Heussner, A.H., et al., Toxin content and cytotoxicity of algal dietary supplements. *Toxicology and Applied Pharmacology*, 2012. 265(2): p. 263-271.

Pereira, P., et al., Taxonomy and production of paralytic shellfish toxins by the freshwater cyanobacterium Aphanizomenon gracile LMECYA40. *European Journal of Phycology*, 2004. 39(4): p. 361-368.

Wiberg, G.S. and N.R. Stephenson, Toxicologic studies on paralytic shellfish poison. *Toxicology and Applied Pharmacology*, 1960. 2(6): p. 607-615.

Anderson, D.M., et al., Biogeography of toxic dinoflagellates in the genus Alexandrium from the northeastern United States and Canada. *Marine Biology*, 1994. 120(3): p. 467-478.

Merel, S., et al., State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. *Environment International*, 2013. 59: p. 303-327.

Van Apeldoorn, M.E., et al., Toxins of cyanobacteria. *Molecular Nutrition and Food Research*, 2007. 51(1): p. 7-60.

Singh, S., B.N. Kate, and U.C. Banecjee, Bioactive compounds from cyanobacteria and microalgae: An overview. *Critical Reviews in Biotechnology*, 2005. 25(3): p. 73-95.

Watanabe, R., et al., Quantitative determination of paralytic shellfish toxins in cultured toxic algae by LC-MS/MS. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, 2013. 30(8): p. 1351-1357.

Costa, I.A.S., et al., Occurrence of toxin-producing cyanobacteria blooms in a Brazilian semiarid reservoir. *Brazilian Journal of Biology*, 2006. 66(1 B): p. 211-219.

Graham, J.L., et al., Cyanotoxin mixtures and taste-and-odor compounds in cyanobacterial blooms from the midwestern united states. *Environmental Science and Technology*, 2010. 44(19): p. 7361-7368.

Ledreux, A., et al., Evidence for saxitoxins production by the cyanobacterium Aphanizomenon gracile in a French recreational water body. *Harmful Algae*, 2010. 10(1): p. 88-97.

Rapala, J., et al., First report of saxitoxin in Finnish lakes and possible associated effects on human health. *Environmental Toxicology*, 2005. 20(3): p. 331-340.

Wörmer, L., et al., First detection of cyanobacterial PSP (paralytic shellfish poisoning) toxins in Spanish freshwaters. *Toxicon*, 2011. 57(6): p. 918-921.

Gkelis, S., P. Fernández Tussy, and N. Zaoutsos, Isolation and preliminary characterization of cyanobacteria strains from freshwaters of Greece. *Open Life Sciences*, 2015. 10(1): p. 52-60.

Gkelis, S., et al., Anthropogenic and climate-induced change favors toxic cyanobacteria blooms: Evidence from monitoring a highly eutrophic, urban Mediterranean lake. *Harmful Algae*, 2014. 39: p. 322-333.

Gkelis, S. and N. Zaoutsos, Cyanotoxin occurrence and potentially toxin producing cyanobacteria in freshwaters of Greece: A multi-disciplinary approach. *Toxicon*, 2014. 78: p. 1-9.

Katikou, P., et al., First report on toxicity assessment of the Lessepsian migrant pufferfish Lagocephalus sceleratus (Gmelin, 1789) from European waters (Aegean Sea, Greece). *Toxicon*, 2009. 54(1): p. 50-55.

Moustaka-Gouni, M., et al., First report of Aphanizomenon favaloroi occurrence in Europe associated with saxitoxins and a massive fish kill in Lake Vistonis, Greece. *Marine and Freshwater Research*, 2016.

Gkelis, S., et al., Monitoring a newly re-born patient: Water quality and cyanotoxin occurrence in a reconstructed shallow Mediterranean lake. *Advances in Oceanography and Limnology*, 2017.

Halme, M., et al., Verification and quantification of saxitoxin from algal samples using fast and validated hydrophilic interaction liquid chromatography-tandem mass spectrometry method. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 2012. 880(1): p. 50-57.

Zhuo, L., et al., Determination of paralytic shellfish poisoning toxins by HILIC-MS/MS coupled with dispersive solid phase extraction. *Food Chemistry*, 2013. 137(1-4): p. 115-121.

Dell'Aversano, C., P. Hess, and M.A. Quilliam, Hydrophilic interaction liquid chromatography-mass spectrometry for the analysis of paralytic shellfish poisoning (PSP) toxins. *Journal of Chromatography A*, 2005. 1081(2): p. 190-201.

Dell'Aversano, C., G.K. Eaglesham, and M.A. Quilliam, Analysis of cyanobacterial toxins by hydrophilic interaction liquid chromatography-mass spectrometry. *Journal of Chromatography A*, 2004. 1028(1): p. 155-164.

Lajeunesse, A., et al., 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. *Journal of Chromatography A*, 2012. 1219: p. 93-103.

Turner, A.D., et al., Comparison of AOAC 2005.06 LC official method with other methodologies for the quantitation of paralytic shellfish poisoning toxins in UK shellfish species. *Analytical and Bioanalytical Chemistry*, 2011. 399(3): p. 1257-1270.

Foss, A.J., et al., Characterization of paralytic shellfish toxins from Lyngbya wollei dominated mats collected from two Florida springs. *Harmful Algae*, 2012. 16: p. 98-107.

Jaime, E., et al., Determination of paralytic shellfish poisoning toxins by high-performance ion-exchange chromatography. *Journal of Chromatography A,* 2001. 929(1-2): p. 43-49.

Hummert, C., et al., Analysis of the characteristic PSP profiles of Pyrodinium bahamense and several strains of Alexandrium by HPLC based on ion-pair chromatographic separation, post-column oxidation, and fluorescence detection. *Chromatographia*, 1997. 45: p. 312-316.

Leão, J.M., et al., Solid-phase extraction and high-performance liquid chromatography procedures for the analysis of paralytic shellfish toxins. *Journal of Chromatography A*, 1998. 798(1-2): p. 131-136.

Vale, P. and H. Taleb, Assessment of the quantitative determination of paralytic shellfish poisoning toxins by pre-column derivatization and elimination of interfering compounds by solid-phase extraction. *Food Additives and Contaminants*, 2005. 22(9): p. 838-846.

Chen, D., et al., Determination of Paralytic Shellfish Poisoning Toxins by Liquid Chromatography with Fluorescence Detection Using Pre-column Derivatization with Hydrogen Peroxide Oxidation. *Chinese Journal of Analytical Chemistry*, 2006. 34(7): p. 933-936.

Biré, R., et al., Improved Solid-Phase Extraction Procedure in the Analysis of Paralytic Shellfish Poisoning Toxins by Liquid Chromatography with Fluorescence Detection. *Journal of Agricultural and Food Chemistry*, 2003. 51(22): p. 6386-6390.

Boundy, M.J., et al., Development of a sensitive and selective liquid chromatography-mass spectrometry method for high throughput analysis of paralytic shellfish toxins using graphitised carbon solid phase extraction. *Journal of Chromatography A*, 2015. 1387: p. 1-12.

Sayfritz, S.J., J.A.B. Aasen, and T. Aune, Determination of paralytic shellfish poisoning toxins in Norwegian shellfish by liquid chromatography with fluorescence and tandem mass spectrometry detection. *Toxicon*, 2008. 52(2): p. 330-340.