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ОПРЕДЕЛЕНИЕ САКСИТОКСИНА МЕТОДОМ ВЭЖХ-МС С ПРЕДКОЛОНОЧНОЙ ДЕРИВАТИЗАЦИЕЙ 2,4-ДИНИТРОФЕНИЛГИДРАЗИНОМ

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В настоящей работе предложен оригинальный метод определения сакситоксина (STX) основанный на его выделении из цианобактериальной биомассы путем экстракции 0,05 М уксусной кислотой, дериватизации 2,4-динитрофенилгидразином (DNPH) и последующем анализе производного методом ВЭЖХ-МС. Дериватизация проводилась в смеси ацетонитрил-трифторуксусная кислота (98,5:1,5 об./об.) при 65 °С; период полупревращения составляет ~ 3,6 ч. Разработанный метод был применен для анализа природных цианобактериальных образцов, а также культивированного штамма Nostoc Pruniforme. Детектирование гидразона сакситоксина проводилось путем измерения полного ионного тока и последующего экстрагирования ионного тока при m/z 462,15 ± 0,1. Дериватизация 2,4-динитро-фенигидразином осложнена образованием двух стереоизомеров, E и Z, которые дают два отдельных пика при ВЭЖХ. Выполненные нами квантово-химические расчеты показывают, что в случае сакситоксина образуется только один Z-изомер вследствие стерического фактора и, следовательно, только один хроматографический пик гидразона сакситоксина наблюдается на хроматограмме. Для предложенного метода получены следующие метрологические характеристики: предел определения – 20 нг/г (сухая биомасса), повторяемость – 4%, внутрилабораторная прецезионность – ±7%.
Ключевые слова: *цианобактерии, сакситоксин, дериватизация, ВЭЖХ-МС, 2,4-динитрофенилгидразин*

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A METHOD FOR DETERMINATION OF SAXITOXINS USING HPLC-MS WITH 2,4-DINITROPHENYLHYDRAZINE PRECOLUMN DERIVATIZATION

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A method for saxitoxin (STX) determination is proposed. It consists of target compound extraction with 0,05 M acetic acid, derivatization with 2,4-dinitrophenylhydrazine (DNPH), and subsequent HPLC electrospray ionization MS analysis. The reaction was performed in acetonitrile-trifluoroacetic acid (98,5:1,5 v/v) at 65 °C; the half-life of STX was ca. 3,6 hours. The method was applied to analysis of natural cyanobacteria samples and a cultivated Nostoc Pruniforme strain. Detection was performed by measuring total ion current, and chromatograms for STX were obtained using an extracted ion procedure at m/z 462,15 (STX hydra-

zone). A frequent complication of the derivatization with DNPH is the formation of two stereoisomers, E and Z, giving two separate peaks under HPLC. Quantum-chemical calculation predicted that, due to steric hindrance, only one isomer (Z) of STX adduct is formed, and hence only one peak of the analyte is present in the chromatogram. The following metrological parameters were achieved: limits of determination (20 ng/g of dry cyanobacterial biomass), reproducibility (4%), and medium-term precision (± 7%).
Keywords: *cyanobacteria, saxitoxin, derivatization, HPLC-MS, 2,4-dinitrophenylhydrazine*

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INTRODUCTION

Saxitoxins (STXs) are a large group of hazardous poisons produced by dinoflagellates in marine environments and cyanobacteria in fresh waters. Currently, more than fifty STX analogues are known [1]. These substances are highly toxic to humans. They are neurotoxins that block sodium channels [2]. Mass production of STXs occurs in lakes and reservoirs during toxic microalgal blooms. Humans can be poisoned with STX when they swallow STX-containing water or eat toxin-containing tissues of seafood, such as edible shellfish [3].

There are many methods for STX quantification: HPLC with fluorescent detection with pre- or post-column derivatization, HPLC-MS, capillary electrophoresis with MS detection, enzyme-linked immunoassay, and others [4, 5].

All STXs are highly hydrophilic compounds and are poorly retained by reversed phase HPLC adsorbents [6-8]. Prior to HPLC, STXs are usually enriched using ion-exchange [9], graphite amorphous carbon [8], or polyamide cartridges. [10].

STXs are geminal diols in water solutions and are in equilibrium with their ketone forms [11, 12]. Many compounds which react with the carbonyl group (geminal diol) can be used for chemical modification of aldehydes and ketones to improve their chromatographic properties. It occurred to us that one potentially convenient reagent could be 2,4-dinitrophenylhydrazine (Brady's reagent, DNPH) [13-17]. DNPH is slightly soluble in water and hydrophobic, having a high affinity to reversed-phase chromatographic adsorbents [18].

The purpose of the present study was to develop a method for quantitative analysis of STX by means of HPLC-MS involving pre-column chemical modification with Brady's reagent.

EXPERIMENTAL

The following reagents were used: water (mass-spectrometry grade, Honeywell, USA); acetic acid (analytical grade Reachem, Russia); chloroform (analytical grade Kryochrom, Russia); acetonitrile (UHPLC grade, AppliChem, Germany); trifluoroacetic acid (99%, Panreac, Germany); heptafluorobutyric acid (99%, Fluorochem, UK); 2,4-dinitrophenylhydrazine (99%, twice recrystallized from ethanol, Reachem, Russia); STX calibration solution (63,3 μM, National Research Council, Canada).

Preparation of extracts. Three samples of dry biomass (25 ± 0,1 mg) were placed into 1,5 ml tubes and 1 ml of 0,05 M acetic acid was added. 0,1 μl of STX standard was added into the second tube and 0,2 μl of STX standard were added into the third tube to process quantification using the method of standard addition. The suspension was sonicated using the ultrasonic probe (5 times for 20 s, Bandelin Sonorex ultrasonic bath), and centrifuged at 13400 rpm for 15 min (Eppendorf Minispin centrifuge). The supernatant (600 μl) was sampled and 600 μl of chloroform were added to the supernatant. The mixture was shaken for 5 min and centrifuged at 13400 rpm for 5 min. A sample (100 μl) of water phase was transferred into a microtube and evaporated in a vacuum centrifuge at 60 °C. The residue was dispersed in acetonitrile (100 μl), sonicated in an ultrasonic bath for 30 min, and centrifuged at 13400 rpm for 10 min. The supernatant (80 μl) was evaporated in a vacuum centrifuge (Eppendorf Concentrator Plus at 60 °C to dryness and subjected to derivatization.

Saxitoxin derivatization. For derivatization, a 2,4-dinitrophenylhydrazine solution prepared by mixing of 10 mg of 2,4-dinitrophenylhydrazine and 1 ml of 0,2 M trifluoroacetic acid in acetonitrile (1,5:98,5 v/v) was added to dry residue and the reaction mixture heated at 65 °C for 24 hours. To remove DNPH excess, water (40 μl) and chloroform (150 μl) were added and the mixture was shaken for 5 min. The sample was centrifuged at 6000 rpm for 5 min and a 30 μl sample of water phase was subjected to HPLC-MS analysis.

LC-MS analysis. Mass spectra were measured with an Agilent 6210 time-of-flight mass spectrometer with electrospray ionization (ESI-MS-TOF) coupled to an Agilent 1200 liquid chromatography system (HPLC-DAD) with a Zorbax 300SB-C18 (5 μm; 2,1×150 mm) column. Eluent A was 0,1% heptafluorobuthyric acid (HFBA) in water, and eluent B was 0,1% HFBA solution in acetonitrile. The column temperature was 35 °C. The column was preconditioned with 100% eluent B for 15 min at 0,2 ml/min, then 10% eluent B for 15 min at 0,2 ml/min.

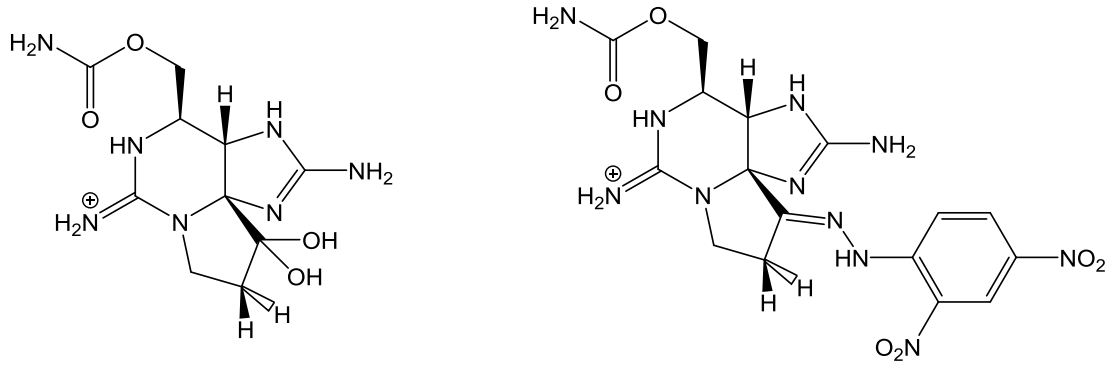

Fig. 1 SXT (left) and its 2,4-dinitriphenylhydrazone (right)

Рис.1 Сакситоксин (слева) и его 2,4-динитрофенилгидразон (справа).

Gradient elution (10% eluent B to 100% eluent B) was performed at a flow rate of 0,15 ml/min for 20 min. The detection mode was electrospray ionization with positive ion registration (ESI+). The range of detection was 100 to 600 Da, and the ion source temperature was set at 250 °C, gas flow was 3,5 l/min, nebulizer was 45 psi.

DISCUSSION

Preparation of extracts. The method is primarily focused on cyanobacteria colonies. The test cyanobacteria were *Dolichospermum sp., Nostoc sp.* and *Gloeotrichia sp.* collected from Lake Baikal, East Siberia, Russia. Sample preparation is partially based on the earlier described methods [6-8, 19]. They were modified with respect to the object specificity under study. The optimised sample preparation procedure incorporated extraction by acetic acid with the subsequent removal of lipophilic components by chloroform. Then the aqueous phase was evaporated to dryness and suspended in acetonitrile. In this case inorganic salts, carbohydrates and polypeptides remained in the sediment and the target compounds passed into the organic phase.

The insoluble fraction was removed by centrifugation, and the supernatant evaporated to dryness and used for chemical modification.

Saxitoxin derivatization. The reaction between STX and DNPH in the presence of acids affords 2,4-dinitrophenylhydrazones (Fig. 1). Derivatization conditions were chosen based on the results of kinetic studies with STX standard solution varying such key parameters as temperature and acidity. These reaction kinetics were measured with a standard of STX, which is delivered as a solution in 3 mM HCl. An appropriate volume of the starting STX solution was evaporated to dryness and dissolved in acetonitrile/trifluoroacetic acid/DNPH taken at different proportions: 0,02; 0,2 and 2 M of trifluoroacetic acid and 10 mg/ml of DNPH. The temperature varied between 55 and 75 °C. The following conditions were chosen: 0,2 M trifluoroacetic acid, 10 mg/ml DNPH, temperature 65 °C, reaction time 24 h. The reaction was found to be pseudo-first order with a rate constant of 0.145 h^{-1} at a concentration of DNPH equal to 10 mg/ml DNPH. The half-life of STX under these conditions was approximately 3,6 hours.

LC-MS analysis. HPLC-MS was performed as described in the Experimental section. The nonpolar nature of STX hydrazone makes it suitable for chromatography under reversed phase conditions. The chromatographic peak is narrow and has a high constant retention time (15,9 min). Native STXs and their derivatives are well ionized under ESI+ conditions due to the high proton affinity of the guanidine moiety. The derivatives were not identified on total ion current chromatograms caused by low concentration. Therefore, their identification and quantification were performed using the extracted-ion procedure. The MS conditions for further derivative separation from natural extracts were optimized utilizing the method of standard addition.

The chromatogram contains a single peak of STX 2,4-dinitrophenylhydrazone (Fig. 2). Hydrazones contain a double bond between the carbon atom formerly belonging to the ketone and the nitrogen atom formerly belonging to the N_β atom of hydrazine. It is known that for this reason, hydrazones can consist of two (*E* and *Z* or *cis*- and *trans*-) stereoisomers. Substituents at the atoms forming the double bond can be directed inside (*E*) and outside (*Z*) the structure formed.

The *E* and *Z* isomer ratio in different hydrazones may vary over a broad range, 0–50%, and can even vary in the same hydrazone, depending on the derivatization conditions. This phenomenon is inconvenient for any subsequent quantitative analysis since it has to deal with two rather than one reaction product. *Z*-Isomers have a greater area in the plane of the double bond and hence have a longer retention time in HPLC due to a greater affinity toward the hydrophobic adsorbent.

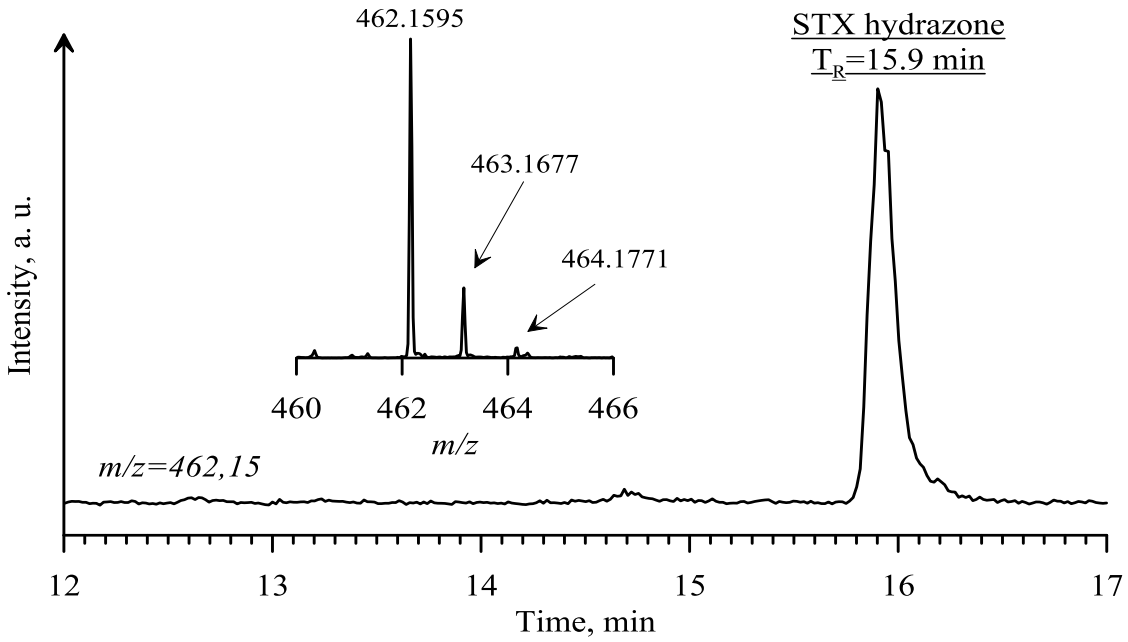

Fig. 2 Extracted ion current (EIC) chromatogram and isotopic distribution of STX hydrazine obtained from its standard solution (21 ng/peak for STX).

Рис. 2 Экстрагированная хроматограмма по характеристическому иону 2,4-динитрофенилгидразона сакситоксина (m/z 462,15±0,1) и его изотопное распределение полученные в результате модификации стандартного образца сакситоксина (21 нг/пик).

To identify the steric isomer, which was detected by HPLC-MS, and to confirm that the second isomer cannot be formed under the conditions employed we used quantum-chemical modeling within the density functional theory (B3LYP/6-31G(d,p) level of theory). Calculations were made by means of Gaussian 09 software. We assume that the reaction path is as follows: at the first stage of reaction, STX with two geminal hydroxy groups reacts with DNPH. Reaction of one of the STX hydroxy groups with the proton of the DNPH amino group results in elimination of a water molecule. This can potentially lead to formation of isomers A and B (Fig. 3). The two pathways are equally possible in terms of kinetics and thermodynamics. The intermediate A is transformed into Product 1 (*Z*) due to the topologically favorable position of the proton at the second amino group with respect to the hydroxyl group. As for the intermediate B, its proton at the amino group is topologically located in a non-favorable position; in order for the reaction to proceed, the intermediate B has to be transformed into intermediate B' to remove the topological restriction. Subsequent elimination of a water molecule from the rotational isomer B' is similar to elimination of water from the intermediate A, and yields Product 1 (*Z*).

Quantitative determination. Quantitative determination of STX was performed by the method of standard addition. The area of the chromatographic peak of the extracted ion current at *m/z* 462,15 was used as the analytical signal. The chromatographic peak of STX hydrazone was identified on the basis of retention time and isotopic distribution of the molecular ion cluster at the maximum of the chromatographic peak at *m/z* 462,15.

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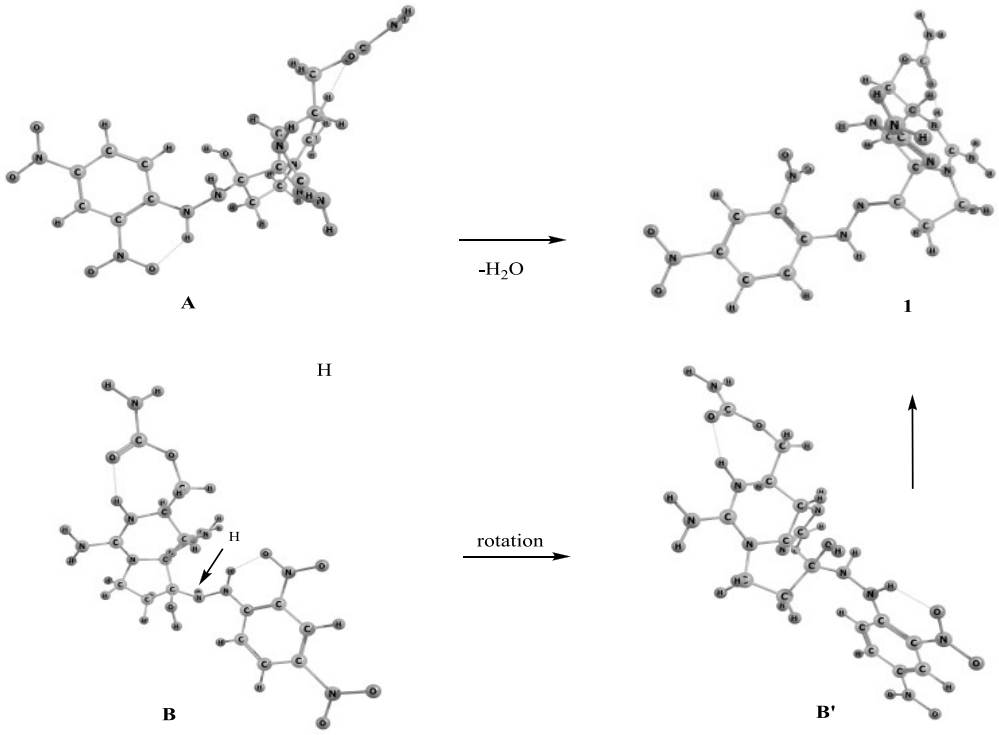

Fig. 3. Proposed paths of STX hydrazone formation from intermediates A and B

Рис. 3. Пути формирования гидразона сакситоксина из промежуточных интермедиатов A и B

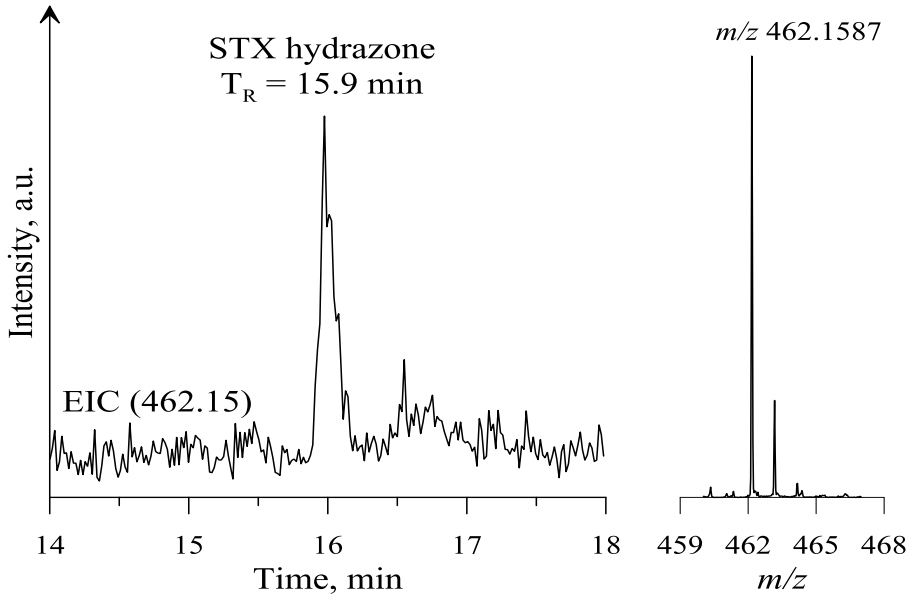

Fig. 4. Extracted ion current (EIC) chromatogram (m/z 462,15) of the Tychonema Distorta sample (May 2017, Lystvyanichny bay near the outlet of the Angara river) and STX hydrazone isotopic distribution

Рис. 4. Экстрагированная хроматограмма по характеристическому иону гидразона сакситоксина (m/z 462,15±0,1) для образца цианобактерий Tychonema distorta (май 2017 г., Лиственичный залив вблизи истока р. Ангара) и его изотопное распределение

Table 1

Analyzed samples data

Таблица 1

Результаты количественного анализа сакситоксина в некоторых видах цианобактерий

Cyanobacterium	Habitat	Presence of *stxA* gene	Sampling place	Sampling date	SXT content, ng/g of dry biomass
Dolichospermum Lemmermanni	Plankton sample	+	Irkutsk HPS reservoir	05.08.17	1000±150
Gloeotrichia Echinulata	Planktonsample (Spherical colony)	+	Kuchelga river, tributary of Maloe more strait	16.08.17	430±100
Nostoc Pruniforme	Natural colony	+	Maloe more strait	16.08.17	3800±600
Tychonema Distorta	Natural sample	+	Listvanichny bay	10.05.17	950±150
Nostoc sp.	Cultivated strain isolated from Lake Baikal	+	Laboratory culture	-	400±100

This step was followed by the extraction procedure and HPLC-MS described above. The level of detection for STX hydrazone was found to be ca. 20 ng/g of dry biomass (LoD, S/N=3) and the limit of quantitation – ca. 65 ng/g (LoQ, S/N=10) of dry biomass. Assessments of day-to-day reproducibility (S_{mtp}=7%, n=3) and reproducibility (S_r=4%, n=15) were achieved on the condition that the calibration was obtained for every series of analyzed samples. Using the developed protocol, we determined the concentrations of STX in the biomass of some other cyanobacteria. The example of chromatogram is shown in Fig. 4. Data for all species studied are shown in Table 1

CONCLUSION

The possibility of chemical modification of STX containing a geminal diol moiety with Brady's reagent (2,4-dinitrophenylhydrazine) is demonstrated in the present study. The final product, STX hydrazone, is amphiphilic. The original STX backbone makes the compound soluble in water, however derived phenyl moiety fundamentally changes its behavior to interact with nonpolars.

The method proposed provides reliable data for the content of saxitoxin in fresh water cyanobacteria, particularly in combination with PCR and sequencing aimed at detection of the *stxA* gene which takes part and the series reaction leading to saxitoxin.

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Contribution

Zubkov I.N., Kuzmin A.V., Tikhonova I.V., Belykh O.I., Smirnov V.I., Ivanov A.V., Shagun V.A., Grachev M.A., Fedorova G.A. carried out the experimental work, on the basis of the results summarized the material and wrote the manuscript. Zubkov I.N., Kuzmin A.V., Tikhonova I.V., Belykh O.I., Smirnov V.I., Ivanov A.V., Shagun V.A., Grachev M.A., Fedorova G.A. have equal author's rights and bear equal responsibility for plagiarism.

Conflict of interests

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