Determination of paralytic shellfish poisoning toxins by high-performance ion-exchange chromatography

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Abstract

An efficient LC method has been developed for the determination of paralytic shellfish poisoning (PSP) toxins based on ion-exchange chromatographic separation of the toxins followed by electrochemical post-column oxidation and fluorescence detection as well as mass spectrometric (MS) detection. The method can be applied to the determination of PSP toxins in phytoplankton and to control seafood for PSP content. © 2001 Published by Elsevier Science B.V.

1. Introduction

Paralytic shellfish poisoning (PSP) is caused by a group of 18 neurotoxins (Fig. 1).

One of the most dangerous intoxications is from seafood contaminated with algal toxins. The charged PSP molecules act as potent sodium channel blockers and cause paralytic symptoms, such as respiratory insufficiency, in serious cases with fatal results [1].
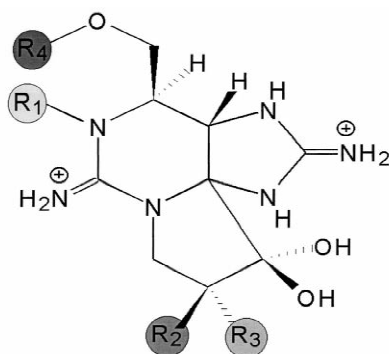
Many efforts have been made to establish methods allowing the rapid and unambiguous determination of PSP toxins. The control of contaminated seafood requires exact quantification of the PSP content with regard to international regulations [2]. In addition,

the broad toxicity range of different PSP toxins, the diverse PSP toxin profiles in dinoflagellates, and the possibility of biotransformation of PSP toxins by marine organisms, have provided challenges for analytical chemists to develop accurate and reliable analytical methods [3–5].

To overcome the analytical problems regarding PSP-producing algae and seafood control, reversed-phase LC methods with different ion-pair reagents and phosphate buffer, pre- or post-column derivatization, and fluorescence detection, have been introduced for the analysis of the PSP species [6–9]. These commonly used methods are able to separate most of the PSP toxins. However, they also have disadvantages. The chemical derivatization, i.e., the post-column oxidation of the PSP toxins to fluorescent derivatives, is often troublesome and the combination of ion-pair reagent, phosphate buffer, and

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Toxin	R1	R2	R3	R4	Toxicity factor [15]	Molecular weight
STX	H	H	H		1.00	301
NEO	OH	H	H		1.10	317
GTX I	OH	H	OSO_3^-	H_2N-CO	0.90	412
GTX II	H	H	OSO_3^-	(Carbamoyl-)	0.48	396
GTX III	H	OSO_3^-	H		0.76	396
GTX IV	OH	OSO_3^-	H		0.90	412
B1	H	H	H		0.07	380
B2	OH	H	H		0.07	396
C3	OH	H	OSO_3^-	^-O_3S-NH-COO	0.01	492
C1	H	H	OSO_3^-	(N-Sulfo-	0.01	476
C2	H	OSO_3^-	H	carbamoyl-)	-	476
C4	OH	OSO_3^-	H		-	492
dc-STX	H	H	H		0.43	258
dc-NEO	OH	H	H		0.43	274
dc-GTX I	OH	H	OSO_3^-	H	0.45	369
dc-GTX II	H	H	OSO_3^-	(Decarbamoyl-)	0.18	353
dc-GTX III	H	OSO_3^-	H		0.18	353
dc-GTX IV	OH	OSO_3^-	H		0.45	369

Fig. 1. Chemical structure of STX and related compounds (see Ref. [15] in Fig. table).

derivatization chemicals makes it difficult to couple the LC device to a mass spectrometer.

Other authors have proposed the application of capillary electrophoresis (CE). The separation conditions developed were found to be entirely compatible with mass spectrometry (MS) [10,11]. However, CE separations demand highly purified extracts for a reproducible separation. In addition, the handling of the extremely small volumes for injection results in about one magnitude higher LOD of CE–MS as compared to LC with fluorescence detection [12].

This paper presents an ion-exchange LC method for the analysis of PSP toxins. Aqueous solution of ammonium acetate is used, and the chromatographic separation of all relevant PSP toxins is achieved in one chromatographic run. Since only volatile components are applied for elution, the method allows the parallel determination of PSP toxins with fluorescence detection after electrochemical oxidation and with MS detection.

2. Experimental

2.1. PSP toxin standards

Saxitoxin (STX), neosaxitoxin (NEO) and gonyautoxins (GTXs) were purchased from the National Research Council, Marine Analytical Chemistry Standards Program (NRC-PSP-1B), Halifax, Nova Scotia, Canada. The standard solutions of GTX2 and GTX3 contained dcGTX2 and dcGTX3 as minor components, but the exact content of these toxins was not given. dcSTX was provided by the European Commission (BCR, The Community Bureau of Reference, Brussels), for use as a standard during an intercalibration exercise from 1995 to 1996.

Extract of *Alexandrium* sp. (BAH-ME 220, isolated by M. Delgado, Spain) containing *N*-sulfocarbamoyltoxins (especially C1 and C2) was used as standard solution for these PSP toxins, because C toxins are not commercially available.

2.2. Solvents

All chemicals used were analytical grade. Ammonium acetate was obtained from Merck (Darmstadt, Germany; No. 1.01115). Water was purified to HPLC-grade quality with a Millipore-Q RG Ultra Pure Water System (Millipore, Milford, USA).

2.3. Extraction

Cells of cultivated *Alexandrium* sp. (BAH-ME 220) were stored in seawater at 4°C. The sample preparation protocol by Hummert et al. [13] was applied for extraction. The well-shaken algal mixture (ca. 10 mg ml^{-1}) was mixed with 2 ml acetic acid (0.03 N), homogenized for 2 min using a Sonopuls HD 70 ultrasonic probe (Bandelin, Berlin, Germany), and centrifuged for 10 min (2980 *g*). The supernatant was passed through a 0.45-μm, 25-mm diameter PTFE filter (No. H250.1, Carl Roth, Karlsruhe, Germany) and subsequently injected into the LC–fluorescence–MS device.

Homogenized shellfish tissue (1.0 g) was mixed with acetic acid (0.03 N; 3 ml), and again homogenized using the ultrasonic probe for 2 min. The raw extract was centrifuged for 10 min (2980 *g*). The

supernatant was passed through a 0.45-μm, 25-mm diameter PTFE filter.

2.4. Liquid chromatography with fluorescence detection

LC was performed with an SIL-10A intelligent autosampler, an LC-10ATvp intelligent pump, an SCL-10Avp system controller, an RF-10Axl fluorescence detector, and using Class-vp 5.3 software (Shimadzu, Duisburg, Germany). An ESA Colouchem detector 5100A consisting of a potentiostat with the guard cell 5020 (ESA, Bredford, USA) was used as electrochemical oxidation system. The analytical columns were: a cation-exchange column Source 15S PE 4.6/200 and an anion-exchange column Source 15Q PE 4.6/100 (both from Pharmacia Biotech, Uppsala, Sweden).

LC was performed with two aqueous eluents. Eluent A consisted of 20 mM ammonium acetate and eluent B of 450 mM ammonium acetate. The pH was adjusted to 6.9 with 25% ammonia solution. The gradient used for chromatography starts with eluent A (100%) for 5 min, followed by a linear gradient to 100% eluent B for 25 min and 8 min isocratic elution with eluent B. Eluent A was used isocratically for 27 min to equilibrate the chromatographic system; the flow-rate was continuously 0.8 ml min^{-1}.

The potential for electrochemical post-column oxidation was set to +1050 mV. Fluorescence detection of the oxidation products was employed with excitation and emission wavelengths of 330 and 395 nm, respectively.

2.5. Liquid chromatography with MS detection

Liquid chromatography with MS detection was performed with a PE Series 200 Quaternary Pump and a PE Series 200 autosampler (Perkin-Elmer, Rodgau, Germany). The chromatographic separation was achieved by gradient elution.

A single quadrupole API 165 PE Sciex mass spectrometer equipped with turbo ion spray (TIS) (PE Sciex, Halifax, Canada) was applied for PSP determination. The eluate from the column was transferred into the MS using a split ratio of 3:1 (volume wasted:volume transferred). Nitrogen heated to 400°C (7.5 l min^{-1}) was used to dry the ion spray.

The ionization voltage of the TIS interface was set to 5.2 kV. Nitrogen was also used as the nebulizer gas at a flow of approximately 0.6 l min^{-1}.

Measurements were carried out in selected-ion monitoring (SIM) mode. The MS responses were obtained from the protonated molecules [M+H]$^+$ of STX (m/z 300.1), NEO (m/z 316.1), GTX3 (m/z 396.2), GTX4 (m/z 412.2), dcGTX2/3 (m/z 352.1), and C3/4 (m/z 492.3). Due to the elimination of SO$_3$ m/z 332.1 and m/z 316.1 were used for GTX1 and GTX2 determination, while m/z 396.2 was applied for determination of C1 and C2, respectively.

Fig. 2 shows the schematic draft of an automatically operated system including the option for the collection of substances of interest. The PSP toxins eluting from the ion-exchange columns are transferred into both the electrochemical cell for oxidation and fluorescence detection and into the mass spectrometer.

3. Results and discussion

In order to separate the PSP toxins in a single chromatographic run, we used a combination of anion- and cation-exchange chromatography with aqueous ammonium acetate as mobile phase. As the mobile phase did not contain ion-pair reagents or phosphate buffer, an electrochemical cell could be used for post-column oxidation of the toxins instead of a chemical post-column oxidation unit. In addition, this mobile phase allowed the use of on-line LC–MS,

Quantitative calibration was performed by injecting a range of standard solutions of known concentration. The peak areas (mean of three) were plotted against the concentrations, and the equations of the resulting calibration lines were calculated. Table 1 gives detailed statistical information obtained by application of gradient elution, electrochemical oxidation, and fluorescence detection, whereas Table 2 shows linearity ranges and the LODs of the PSP determination with MS detection. The LODs obtained for the individual PSP toxins were comparable to those obtained by other methods based on ion-pair chromatography with chemical oxidation and fluorescence detection [9,13].

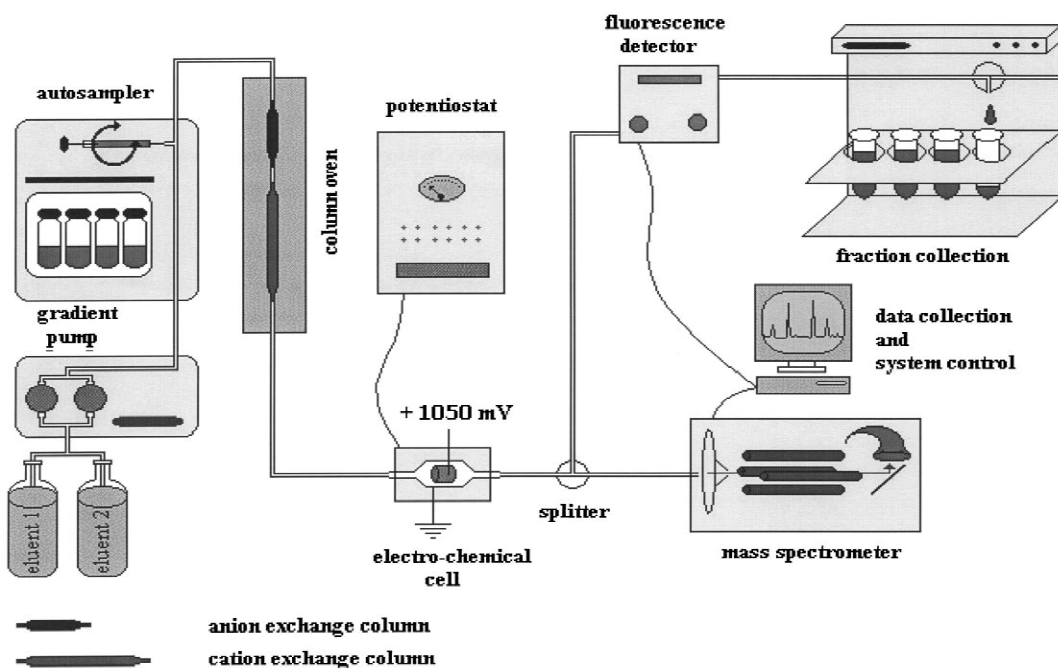The toxins were eluted in the following order: C1,

Fig. 2. Automated HPLC system with ion-exchange columns, electrochemical cell with fluorescence detector and mass spectrometer (fraction collection as additional option).

Table 1
Linearity and LODs ($S/N=3$:1) of PSP determination by application of ion-exchange chromatography and fluorescence detection (for conditions see text)

Toxin	Concentration range (ng)	Calibration curve (five data points)	Correlation coefficient	LOD (ng)
GTX1	3.5–15.5	$28976x+0$	0.9378	0.8
GTX2	0.6–4.8	$2376510x+0$	0.9958	0.01
GTX3	0.1–1.2	$2864030x+0$	0.9945	0.01
GTX4	1.7–6.1	$46144x+0$	0.9649	0.5
Neo	7–56	$36891x+0$	0.9996	0.6
dcSTX	0.5–4	$2261770x+0$	0.9963	0.02
STX	0.7–5.6	$954390x+0$	0.9963	0.03

Table 2
Linearity and LODs ($S/N=3$:1) of PSP determination by application of ion-exchange chromatography and MS detection (for conditions see text)

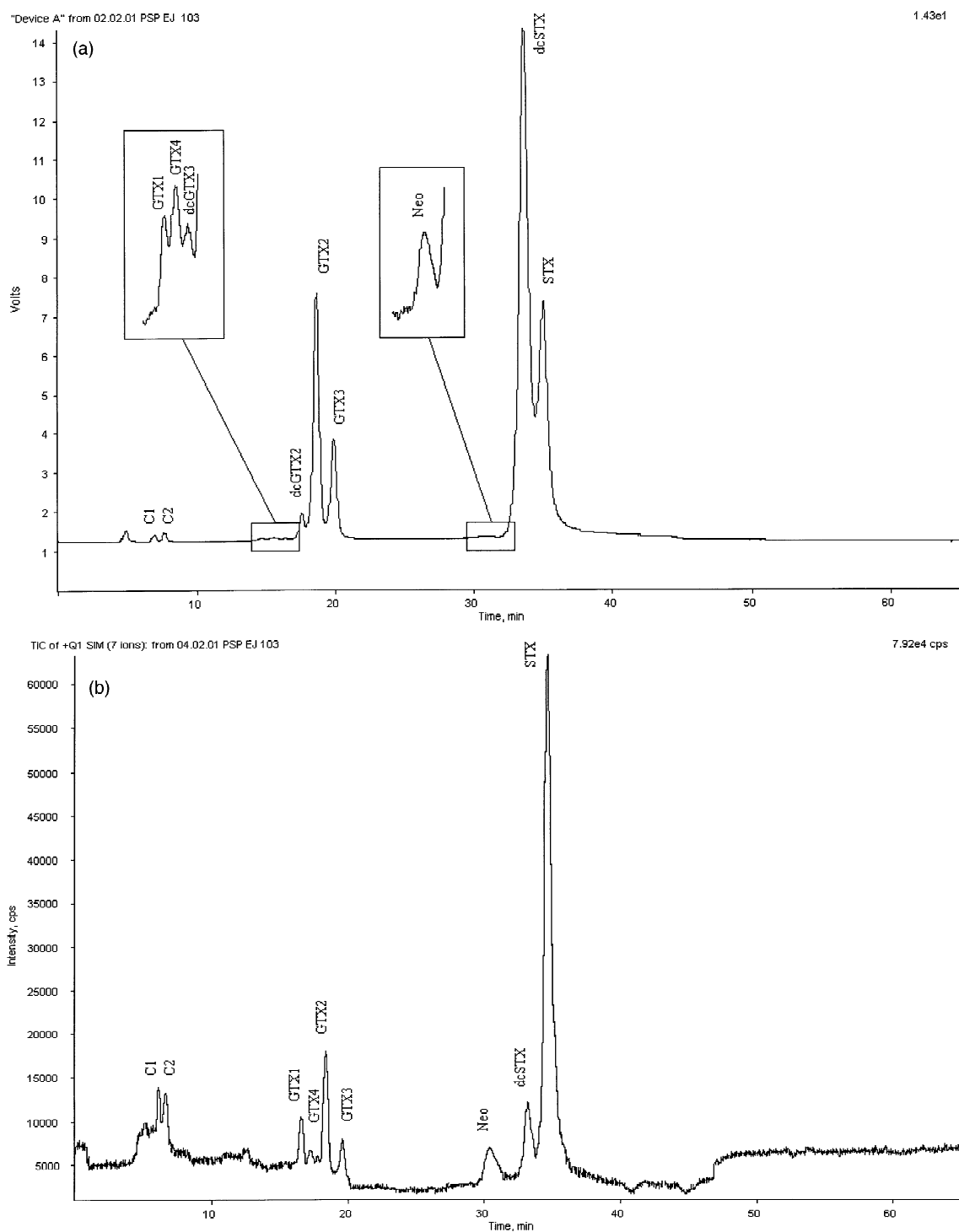| Toxin	Concentration range (ng)	Calibration curve (five data points)	Correlation coefficient	LODs (ng)
GTX1	0.8–33.5	$61508x+0$	0.9936	1
GTX2	1.4–54	$114890x+0$	0.9992	1.5
GTX3	0.3–13	$160160x+0$	0.9981	0.5
GTX4	1.4–14.5	$30174x+0$	0.9999	1.5
Neo	1.6–64	$96729x+0$	0.9995	2
dcSTX	1.0–7	$56299x+0$	0.9980	1
STX	0.3–64	$396710x+0$	0.9987	0.5

Fig. 3. LC–fluorescence–MS determination of PSP toxins: C1/C2, GTX1 (17.2 ng), GTX4 (8.9 ng), dcGTX3, dcGTX2, GTX2 (28.7 ng), GTX3 (10.4 ng), Neo (34.8 ng), dcSTX (25.0 ng), STX (39.9 ng). (a) Fluorescence chromatogram, (b) TIC chromatogram.
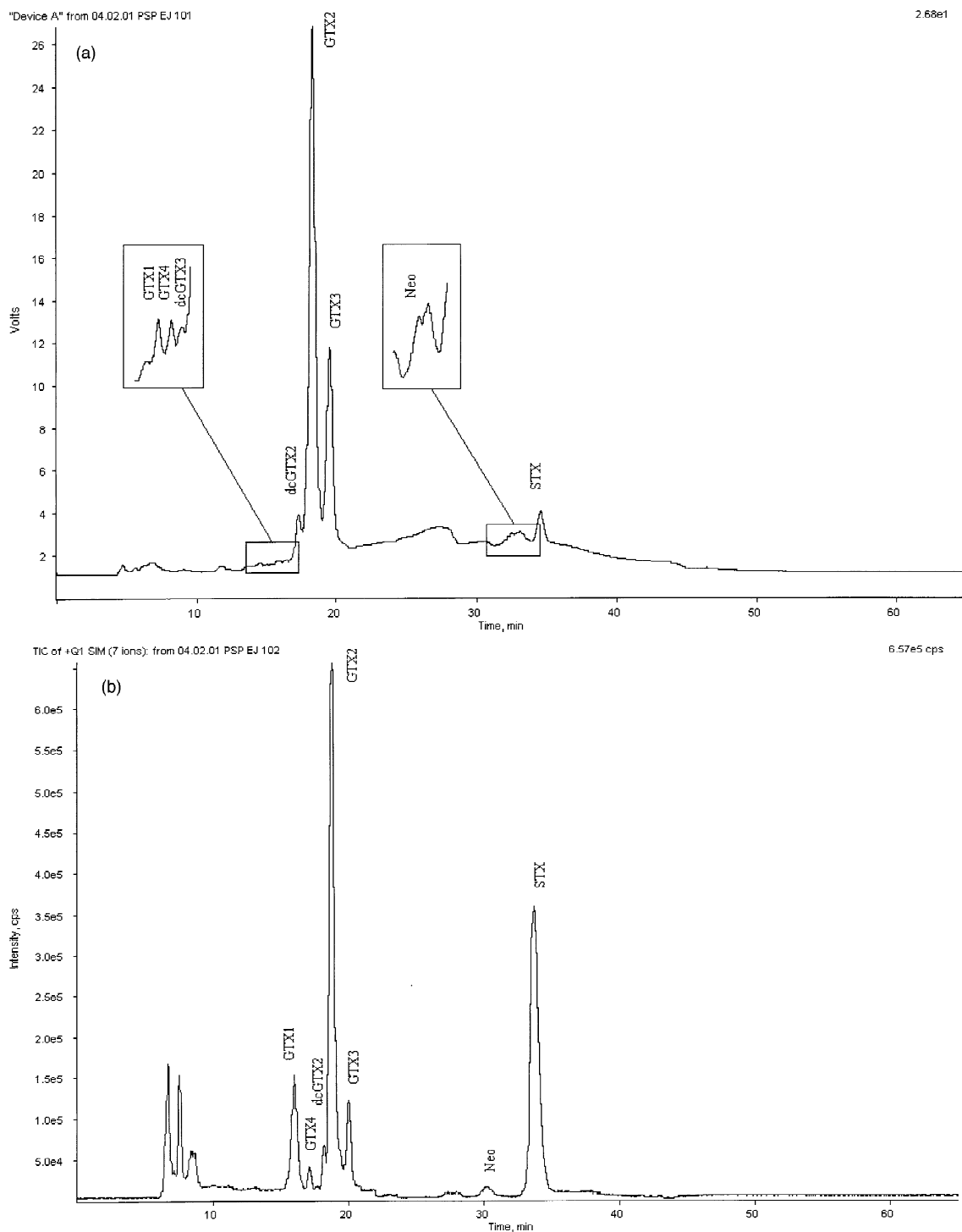
Fig. 4. Determination of PSP toxins in mussels (*Mytilus chilensis*, Fjord Aysen, Chile XI, 2000) by ion-exchange chromatography, gradient elution, and parallel fluorescence and mass spectrometric detection (100 µl injected into the LC–fluorescence–MS device; for chromatographic conditions see text). (a) Fluorescence chromatogram, (b) TIC chromatogram.

C2, GTX1, GTX4, dcGTX3, dc GTX2, GTX2, GTX3, Neo, dcSTX, STX (Fig. 3).

The method is well suited for determination of PSP toxins in biological materials (Fig. 4). Here, the combination of both fluorescence and MS detection often reveals the presence of PSP toxins exceeding the regulation limit of 800 μg PSP/kg for mussels and shellfish [14]. In such cases, PSP contamination (expressed as STX equivalents per kg) has to be checked by application of an analytical methodology which enables reliable quantification of all regulation relevant PSP toxins. Therefore, the application of LC–MS coupling for unambiguous PSP determination and for confirmation of the results obtained by methods with fluorescence detection is proposed. In addition, the LC/Fluorescence/MS device can be used to collect unidentified substances or individual PSP toxins, and the up-scaling to preparative ion-exchange chromatography offers a powerful tool to obtain individual PSP toxins.

Acknowledgements

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