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Capillary electrophoresis with diode array detection as an alternative analytical method for paralytic and amnesic shellfish toxins

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Abstract

In recent years the marine environment has been seriously damaged by the presence of several toxic phytoplanktonic species, such as dinoflagellates and other toxic algae, which contaminate shellfish and other marine products. Amnesic and paralytic shellfish toxins are examples of these contaminants. The search for sensitive methodologies for the analysis of such compounds is one of the aims of researchers working in the marine environment. High-performance liquid chromatographic methods have been used for this purpose, allowing the detection of very low levels of these toxins. Recently, capillary electrophoresis (CE) has been used as an alternative for the separation and analysis of these compounds. In this paper, we report the optimization of CE procedures for their analysis. Due to the complexity of the matrix, clean-up procedures are required for removing interferences which affect the electrophoretic resolution. The influences of electrophoretic parameters such as voltage, buffer concentrations and organic modifiers, were studied in order to optimize the electrophoretic system to achieve high resolution as well as an accurate quantitation. Extraction and other steps such as clean-up of samples prior to the electrophoretic analysis have been also studied. Different buffers and organic modifiers were used in order to improve the separation of the toxic components, and consequently to obtain accurate quantitative information about the amount of toxins present in the contaminated samples.

Keywords: Shellfish poisoning; Food analysis; Toxins

1. Introduction

Increasing incidents of poisoning caused by the consumption of seafood contaminated with marine toxins are causing serious problems to public health and fisheries industries worldwide. The main toxins that will be discussed here are those causing amnesic and paralytic shellfish poisoning (ASP, PSP). The structures of these compounds are shown in Fig. 1. Both these toxins are present in the marine environment and they are produced by phytoplanktonic species. While ASP toxins are produced by diatoms such as *Nitzschiapungens* f. *multiseries* [1], PSP toxins have their origin in toxic dinoflagellates, such as *Gymnodinium catenatum* or several species of *Alexandrium* [2].

The main chemical properties of these compounds are based on their possible charge states. Domoic acid has three carboxyl groups and one amino group, therefore there are five charge states depending on pH values. The pK_a values are 2.10, 3.72, 4.97 and 9.82. On the other hand, PSP compounds contain

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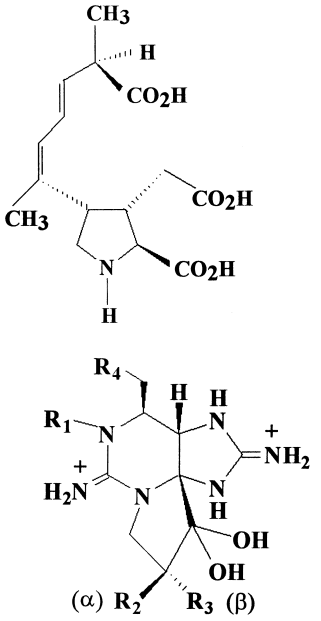

			R4		
R1	*R2*	*R3*	Carbamoyl toxins	N-Sulfocarbamoyl toxins	Decarbamoyl toxins
H	H	H	STX	GTX5	dcSTX
H	H	OSO_3^-	GTX2	epiGTX8	dcGTX2
H	OSO_3^-	H	GTX3	GTX8	dcGTX3
OH	H	H	neoSTX	GTX6	dcneoSTX
OH	H	OSO_3^-	GTX1	C3	dcGTX1
OH	OSO_3^-	H	GTX4	C4	dcGTX4

Fig. 1. Chemical structure of (a) domoic acid and (b) PSP toxins.

two guanidinium groups which are protonated, with charge fully delocalized over each. In the case of STX, this toxin has two proton dissociations at pK_a 8.22 and 11.28 while neosaxitoxin reveals a dissociable proton with a pK_a of 6.75.

Bivalve molluscs, such as mussels, razor clams and scallops can accumulate these toxic compounds by filter-feeding. Herbivorous finfish also accumulate ASP and PSP toxins, so they become part of the food chain of marine mammals, and ultimately the food human chain. Due to the relevance of the symptomatology caused by these toxic compounds as well as the low regulatory levels, a sensitive analytical methodology is required for their control in seafood products.

Several analytical methodologies have been proposed for the analysis of such compounds: high-performance liquid chromatography (HPLC), immunochemical, enzymatic and radioimmunoassay [3,4]. Chemical methods, specially those that involve

separation prior to detection have been used. While UV is a sensitive detection method for ASP toxins [5], to obtain a sensitive response for PSP toxins, fluorescence detection (FLD) is required. In order to convert the PSP components into the correspondent fluorescent derivatives, an oxidation reaction is required; this oxidation can be carried out before or after the chromatographic separation and the oxidation products are subsequently detected by fluorescence detection [6,7]. The HPLC–FLD technique provides high resolution separations and high selectivity to deal with complicated sample matrices. HPLC also provides excellent quantitative precision and it could be easily automatized.

Capillary electrophoresis (CE) is an alternative separation technique, which has been recently applied for the analysis of marine toxins [8]. This technique is based on the different mobilities of polar substances in an electric field depending on the charge and the size of the molecule [9]. ASP and PSP have chemical structures with functional groups which are capable of protonation (Fig. 1), so that they produce charged molecules and can be easily analyzed by CE with the exception of C's toxin group, which cannot be analyzed by CE due to their neutral global charge in acidic media.

In both cases, the charge states depend on the pK_a values and the pH of the solution containing these toxins. It is possible to apply CE as an alternative to HPLC for the analysis of ASP [10] and PSP toxins [8].

In this work we show an example of the application of the CE technique, for the analysis of ASP and PSP toxins under the conditions previously optimized.

Sample preparation, including extraction and clean-up of the samples is crucial for achieving the highest resolution in the analysis of the samples. Solid-phase extraction (SPE) methods were applied for this clean-up to remove interferences, thus increasing the selectivity and also the quantitation accuracy. The influence of the buffer composition in the electrophoretic resolution has been studied and as result, the best conditions obtained were used for the analysis of such compounds in order to achieve the highest electrophoretic resolution. These optimized conditions were applied to the analysis of ASP and PSP toxins in real samples.

2. Materials and methods

2.1. Toxin standard and samples

Domoic acid calibration solution (DACS-1B) and mussel tissue reference material (MUS-1) containing 100 μg DA/ml and 100 μgDA/g respectively, were provided by the Marine Analytical Chemistry Standards Program, National Research Council of Canada. Acetic acid solutions (0.03 *M*) of STX and dcSTX (20 μg/ml) were provided by RIVM (Bilthoven, The Netherlands) for BCR standard measurements and testing program certification study, were used.

ASP contaminated samples of razor clams and mussels from Ría de Vivero (Lugo), collected in May 1997 were kindly provided by Delegación Provincial de Pesca de Lugo, Consellería de Pesca, Xunta de Galicia. PSP contaminated mussel samples from Ría de Vigo, collected in September–October 1993, were provided by Consellería de Sanidade, Xunta de Galicia. These samples were kept frozen (−18°C) until analysis.

2.2. Reagents

Sodium borate (50 m*M*, pH 9.3) was obtained from Hewlett-Packard. Other reagents such as acetonitrile, methanol, were analytical grade, and Milli-Q grade water (Millipore) was used for the analysis of ASP toxins. Guaranteed grades of formic acid from Prolabo and morpholine from Sigma were used.

2.3. CE system

A CE system model HP ^{3D}CE (Hewlett-Packard), equipped with a diode array detection (DAD) system and a HP ^{3D}CHEMSTATION data system was used for the analysis.

2.4. Analysis of ASP toxins

2.4.1. Extraction of domoic acid

To 4 g homogenate tissue, 16.0 ml methanol–water (1:1, v/v) were added. This mixture was homogenized for 3 min and then centrifuged at 4500 rpm for 10 min. The supernatant was filtered through

a 0.45-μm filter (Millex-HV) and kept in the fridge until analysis.

2.4.2. Clean-up for ASP toxins

The conditions used for this clean-up were as described by Zhao et al. [10] with some slight modifications.

Step 1: 5.0 ml of extract were passed through a strong anion-exchange (SAX) cartridge (part No. 1210-2044, lot No. 182639, 3 ml of capacity, 500 mg, Varian), previously conditioned with methanol, water and methanol–water (1:1, v/v). The extract was washed with methanol–water (1:1) and eluted with 5 ml 0.1 *M* formic acid.

Step 2: Through a strong cation-exchange (SCX) cartridge (part No. 1211-3039, 10 ml/500 mg of size, lot No. 171069) preconditioned with methanol, water and 0.1 *M* formic acid, 5 ml of SAX eluate were loaded. The cartridge was washed with 5 ml of 0.01 *M* formic acid, eluted with 0.5 ml of 25 m*M* sodium tetraborate (pH 9.2)–acetonitrile (9:1, v/v). Elute with six portions of 2 ml of 25 m*M* sodium tetraborate (pH 9.2)–acetonitrile (9:1) and domoic acid starts to appear in the third eluate.

2.5. CE–UV analysis of ASP toxins

CE analysis of ASP toxins were performed using bare fused-silica capillaries 66 cm×363 μm O.D., 50 μm I.D. with a UV window located 15 cm from the exit end of the capillary at room temperature. The UV detection was performed at a wavelength of 242 nm.

Injections used a 50 mbar push for 12 s and the voltage applied for the separation was 30 kV. Different buffer electrolyte concentrations in a range 10, 25 and 50 m*M* in borate buffer were used.

2.6. Analysis of PSP toxins

2.6.1. Extraction and clean-up of PSP toxins

PSP toxins were extracted from mussel samples according to the official method of the American Association of Official Analytical Chemists [11] for the analysis of paralytic shellfish poisoning toxins in seafood. A 3.0-ml volume of supernatant obtained in the extraction procedure was passed through a C_{18} cartridge and 1.5–2.0 ml of eluate were collected for the analysis. The cartridge was previously conditioned with methanol and water under the conditions described in Ref. [12]. After purification on a C_{18} cartridge, the extracts were ultrafiltered in 0.45-μm membrane (Ultrafree-MC, Millipore) and then analyzed by CE–UV.

2.7. CE–UV analysis of PSP toxins

CE analyses of PSP toxins were performed under the conditions described by Locke and Thibault [8] with some modifications. The separations were performed in a polyvinylalcohol (PVA) capillary (104 cm×75 μm I.D.) under a constant voltage of 20 kV at the injector end of the capillary. The sample was applied under constant pressure (50 mbar) such that 20% volume of the capillary was introduced. The UV detector was operated at 200 nm. The CZE background electrolyte under capillary isotachophoretic (cITP) preconcentration was 50 m*M* morpholine in water adjusted to pH 5 with formic acid. The cITP terminating electrolyte was 10 m*M* formic acid.

3. Results and discussion

3.1. ASP toxins

The structure of domoic acid with three carboxyl groups and one amino group clearly justify its ability for protonation, (see Fig. 1a). Due to the distinct charge states, their proportions in solution are determined by the pK_a values and also by the pH; for this reason different modes of operation could be applied using either acidic or basic conditions.

CE conditions developed by Zhao et al. [10] were used in this study with some slight modifications, as described in Section 2. Since borate seems to be the optimum buffer for such analysis, it was used under basic conditions. These conditions allowed us to identify clearly the presence of domoic acid in some contaminated real samples by comparison with standard solutions of domoic acid and also mussel tissue reference material (MUS-1). Different buffer concentrations were tested (10, 25, 50 m*M*) in order to find the optimised conditions for the analysis in terms of efficiency and resolution. When high buffer

concentrations were used, an increase on the peak width as well as the Joule heating effect in the capillary were observed due to high salinity content in the electrolyte buffer [13], this last effect could affect to the vaporisation of the solvent and also a loss of sample prior the electrophoretic separation. An increase in the ionic strength could produce longer migration times. Shorter migration times were observed when 10 m*M* buffer concentration was used, but as a consequence, domoic acid was not clearly identified due to the coelution with some other compounds. This lack of selectivity was overcome by using 25 m*M* borate concentration, which also provides higher electrophoretic resolution; at the same time sharper peaks and an increase in the separation efficiency were obtained under these conditions (Fig. 2).

Calibration of the system was carried out for quantitative purposes by using standard solutions of domoic acid (Fig. 3), the results obtained for this calibration are summarized in Table 1.

Optimized conditions were applied for the analysis of contaminated razor clams, these samples were previously cleaned-up through SAX and SCX cartridges following the procedure above described under the conditions described by Zhao et al. [10]. Recovery experiments were carried out, in order to evaluate the efficiency of this procedure. The mean

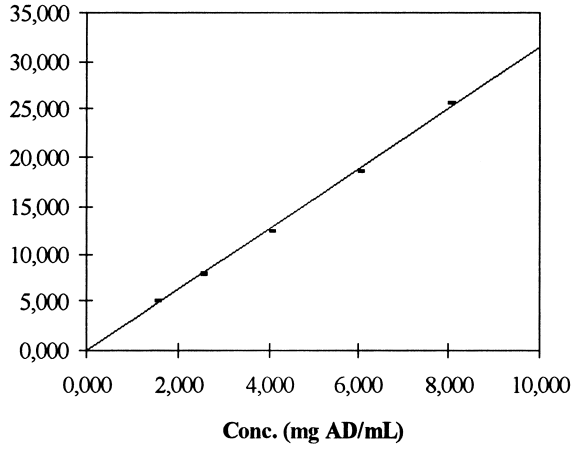

Fig. 3. Calibration curve for domoic acid by CE–UV/DAD. *y*-axis: Peak area.

Table 1
Calibration parameters obtained for ASP toxins by CE–UV/DAD

Parameters	Domoic acid
Range (μg/ml)	1.5–8
Slope	3.152
b	0.015
r	0.998
Detection ($S/N=3$) (μg/ml)	0.75
RSD areas (%, $n=10$)	0.582
RSD migration times (%, $n=10$)	0.158

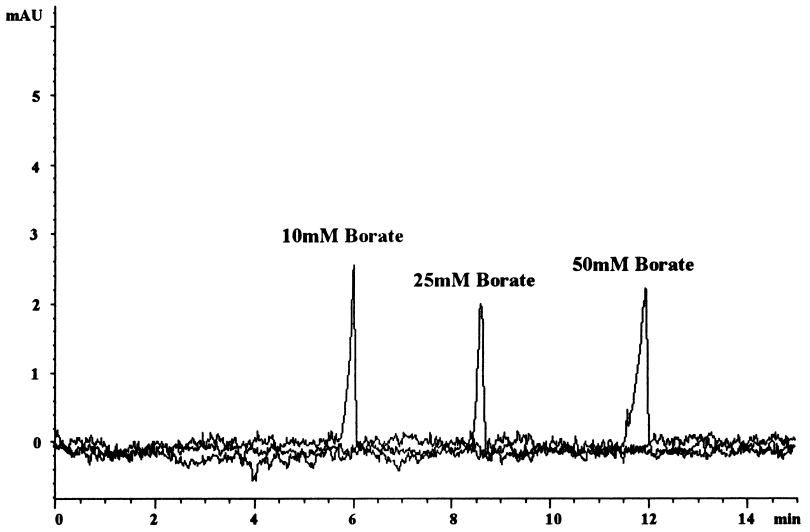

Fig. 2. Effect of buffer concentration in the separation of Domoic acid by CE–UV/DAD.

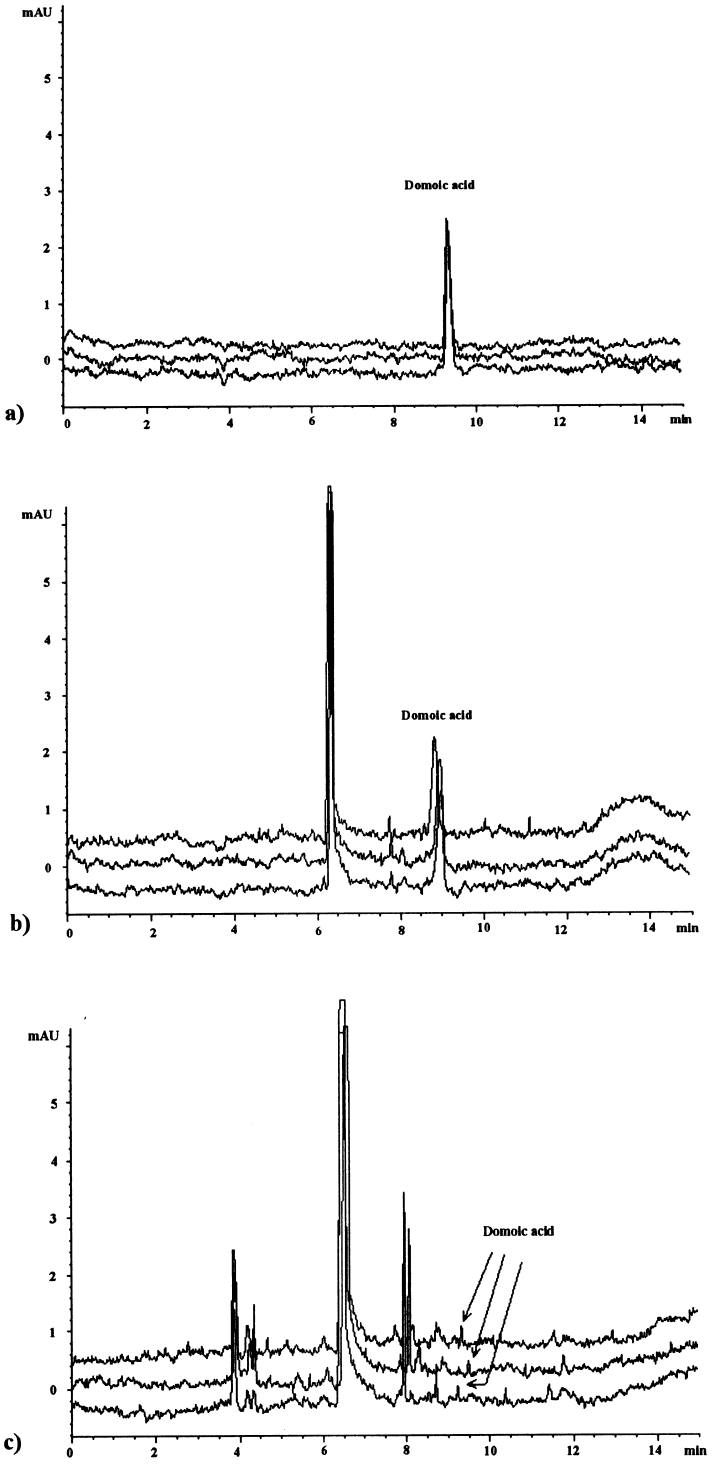

Fig. 4. CE–UV/DAD analysis of: (a) Domoic acid standard, (b) MUS-1 Reference material after SAX-SCX clean-up and (c) Galician razor clam sample after SAX-SCX clean-up.

of the recovery values achieved in these experiments was 103%. The material used for such a clean-up clearly affects the values of the efficiency.

The presence of domoic acid was confirmed by spiking the samples with domoic acid standard, these analysis were carried out in triplicate to check reproducibility between injections (Fig. 4).

3.2. PSP toxins

Based on the structure of PSP toxins with a global charge +2, +1, 0, depending on the pH of the medium, these toxic compounds can migrate in a electric field, being separated by CE, with the exception of C's toxins group, which due to their neutral average charge in acidic medium are not able to migrate in an electric field. CE offers good potential for the analysis of most of PSP toxins, nevertheless sample preparation prior the electrophoretic analysis plays an important role in order to achieve the highest resolution. Because of this, extraction and clean-up are critical steps which need to be optimized. The optimal conditions for these steps are described in Section 2. These optimization studies were reported in [14], because the efficiency of CE separation is related to the extraction and clean-up in sample preparation. The study of the efficiency of the extraction was carried out taking into account the effect of acidic conditions and temperature on this step.

CE conditions were also optimized taking into account previous studies of Locke and Thibault [8]. The influence of buffer concentration and voltage in the electrophoretic resolution was carefully studied; as result of this, 50 m*M* morpholine buffer and 20 kV at pH 5 were the optimal conditions found for this analysis [15]; a good efficiency in the separation for all toxins studied, STX, dcSTX, neoSTX and GTX1-5, was observed and these results are shown in Fig. 5. The effect of organic modifiers in the background buffer was also studied, a decrease in the mobility of PSP toxins being detected, as well as an increase in the migration times. As a consequence of this, an important loss in the efficiency and electrophoretic resolution was noted.

An increase in the organic modifier concentration causes a loss of neoSTX, and losses of GTX compounds were also observed under these conditions (Fig. 6). The optimal conditions for buffer composition were reported in [15], and the these results were obtained with 50 m*M* morpholine at pH 5.

Calibration of the CE system was carried out by using individual standard solutions of STX and dcSTX. Results of this calibration are shown in Table 2 and Fig. 7.

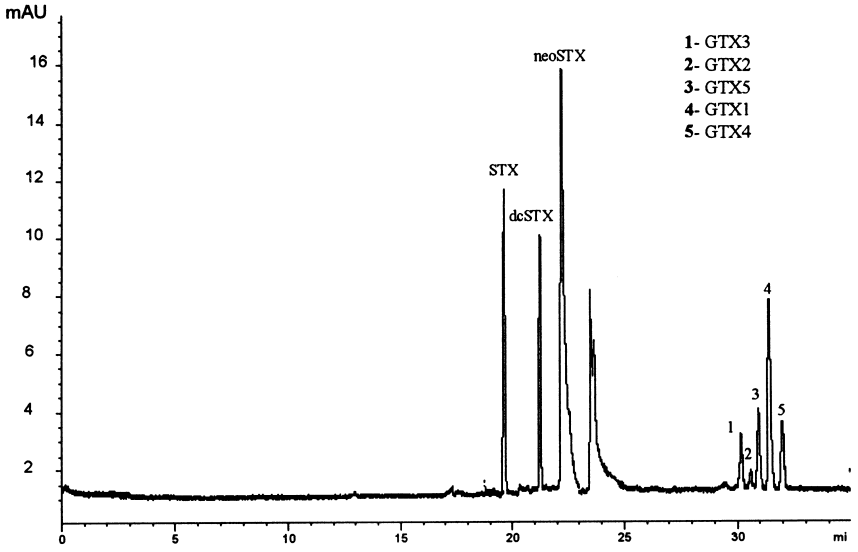

Fig. 5. Standard of PSP toxins, 20 kV, 50 m*M* morpholine, pH 5.

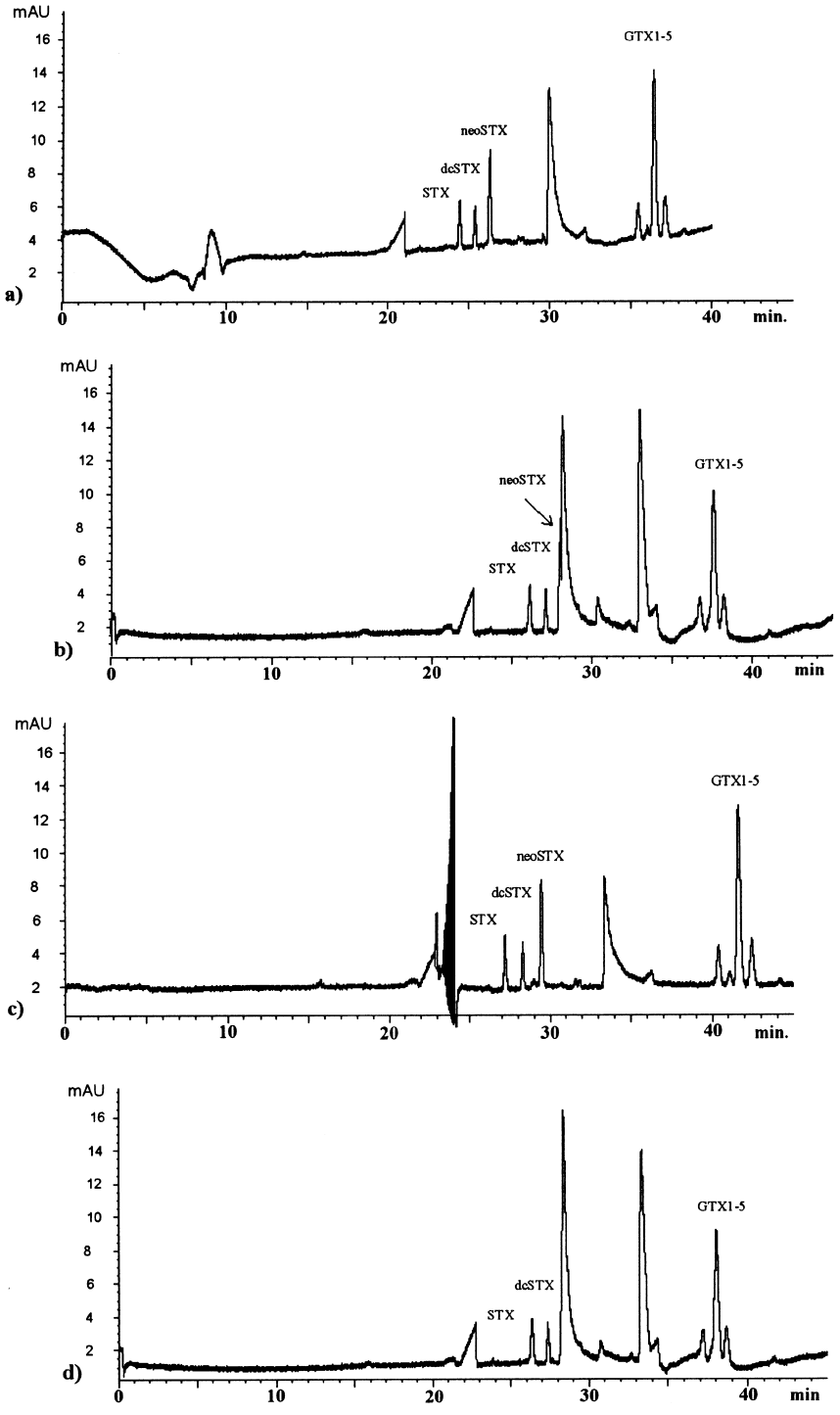

Fig. 6. Effect of organic modifier in buffer composition for the analysis of PSP toxins by CE–UV, (a) morpholine 50 m*M*, pH 5, 10% v/v CH_3CN, (b) morpholine 50 m*M*, pH 5, 20% v/v CH_3CN, (c) morpholine 50 m*M*, pH 5, 10% (v/v) MeOH, (d) morpholine 50 m*M*, pH 5, 20% (v/v) MeOH; voltage: 20 kV

Table 2
Calibration parameters obtained for PSP toxins by CE–UV/DAD

Parameters	dcSTX	STX
Range (μg/ml)	0.2–1.0	0.2–1.0
Slope	91.14	88.01
b	10.129	−0.694
r	0.991	0.994
Detection limit (*S*/*N*=3) (μg/ml)	0.06	0.05
RSD areas (%, *n*=10)	1.1	0.5
RSD migration times (%, *n*=10)	1.5	0.5

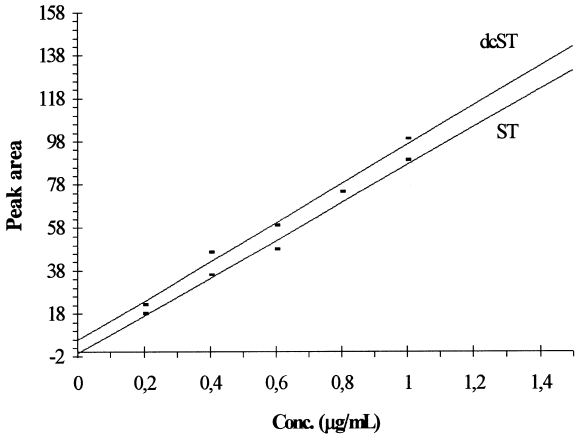

Fig. 7. Calibration curves for STX and dcSTX obtained by CE–UV/DAD method.

The optimized conditions were applied to the analysis of some contaminated real mussel samples; the results obtained in these analysis are shown in Fig. 8. A loss of resolution in the GTX group was observed, while an efficient separation was observed for STX group. This low resolution could be associated with the complexity of the matrix, high salt content and also the presence of interferences. The loss of resolution could be also related to the presence of some unknown isomeric GTX components; this hypothesis was later confirmed by using MS detection [16].

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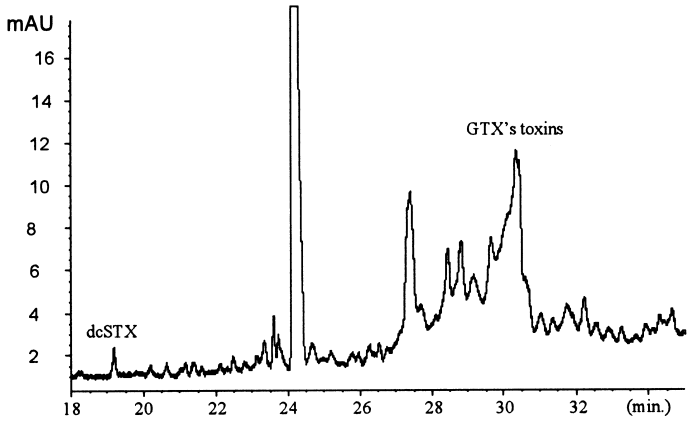

Fig. 8. CE–UV analysis of PSP toxins in contaminated Galician mussel sample.

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