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Hydrophilic interaction liquid chromatography–mass spectrometry for the analysis of paralytic shellfish poisoning (PSP) toxins

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Abstract

Hydrophilic interaction liquid chromatography (HILIC) was examined for the separation of paralytic shellfish poisoning (PSP) toxins using the stationary phase TSK-gel Amide-80®. The parameters tested included type of organic modifier and percentage in the mobile phase, buffer concentration, pH, flow rate and column temperature. Using mass spectrometric (MS) detection, the HILIC column allowed the determination of all the major PSP toxins in one 30 min analysis with a high degree of selectivity and sensitivity. The high percentage of organic modifier in the mobile phase and the omission of ion pairing reagents, both favored in HILIC, provided limits of detection (LOD) in the range 50–1000 nM in selected ion monitoring (SIM) mode on a single quadrupole LC–MS system. LOD in selected reaction monitoring (SRM) mode on a sensitive triple quadrupole system were as low as 5–30 nM. Excellent linearity of response was observed.

Keywords: Hydrophilic interaction liquid chromatography; Mass spectrometry; HILIC–MS; Paralytic shellfish poisoning toxins; Saxitoxins; Marine toxins

1. Introduction

Paralytic shellfish poisoning (PSP) toxins (Fig. 1) are produced by marine dinoflagellates belonging to *Alexandrium*, *Pyrodinium* and *Gymnodinium* genera as well as by freshwater cyanobacteria such as *Aphanizomenon flos-aquae*, *Anabaena circinalis* and *Lyngbya wollei* [1,2]. These toxins cause persistent problems due to their accumulation in filter feeding shellfish [3], but they can also move through the food chain, affecting zooplankton, fish, birds and marine mammals [4]. PSP poses a serious hazard to public health and threatens the shellfish industry throughout the world. Deaths of wildlife and domestic animals following the ingestion of contaminated freshwater supplies have also been reported.

PSP toxins are potent, reversible blockers of voltage-activated sodium channels on excitable cells [5]. The PSP syndrome is characterized by neurological distress, which typically appears within 15–30 min after consumption of contaminated shellfish and can result in death. PSP toxins are tetrahydropurine derivatives and can be divided into three groups based on the nature of the side chain: carbamoyl (R_4 = –$OCONH_2$), *N*-sulfocarbamoyl (R_4 = –$OCONHSO_3^-$) and decarbamoyl (R_4 = –OH). They all bind to site 1 of sodium channels but with different affinities resulting in different toxicities, the carbamoyl toxins being the most toxic and the *N*-sulfocarbamoyl derivatives the least toxic [6]. Thus, for assessment of health risk it is necessary to determine the level of each toxin individually, unless a toxicity-based assay is used.

The AOAC official mouse bioassay method [7] is used widely for routine monitoring of shellfish for overall toxicity. Although this approach has the advantage of providing a single integrated response from all the toxins, variation in toxin profiles cannot be monitored. The method also suffers

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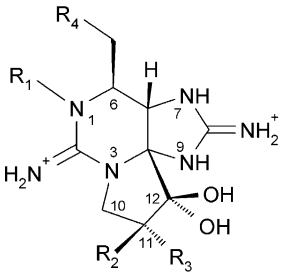

STX = saxitoxin
NEO = neosaxitoxin
GTX = gonyautoxins

R_1	R_2	R_3	R_4	Toxin
H	H	H	$-O-C(=O)-NH_2$	STX
H	H	OSO_3^-		GTX2
H	OSO_3^-	H		GTX3
OH	H	H		NEO
OH	H	OSO_3^-		GTX1
OH	OSO_3^-	H		GTX4
H	H	OH		11α-OH-STX
H	OH	H		11β-OH-STX
H	H	H	$-O-C(=O)-NHSO_3^-$	B1 (GTX5)
H	H	OSO_3^-		C1
H	OSO_3^-	H		C2
OH	H	H		B2 (GTX6)
OH	H	OSO_3^-		C3
OH	OSO_3^-	H		C4
H	H	H	$-OH$	dcSTX
H	H	OSO_3^-		dcGTX2
H	OSO_3^-	H		dcGTX3
OH	H	H		dcNEO
OH	H	OSO_3^-		dcGTX1
OH	OSO_3^-	H		dcGTX4

Fig. 1. Structures of principal toxins associated with paralytic shellfish poisoning (PSP) syndrome.

from poor reproducibility, low sensitivity and interferences from other components in the extract and there are ethical arguments against the continued use of this live animal assay. Alternative assays include those based on in vitro cell toxicity, receptor binding, and immunological response. None of these assays provide detailed information on the toxin profile in samples and in most regulatory situations positive results with bioassays require further confirmation.

Instrumental methods can overcome the above drawbacks and offer the possibility of precise, sensitive, and automated quantitative analyses for both monitoring and research work [8–11]. However, PSP toxins have posed a significant challenge to the development of instrumental methods for their detection and quantitation: they are present in nature in a great variety of closely related structures with three different charge states (0, +1 and +2); they lack a useful chromophore; and they are nonvolatile and thermally labile. These characteristics, coupled with the very low levels found in most samples, eliminate most traditional chromatographic techniques such as gas chromatography (GC) and liquid chromatography (LC) with ultraviolet (UV) detection.

The most common technique for the analysis of PSP toxins is ion-pair LC using reversed phase columns coupled with post-column oxidation and fluorescence detection (LC-ox-FLD) [11]. It is based on conversion of PSP toxins into fluorescent derivatives under alkaline conditions. Even though the technique yields high sensitivity, it typically requires a complex set-up and operation of equipment along with demanding daily maintenance. In addition, interfering compounds have been identified for this technique [12]. An alternative method is the pre-column oxidation approach (ox-LC-FLD) developed by Lawrence et al. [13]. This method does not require the complex post-column reaction system and is rapid, sensitive, and fully automated [14]. However, the interpretation of quantitative results is complex because some toxins give the same oxidation product, while others give two or three products [15].

Electrospray ionisation–mass spectrometry (ESI–MS) is effective for detection of PSP toxins, which are quite basic and therefore give strong $[M+H]^+$ ions [16]. Thus, direct detection of PSP toxins is possible by using a mass spectrometer (MS) as detector for either liquid chromatography or capillary electrophoresis (CE). Although CE–MS [17,18] is ideally suited to the analysis of the highly charged PSP toxins, it is not possible to analyze all the toxins in a single analysis due to their different charge states and the technique is susceptible to interference from co-extracted salt in samples.

The most common LC method, reverse-phase LC, requires ion-pairing reagents, such as heptanesulfonic acid, heptafluorobutyric acid or tetrabutylammonium sulfate, in the mobile phase to provide sufficient retention of charged PSP toxins [11]. Such agents seriously interfere with MS detection by causing suppression of ionization and ion source contamination. In addition, C toxins must be analyzed in a separate run from STX, NEO and GTX toxins due to their different charge states. An alternative approach based on ion-exchange chromatography with fluorescence and MS detection has been proposed recently by Jaime et al. [19].

In this paper, we present a new approach to the analysis of PSP toxins that is based on hydrophilic interaction liquid chromatography (HILIC) coupled with electrospray ionization tandem mass spectrometry (MS/MS). HILIC is a valuable tool in the separation of polar compounds introduced by Alpert for separation either of peptides and nucleic acids [20]. Strege made effective use of HILIC–MS in drug research [21]. In this type of chromatography, a

hydrophilic stationary phase is combined with a mobile phase that has a high percentage organic component. The mechanism involves hydrophilic interaction of either polar or ionic compounds with a stagnant aqueous phase at the packing surface, as well as ion exchange interactions.

The developed HILIC–MS method overcomes many drawbacks of the above assays and instrumental methods. The mobile phase does not use ion pair agents, so it does not reduce ionization efficiency, and is high in organic modifier, so it enhances ionization yield. It allows the simultaneous determination of all the major PSP toxins listed in Fig. 1 in a single 30 min analysis with a high degree of selectivity and no need for further confirmation. While some preliminary results of this work have been communicated previously [22], this paper reports on the systematic investigation of parameters leading to an optimized analytical technique. Application to plankton and shellfish samples is also demonstrated.

2. Experimental

2.1. Chemicals

All organic solvents were of distilled-in-glass grade (Caledon Labs, Georgetown, ON, Canada). Water was distilled and passed through a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA) to 18 MΩ quality or better. Formic acid (90%, laboratory grade), acetic acid, ammonium formate (AR grade) and ammonium acetate (AR grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Standard solutions of PSP toxins were provided by the NRC Certified Reference Materials Program (Institute for Marine Biosciences, Halifax, NS, Canada).

2.2. Plankton sample

The plankton sample was collected on June 2000 in a Nova Scotian harbour [23]. Extraction was performed by suspending 1 g (wet weight) of cell pellet in 1.5 mL of acetonitrile/water/formic acid (80:19.9:0.1), mixing well and allowing to sit undisturbed for 10 min. The mixture was centrifuge filtered through a 0.45 μm membrane (Ultrafree-MC, Millipore, Bedford, MA) at 6000 rpm for 10 min. The filter was washed twice with 1.5 mL of the extracting solvent mixture. The filtrates were combined, adjusted to 5 mL final volume, and directly analyzed by HILIC–MS.

2.3. Mussel sample

Wild blue mussels (*Mytilus edulis*) attached to a salmon cage were collected from the same location as the plankton sample above. Whole mussel soft tissues (100 g) were removed from a large number of animals, pooled, and homogenized. A portion of homogenate (5 g) was extracted with three portions (10, 5 and 5 mL) of acetonitrile/water (80:20, v/v) with 0.1% formic acid, homogenizing each time at 10,000 rpm for 5 min, and centrifuging at 7000 rpm for 10 min. The extracts were pooled and adjusted to 25 mL final volume. The extract was cleaned using a PolyHydroxyEthyl Aspartamide® (PHEA) SPE cartridge (PolyLC inc., Columbia, MD, USA), which had been previously conditioned with 5 mL acetonitrile/water (10:90, v/v) with 0.1% formic acid and 5 mL acetonitrile/water (90:10, v/v) with 0.1% formic acid. A 1.0 mL aliquot of the crude extract was loaded, followed by a wash of 1 mL acetonitrile/water (80:20, v/v) with 0.1% formic acid and 0.4 mL acetonitrile/water (10:90, v/v) with 0.1% formic acid. The PSP toxins were eluted into a 2.0 mL volumetric tube with acetonitrile/water (10:90, v/v) with 0.1% formic acid.

2.4. Equipment

Mass spectral experiments were performed using the following systems: (i) a PE-SCIEX (Concorde, ON, Canada) API-165 single quadrupole mass spectrometer equipped with either a pneumatically assisted electrospray (Ionspray®) ionization source or a Turbospray® source coupled to an Agilent (Palo Alto, CA, USA) model 1100 LC; (ii) a PE-SCIEX API-III+ triple quadrupole mass spectrometer equipped with an Ionspray® source coupled to an Agilent model 1090 LC; or (iii) a PE-SCIEX API-4000 triple quadrupole mass spectrometer equipped with a TurboSpray® interface coupled to an Agilent model 1100 LC. The LC equipment included a solvent reservoir, in-line degasser, binary pump, refrigerated autosampler, and temperature-controlled column oven.

2.5. Methods

HILIC–MS analyses were carried out using a 5 μm TSK-gel Amide-80® column (250 mm × 2 mm or 4.6 mm i.d.) (Tosoh Bioscience LLC, 156 Keystone Drive, Montgomeryville, PA, USA). A 5 μm PolyHydroxyEthyl Aspartamide® column (200 mm × 2 mm i.d.) (PolyLC Inc., 9151 Rumsey Road, Ste. 180, Columbia, MD, USA) was also tested in preliminary work.

Key parameters such as type and percentage of organic modifier, pH, buffer character and concentration, flow rate, and column temperature were tested to improve peak shape, resolution and sensitivity. For these experiments, all mobile phases were made up as binary phases. One part (A) was kept 100% aqueous and the other part (B) contained 95% organic and 5% aqueous (v/v). Both A and B were made up by adding 5% (v/v) of a concentrated buffer (100% aqueous) to either deionised water or organic solvent (acetonitrile or methanol). This system allowed a constant buffer strength to be maintained throughout the run, even if the percent B was changed as a gradient. It also allowed rapid changes in the organic content to evaluate the influence of the percent organic modifier on the separation.

Changes in the buffer concentration of the mobile phase were made using a concentrated stock solution (ammonium formate or acetate) of different concentration. Final buffer

concentrations ranging from 0 to 10 mM in both aqueous and organic parts were tested. Addition of either concentrated formic or acetic acid to the diluted mobile phases facilitated testing the effect of the pH in the range 2.5–4. Changes in the pH of the mobile phase were made through addition of either concentrated formic or acetic acid to the diluted mobile phase. The amount of acid added to the aqueous phase A to achieve a particular pH was recorded and the same amount was added to phase B. Changes in percent B and different flow rates were programmed through the binary pump.

The influence of the flow rate was tested using the 250 mm × 4.6 mm i.d. Amide-80 column. Thereafter, all work with the 250 mm × 2 mm i.d. column used a flow rate of 0.2 mL/min. The column temperature was tested in the range 10–45 °C. A sample injection volume of 5 μL was used for the 2 mm i.d. column while 20 μL was used in the case of the 4.6 mm i.d. column.

The final conditions recommended for routine operation are the following: the 5 μm Amide-80 column (250 mm × 2 mm i.d.) maintained at 20 °C and eluted isocratically at 0.2 mL/min with 65% B, where eluent A was water and B was acetonitrile/water (95:5), both containing 2.0 mM ammonium formate and 3.6 mM formic acid (pH 3.5).

LC–MS analyses were performed in the positive ion mode with an electrospray voltage of 5000 V. Orifice voltages (OR) were set at 50 V and 10 V on the API-III+ and API-165 instruments, respectively. The corresponding declustering potential (DP) setting on the API-4000 was 50 V. Nitrogen was used as both nebulizer and curtain gas. A post-column split was employed to deliver, approximately 20 μL/min of mobile phase to the Ionspray® interface. The entire flow of the 250 mm × 2 mm i.d. column was transferred to the Turbospray® sources. The Turbospray® sources were operated with a drying nitrogen flow rate of 8 L/min and a gas temperature of 275 °C.

Full scan (Q1) spectra were collected in the mass range *m/z* 250–550. MS/MS (Q3) product ion spectra on the API-III+ were acquired at a collision energy of 20 V using either the protonated or ammoniated ions as precursor ions. A collision energy of 35 V was used on the API-4000. Argon was used as collision gas in the second radio-frequency only quadrupole (Q2) of the API-III+, while nitrogen was used in the API-4000. Selected ion monitoring (SIM) and selected reaction monitoring (SRM) detection was carried out selecting ions (boldfaced in Table 1) and transitions (Table 2), respectively. Ion dwell times were adjusted to give a total cycle time of 1 s.

Molecular modeling was performed with the program HyperChem (Hypercube Inc., Gainesville, FA).

3. Results and discussion

3.1. Optimization of mass spectrometry

As demonstrated previously [16], STX and its analogues are well suited to electrospray ionization, giving abundant $[M+H]^+$ ions. Since spectra for only a few PSP toxins have been reported, the Q1 mass spectra and Q3 product ion spectra of all saxitoxin analogues available to us were examined. The data are reported in Table 1, while Fig. 2 shows spectra of selected analytes.

It should be noted that for *N*-sulfocarbamoyl derivatives (B1, C1, C2, B2, C3, C4), the spectra vary strongly with instrument type. Orifice voltage settings of 10 and 50 V on the API-165 and API-III+ instruments, respectively, and a declustering potential of 50 V on the API-4000 instrument, provided minimum fragmentation of analytes while still maintaining a good background spectrum. The API-165 instrument gave a higher degree of fragmentation than the API-III+ and the API-4000, which could result in a lower sensitivity. The Turbospray® interface, which applies heated nitrogen to assist nebulization, can also give more fragmentation if too high temperatures are used. Thus, a minimum temperature (275 °C) had to be used to minimize temperature-induced fragmentation.

As shown in Fig. 2a and Table 1, STX, NEO, dcSTX and dcNEO gave $[M+H]^+$ ions as the base peak in their spectra, with a small fragment ion corresponding to the loss of a water molecule. Interestingly, these compounds, which exist as dications in solution, do not produce doubly charged ions in electrospray.

A different fragmentation pattern was observed with the two epimeric pairs of gonyautoxins, GTX1 and GTX4, GTX2 and GTX3 (Fig. 2a). The $[M+H]^+$ ion was still the most abundant ion in the Q1 spectra of gonyautoxins with the hydroxysulfate group in an α-orientation (GTX1 and GTX2), while an $[M+H\text{-}80]^+$ fragment ion, corresponding to loss of SO_3, dominated the spectra of 11β-hydroxysulfate toxins (GTX3 and GTX4). The same behavior was observed for the decarbamoyl gonyautoxins, dc-GTX1-4 (Table 1).

B1 gave abundant $[M+H]^+$ and $[M+H\text{-}80]^+$ ions (Fig. 2a). C1-4, the *N*-sulfocarbamoyl derivatives of GTX1-4, gave both protonated and ammoniated ions, as well as associated fragment ions due to loss of SO_3 and/or H_2O molecules.

The protonated molecules or adduct ions and the main fragment ions of each toxin were selected for selected ion monitoring experiments, taking into account an important aspect. For some toxins, fragment ions caused by elimination of SO_3 from $[M+H]^+$ ions could potentially interfere with protonated molecules of other compounds (e.g., B1 with STX; C1, C2, GTX2, GTX3 and B2 with each other and NEO). This actually reduced the number of ions required for SIM, thus increasing sensitivity, but it also posed the need for a good chromatographic separation of the toxins. Table 1 has the recommended SIM ions boldfaced.

Since more selective detection of compounds is possible through selected reaction monitoring, Q3 product ion spectra of PSP toxins were also examined. The spectra of selected toxins are shown in Fig. 2b and the results are summarized in Table 1 for all compounds tested. Significant differences were observed between fragmentation patterns of epimeric pairs of toxins (e.g., GTX2 and GTX3). Thus, more than

Table 1
Mass spectral data for paralytic shellfish poisoning (PSP) toxins

Toxin	Q1 spectra (%RI)[a,b]				Q3 product ion spectra (%RI)[c]		
	RT (min)[d]	$[M + NH_4]^+$	$[M + H]^+$	Fragment ion	Precursor ion	Product ion	Loss of
STX	20.3		**300** (100)	282 (10)	300 (40)	204 (100)	$-2H_2O-NH_3-NHCO$
						138 (75)	$-H_2O-NH_3-CO_2-HNCNH-H_2C_2NH$
						179 (60)	$-H_2O-NH_3-CO_2-HNCNH$
						186 (45)	$-3H_2O-NH_3-NHCO$
						282 (40)	$-H_2O$
						221 (25)	$-H_2O-NH_3-CO_2$
GTX2	9.6		396 (5)	**316** (100)	396 (0)	316 (100)	$-SO_3$
						298 (10)	$-SO_3-H_2O$
GTX3	10.7		**396** (100)	298 (38)	396 (0)	298 (100)	$-SO_3-H_2O$
				316 (27)		316 (10)	$-SO_3$
				378 (23)		220 (10)	$-SO_3-2\ H_2O-NH_3-NHCO$
NEO	21.0		**316** (100)	298 (12)	316 (100)	220 (68)	$-2\ H_2O-NHCO-NH_3$
						138 (65)	$-H_2O-NH_3-CO_2-HNCNH-H_2C_2NOH$
						298 (62)	$-H_2O$
						177 (60)	$-2H_2O-NH_3-CO_2-HNCNH$
						237 (42)	$-H_2O-NH_3-CO_2$
GTX1	9.8		412 (8)	**332** (100)	412 (0)	332 (100)	$-SO_3$
						314 (15)	$-SO_3-H_2O$
GTX4	10.9		**412** (100)	394 (55)	412 (0)	314 (100)	$-SO_3-H_2O$
				332 (12)		332 (10)	$-SO_3$
				314 (9)		253 (5)	$-SO_3-H_2O-NH_3-CO_2$
11(α, β)-OH-STX	24.9		**316** (100)		316 (27)	148 (100)	
						108 (50)	
						220 (45)	$-2\ H_2O-NHCO-NH_3$
						298 (40)	$-H_2O$
						196 (38)	$-H_2O-NHCO-NH_3-HNCNH$
						237 (10)	$-2\ H_2O-NHCO$
B1 (GTX5)	13.1		**380** (100)	300 (98)	380 (0)	300 (100)	$-SO_3$
				282 (10)		282 (38)	$-SO_3-H_2O$
				257 (8)		204 (30)	$-SO_3-2\ H_2O-NHCO-NH_3$
						221 (18)	$-SO_3-H_2O-CO_2-NH_3$
C1	7.2	493 (50)	476 (18)	**396** (100)	493 (0)	316 (100)	$-NH_3-2\ SO_3$
				413 (38)		396 (20)	$-NH_3-SO_3$
				316 (25)		298 (2)	$-NH_3-2\ SO_3-H_2O$
C2	8.0	493 (45)	476 (15)	**396** (100)	493 (0)	298 (100)	$-NH_3-2\ SO_3-H_2O$
				378 (50)		316 (68)	$-NH_3-2\ SO_3$
				413 (42)		378 (30)	$-NH_3-SO_3-H_2O$
				316 (5)		396 (12)	$-NH_3-SO_3$
B2 (GTX6)	14.6		**396** (100)	316 (30)	396 (0)	316 (100)	$-SO_3$
						220 (5)	$-SO_3-2\ H_2O-NH_3-NHCO$
						298 (4)	$-SO_3-H_2O$
						237 (3)	$-SO_3-2\ H_2O-NHCO$
						177 (3)	$-SO_3-2H_2O-NH_3-HNCNH-CO_2$
C3	7.9	509 (12)	492 (17)	**412** (100)	509 (0)	332 (100)	$-NH_3-2\ SO_3$
				332 (11)		412 (22)	$-NH_3-SO_3$
						314 (13)	$-NH_3-2\ SO_3-H_2O$
						394 (2)	$-NH_3-SO_3-H_2O$
C4	8.8	509 (10)	492 (8)	**412** (100)	509 (0)	314 (100)	$-NH_3-2\ SO_3-H_2O$
				332 (1)		394 (95)	$-NH_3-SO_3-H_2O$
						332 (77)	$-NH_3-2\ SO_3$
						412 (38)	$-NH_3-SO_3$
dcSTX	21.1		**257** (100)	239 (17)	257 (20)	126 (100)	
						138 (65)	$-H_2O-NH_3-2\ HNCNH$
						222 (50)	$-H_2O-NH_3$
						180 (48)	$-H_2O-NH_3-HNCNH$

Table 1 (*Continued*)

Toxin	Q1 spectra (%RI)[a,b]				Q3 product ion spectra (%RI)[c]		
	RT (min)[d]	$[M + NH_4]^+$	$[M + H]^+$	Fragment ion	Precursor ion	Product ion	Loss of
						156 (45)	$-NH_3-2$ HNCNH
						239 (25)	$-H_2O$
dcGTX2	10.2		353 (4)	**273** (100)	353 (0)	273 (100)	$-SO_3$
						255 (15)	$-SO_3-H_2O$
						126 (12)	
						148 (10)	
						238 (8)	$-SO_3-H_2O-NH_3$
						196 (8)	$SO_3-H_2O-NH_3$–HNCNH
dcGTX3	11.3		**353** (100)	335 (55)	353 (0)	255 (100)	$-SO_3-H_2O$
				273 (42)		196 (25)	$-SO_3-H_2O-NH_3$–HNCNH
				255 (30)		273 (12)	$-SO_3$
						238 (12)	$-SO_3-H_2O-NH_3$
						335 (7)	$-H_2O$
dcNEO	20.8		**273** (100)	255 (20)	273 (40)	126 (100)	
						225 (87)	
						180 (60)	
						138 (50)	
						207 (40)	
						255 (31)	$-H_2O$
						220 (25)	$-2\ H_2O-NH_3$
dcGTX1	10.1		369 (5)	**289** (100)	369 (0)	289 (100)	$-SO_3$
						271 (13)	$-SO_3-H_2O$
						126 (7)	
						195 (5)	
dcGTX4	11.4		**369** (100)	**289** (1)	369 (0)	271 (100)	$-SO_3-H_2O$
						195 (33)	
						289 (15)	$-SO_3$
						178 (15)	
						351 (10)	$-H_2O$

[a] Q1 spectra were obtained using a PE-SCIEX API-165 mass spectrometer. Percentage relative intensities (%RI) are reported in brackets.
[b] The bold-faced ions are recommended as ions to monitor in selected ion monitoring (SIM) experiments.
[c] Q3 product ion spectra were obtained using a PE-SCIEX API-4000 mass spectrometer. Percentage relative intensities (%RI) are reported in brackets.
[d] Retention times (RT) are for optimized chromatographic conditions (see Section 2).

one transition for each of these toxins had to be selected for SRM experiments. The recommended transitions for all compounds are shown in Table 2.

3.2. Optimization of chromatography

To investigate chromatographic conditions, a standard mixture of most of the known PSP toxins was analysed under various conditions. The ions and transitions reported in Tables 1 and 2 were selected for performing either SIM or SRM experiments, respectively.

3.2.1. Stationary phase

Suitable HILIC stationary phases available at the time of initial experiments were TSK-gel Amide-80, polyhydroxyethyl aspartamide, cyclodextrin, cyano, and amino-based column materials. The PHEA and Amide-80 columns were selected for this study because they had been reported to retain guanine, a compound very similar to PSP toxins, at various buffer concentrations [21].

The PHEA column provided a good separation between epimeric pairs (C1 and C2, GTX2 and GTX3, GTX1 and GTX4) when using water as eluent A and acetonitrile/water (95:5) as eluent B, both containing 2.0 mM ammonium formate, 3.6 mM formic acid, pH 3.5. However, selective detection of the major PSP toxins was possible only over a 120 min analysis time with poor peak shape (gradient: 90% B for 80 min, 90–65% B over 15 min and hold 25 min). Shorter analysis time (35 min) resulted in poor separation between potentially interfering compounds, namely C1/GTX2 and STX/B1 (gradient: 80–65% B over 20 min and hold 15 min). Several mobile phase systems were tried but separation selectivity could not be improved. Furthermore, extreme column bleed was observed at pH 2.5–3.5 in the mass range 200–500, which greatly hampered the determination of PSP toxins. Due to background and selectivity problems no further development on the PHEA column was considered.

The 5 μm Amide-80 column was reported to have stability from pH 2 to 7.5 and most importantly showed no significant bleed at low pH. This column provided superior

Table 2
Recommended SRM transitions for HILIC–MS analysis of major PSP toxins[a]

Toxin	*m/z* > *m/z*	509 > 332	509 > 314	493 > 316	493 > 298	412 > 332	412 > 314	396 > 316	396 > 298	380 > 300	369 > 289	369 > 271	353 > 273	353 > 255	316 > 220	316 > 298	300 > 204	300 > 282	273 > 255	257 > 239
	RRT[c]																			
C1[b]	1,00			100	3			65	15							5				
C3[b]	1,10	100	8			50	13													
C2[b]	1,11			45	99			3	100							5				
C4[b]	1,22	19	46			1	100													
GTX2	1,33							100	5						10	30				
GTX1	1,36					100	5													
dcGTX1	1,40										100	15								
dcGTX2	1,42												100	15					30	
GTX3	1,49							20	100							10				
GTX4	1,51					10	100													
dcGTX3	1,57										15	100							5	
dcGTX4	1,58									10		100								
B1	1,82									100							5	5		
B2	2,03							100	20						5	5				
STX	2,82																80	100		
dcNEO	2,89																		100	
NEO	2,91														50	100				
dcSTX	2,93																			100
11-OH-STX	3,46														30	100				

[a] Reported data for most PSP toxins were obtained using a PE-SCIEX API-III+ MS. Percentage relative intensity is provided for each transition. The most abundant transitions (1 0 0) are recommended for quantitative studies, although other transitions may be more suitable on different instruments.
[b] Data for C1-4 toxins were obtained using a PE-SCIEX API-4000 mass spectrometer. The $[M+H]^+$ or $[M+NH_4]^+$ ions for C toxins were not sufficiently abundant on the API-III+ MS system, in which case transitions associated with the $[M-SO_3+H]^+$ ions were used for quantitation.
[c] Retention times relative to C1 (RRT) are referred to the optimized chromatographic conditions (see Section 2).

retention within the chromatographic window and was therefore employed for further optimization studies.

3.2.2. Mobile phase

The eluting system proposed by Strege [21] for the Amide-80 column was used initially (gradient: 90–60% B over 20 min, hold for 60 min, with A being water and B acetonitrile/water (95:5), both containing 6.5 mM ammonium acetate, pH 5.5). Under these conditions, the early eluting peaks of GTX1-4 showed tailing and later eluting peaks of STX and NEO showed fronting. A number of eluting systems were tested on this column. Particular attention was paid to organic modifier character and percentage, buffer character and percentage and pH.

As found by Yoshida [24] for the separation of peptides, the percentage of organic modifier in the mobile phase was a dominant factor for the absolute retention time of PSP toxins. HILIC behaves like normal phase chromatography, so retention times increase proportional to the percentage of organic modifier and to the polarity of the solute. The neutral C toxins eluted first, followed by the single-charged gonyautoxins, then the double-charged STX and NEO, and finally the decarbamoyl derivatives.

Both acetonitrile and methanol were tested as possible organic modifiers. Acetonitrile provided sharper peaks than methanol and was thus preferred. Methanol resulted in a dramatic change in the relative order of elution and poorer separation.

The ammonium formate buffer concentration had significant influence on the retention time in the range 0–2 mM (>3 min) while small shifts (1 min maximum) were observed for buffer concentration 2–10 mM. Generally, as the concentration of buffer increased, retention times decreased which corresponds well to the observation by Strege [21] on the retention of guanine. However, when no aqueous buffers were used, PSP toxin retention times exceeded 180 min and the peak widths were unacceptably broad. Therefore, an aqueous buffer was required to modify the mobile phase. Both ammonium acetate or formate were tested. The latter provided better peak shape and was thus preferred.

The pH of the mobile phase had the greatest influence on the separation of PSP toxins (Fig. 3). As pH increased, retention times and separation selectivity increased. At low pH the peaks were broader. The neutral C toxins did not change their absolute retention time when the pH was changed whereas the other toxins showed large changes in retention time (1–2 min) for relatively small changes in pH (0.1–0.2 pH units). Therefore, the retention times in Fig. 3 were calculated relative to C1.

3.2.3. Flow rate

In a HILIC separation, the column efficiency was dependent on flow rate and varied between toxins. Initial work was performed on a 250 mm × 4.6 mm i.d. Amide 80 column. The highest plate number was generally obtained at flow rates 0.8–1 mL/min. The later eluting toxins, namely STX and

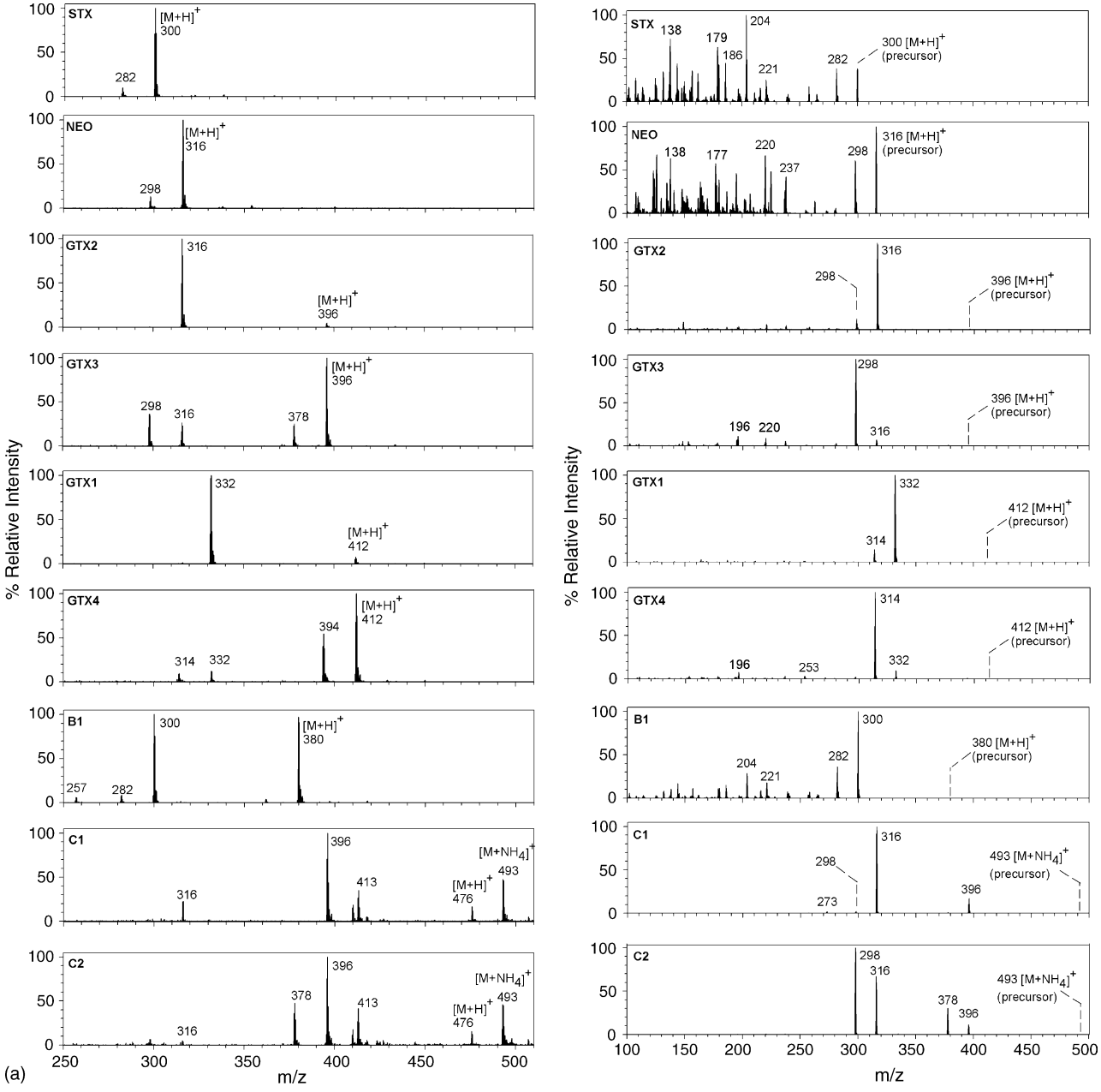

Fig. 2. (a) Q1 mass spectra in positive ion mode of assorted PSP toxins acquired using the single quadrupole API-165 MS. (b) Q3 product ion spectra of the $[M+H]^+$ ions of STX, NEO, GTX2, GTX3, GTX1, GTX4, B1, and of the $[M+NH_4]^+$ ions of C1 and C2 obtained on the API-4000 system using a collision energy of 35 V. Assignments of labeled product ions are shown in Table 1.

NEO, showed higher performance at 0.6–0.8 mL/min but the greatly increased analysis time was considered a serious disadvantage. Therefore, a flow rate of 1 mL/min was selected. When work switched to the 250 mm × 2 mm i.d. column, a 0.2 mL/min flow was selected.

3.2.4. Column temperature

The effect of column temperature on retention of PSP toxins was investigated in the range 10–45 °C and it was found to be slight. In particular, retention times of mono-cationic (GTX1-4 and their decarbamoyl derivatives, B1 and B2) and di-cationic (STX, NEO and their decarbamoyl derivatives) toxins increased as a function of the temperature. No effect was observed upon retention of the neutral C toxins. Similarly, selectivity increased linearly with temperature for all PSP toxins except neutral ones. Retention and selectivity are known to be temperature dependent in ion exchange chromatography [25] so the obtained results support a presumed role of ion exchange in the HILIC separation of PSP toxins. It was also observed that as temperature increased, resolution of most peaks increased but the peak shapes of later eluting toxins broadened. A temperature of 20 °C was selected as optimum for retention, selectivity and peak shape.

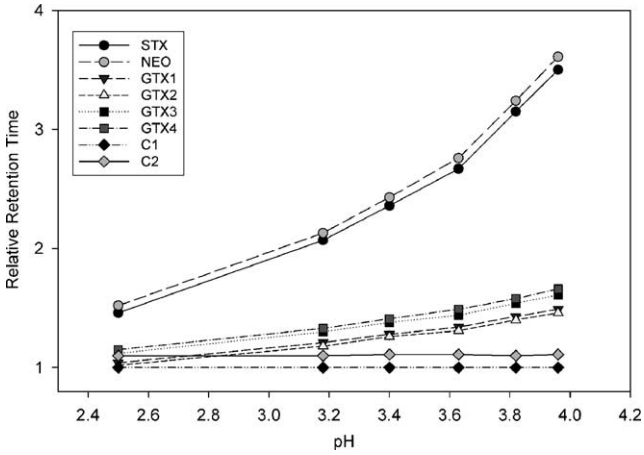

Fig. 3. Influence of pH on separation of assorted PSP toxins on the 5 μm TSK gel Amide-80 (250 mm × 4.6 mm i.d.) column. All other parameters were kept constant, namely mobile phase, 65% B isocratic with eluent A being water and B acetonitrile/water (95:5); buffer concentration and character, 10 mM ammonium formate; column temperature, 20 °C; and flow rate, 1 mL/min. Retention times were calculated relative to C1.

3.3. Analysis of PSP toxin standards

On the basis of all of the above findings, the best results were obtained using the 5 μm Amide-80 column (250 mm × 2 mm i.d.) maintained at 20 °C and eluted isocratically at 0.2 mL/min with 65% B, where eluent A was water and B was a acetonitrile/water (95:5) solution, with both eluents containing 2.0 mM ammonium formate and 3.6 mM formic acid (pH 3.5 in the aqueous phase).

Figs. 4 and 5 show a HILIC–MS analysis in SIM and SRM modes, respectively, of a standard mixture of PSP toxins under the optimized chromatographic conditions. Some compounds could not be chromatographically resolved: GTX1 and GTX2, GTX4 and GTX3 and the associated decarbamoyl derivatives eluted in a 2 min range; C1 and C2 substantially co-eluted with C3 and C4; B1 partially co-eluted with B2; STX partially co-eluted with NEO, and dcSTX with dcNEO. However, the additional detection selectivity provided by different channels of detection in SIM or SRM, made it possible to individually detect all PSP toxins in a reasonable period of time, namely 25–30 min.

Alpert [20] considered HILIC to have a separation mechanism similar to normal phase partition chromatography, where a stagnant mobile phase (mostly aqueous) is in contact with the stationary phase and a dynamic mobile phase (mostly organic) is separated from the stationary phase. The analyte partitions between the two mobile phases and may be orientated in space to interact with functions of the stationary phase. Ion exchange or electrostatic interactions can also have a role [26,27]. As for PSP toxins, the mechanism of separation appears to be primarily electrostatic in nature. This is supported by the short retention times of the neutral C toxins, the intermediate retention times of the single-charged gonyautoxins, and the long retention times of the double-charged toxins.

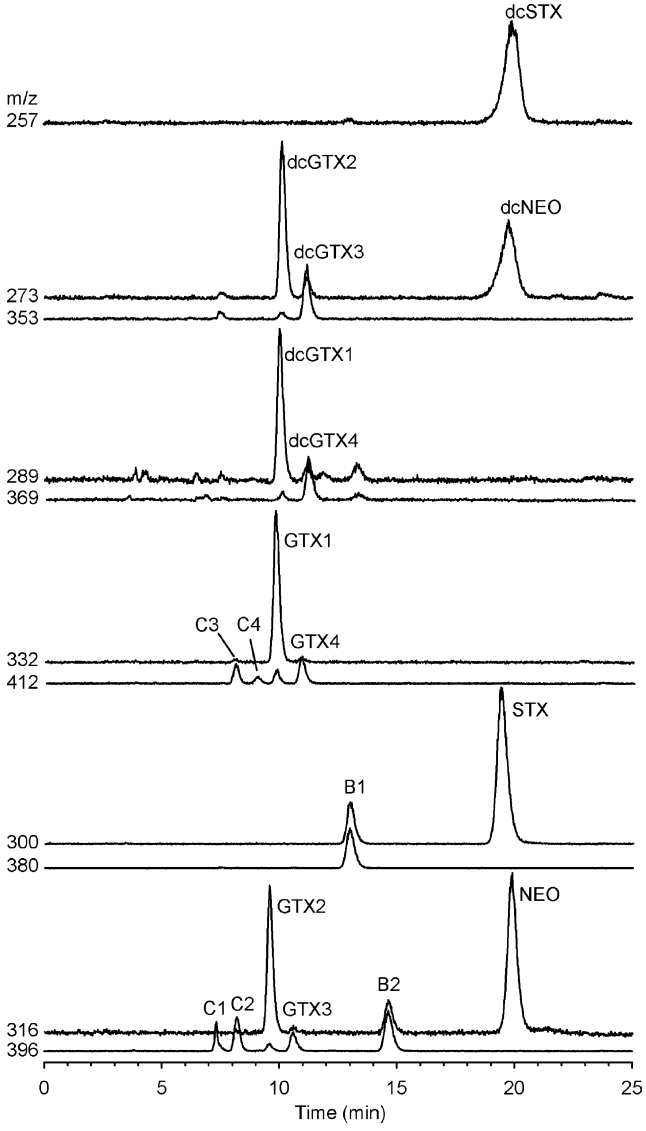

Fig. 4. HILIC–MS analyses of a standard mixture containing assorted PSP toxins. Experiments were carried out in SIM mode on API-165 MS system. Protonated and/or fragment ions were selected for monitoring (Table 1). Separations were carried out on a 5 μm Amide-80 column (250 mm × 2.0 mm i.d.), isocratically eluted with 65% B with eluent A being water and B acetonitrile-water (95:5), both containing 2.0 mM ammonium formate and 3.6 mM formic acid (pH 3.5). Column temperature was 20 °C and flow rate 0.2 mL/min.

The excellent separation achieved between epimeric pairs such as GTX1 and GTX4, or GTX2 and GTX3, may be explained by considering the effect that α- or β-orientation of the 11-hydroxysulfate function has on the charge states of individual functional groups. Molecular modeling showed that when the 11-hydroxysulfate group is in the α-orientation (GTX1 and GTX2), it can establish an intra-molecular interaction with the guanidinium function at C-8. This would reduce the number of positively charged functions on the molecule available for interaction with the stationary phase. On the other hand, both guanidinium groups are available for interaction with the stationary phase

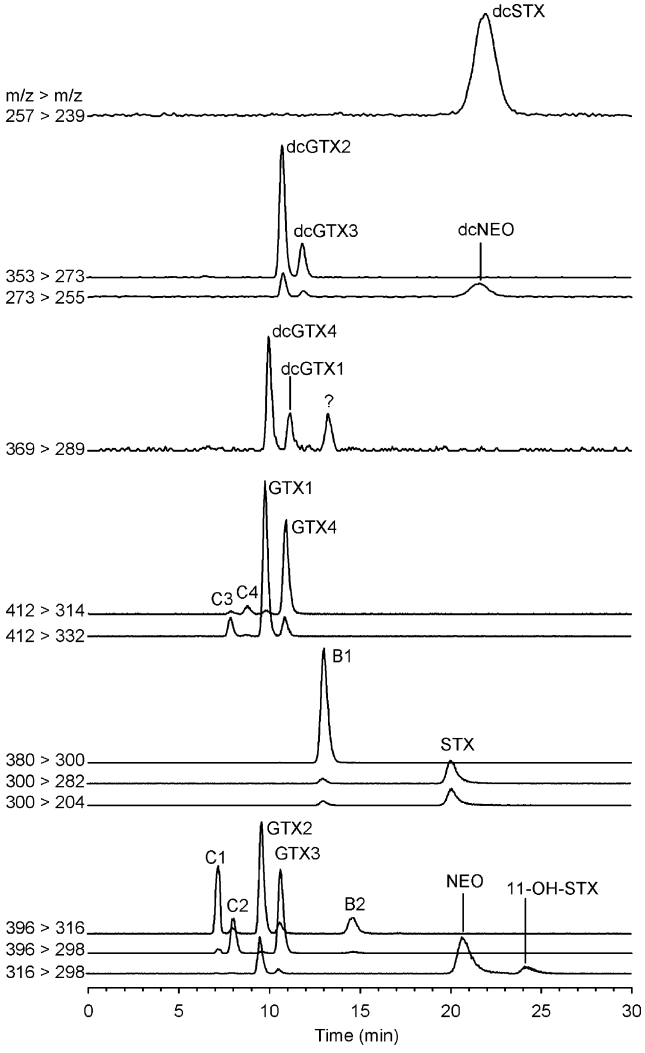

Fig. 5. HILIC–MS analyses of a standard mixture containing assorted PSP toxins. Experiments were carried out in SRM mode on API-III+ system using a collision energy of 20 V. Selected ion transitions (Table 2) were consistent with the fragmentation pattern of each toxin (Table 1). For LC conditions see Fig. 4.

when the 11-hydroxysulfate function is β-oriented (GTX3 and GTX4). It should also be noted that 11(α,β)-OH-STX epimers do not resolve under the same conditions. This supports the argument that the 11-hydroxysulfate group and its interaction with the guanidinium function is important for separation of epimers.

3.4. Quantitation

Six-point calibration curves were generated for STX, GTX2, GTX3, NEO, GTX1, GTX4, B1, C1 and C2. Over the tested concentration ranges, linear regression of observed peak areas versus concentration gave excellent linearity with R^2-values of 0.999 or greater. Limits of detection (LOD) for matrix-free toxins with 5 μL injected on a 2 mm i.d. column, were determined from the data for low level samples extrapolated to a signal to noise ratio of 3. They ranged from 50 to 1000 nM in SIM and 40 to 7000 nM in SRM mode on the API-III+ instrument (Table 3). Unfortunately, a single collision energy was used for the entire group of SRM ions on the API-III+ system. The compromise value of 20 V was too low for good fragmentation of STX and NEO, resulting in poor detection limits. LOD values for such toxins could be lowered by at least five-fold by using a 30 V collision energy on $[M + H]^+$ ions of STX and NEO, which is possible if time programming of SRM transitions is used.

The method was implemented on the API-4000 MS system, which had much better sensitivity and the ability to use optimized collision energies for each ion transition. The results of a similar linearity experiment on the API-4000 MS were impressive with LOD values that ranged from 5 to 30 nM (Table 3) and excellent linearity, with R^2-values of 0.999 or greater. Comparing to LOD for the LC-ox-FLD method [11] (Table 3) reveals that the HILIC–MS method on the API-4000 system has better sensitivity than the LC-ox-FLD method and should therefore be suitable for analysis of shellfish samples near the maximum acceptable regulatory limits for PSP toxins (currently 0.8 mg of saxitoxin equivalent per kilogram of edible tissue).

3.5. Application to plankton and shellfish samples

In order to test the suitability of the method for real samples, we selected plankton and mussel samples collected during an intense bloom of *Alexandrium tamarense*, which occurred in June 2000 in a Nova Scotian harbour [23]. Extracts of these samples were analyzed in both SIM and SRM modes. Simple extraction methods were used and only a simple clean-up with a HILIC–SPE cartridge was performed on the mussel tissue extract in order to demonstrate rapid analysis. The SIM mode proved suitable for detection of most of the PSP toxins present, particularly in the plankton samples, but unambiguous interpretation of the results and good quantitation was prevented by the presence of many extra peaks from other components in the crude extract, a high background signal in some ion traces, and a matrix-related hump in the chromatograms at about 20 min. The higher selectivity of the SRM mode made interpretation of the results much easier due to elimination of signals from other co-extractives. Fig. 6 shows the results of the HILIC–SRM analysis of the crude extract of the plankton sample, while Fig. 7 shows the results of the HILIC–SRM analysis of the crude extract of the mussel sample.

The plankton sample showed a complex array of toxins with the major toxins being GTX4, C2, C4, GTX3, B1, NEO and STX. Only low levels of the corresponding epimeric C and GTX toxins were observed. This is consistent with the fact that most plankton produce only a single epimeric form and others are formed through equilibration in solution [28] or in shellfish [29]. The identities of the toxins were also confirmed by LC-ox-FLD analyses [23].

The same toxins can be observed in the mussel extract as in the plankton sample, which was not surprising, as these

Table 3
Estimated limits of detection (LOD, $S/N = 3$) for major PSP toxins on different PE-SCIEX LC–MS systems in the selected ion monitoring (SIM) and selected reaction monitoring (SRM) modes

Toxin	SIM				SRM		
	API-165		API-III+		API-4000		LC-ox-FLD [11]
	m/z	LOD (nM)	*m/z* > *m/z*	LOD (nM)	*m/z* > *m/z*	LOD (nM)	LOD (nM)
STX	300	800	300 > 282	7000	300 > 204	20	60
GTX2	316	300	396 > 316	1000	396 > 316	20	20
GTX3	396	400	396 > 298	300	396 > 298	10	5
NEO	316	900	316 > 298	7000	316 > 220	30	60
GTX1	332	200	412 > 332	800	412 > 332	10	20
GTX4	412	800	412 > 314	400	412 > 314	5	30
B1	380	1000	380 > 300	700	380 > 300	10	100
C1	396	50	396 > 316	40	493 > 316	20	30
C2	396	60	396 > 298	50	396 > 298	10	20

mussels had consumed this same plankton material. It was also not unexpected that there was some conversion of the C toxins and gonyautoxins to their corresponding epimers, C1, GTX1 and GTX2, since this occurs readily in mussel digestive glands. It was noted, however, that the relative level of C1 + C2 was lower in the mussel sample than in the plankton and it is hypothesized that the C toxins have been metabolized or degraded in the mussel. Interestingly, some new saxitoxin analogues were observed in the mussel sample. The new compounds, labeled as M1, M2 and M3 in Fig. 7, appear to be metabolites and/or degradation products formed in the mussel, since they were absent in the plankton sample. The M2 peak had an exact match of retention time and product ion spectrum for 11-hydroxy-STX. The structure elucidation of compounds M1 and M3, which required preparative isolation work and NMR spectroscopy, will be reported elsewhere [30].

A slight shift of retention times for the sample extract versus those for the standards could be observed. This is due to a matrix effect that gets worse as more concentrated crude extracts are used. In SIM analyses of sample extracts,

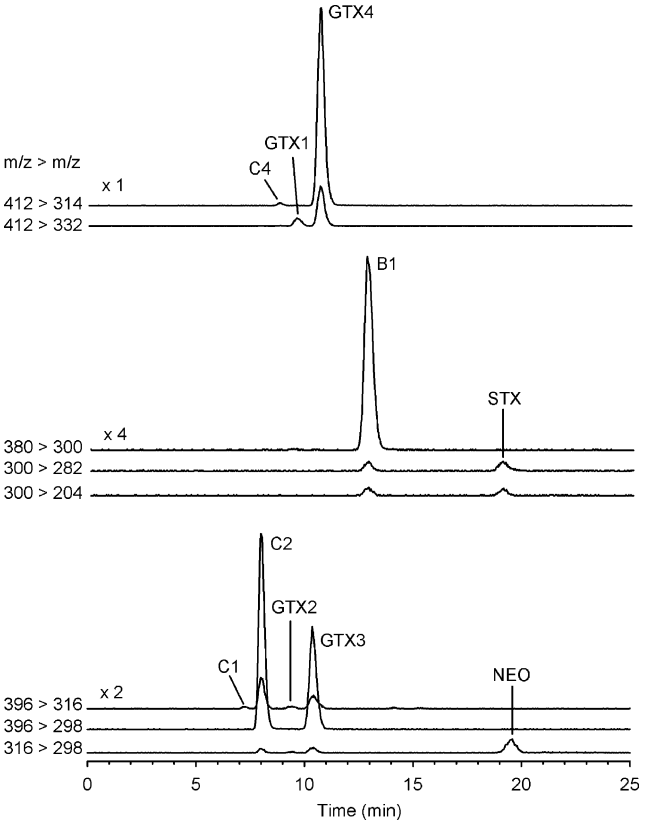

Fig. 6. HILIC–MS analyses of an *Alexandrium tamarense* extract containing various PSP toxins. Experiments were carried out in SRM mode on API-III+ MS. For LC conditions see Fig. 4. Some traces are plotted with expanded scale as indicated.

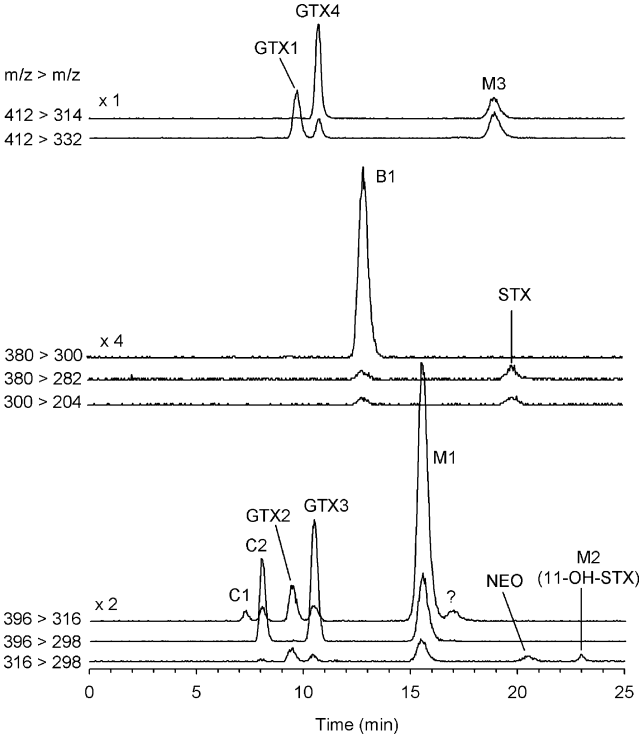

Fig. 7. HILIC–MS analyses of a *Mytilus edulis* extract containing various PSP toxins. Experiments were carried out in SRM mode on API-III+ MS. For LC conditions see Fig. 4. Some traces are plotted with expanded scale as indicated.

a hump near the end of the chromatogram appeared to force off the column late eluting compounds (STX and NEO). This resulted in sharper peaks for these compounds but made it difficult to match retention times of sample peaks with those of standards. The nature of the material that causes this effect is unknown at this time. Dilution of the extract minimizes the problem but reduces the sensitivity of the method. In addition, high levels of salt in some crude extracts can partially suppress ionization of the C toxins. We are currently investigating clean-up procedures that will eliminate both the salt suppression effect and the retention time shift effects. Following that development, a full validation of the method for quantitative analysis will be conducted.

4. Conclusions

The developed HILIC–MS method allows the separation and selective detection of the principal PSP toxins in a single 30 min analysis with no need for further confirmatory analyses. The mobile phase does not use ion pair agents and is high in organic modifier, which results in high ionization efficiency. The detection limits in SRM mode can vary between instruments and MS tuning, but for a sensitive instrument, such as the triple quadrupole API-4000, detection limits as low as 5–30 nM have been demonstrated. The latter LOD values compete favorably with those achieved by the LC-ox-FLD method and should allow the analysis of shellfish samples near the regulatory limit. In this manuscript, the application of HILIC–MS/MS to the qualitative analysis of complex plankton and shellfish samples has been demonstrated, along with its power to detect the presence of new toxin analogues. Future work will deal with validation of this technique for the quantitative analysis of shellfish samples. The HILIC–MS/MS method appears suitable for the analysis of other polar toxins and natural products. Its application to cyanobacterial toxins has been presented recently [31].

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