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Verification and quantification of saxitoxin from algal samples using fast and validated hydrophilic interaction liquid chromatography–tandem mass spectrometry method

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ABSTRACT

Hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS) method was validated with algal samples for verification and quantification of saxitoxin (STX), a potent neurotoxin which is listed in the Chemical Weapons Convention (CWC) in Schedule 1A. Isocratic elution, conventional bore HILIC column and high flow rate together with accurate post-column splitter provided detection of STX in 6.5 min with total analysis time of 9 min per sample. STX analogue, gonyautoxin 1 (GTX 1) was used as an internal standard. Sample preparation of freeze-dried algae included liquid extraction and centrifugal filtering with mean recovery of 99.9% at concentration level of 10 ng/ml ($n = 3$). Retention times for STX and GTX 1 were 6.47 ± 0.03 min and 4.44 ± 0.01 min ($n = 45$), respectively. Four diagnostic product ions were used for reliable verification of saxitoxin. Method was found to be precise and linear ($R^2 = 0.9714$ and $R^2 = 0.9768$) in concentration ranges of 5–50 ng/ml and 25–200 ng/ml, respectively. For saxitoxin, calculated LOD was 3 ng/ml and LLOQ 11 ng/ml. Validation was conducted using spiked algal matrix since this method is not only needed for verification analysis for the CWC but also for safety analysis of other environmental samples for presence of STX. Identification criteria for verification of STX with HILIC–MS/MS method are discussed.

1. Introduction

Saxitoxin (STX) is a potent neurotoxin produced by marine algae and freshwater cyanobacteria and belongs to a group of paralytic shellfish poisoning (PSP) toxins [1]. It is mostly known for causing severe food poisoning when eating shellfish or other seafood. STX reversibly blocks the voltage activated sodium channels and stops the flow of nerve impulses causing tingling, numbness and weakness and at the worst case, respiratory failure. This toxin is highly lethal and there exist no antidote [2]. STX is the most toxic PSP variant, with LD_{50} value of 10 μg/kg by intraperitoneal injection (i.p.) in mice [3].

STX have been known since the mid-nineteenth century, when it was first extracted from shellfish [4]. After two decades it was discovered to be a metabolic product of marine dinoflagellates [5] and few years' later freshwater cyanobacteria [6]. This toxin accumulates in filter-feeding bivalve molluscs like mussels, oysters and scallops and therefore can end up to human or animal consumption.

As a potent neurotoxin, STX is listed in the Chemical Weapons Convention (CWC) [7]. The CWC prohibits the development, production, stockpiling and use of chemical weapons. The interest of using STX as a chemical warfare agent (CWA) may be possible. Highly lethal STX could be used to contaminate food supplies or drinking water as well as in small arms ammunitions [8]. On the other hand, PSP toxins themselves cause a serious hazard to public health and threat the shellfish industry through the world.

The chemical analysis of STX has been a challenge because of its chemical structure and physical properties. All PSP toxins have the same tetrahydropurine backbone but different functional groups. STX is highly polar so it has no retention to reversed-phase liquid chromatography column (RPLC) without ion-pair reagents. On the other hand, the ion-pair reagents are not suitable for mass spectrometry because they interfere with detection by causing ion suppression and contamination of the ion source. STX occurs

Abbreviations: STX, saxitoxin; GTX1, gonyautoxin 1; ISTD, internal standard; HILIC, hydrophilic interaction liquid chromatography; MS/MS, tandem mass spectrometry; LOD, limit of detection; LLOQ, lower limit of quantification; QC, quality control.

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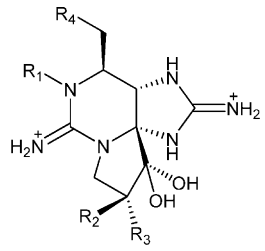


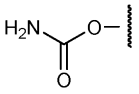
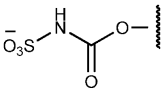
			Carbamate toxins	*N*-Sulfocarbamoyl toxins	Decarbamoyl toxins	Deoxydecarbamoyl toxins
					OH	H
R_1	R_2	R_3	R_4	R_4	R_4	R_4
H	H	H	STX	B1	dcSTX	doSTX
OH	H	H	NEO	B2	dcNEO	
OH	H	OSO^-_3	GTX 1	C3	dcGTX 1	
H	H	OSO^-_3	GTX 2	C1	dcGTX 2	doGTX 2
H	OSO^-_3	H	GTX 3	C2	dcGTX 3	doGTX 3
OH	OSO^-_3	H	GTX 4	C4	dcGTX 4	

Fig. 1. Chemical structures of most common PSP toxins.

mainly in ionic form and is stable only in acidic conditions but in basic conditions, it oxidizes easily. Structures of STX and other PSP toxins are presented in Fig. 1.

Hydrophilic structure separates STX and other PSP toxins clearly from other algal toxin groups. There is no absorptive chromophore in STX but with pre- or post-column oxidation it is possible to form aromatic aminopurine structure which can be identified with fluorescence detector after ion-pair liquid chromatography (LC-FLD) [9–12]. This technique provides high sensitivity but it is time-consuming, fluorescent matrix compounds can interfere with quantification of other PSP toxins and for routine monitoring purposes it has low daily sample throughput [12]. Besides the LC-FLD methods, enzyme-linked immunosorbent assay (ELISA) and mouse bioassay (MBA) are currently used for monitoring of STX in food supplies and drinking water [13]. The latter methods lack of specificity.

The hydrophilic interaction liquid chromatography coupled with mass spectrometry (HILIC–MS) is efficient technique for separation and identification of polar and hydrophilic compounds [14]. Using HILIC there is no need to use ion-pair reagents or derivatization for polar and ionic analytes. HILIC–MS method has been used before for PSP toxins [15,16] but the lack of isotopically labelled internal standard for algal toxins has slowed down the development of validated quantification analysis methods. In the study of Johnson et al. [17] a novel $^{15}N_7$-isotopically enriched STX was used as an internal standard when quantifying STX. However, any isotopically labelled standard is still not commercially available.

Fast and quantitative analysis method is needed for STX for verification purposes for CWC and also for food analyses and environmental monitoring. STX have also been detected as a newcomer in Finland both in freshwater and marine samples during the past decade [18,19]. In this study we have optimized and validated fast HILIC–MS/MS method for reliable verification of STX from algal matrix using full scan MS/MS, isocratic elution, conventional bore HILIC column and high flow-rate with accurate post-column splitter. Gonyautoxin 1 (GTX 1) was used as an internal standard (ISTD) (see Fig. 1). The developed method separates STX from other PSP toxins which are also identified by full scan MS/MS analysis. For validation of HILIC–MS/MS method, Food and Drug Administration's (FDA's) bioanalytical method validation recommendations were used [20]. Various criteria, namely precision and accuracy were used to evaluate quantitative performance of the method. For identification, WADA criteria [21] have been evaluated for verification of STX by using four diagnostic ions in MS/MS analysis. Advantage of the developed method is that it could be used to quantification and identification of trace levels of STX and it provides full scan MS/MS spectra of most common PSP toxins.

2. Experimental

2.1. Materials

Certified reference chemicals of PSP toxins were purchased from the NRC Certified Reference Materials Program (Institute for Marine Bioscience, Halifax, Canada). Acetonitrile (HPLC purity), formic acid (98–100%) and ammonium formate (≥97%) were obtained from VWR International (Belgium), Merck (Germany) and from Fluka (Switzerland), respectively. Water was purified using Elgastat option 3A and Elgastat UHQ PS equipments (Elga, UK). Freeze-dried blank dinoflagellate samples from field (Baltic Sea) and cultured *Alexandrium Ostenfeldii* samples (from algal strains AOTV-A4/3 and AOTV-B4/6 from Föglö/Åland [19]) were obtained from Finnish Environment Institute (SYKE). Centrifuge filters were purchased from Millipore (USA).

2.2. Extraction of algal samples

Cultivation, collection on filter papers (47-mm GF/C filter, Whatman, England) and freeze-drying of algae were conducted at Finnish Environment Institute [19] and delivered frozen to VERIFIN. The freeze-dried samples with filter papers were wetted in large vials with 2 ml of analysis buffer mixture containing (A) 4 mM ammonium formate buffer, pH 3.5 and (B) acetonitrile (ACN) with buffer (95:5, v/v) in ratio 40:60 (A:B). The vials were then shaken roughly and let stand on ice for 10 min and centrifuged at 4000 rpm for 10 min at 4 °C (Eppendorf R5810R centrifuge and Eppendorf F 45-30-11 rotor, Germany). Then the samples were filtered with Ultra free MC Duvapore PVDF 0.45 μm (Millipore) centrifuge filters at

14,000 rpm for 5 min at 4 °C. Prepared samples were stored in refrigerator until spiked with the chemicals.

2.3. Preparation of validation samples

Stock solutions of STX and GTX 1 standards were prepared in mobile phase containing 4 mM ammonium formate and acetonitrile with formic acid to achieve the pH 3.5. They were diluted into pooled and STX-free, extracted algal matrix (extraction method is described above). The standards were spiked with STX in levels 0, 5, 10, 25, 50, 100 and 200 ng/ml for HILIC–MS/MS method validation. GTX 1 was spiked as an internal standard (ISTD) into all samples to achieve the concentration of 150 ng/ml. STX sample at concentration level of 25 ng/ml was used as quality control (QC) for monitoring the performance of the instrument. For recovery studies, samples ($n = 3$) were made in 0.5 ml of pooled extracted algal matrix and spiked with STX to have final concentrations of 10 ng/ml and 200 ng/ml. The ISTD was also spiked to samples at concentration level of 150 ng/ml before filtering. These samples were filtered with centrifuge filters and results were compared to those from the calibration curves.

2.4. Instrumentation

HILIC–MS/MS was performed on a Finnigan LXQ linear ion trap mass spectrometer equipped with electrospray ionization (ESI) source interfaced to a Finnigan Surveyor Autosampler Plus Liquid Chromatograph (ThermoFinnigan, Hemel Hempstead, UK). Accurate post-column splitter (LC Packings, Norlab, Finland) was set up between LC and MS. Optimization of chromatographic conditions for all PSP toxins was conducted with Tosoh Bioscience 5 μm TSK-gel Amide-80® (250 mm × 2.0 mm) and Waters 3 μm HILIC Silica (150 mm × 2.1 mm) columns. The validation of quantitative method for STX was carried out on a TOSOH Bioscience 3 μm HILIC TSK-gel Amide-80® column (150 mm × 4.6 mm).

The mobile phase was (A) 4 mM ammonium formate and (B) acetonitrile (with 5% of eluent A, v/v %), with both containing the same amount of formic acid to achieve the pH 3.5. This allowed constant buffer strength and pH to be maintained through the run. Isocratic elution was used with eluent ratio 40:60 (A:B, v/v). In preliminary studies the flow rate of 0.2 ml/min was used without post-column splitter. In validation study, flow rate was 1 ml/min with a post-column splitter. The latter arrangement improved the sensitivity of the method. The injection volume was 20 μl. The column was maintained at 30 °C.

The ESI ionization source and the linear ion trap analyzer were operated at positive polarity, spray voltage of 5 kV and nitrogen used as a sheath gas. Full scan mode was used in range of m/z 150–500 for MS analyses. Detection of STX and GTX 1 were conducted by MS/MS mode for precursor ions at m/z 300 and m/z 332, respectively. For GTX 1, the base peak (m/z 332) was chosen for the precursor, not the protonated molecule ion, since it separates GTX 1 from its epimeric pair, GTX 4. One quantitative product ion at m/z 282 and three qualitative product ions at m/z 221, m/z 204 and at m/z 186 for STX were used, respectively. For GTX 1, one quantitative ion at m/z 314 was used. Capillary temperature used for validation studies and for analysis of cultured algal samples were 300 °C and 350 °C, respectively.

3. Results and discussion

3.1. Optimization of separation of STX analogues

Aim of this study was to develop a fast verification method for all commercially available PSP toxins. To optimize the method of Dell'Aversano et al. [15] for separation of all PSP toxins, this study was started with TSK-gel Amide-80® (250 mm × 2.0 mm) and HILIC Silica (150 mm × 2.1 mm) columns at low flow rate without post-column splitter. TSK-gel Amide-80® column was chosen for further studies. When started with a new column, some adsorption effects were noticed, e.g. poor peaks of PSPs. After injections of dirty algal samples (with limited sample preparation) filling of adsorption sites took place. Peak shapes and intensities improved drastically.

Many different gradient ratios were tested but isocratic elution was chosen since the stabilization of HILIC columns is slow after the gradient. The best chromatographic peak shape and peak resolution for epimeric pairs (e.g. GTX 1 and GTX 4) were obtained with isocratic elution ratio of 40:60 (A:B). Three different pH values 3, 3.5 and 4 were tested. With pH 3.5 the retention times were short and peak shapes good, respectively. Three different buffer concentrations 2, 4 and 10 mM were also tested. Using 2 mM buffer concentration, peak resolution for epimeric pairs of PSP toxins i.e. GTX 1/GTX 4, GTX 2/GTX 3 and dcGTX 2/dcGTX 3 were 1.26, 1.36 and 1.18, respectively. Using 4 mM buffer concentration, resolution was not affected but retention times were shorter compared to 2 mM buffer. The effect of column temperature was tested at 20, 30 and 40 °C for STX. The effect of the column temperature was found to be slight as in earlier study [15]. For validation studies 4 mM buffer concentration and 30 °C temperature for column were chosen. In preliminary experiments retention time of STX was 9.8 min with TSK-gel Amide-80® column which is already 50% faster than in the study of Dell'Aversano et al. [15].

3.2. HILIC–ESI-MS/MS method optimization for quantification of STX and identification of other PSP toxins

Quantification of STX is important especially for food and environmental monitoring for its high toxicity. For verification and quantification of STX, further method optimization was conducted. TSK-gel Amide-80® column was proved to be viable for analysing PSP toxins. For quantification of STX, the same column was chosen with conventional bore, shorter length and smaller particle size (150 mm × 4.6 mm, 3 μm). This choice made possible to use much higher flow-rate in LC without sacrificing efficiency. However, the high flow-rate (i.e. 1 ml/min) is not optimal for ESI and the eluent flow was split accurately one to twenty (1:20) before the mass spectrometer. In addition, the retention time of STX moved from 9.8 min to 6.5 min, respectively.

Two different ionization methods were tested during this study; ESI and atmospheric pressure chemical ionization (APCI), both in the positive ion modes. ESI proved to be optimal for quantitative analysis because it gave abundant protonated molecule $[M+H]^+$ of STX in full scan MS mode and there was only little fragmentation in the full scan MS spectrum. In APCI analysis there was no protonated molecule of STX in MS spectrum after loss of water. Most abundant product ions from the product ion spectrum were selected for quantification (Q) and for qualification (q), respectively. MS/MS chromatograms and product ion spectra of STX and GTX 1 (ISTD) from a 25 ng/ml QC standard run are displayed in Fig. 2.

With optimized conditions and arrangements, the analysis of STX got faster and more sensitive. The retention times (Rt) of STX and GTX 1 are 6.47 ± 0.03 min and 4.44 ± 0.01 min, respectively. This means that the retention times have diminished into third compared to earlier study [15]. Dramatically better signal-to-noise ratio (S/N) was achieved compared to the previous one—previously it was three to one (3:1) at sample concentration of 20 μg/ml and now ca 60:1 at sample concentration of 25 ng/ml in LC–MS/MS analysis. Splitting the flow rate does not decrease the signal to noise ratio (S/N) when using concentration-sensitive detector (ESI-MS), because the signal is directly proportional to the concentration of the analyte, not the amount of sample component [22].

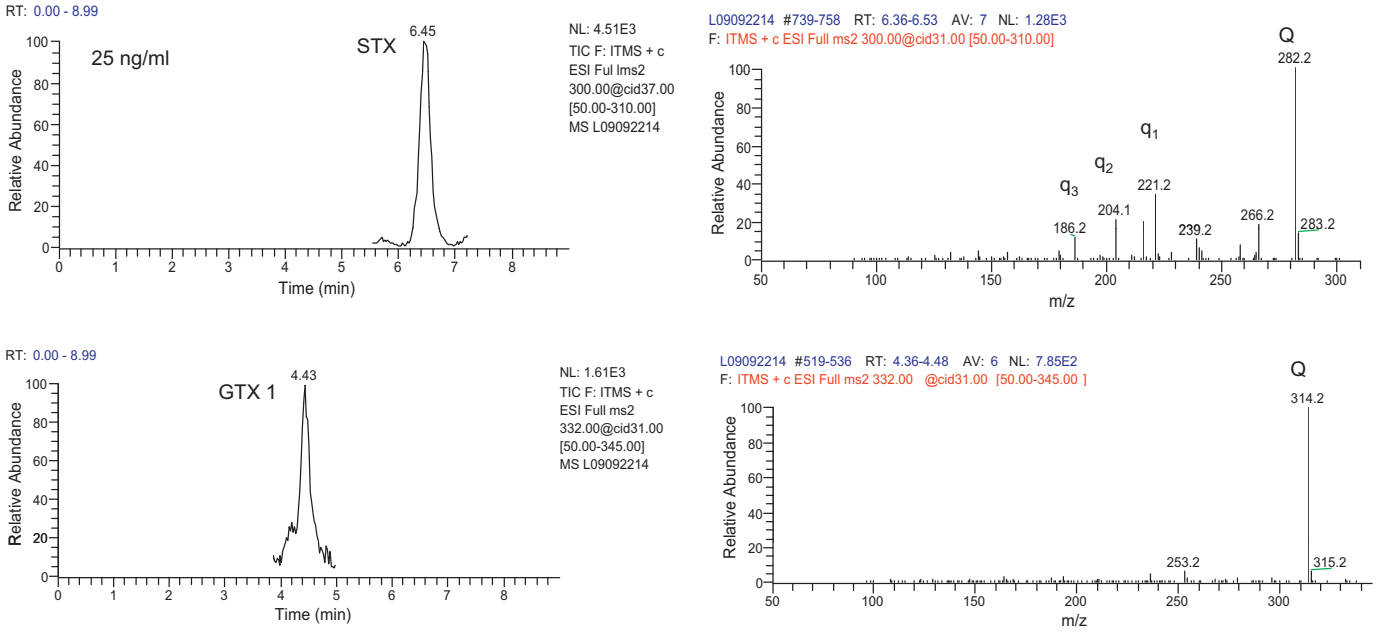


Fig. 2. Chromatograms and spectra of full scan MS/MS analysis of STX and GTX 1 (ISTD). Concentration of STX is 25 ng/ml.

The other common PSP toxins (Table 1) could be also identified with this analysis method. C1 and C2 toxins could not be detected due to their low ionization efficiency. Retention times, precursor ions and main product ions are given in Table 1 and the full scan MS/MS chromatograms and spectra are presented in Fig. 3. Even the retention times overlap it is not a problem since the MS/MS spectra are pure and toxins identifiable.

3.3. Optimization of sample preparation for algal samples

STX occurs in algal and cyanobacterial cells and is accumulated in shellfish but is also extracted in surrounding water [23]. The isolation of STX could be made from any matrixes but the concentration of toxin and their stability is much lower in water samples than in algal or cyanobacteria samples. Algal samples are planned

Table 1
Chromatographic and mass spectrometric parameters for PSP toxins.

Toxin	Retention time (min)	$[M+H]^+$	Precursor ion, *m/z*	Product ions, *m/z*
STX	6.5	300	300	282 221 204 186
NEO	6.7	316	316	298 238 220
dcSTX	6.8	257	257	239 222 198
dcNEO	6.6	273	273	255 225 179
GTX 1	4.5	412	332	314
GTX 4	4.9	412	412	394 332 314
GTX 2	4.2	396	396	316
GTX 3	4.8	396	396	378 316 298
GTX 5 (B1)	5.4	380	380	300 282
dcGTX 2	3.8	353	535	335 273
dcGTX 3	5.0	353	535	335 273 255

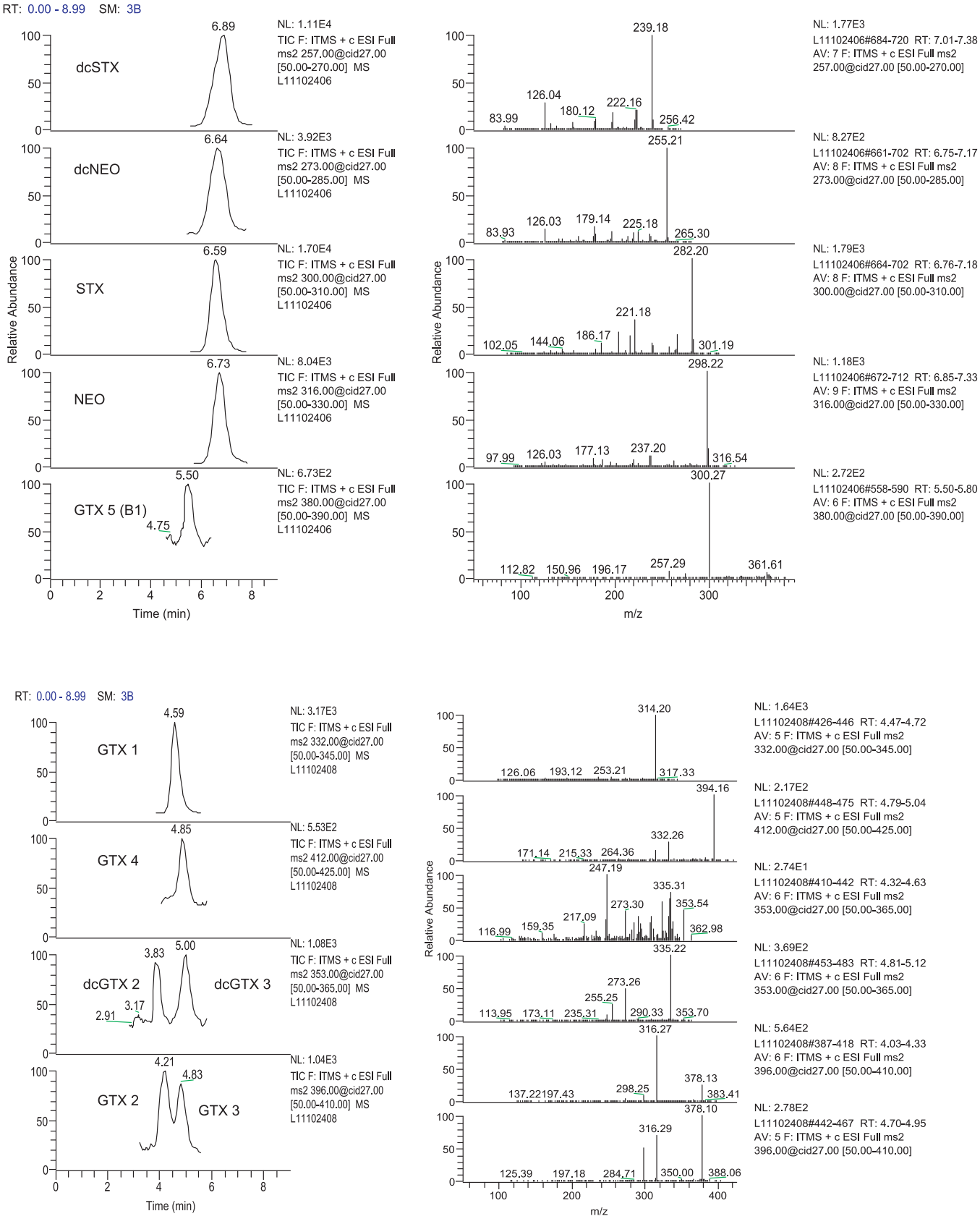


Fig. 3. Chromatograms and spectra of full scan MS/MS analysis of mixture of PSP toxins. Precursor ions are given in Table 1.

to be used for monitoring STX in the Baltic Sea. STX and its analogues can be extracted with acidic aqueous solutions from algal matrix, because of their hydrophilic structure. For example, acetic acid [11,18,24,25], hydrochloric acid [26] or mixture of formic acid and acetonitrile [15] have previously been used. In our study, we modified the extraction solvent from the study of Dell'Aversano et al. [15] and used freeze-dried algae samples for breaking the cells and minimizing the water content in samples. Depending on sample type (e.g. dinoflagellate or cyanobacteria), different homogenization methods have also been used. Dinoflagellates have thin cell walls which break easily when freezing and melting the sample. However, cyanobacteria need harder treatment like ultrasonication to break the thick cell wall.

Three different filters were tested for sample preparation; PVDF 0.45 μm, PTFE 0.2 μm and YM-3 with molecular cut-off 3 kDa (Millipore) to remove the impurities and algal cell remains of the sample. The long centrifugation time (20 min) of sample with YM-3 filter indicated that this filter type was too thick for sample preparation. The results with this filter were poor. The PVDF and PTFE filters gave quite similar results and STX was recovered with both filters. Later in analysis of STX containing cultured algal sample, PTFE filters seemed to absorb compounds produced by algae and that is why the PVDF filter was chosen for the further validation studies to avoid possible losses of other interesting compounds.

3.4. Validation

Food and Drug Administration's (FDA's) bioanalytical method validation recommendations were used in our validation study except in recovery samples [20]. To fulfil FDA recommendations thoroughly, three different concentration levels and at least six parallel samples ($n = 6$) were analysed.

For saxitoxin, four different product ions were taken into account, one being so-called quantifier (Q) at m/z 282 and other three as qualifiers (q) at m/z 221 (q_1), m/z 204 (q_2), m/z 186 (q_3). In the used true ion trap instrument, parent ion is totally dissociated in the optimum conditions for MS/MS. The advantage of full scan MS/MS method compared to previously used SIM or SRM methods [15] is the availability of full product ion spectra for reliable identification of STX and for recognition of false positives.

Validation was performed using a PSP analogue, GTX 1, as an internal standard. It was chosen because of the lack of commercially available isotopically labelled standard and the next best alternative for internal standard is structure analogue of the analyte. GTX 1 has totally different retention time and precursor and product ions compared to STX. Minor weakness of GTX 1 is that its ionization in ESI compared to STX is inferior.

All standards for validation studies were made in blank algal matrix which was extracted as described above. Before validation studies blank algal matrix was verified for absence of STX and GTX 1. Validation runs were performed at 4 days. Three calibration curves were obtained daily during 3 days ($n = 9$). The recovery tests were performed at fourth day. True values for the concentrations of the calibration standards and their accuracy (relative standard deviation, RSD) were calculated from nine calibration curves. Precision (variance within group and between groups), random error, systematic error (*bias*) and estimation of uncertainty were calculated from the true values of the calibration standards. Single-factor ANOVA (Analysis of Variance) was used to calculate these validation results. The validation results were calculated over the linear concentration range from 5 ng/ml to 50 ng/ml (average correlation coefficient $R^2 = 0.9714$, $n = 9$) and from 25 ng/ml to 200 ng/ml (average correlation coefficient $R^2 = 0.9768$, $n = 9$). Average calibration curves are presented in Fig. 4. Linear calibration was not possible

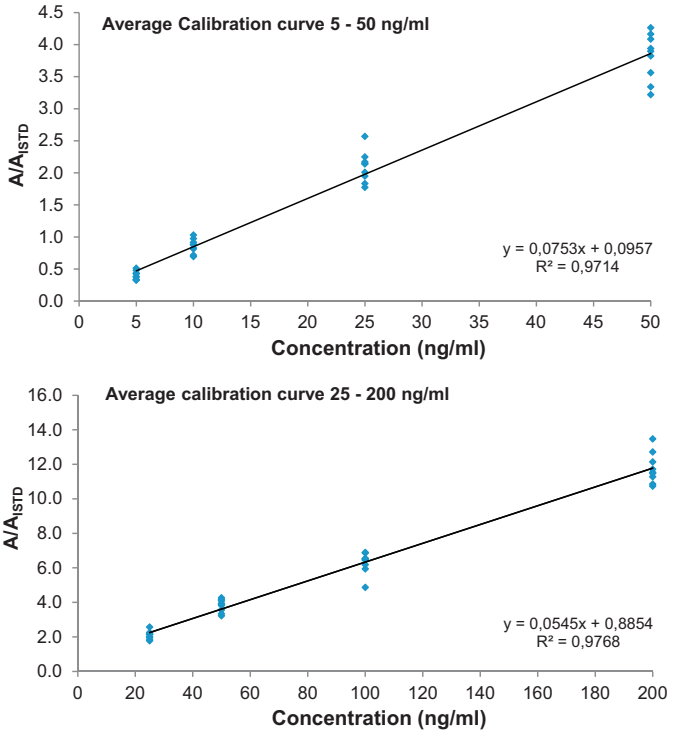


Fig. 4. Average calibration curves for STX in linear ranges (A) 5–50 ng/ml and (B) 25–200 ng/ml.

in whole concentration range of 5–200 ng/ml because of bending of the curves by reason of ion suppression. Ion suppression derives from salty sample matrix [22], physical characteristics of ESI source [22] and also concentration-dependence of the ionization of PSP toxins [24].

Limit of detection (LOD) and lower limit of quantification (LLOQ) were calculated from the range of 5 to 50 ng/ml with Eqs. (1)–(4), where STDEV(n) is the standard deviation of the intersection, m is the slope and n is the intersection of the calibration curves. For STX, calculated LOD was 3 ng/ml and LLOQ 11 ng/ml. Compared to earlier studies, lowest LOD for STX using triple quadruple and SRM method was estimated to 20 nM (with S/N 3) which corresponds 6 ng/ml STX concentration [15].

$$\mathrm{LOD}(y = mx + n)$$

$$\text{If } n > 0, \quad \mathrm{LOD} = \frac{3x\,\mathrm{STDEV}(n)}{m} \tag{1}$$

$$\text{If } n < 0, \quad \mathrm{LOD} = \frac{3x\,\mathrm{STDEV}(n) - n}{m} \tag{2}$$

$$\mathrm{LLOQ}(y = mx + n)$$

$$\text{If } n > 0, \quad \mathrm{LOQ} = \frac{10x\,\mathrm{STDEV}(n)}{m} \tag{3}$$

$$\text{If } n < 0, \quad \mathrm{LOQ} = \frac{10x\,\mathrm{STDEV}(n) - n}{m} \tag{4}$$

In the range of 5–50 ng/ml method was found to be accurate (RSD's < 15%) and precise since variance both within- and between group was <9% except limit of detection level (LOD). These values fall in the FDA recommendations where each concentration level should not exceed 15% except for LLOQ, where it should not exceed 20%. In our study, the LLOQ level was also recovered with RSD lower than 15%. In the range of 25–200 ng/ml, the method was likewise accurate (RSD's < 7%) and precise (both within- and between group

Table 2
Calculated validation results for STX standards spiked in algal matrix in calibration ranges 5–50 ng/ml and 25–200 ng/ml.

Chemical	Standard concentration	Average concentration	SD	RSD (%)	Variance within group (%)	Variance between groups (%)	Random error (%)	Systematic error (%)	Combined uncertainty (%)
STX	5.0 (LOD)	3.9	0.6	14.5	30.7	10.1	32.3	3.76	33
	10.0 (LLOQ)	10	0.6	5.8	8.2	3.4	8.9	0.58	9
	25.0	26.5	0.6	2.4	7.8	3.8	8.7	0.09	9
	50.0	49.2	0.3	0.5	1.8	0.9	2.0	0.01	3
	QC (25 ng/ml)[a]	26.9	2.8	10.4	13.5	9.1	16.3	0.39	18.8
STX	25.0	21.7	1.4	6.3	22.6	11.6	25.4	0.29	25
	500	54.3	1.5	2.7	5.2	1.3	5.3	0.05	7
	100.0	99.3	1.0	1.0	9.0	5.1	10.3	0.01	11
	200.0	199.7	0.5	0.2	1.8	1.0	2.1	0.00	9
	QC (25 ng/ml)[a]	27.6	5.2	18.7	21.9	17.5	28.0	0.68	29.4

[a] QC runs were performed four times a day; before in the middle and after every calibration curve ($n = 12$).

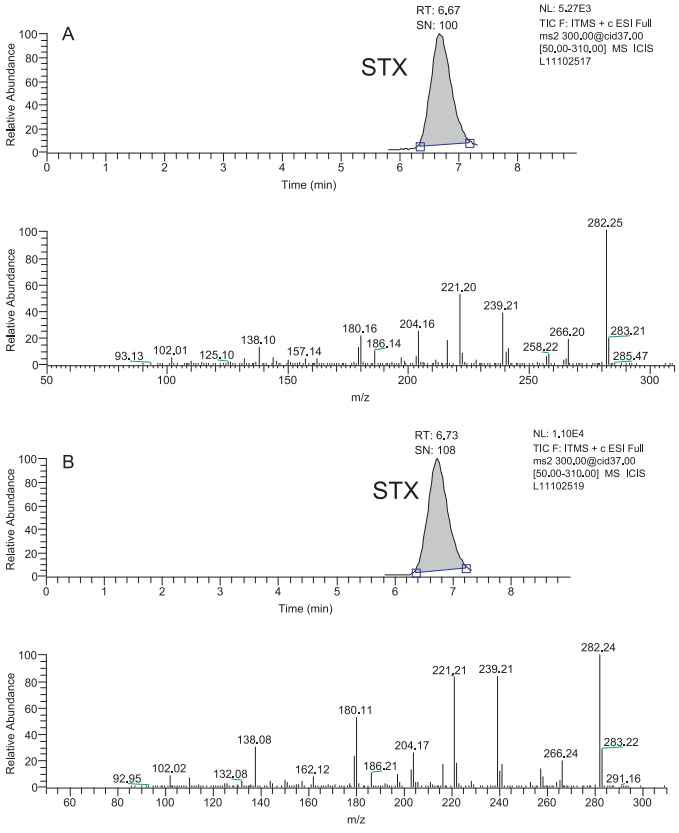


Fig. 5. Chromatograms and MS/MS spectra of extracts after dilution of cultured, authentic STX containing algal samples (A) AOTV-A4/3 and (B) AOTV-B4/6 containing STX.

was <12%) except in the level of 25 mg/ml. The selectivity of the method was tested with blank algal samples and purified water during the validation. No effects were noticed from the early or late eluting compounds during the validation. The validation results are presented in Table 2.

For recovery studies the concentration levels of 10 ng/ml and 200 ng/ml for STX were used. The recoveries of 10 ng/ml samples were calculated from calibration curve of range 5–50 ng/ml and for 200 ng/ml samples from calibration range 25–200 ng/ml. Recoveries were found to be excellent. However, since calibration curve have tendency to bend for the ion suppression, we would recommend that sample containing 200 ng/ml or more of STX should be diluted. Results of recovery test are presented in Table 3.

3.5. Verification and quantification of STX from algal sample

The validated LC–MS/MS method was tested by analysing and quantifying STX from freeze-dried *Alexandrium Ostenfeldii* samples (AOTV-A4/3 and AOTV-B4/6) using MS and MS/MS modes. For this analysis, the mass spectrometer was tuned again with STX solution and capillary temperature was raised from 300 °C to 350 °C. After that, the calibration curves were linear ($R^2 = 0.999$) over the whole range from 5 to 200 ng/ml, and that was used in quantification.

STX was shown to be present in studied algal samples. Ion chromatograms and mass spectrums' of the results are given in Fig. 5 and calibration curve in Fig. 6. The extracts contained STX at high concentrations (>200 ng/ml), so they were diluted with mobile phase into one to five (1:5). Before the dilution, measured concentrations of STX in AOTV-A4/3 and AOTV-B4/6 algal samples were 317 ng/ml and 238 ng/ml, and after dilution 69 ng/ml and 55 ng/ml, respectively. Then the real STX concentrations of the samples were 345 ng/ml and 275 ng/ml, respectively. Slight ion suppression effect is observed when comparing the results of diluted and not diluted samples.

One of the goals of our study was to evaluate criteria of WADA and the Organisation for the Prohibition of Chemical Weapons (OPCW) [27] for identification of STX using validated analysis method. Both WADA criteria and identification criteria for scheduled chemicals, present that retention time shall not differ more than ±0.1 min from reference sample. That criterion was fulfilled in our validation experiments. However, in studies of cultured algal samples the retention times were shifted from 6.5 min (Fig. 2) to 6.7 min (Fig. 5) when diluting the sample. In HILIC analysis, retention time is dependent on salt concentration and this effect was shown by dilution. It is recommended to dilute the samples with mobile phase. If possible, reference chemicals should be prepared in similar matrix than the sample if available or other alternative is

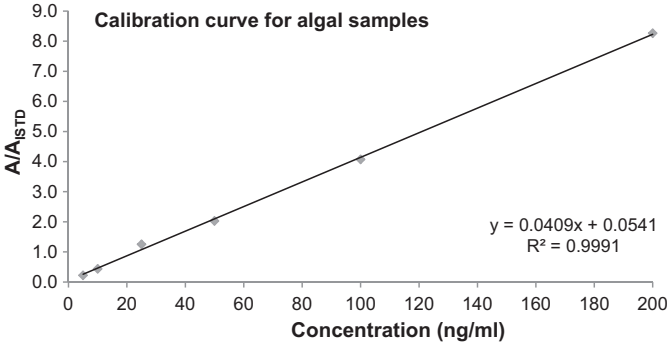


Fig. 6. Calibration curve for quantifying STX from algal samples.

Table 3
Recovery results of STX in spiked algal samples.

Concentration (ng/ml)	Recovery samples (%)			Average recovery (%)	SD (%)	RSD (%)
	$n=1$	$n=2$	$n=3$			
10	100.6	97.3	101.7	99.9	2.3	2.3
200	123.6	106.1	105.3	111.7	10.3	9.2

to spike STX to positive sample to verify its presence by increased peak height/area. However, in HILIC analyses the retention times are comparable within the same sample batch, like in our validation study. Because of the retention time shifting, the spectral identification is crucial in HILIC analysis when the method is used for verification purposes.

According to the current OPCW identification criteria, intensity of precursor ion must be at least 10% [27]. In true ion trap, no precursor ion ($[M+H]^+$) is left in the MS/MS spectrum but four product ions could be easily monitored and identified. In WADA criteria, there is not defined number of diagnostic ions in full scan MS/MS experiments but the abundance of all diagnostic ions shall be greater than 10% of the base peak [19]. In our experiments even the least abundant product ion was more than 10% of the base peak. S/N ratios of quantifier ions were always higher than eighteen to one ($\geq$18:1), even at the concentration level at 5 ng/ml. The WADA criteria were fulfilled in our validation studies.

In this study, HILIC–MS/MS was used for verification and quantification of STX. According to OPCW, identification of each chemical must be based on at least two different analytical techniques, one being preferably spectrometric. In case of STX, the second technique might be HPLC–ox–FLD or biochemical technique, e.g. ELISA.

4. Conclusion

The optimized and validated HILIC–MS/MS method is suitable for verification and quantification of STX from algal samples. It has proven to be linear in concentration range from 5 to 50 ng/ml and from 25 to 200 ng/ml, and accurate and precise in both LLOQ and ULOQ levels. In comparison to previous studies, the validated method is fast and identification criteria were fulfilled using the HILIC–MS/MS method. The identification is based on full scan MS/MS spectrum and four diagnostic product ions. Other most common PSP analogues are separated and identified using the developed method. The presented HILIC–MS/MS method has been found to be applicable in proficiency testing of STX and quantitative analysis of algal samples.

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References

[1] Y. Shimizu, Chem. Rev. 93 (1993) 1685.
[2] Y. Shimizu, in: L.M. Botana, M. Dekker (Eds.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, ACS Publications, Washington, DC, 2000.
[3] G.S. Wiberg, N.R. Stephenson, Toxicol. Appl. Pharmacol. 2 (1960) 607.
[4] E.J. Schantz, J.M. Mold, D.W. Stanger, J. Shavel, F.J. Riel, J.P. Bowden, J.M. Lynch, R.S. Wyler, B.R. Riegel, H. Sommer, J. Am. Chem. Soc. 79 (1957) 5230.
[5] P. Vale, Phytochem. Rev. 9 (2010) 525.
[6] M.E. van Apeldoorn, H.P. van Egmond, G.J.A. Speijers, G.J.I. Bakker, Mol. Nut. Food Res. 51 (2007) 7.
[7] http://www.opcw.org/chemical-weapons-convention/annex-on-chemicals/b-schedules-of-chemicals/schedule-1/ (01.08.11).
[8] SIPRI (Stockholm International Peace Research Institute), The Problem of Chemical and Biological Warfare: A Study of the Historical, Technical, Military, Legal and Political Aspects of CBW, and Possible Disarmament Measures, vol. 2, CB Weapons Today, Almqvist and Wiksell, Stockholm, 1973.
[9] T.N. Asp, S. Larsen, T. Aune, Toxicon 43 (2004) 319.
[10] J. Franco, Chromatographia 35 (1993) 613.
[11] C. Hummert, Chromatographia 45 (1997) 312.
[12] E.A. Turrell, J.P. Lacaze, L. Stobo, Harmful Algae 6 (2007) 438.
[13] A.R. Humpage, V.F. Magalhaes, S.M. Froscio, Anal. Bioanal. Chem. 397 (5) (2010) 1655.
[14] P. Hemström, K. Irgum, J. Sep. Sci. 29 (2006) 1784.
[15] C. Dell'Aversano, P. Hess, M.A. Quilliam, J. Chromatogr. A 1081 (2005) 190.
[16] P. Blay, J.P.M. Hui, J. Chang, J.E. Melanson, Anal. Bioanal. Chem. 400 (2011) 577.
[17] R.C. Johnson, Y. Zhou, K. Statler, J. Thomas, F. Cox, S. Hall, J.R. Barr, J. Anal. Toxicol. 33 (2009) 8.
[18] J. Rapala, A. Robertson, A.P. Negri, K.A. Berg, P. Tuomi, C. Lyra, K. Erkomaa, K. Lahti, K. Hoppu, L. Lepistö, Environ. Toxicol. 20 (2005) 331.
[19] A. Kremp, T. Lindholm, N. Dreßler, K. Erler, G. Gerdts, S. Eirtovaara, E. Leskinen, Harmful Algae 8 (2009) 318.
[20] Bioanalytical Method Validation, Guidance for Industry, US Department of Health and Human Services, Food and Drug Administration, 2001.
[21] World Anti-Doping Agency, WADA Technical Document TD2010IDCR, World Anti-Doping Agency, Montreal, Canada, 2010.
[22] J.T. Watson, O.D. Sparkman, Introduction to Mass Spectrometry, 4th ed., Wiley, England, 2007.
[23] K.A. Lefebvre, B.D. Bill, A. Erickson, K.A. Baugh, L. O'Rourke, P.R. Costa, S. Nance, V.L. Trainer, Mar. Drugs 6 (2008) 103.
[24] E. Jaime, C. Hummert, P. Hess, L. Bernd, J. Chromatogr. A 929 (2001) 43.
[25] B. Krock, C.G. Seguel, A.D. Cembella, Harmful Algae 6 (2007) 734.
[26] M. Diener, K. Erler, S. Hiller, B. Christian, B. Luckas, Eur. Food Res. Technol. 224 (2) (2006) 147.
[27] Organisation for the Prohibition of Chemical Weapons (OPCW), Work instruction for the evaluation of the results of OPCW Proficiency Tests (QDOC/LAB/WI/PT03), Issue 1, 31.3.2010. Available from the OPCW Laboratory.