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

Manolıdı K.1, 2, Triantıs T.1, And Hıskıa A.1.*

1 NCSR “Demokritos”, INN, 15310, Agia Paraskevi, Athens, Greece
2 NKUA, Faculty of Chemistry, Panepistimiopolis, 15784
*e-mail: a.hiskia@inn.demokritos.gr

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-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, LD₅₀, 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].

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 3.6 mM 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 chromatophores 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.
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


