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Analysis of paralytic shellfish poisons by capillary electrophoresis[a]

P. THIBAULT*, S. PLEASANCE[b] and M. V. LAYCOCK

Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1 (Canada)

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ABSTRACT

A capillary electrophoresis (CE) method with UV detection is described for the separation and determination of underivatized toxins associated with paralytic shellfish poisoning (PSP). Confirmation of the electrophoretic peaks was facilitated by mass spectrometric (MS) detection using an ionspray CE–MS interface and by high-performance liquid chromatography with fluorescence detection. The determination of PSP toxins, such as saxitoxin and neosaxitoxin, in toxic dinoflagellates and scallops is demonstrated and comparisons are made with existing techniques.

INTRODUCTION

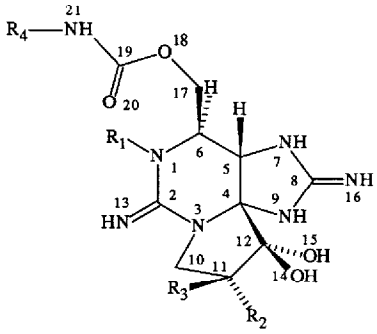
Paralytic shellfish poisoning (PSP) is a severe form of seafood poisoning which results from the ingestion of contaminated shellfish [1–4]. It presents a real threat to public health as no antidote has yet been found and it is not possible to detoxify the contaminated shellfish efficiently. Symptoms of PSP are primarily neurological and can vary from facial paresthesis, nausea and vomiting to death resulting from respiratory paralysis, which can occur within 12 h after ingestion of toxic shellfish.

Toxic phytoplankton blooms, which occur sporadically along many coasts around the world, are the primary source for the toxification of filter-feeding shellfish [3]. Toxicity and toxin profiles vary widely among PSP-producing dinoflagellates and as much as 20 fmol per cell of the potent saxitoxin (STX) has been reported for a Bay of Fundy strain of *Alexandrium tamarensis* [5]. Toxins associated with PSP include STX, neosaxitoxin (NEO) and a complex suite of sulfate and N-sulfonate analogues [1]. Their structures are characterized by a five-membered ring fused on a perhydropurine skeleton (Fig. 1).

The development of reliable analytical techniques for the detection of these toxins has become important not only in view of the small amounts of toxins reported to cause illness (6–40 μg/kg [6,7]) but also for a greater understanding of the biogen-

[a] NRCC No. 31946.

[b] Under contract from SCIEX, 55 Glen Cameron Road, Thornhill, Ontario, L3T 1P2.



R_1	R_2	R_3	R_4	
H	H	H	H	STX
OH	H	H	H	NEO
H	H	H	SO_3H	B_1
OH	H	H	SO_3H	B_2
H	H	OSO_3H	H	GTX_2
H	OSO_3H	H	H	GTX_3
OH	H	OSO_3H	H	GTX_1
OH	OSO_3H	H	H	GTX_4
H	H	OSO_3H	SO_3H	C_1
H	OSO_3H	H	SO_3H	C_2
OH	H	OSO_3H	SO_3H	C_3
OH	OSO_3H	H	SO_3H	C_4

Fig. 1. Structures of toxins associated with paralytic shellfish poisoning.

esis and metabolic pathways of the toxins involved. At present the official analytical procedure which supports the majority of toxin-monitoring programs in shellfish is the mouse bioassay [8,9]. This procedure allows the determination of the "total toxicity" of a biological extract and provides adequate sensitivity with respect to the permissible limit for human consumption (80 μg per 100 g of shellfish meat) [3]. However, its narrow dynamic range, coupled with its wide variability (effects of salts, susceptibility to mouse strains), difficulties in the supportive logistics and increasing pressure to discontinue the use of animal bioassays have stimulated the development of alternative methods [10].

The detection of PSP toxins has provided a considerable challenge to the development of analytical techniques. The lack of useful UV chromophores absorbing above 220 nm [11] and the low volatility and highly polar nature of these compounds are some of the difficulties that prevent their separation and detection by conventional techniques. In spite of these obstacles, a variety of separation procedures have been reported for the isolation and determination of PSP toxins, including column chromatography [12,13], thin-layer chromatography [14] and cellulose acetate electrophoresis [15]. The instrumental technique most commonly used for the routine determination of PSP toxins involves reversed-phase high performance liquid chromatography using ion-pairing reagents and post-column reaction permitting fluorescence detection (HPLC–FLD) [16]. The post-column reaction system consists of an alkaline oxidation reaction which converts the native toxins into their corresponding fluorescent derivatives [16]. Although the method offers good sensitivity and dynamic range for the separation and detection of different PSP toxins, the sensitivity is dependent on parameters such as reagent concentrations, reaction times, pH and temperature of the oxidation reaction [17]. In addition to the elaborate procedure required to achieve reliable and reproducible results, a significant drawback of the alkaline oxidation reaction is the reliance on fluorescence response factors for the different PSP toxins based on that of the only commercially available standard, STX [16,18].

The successful separation of these marine toxins by both ion-exchange chromatography and cellulose acetate electrophoresis [15] prompted us to investigate the

application of capillary electrophoresis (CE) for their analysis. Also, the recent investigation of ionspray mass spectrometry of marine toxins [19] using flow-injection analysis indicated a useful detection limit for STX (30 pg) and suggested that mass spectral detection of PSP toxins following separation by CE would be practical, provided that a suitable interface is built. Owing to its high-resolution separation capability (up to 10^6 theoretical plates in less than 20–25 min [20–22]) and its ease of operation, the CE technique has aroused considerable interest for the analysis of complex polar biomolecules including marine toxins. Wright *et al.* [23] recently demonstrated the applicability of CE using laser-induced fluorescence detection for the analysis of derivatives of marine toxins including STX. The method has permitted high-resolution separations, with an attomole-range detection limit for the *o*-phthaldialdehyde derivative of STX [23]. However, in view of the desired application to the analysis of biological extracts, the use of chemical derivatives could potentially lead to problems of highly variable response factors, interferences due to reagents and other non-PSP compounds and complications arising from the instability of the derivative and the extensive work-up required.

This paper describes the application of CE with UV detection to the determination of PSP toxins in marine samples. Confirmation of the chemical identities of electrophoretic peaks is made using an improved mass spectrometric interface. A preliminary evaluation of the factors affecting the separation and detection of these toxins is also reported.

EXPERIMENTAL

Chemicals

Saxitoxin was obtained from Calbiochem Biochemicals (San Diego, CA, U.S.A.) as a 1 μmol/ml solution in 0.01 *M* acetic acid and used without further purification. Analytical reagent grade acetic acid (Caledon Laboratories, Georgetown, Ont., Canada), sodium citrate (Sigma, St. Louis, MO, U.S.A.) and water purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.) were used to prepare the buffer solutions.

Biological samples

Toxins from either cultured algal cells (1–10 g wet weight) or homogenized scallop livers (15 g wet weight) were extracted by sonication in 0.1 *M* acetic acid. Freeze-dried cells of the cyanobacterium *Aphanizomenon flos-aquae* were provided from Dr. W. W. Carmichael (Wright State University, Dayton, OH, U.S.A.). Cell and tissue debris were removed by centrifugation at 20 000 *g* for 30 min and the supernatant fluid was then freeze-dried. The resulting solid was dissolved in 5 ml of 0.1 *M* acetic acid, adjusted to pH 5 and then passed through a 90 × 1.6 cm I.D. Bio-Gel P-2 column (Bio-Rad Labs., Mississauga, Ont., Canada) previously equilibrated with water and eluted with 0.1 *M* acetic acid. Fractions of 1.5 ml were collected and subsequently analysed by high-voltage paper electrophoresis (HVPE).

High-voltage paper electrophoresis

HVPE was conducted in a similar manner to that described by Leggett-Bailey [24]. Aliquots of 10–20 μl of each fraction were spotted across a sheet of Whatman

No. 1 paper (46 × 57 cm). After drying of the sample line, the paper was wetted with the electrolyte [10% (v/v) acetic acid] on either side of the origin and excess of electrolyte was removed with blotting paper. A glass chromatographic tank (Shandon, London, U.K.) containing 5 l of the electrolyte as the anode reservoir was used for electrophoresis. The paper was suspended from the cathode at the top of the tank. The remainder was filled with toluene cooled by a convoluted glass tube through which cold tap-water flowed. Electrophoresis was conducted at 3 kV for 30 min, after which the paper was dried in a vented oven at 100°C. PSP toxins were detected with a long-wavelength UV light source after spraying with 1% hydrogen peroxide solution and heating for 5 min at 100°C.

Capillary electrophoresis

An Applied Biosystems (Foster City, CA, U.S.A.) Model 270A capillary electrophoresis system was used for all CE experiments. An untreated fused-silica capillary column (Polymicro Technologies, Phoenix, AZ, U.S.A.) of 50 μm I.D. and length 90 cm (68 cm to the detector) was used in all separations. Sample introduction was performed using either hydrodynamic (vacuum) or electrokinetic (voltage) injections. In hydrodynamic injection, a preset vacuum (17 kPa) was applied to the detector end of the capillary for 3 s, whereas electrokinetic injection used a potential of 5 kV applied to the capillary end for 30 s. Unless specified otherwise, electrophoretic separations were carried out in either 20 m*M* sodium citrate buffer (pH 2) or 0.1 *M* acetic acid (pH 2.9). UV detection was performed at 200 nm. Data acquisition and handling were accomplished using a Hewlet-Packard HP 3396A integrator linked to an MS-DOS microcomputer using the Chromperfect data acquisition–processing software package (Justice Innovations, Palo Alto, CA, U.S.A.).

Mass spectrometry

All mass spectrometric experiments were performed on a SCIEX (Thornhill, Ont., Canada) API III triple quadrupole mass spectrometer equipped with an atmospheric pressure ionization (API) source operated in the ionspray mode. For flow-injection analyses (FIA) samples were injected via a 0.1-μl loop Valco injector (Chromatographic Specialties, Brockville, Ont., Canada) into a stream of acetonitrile–water (1:1) containing 0.1% trifluoroacetic acid, delivered by a Brownlee syringe pump operating at a flow-rate of 50 μl/min.

The CE–MS interface is shown schematically in Fig. 2. The interface was constructed from a modified commercial IonSpray (ISP) probe and was based on a co-axial column arrangement, similar in design to that described by Smith *et al.* [25]. The CE fused-silica column (90 cm × 50 μm I.D. × 350 μm O.D.) was connected to the interface via a zero dead volume (ZDV) tee, mounted externally on the pressurized probe handle. A make-up solution (0.1 *M* acetic acid) was delivered at a flow-rate of 20 μl/min to the probe tip via the interspace between the CE capillary and the outer capillary (42 cm × 400 μm I.D. × 530 O.D.) using a syringe pump (Harvard Apparatus, Southnatick, MA, U.S.A.). Optimization of the CE–MS system was achieved by injecting a standard into the make-up flow stream using a 0.1-μl loop Valco injector as described above. The ISP probe tip consisted of a 20-gauge stainless-steel tube and was held at a potential of *ca.* 6 kV. Air was used as a nebuliser gas at a flow-rate of 1.5–2.0 l/min. The potential difference between the CE electrode and the ISP needle

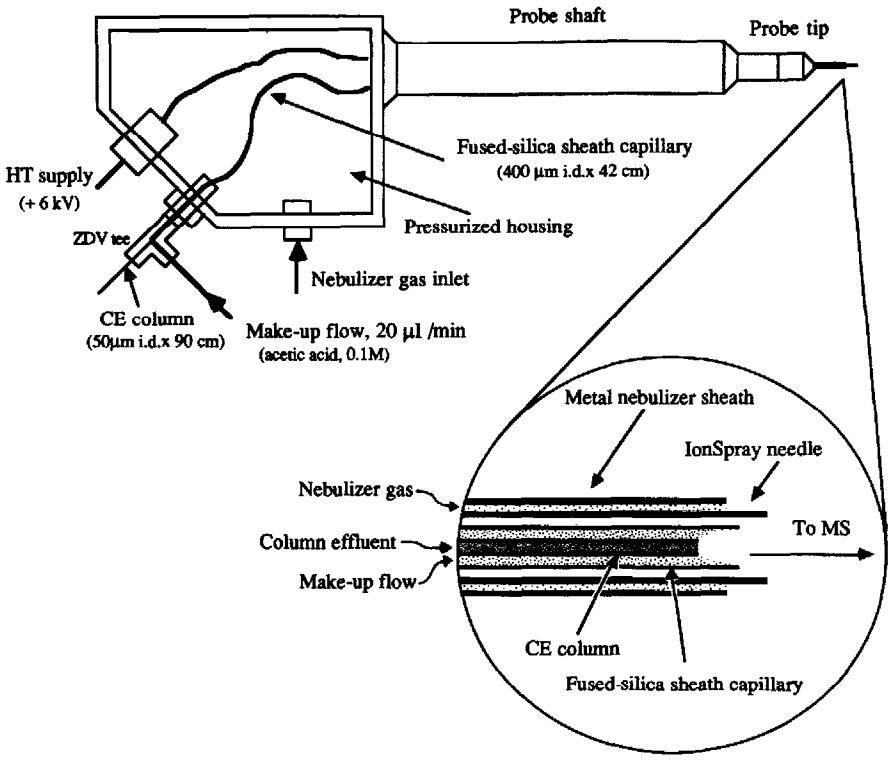


Fig. 2. Schematic diagram of the CE–MS interface.

therefore defined the CE field gradient. Electrokinetic injections (30 s) were made in all CE–MS experiments. The CE column terminated *ca.* 0.5 mm inside the ISP needle. This arrangement minimized column peak broadening and ensured good electrical contact between the ISP needle and the CE electrolyte. Mass spectral acquisition was performed using dwell times of 5 and 200 ms per channel for full mass scan (either 50-350 dalton in CE–MS or 200–500 dalton in FIA–ISP-MS analyses) and selected ion monitoring (SIM) experiments, respectively. A Macintosh IIx computer was used for instrument control, data acquisition and data processing.

RESULTS AND DISCUSSION

Determination of PSP toxins by CE–UV following sample fractionation

With the exception of C compounds (Fig. 1), PSP toxins have an overall positive charge under acidic conditions, making them ideal candidates for separation based on ionic state. Their basic nature is attributed to the delocalized guanidinium groups, centered on C_8 and C_2, which have pK_a values of 8.22 and 11.28 in the case of STX [26]. In this study, electrophoretic separation of PSP toxins by HVPE was conducted to establish toxin profiles as they eluted from the Biogel P-2 gel permeation column. This method is similar to that previously reported using cellulose acetate

electrophoresis [15], except that the acetic acid buffer system chosen here facilitates solvent evaporation and its larger scale allows 50–100 samples to be run simultaneously. Further, the time for electrophoresis was typically 30 min compared with 3 h on cellulose acetate [15]. The P-2 column was used prior to electrophoresis as PSP toxins could easily be separated from salts, proteins, carbohydrates and most non-PSP compounds present in plankton and more particularly in scallop extracts.

A typical two-dimensional analytical chromatogram (P-2 column fractions on the horizontal axis with electrophoretic separation on the vertical axis) of PSP toxins is presented in Fig. 3 for the analysis of an extract isolated from 10 g of sonicated cells of *Alexandrium tamarensis*. Most non-PSP compounds were found in fractions 15–30 and did not show any fluorescence on oxidation with hydrogen peroxide but stained strongly with ninhydrin. PSP toxins eluted later in fractions 40–104, which exhibited a blue fluorescence on oxidation and drying. Based on a previous report [27], these different migration bands can be separated into three main toxin groups in descending order of net charge: fractions 40–58 containing both STX and NEO (2+), fractions 52–66 containing the GTX toxins (1+) and fractions 80–104 containing the C

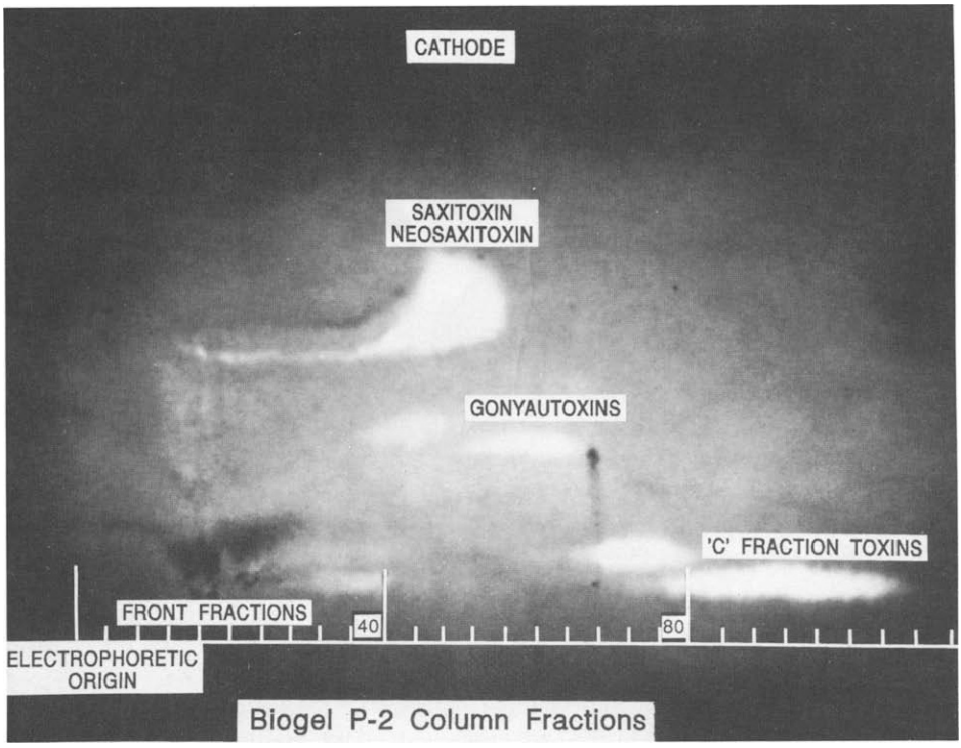


Fig. 3. High-voltage paper electrophoretic separation of PSP toxins following Bio-Gel P-2 column fractionation. Electrophoresis was conducted at 3 kV for 30 min on Whatman No. 1 paper (46 × 57 cm) using 10% (v/v) acetic acid electrolyte. PSP toxins were identified as a blue fluorescent band after H_2O_2 oxidation and heating at 100°C for 5 min. Non-PSP compounds were found in fractions 14–18. STX and NEO were found in fractions 21–24 and did not separate from each other. GTX toxins eluted in fractions 24–27 and the C toxins appeared in fractions 30–37.

toxins (no net charge). Although the C toxins have no net charge in dilute acetic acid, they do migrate from the electrophoretic origin owing to the electroendosmotic flow. Fractions corresponding to each of these three groups were also analyzed by ionspray mass spectrometry in order to validate this qualitative assignment (see below). The fluorescent band seen in fractions 68–80, having a mobility slightly higher than the electroendosmotic flow, was not identified but may be due to a PSP degradation product.

Although the separation of PSP toxins into three groups was readily achieved on the P-2 column and by HVPE, it was anticipated that CE would provide more efficient separation. However, some of the inherent difficulties in analyzing such compounds are the lack of a chromophore absorbing in the usual UV range and the unavailability of standards to confirm the electrophoretic peak identity. With respect to the detection capability, it is expected that PSP detection using a conventional deuterium lamp would be difficult if not impossible, in view of the low molar absorptivities (e.g., $< 1 \cdot 10^{-3}$ mol/cm above 220 nm for STX [11]). However, STX shows a rise in its molar absorptivity below 220 nm [11]. This, coupled with the fact that the signal-to-noise characteristics of the UV detector used in this present study provide better sensitivity at 200 nm on compounds such as peptides [28], prompted us to investigate the practical applications of this instrument to PSP toxins.

In situations where the electrophoretic peak identity could present some uncertainties, or if standards are not available to correlate with the peak of interest, it is desirable to use a complementary technique. Mass spectrometric detection using nebulizer-assisted electrospray ionization (ionspray) was chosen for this purpose, as its excellent sensitivity observed for PSP and other marine toxins [19] renders detection of STX possible within the concentration and injection volumes used in the CE experiment. A CE–MS interface inspired by a co-axial capillary arrangement carrying both the CE buffer and make-up flow initially described by Smith *et al.* [25] was designed and constructed in this laboratory. Although other CE-MS interfaces using a "liquid junction" remote from the ion source have been demonstrated successfully [29,30], the co-axial design has the advantage of removing dead volume associated with the "liquid junction" in addition to minimizing band broadening arising from the dynamic mixing of the make-up flow and CE stream [31].

Initial investigations of PSP toxin analysis by CE were made using a buffer system similar to that used in the HVPE experiment. In addition to enhancing protonation, the acetic acid buffer has the advantage of being compatible with the CE–MS interface and can also be used as make-up flow, thus preventing changes in the CE buffer composition during a run. The results of analyses of an extract of *A. tamarensis* by CE–UV and CE–MS are presented in Fig. 4. The sample chosen for the CE experiment originated from fraction 52 from the Bio-Gel P-2 column separation and showed a fluorescent band in the NEO–STX region of the HVPE analysis. The electropherogram of fraction 52, using UV detection at 200 nm, is shown in Fig. 4a. This separation corresponds to an electrokinetic injection of 30 s and was achieved at a field strength of 220 V/cm. As indicated in Fig. 4a, three major components are observed at 7.41, 7.46 and 7.58 min. The resolution of the last two peaks was estimated to be only 0.50.

The analysis of fraction 52 by CE–MS is presented in Fig. 4b and c, corresponding to the reconstructed ion electropherograms of the protonated molecules (MH^+)

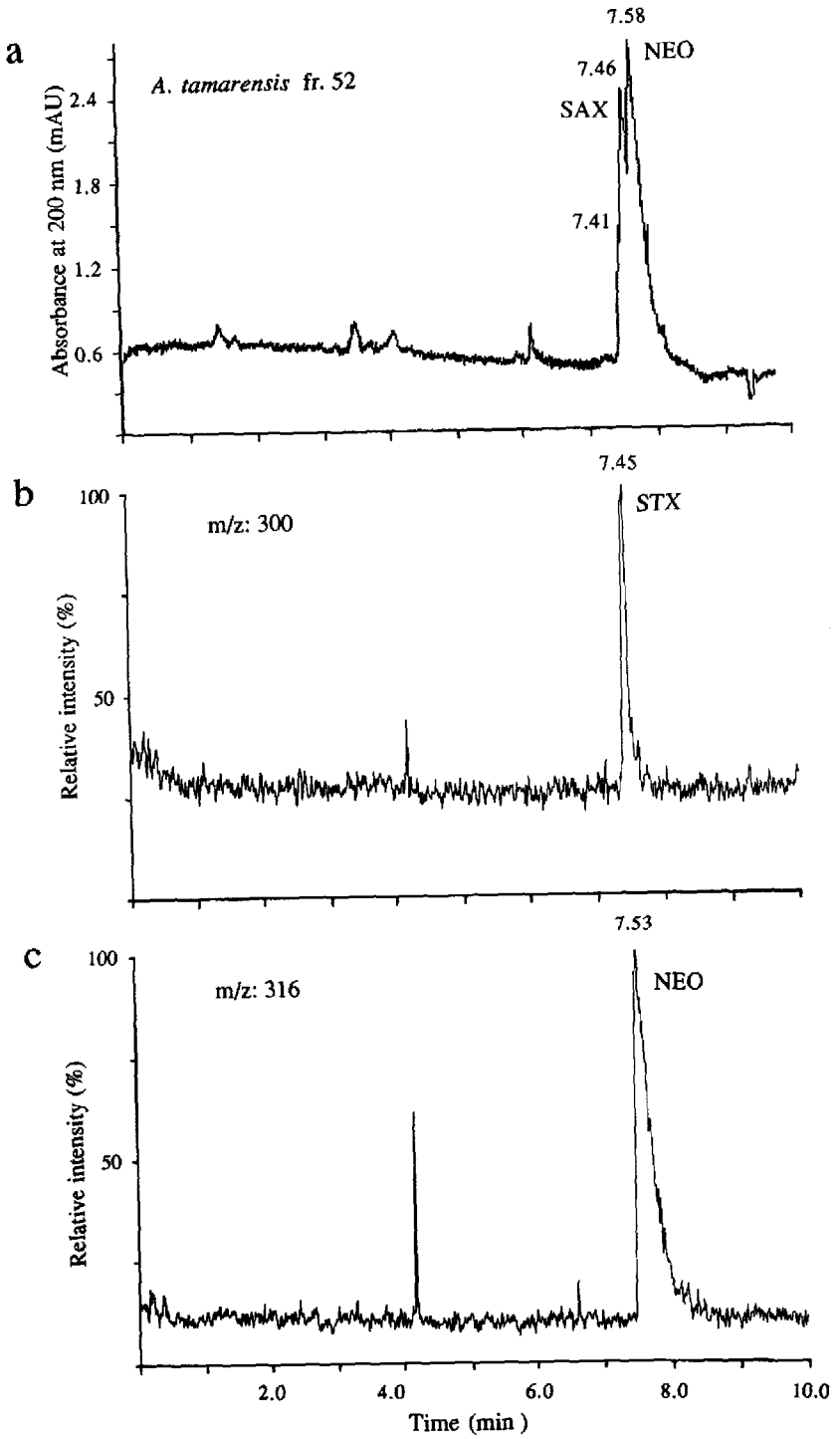


Fig. 4. Capillary electrophoresis of fraction 52 from the *A. tamarensis* extract. (a) Analysis performed using UV detection at 200 nm, 30-s electrokinetic injection, +20 kV, 0.1 *M* acetic acid buffer. Injection of *ca.* 0.5 and 1.3 ng of STX and NEO, respectively. Reconstructed ion electropherogram for (b) *m/z* 300 and (c) *m/z* 316, 30-s electrokinetic injection, +25 kV applied at injector end, +5.6 kV on ionspray probe tip.

of STX and NEO, respectively. The migration times observed for the two toxins are remarkably close to those observed in the CE–UV experiment and confirmed the identity of the two electrophoretic peaks. Independent injections of STX standard, using both CE–UV and CE–MS with full mass acquisition of m/z 100–400 dalton (data not shown), provided additional evidence of the peak identity. In addition to the good correlation of migration times observed in the two experiments, it is noteworthy that the peak widths at half-height differed by at most 3 s in the two experiments, indicating that loss of separation efficiency due to large dead volume was minimal with this CE–MS interface. As a result, the resolution for STX and NEO obtained in the CE–MS analysis was similar to that observed with the CE–UV; a value of *ca.* 0.7 was obtained for the former technique as opposed to 0.5 in the latter.

Although no effort was made to improve the detection limit of the CE–MS interface, adequate signal-to-noise ratios were obtained in the analysis of fraction 52. As will be described later, this fraction contained STX and NEO in concentrations of 32 and 83 μg/ml, respectively. The amount of sample loaded on the CE column by electrokinetic injection was established by comparing area counts with those obtained using vacuum injection, the latter being calibrated by measuring the amount of sample drawn into the cappillary using a coloring agent prepared with the sample solvent. These experiments indicated that *ca.* 500 pg of STX were loaded on the column during the CE–MS experiment. For such amounts a signal-to-noise ratio of 15:1 was obtained, suggesting that an instrumental detection limit of *ca.* 100 pg is achievable. This result is consistent with data obtained independently with flow-injection analysis of STX using ionspray [19], where a detection limit of 30 pg was obtained with a signal-to-noise valid ratio of 2. The difference in sensitivity can be explained by the larger peak width observed with the combined CE–MS technique.

Determination of STX and NEO concentrations in biological extracts

Further to the preliminary work on the separation of NEO and STX described above, work was needed to enhance the separation efficiency of the CE technique and to facilitate the confirmation and determination of these toxins in natural extracts. Buffer composition and acidity were investigated to establish their effects on plate counts, resolution and background contribution.

Variation of the buffer acidity had a considerable effect on the separation of NEO and STX. Increasing the acetic acid content of the buffer from 0.1 to 5.0 *M* resulted in a pH change from 2.9 to 1.9. Using a field strength and capillary identical with those described in Fig. 4, it was observed that the resolution improved progressively with decreasing pH up to the point where these peaks were resolved completely. For example, using solutions of approximately equal concentration of STX and NEO, pH values of 2.9, 2.4 and 1.9 resulted in resolution values (R_s) of 0.5, 1.6 and 2.7, respectively. At pH 1.9 the efficiency of separation (N) obtained from the conventional equation for plate numbers using peak widths at half-height was 53 800, an improvement of *ca.* 2-fold over the same analysis conducted at pH 2.9.

Lowering the pH of the buffer also affected the analysis time. At pH 1.9 the migration of both STX and NEO was completed in 24 min as opposed to 8 min at pH 2.9. This increase in migration time is due to electroendosmosis where the bulk flow of liquid progressively increases with increasing pH. The electroendosmotic flow arises from solvated cations from the double layer adjacent to the capillary wall migrating

to the cathode. At low pH, where the silanol groups of the capillary wall are protonated, there is very little coulombic interaction between the analyte cations, buffer ions and the capillary wall, hence decreasing the electroendosmotic flow [32,33].

Other buffer systems were also investigated in an attempt to improve the separation efficiency. Better resolution and sensitivity were achieved by changing the buffer to a 20 m*M* solution of sodium citrate adjusted to pH 2.1. An example of such a separation is presented in Fig. 5 for the analysis of fraction 52 from the *A. tamarensis* extract. The CE analysis performed under these conditions yielded three distinct peaks with an R_s value of 2.6 for NEO and STX. The identity of the first peak observed at 14.68 min is unknown. However, this compound is not an isomer of either STX or NEO as no corresponding signal was observed in the SIM traces in Fig. 4b or c. Owing to the low concentration of this component and the amount of sample that could be loaded on the capillary, the identification of this compound using CE–MS with full mass scan acquisition was not possible. It is possible that this compound is a decarbamoyl derivative of STX which was previously found to have a mobility slightly higher than that of STX [15].

In terms of separation efficiencies, the number of theoretical plates achieved with the sodium citrate buffer was *ca.* 46 000. Although this is less than that with the acetic acid buffer of pH 1.9, the use of sodium citrate buffer has the advantage of reducing the analysis time to 18 min, in addition to providing better signal-to-noise ratios (typically four times better than with acetic acid at pH 1.9). It is noteworthy that a gain of a factor of 2 in sensitivity could be achieved by setting the UV detector

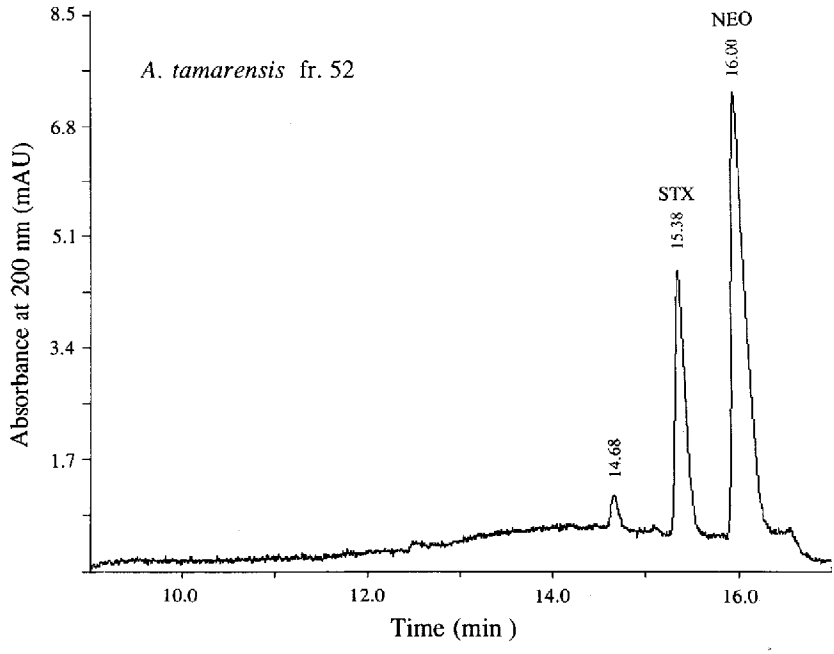


Fig. 5. Capillary electrophoresis of fraction 52 from the *A. tamarensis* extract using UV detection at 200 nm, 3-s vacuum injection, +20 kV, 20 m*M* sodium citrate buffer. The electropherogram represents an injection of 320 and 830 pg of STX and NEO, respectively.

to 190 nm instead of 200 nm. However, in view of the stability of the baseline noise over a day-long operation, analysis at 200 nm was preferred.

In order to improve the separation efficiency and reduce the analysis time still further for routine analysis, the CE separation was carried out at 30 kV using the sodium citrate buffer. Under these conditions, migration of STX was achieved within 10 min with N values at least 4–5 times greater than those obtained in the same experiment performed at 20 kV. Reproducibility of migration times was adequate to ensure proper peak assignment; extremes of migration times varied by no more than 2% on a daily basis. These fluctuations could also be corrected by using a mobility standard such as lysine, thereby reducing these variations to less than 1%. In terms of reproducibility for determination, replicate injections (n = 6) of solutions containing both STX and NEO indicated that the standard deviation of the integrated areas was *ca.* 4% for each compound.

The CE separation developed here for the determination of STX and NEO also provided good linearity over the concentration range 1.5–300 μg/ml. A typical calibration graph for STX is presented in Fig. 6. This graph shows a good linear response over at least two orders of magnitude, with a correlation coefficient of 0.998. The mass detection limit was reached for a *ca.* 15-pg injection of STX. The inset in Fig. 6 shows the CE analysis achieved at this detection limit for STX with a signal-to-noise ratio of 2.

We found that the response for NEO at 200 nm is slightly lower than that for STX. Studies performed using NMR [34] established the molar ratio of NEO and STX in a synthetic mixture of these two toxins. Results from analytical techniques such as FIA–ISP-MS and CE–UV were compared with data obtained from NMR

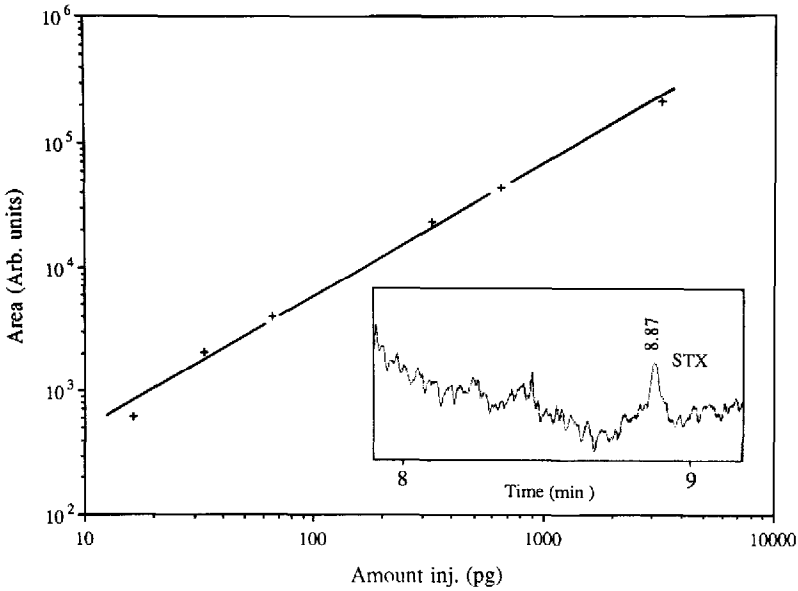


Fig. 6. Calibration curve for STX in the concentration range 1.5–300 μg/ml. Conditions: UV detection at 200 nm, 3-s vacuum injection, + 30 kV, 20 mM sodium citrate buffer. The mass detection limit was reached for a *ca.* 15-pg injection of STX. The inset shows the CE analysis achieved at the detection limit for STX with a signal-to-noise ratio of 2.

spectroscopy. Correlations amongst these different techniques indicated that the response observed for NEO should be corrected by a factor of 1.18 in order to compensate for the lower sensitivity of this compound with UV detection at 200 nm. Based on this information, it was possible to determine the amount of STX and NEO present in fraction 52 from the *A. tamarensis* extract (Fig. 5). This quantitative analysis revealed that the concentrations of STX and NEO were 32 ± 1 and 83 ± 2 μg/ml, respectively.

The results from applying the CE–UV determination of STX and NEO to fractions from phytoplankton, a cyanobacterium and contaminated scallops are presented in Fig. 7. The sample of contaminated scallop livers was from a material

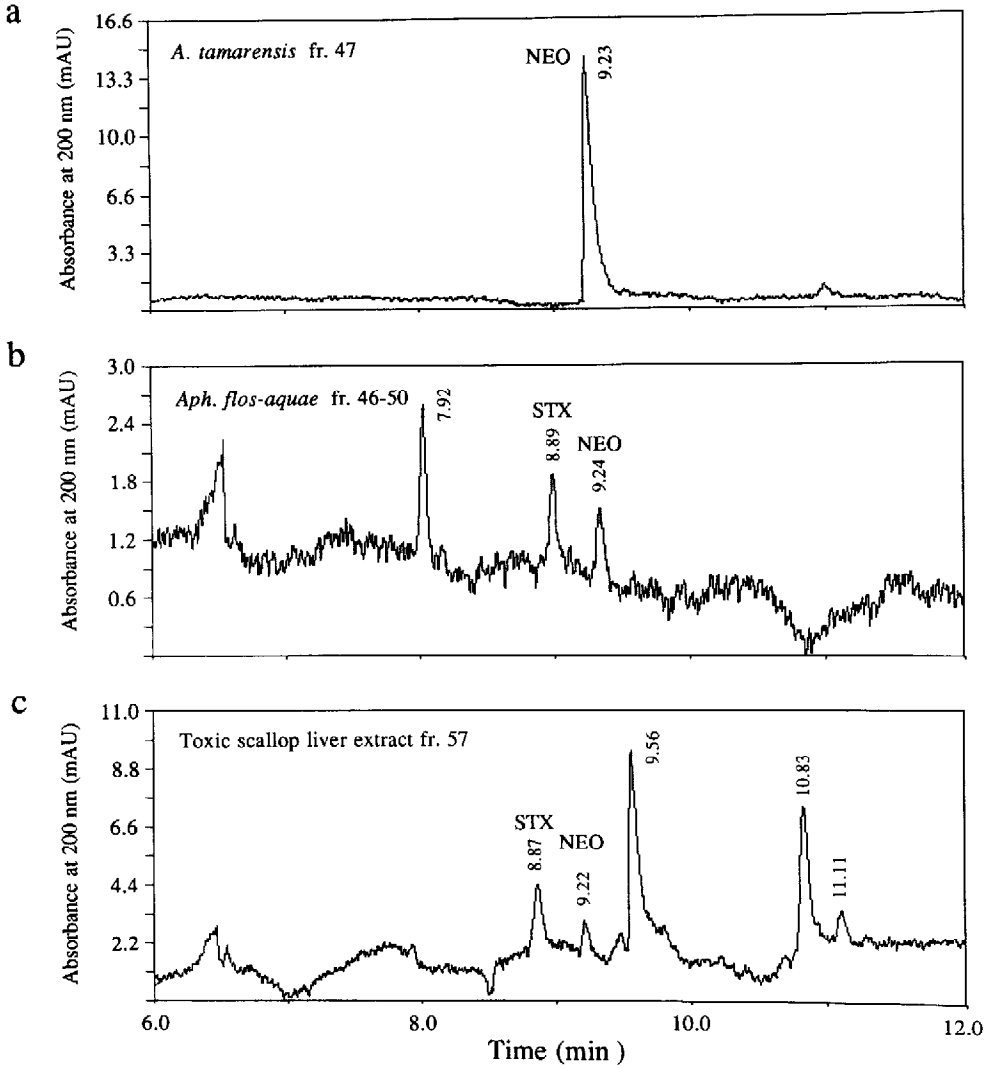


Fig. 7. CE analysis of toxin fractions isolated from different matrices. (a) Fraction 47 from an extract of sonicated cells of *A. tamarensis*; (b) combined fractions 46–50 from an extract of sonicated cells of *Aph. flos-aquae*; (c) fraction 57 from toxic scallop liver extract. Conditions: detection at 200 nm, 3-s vacuum injection, +30 kV, 20 m*M* sodium citrate buffer.

currently under evaluation for its suitability as a PSP reference material. In each instance extracts from sonicated cells were fractionated using a Bio-Gel P-2 column as described previously. Fig. 7 illustrates the electropherograms obtained for three arbitrarily chosen fractions of each biological matrix. The migration times observed for STX and NEO were consistent with those previously obtained on different samples. Although the presence of a high salt content (possibly sodium) could be detected easily by the fronting peak at 6.5 min in Fig. 7b and c, the migration times were reproducible to within 2% on any given day. In addition to observing STX and NEO at the correct migration times, several unknown compounds (possibly amino acids or other dicationic PSP toxins) were also detected, as illustrated in Fig. 7b and c.

Although the migration times for STX and NEO were consistent for all samples analysed, it was observed that high salinity of the sample could complicate the peak assignment. In experiments using solutions of STX prepared in buffers of various salinities, ranging from 0 to 0.5 *M* NaCl, it was found that variation of migration times of more than 2% was observed only when the sample contained more than *ca.* 0.3 *M* NaCl. Above this concentration, loss of resolution between STX and NEO and increased background contribution were also noted. These observations can be explained by the rise in electrophoretic current with increasing salt concentration, resulting in changes in temperature and consequent effects including convection.

The determination of STX and NEO in the fractions from biological extracts presented in Fig. 7 is summarized in Table I. Where possible, the results from the quantitative analysis obtained using CE–UV were correlated with those given by FIA–ISP-MS [19]. However, the latter technique can be used only with relatively pure samples of STX and NEO, as salts and other contaminants present in the fraction interfere in the analysis by forming adduct ions, which reduce the abundance of the protonated molecular ion. In spite of this limitation, useful comparisons were obtained for samples of low salt content, such as fraction 47 from the *A. tamarensis* extract (Fig. 7a). In this instance the two techniques yielded comparable results for the determination of NEO, *i.e.*, 115 ± 5 μg/ml by CE–UV compared with 121 ± 2 μg/ml by FIA–ISP-MS.

Table II illustrates the detection limits for NEO and STX using different analytical techniques for PSP toxin monitoring. It can be seen that in terms of mass detec-

TABLE I

DETERMINATION OF STX AND NEO IN ALGAL AND MOLLUSC EXTRACTS USING CE–UV

Detection at 200 nm, 3-s vacuum injection, +30 kV, 20 m*M* sodium citrate buffer.

Sample	Concentration (μg/ml)	
	NEO[a]	STX
A. tamarensis, Fraction 47	115 ± 5[d]	n.o.[b]
Aph. flos-aquea, Fractions 46-50	4.1 ± 0.1[d]	3.7 ± 0.1[d]
Toxic scallop liver, Fraction 57	6.4 ± 0.3[c,d]	14.9 ± 0.8[d]

[a] Corrected response for NEO.
[a] Corresponds to *ca.* 5.1 μg/g of wet liver tissue.
[c] Not observed (< 1.5 μg/ml).
[d] Standard deviation, $n = 3$.

TABLE II

DETECTION LIMITS OF STX AND NEO USING DIFFERENT TECHNIQUES

Technique	NEO		STX	
	Concentration (μM)	Amount injected (ng)	Concentration (μM)	Amount injected (ng)
Mouse bioassay [35][a]	0.4	120	0.5	150
HPLC–FLD [35][b]	0.065	0.39	0.014	0.08
FIA–ISP-MS [19]	–	–	0.1	0.03
CE–UV (this study)	5.6	0.018	5.0	0.015

[a] Based on a 1-ml injection.
[b] Based on a 20-μl injection.

tion limits CE–UV is considerably more sensitive than the mouse bioassay. It also offers better detection limits than HPLC–FLD [16,35] and flow-injection analysis using FIA–ISP-MS. However, owing to the limited sample (a few nanolitres) which can be loaded on the CE column, the CE–UV technique lacks the ability to provide good detection limits for samples of low concentration (sub-micromolar range). From a practical standpoint, this limitation can be circumvented by concentrating the sample before analysis by CE–UV, as only a limited amount of sample is required. Further, in situations where the sample is dissolved in a solution of lower conductivity than the running buffer, one can apply sample "stacking", where a large volume of a dilute solution is injected on the CE capillary [28]; this permits injection of a solution typically 3–5 times less concentrated without degradation of signal-to-noise ratios or loss of resolution [28].

Application of CE–UV to the determination of GTX and C-type PSP toxins

In addition to STX and NEO, GTX toxins (Fig. 1), more specifically GTX_2 and GTX_3, are often found in toxic shellfish and dinoflagellates [2,14]. During the analysis of biological extracts by CE–UV, GTX toxins migrated more slowly than STX and NEO as they have an overall charge of +1 under the electrophoretic conditions used. An electropherogram corresponding to the analysis of a purified fraction of GTX toxins isolated from *A. tamarensis* is presented in Fig. 8a. This sample contained a high proportion of GTX toxins, as indicated by the peaks in the region of 16 min. A small amount of NEO (4.4 μg/ml) was also found in the same sample, as evidenced by a peak at 9.17 min. The ionspray mass spectrum of the same fraction is presented in Fig. 8b. An abundant protonated molecular ion was observed at m/z 396, which confirmed the presence of the GTX toxins. Fig. 8a also shows a prominent ion at m/z 316, thought to be a fragment ion corresponding to the loss of an SO_3 group from m/z 396. Although the ion at m/z 316 also corresponds to the MH^+ of NEO, the low concentration of this compound found in the CE–UV trace (Fig. 8a) could not account for such an intense peak in the mass spectrum. The assignment of the ion at m/z 316 was confirmed by tandem mass spectrometry (MS–MS), which showed that this ion is indeed a fragment ion of GTX toxins, rather than the MH^+ ion of NEO.

Owing to the unavailability of standards, the assignment of individual GTX

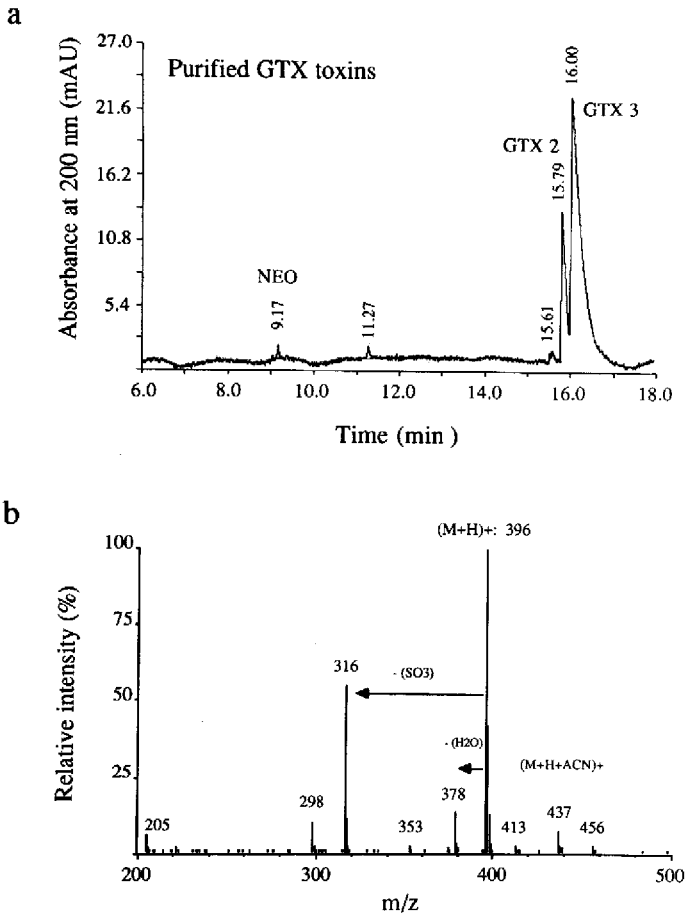


Fig. 8. Analysis of a purified fraction of GTX toxins by (a) CE–UV (conditions as in Fig. 7) and (b) FIA–ISP-MS [0.1-μl injection, flow-rate 50 μl/min, acetonitrile–water (1:1), 0.1% trifluoroacetic acid].

toxins remains difficult at this stage. In order to facilitate the assignments, the peak areas observed in the electropherogram in Fig. 8a were correlated with the results from the HPLC–FLD analysis using post-column reaction modification [16]. Using the latter technique it was found that the purified fraction of GTX toxins contained mainly GTX_3 and GTX_2, at concentrations of 320 and 130 μg/ml, respectively (molar ratio 2.5:1). This result is in reasonably good agreement with that observed in the CE–UV analysis, where a molar ratio of 3.8:1 was obtained for GTX_3:GTX_2. Based on this observation, it was deduced that the early migrating peak at 15.79 min was GTX_2, followed by GTX_3 at 16.00 min.

The preliminary work described above indicates that GTX, NEO and STX toxins can all be identified in a single CE–UV experiment within less than 20 min. However, the conditions chosen here for the analysis of these toxins do not permit the detection of the C toxins as these compounds have no net charge at pH 2.1, and thus

would migrate only with the electroendosmotic flow. The inability to separate the different C toxins is also encountered with the HPLC–FLD method [16], where these compounds are not retained by the column and elute with the solvent front. Work is in progress to establish a direct CE–UV method for the detection of these electrically neutral toxins.

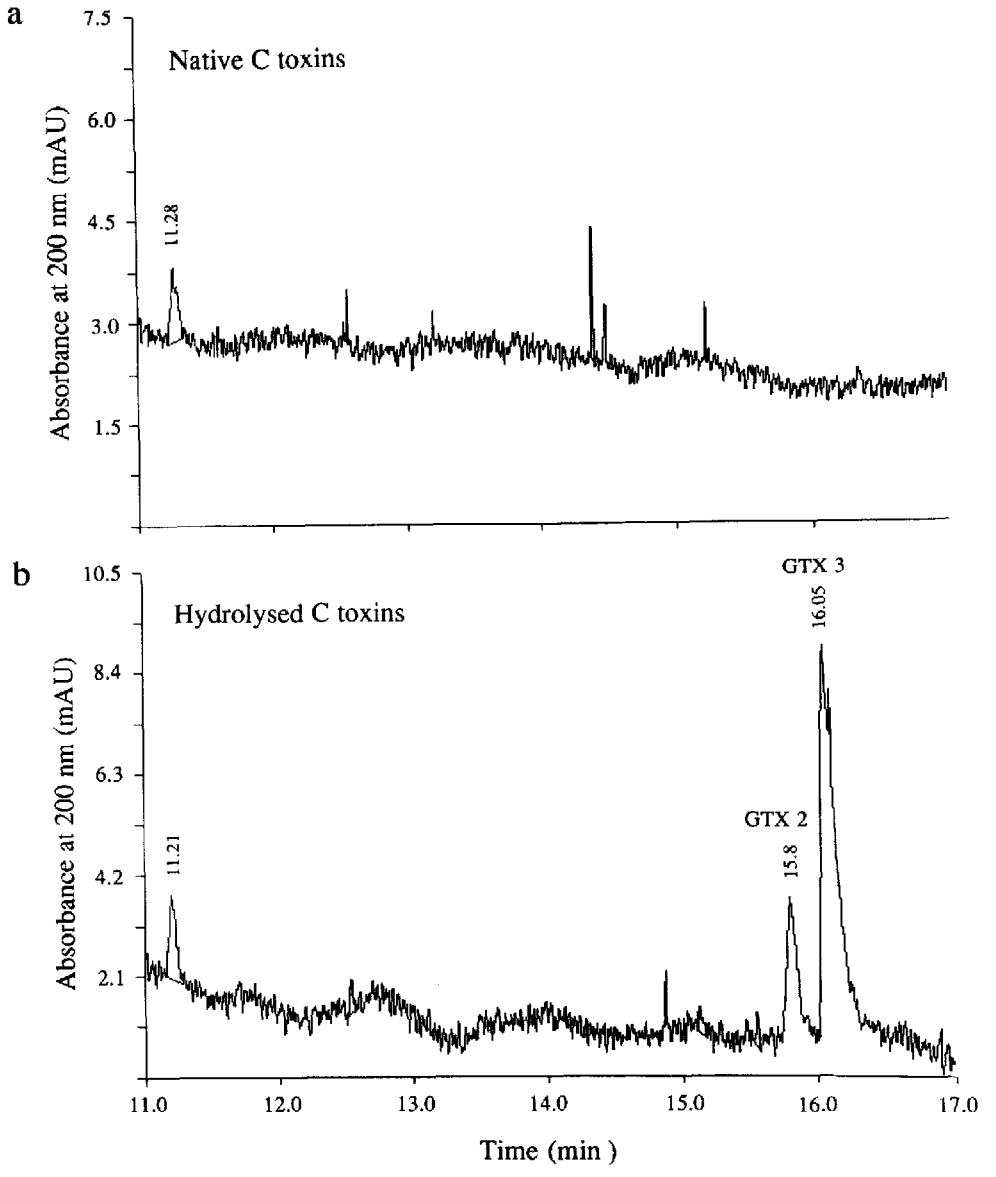


Fig. 9. Analysis of a fraction of C toxins isolated from *A. tamarensis*. Capillary electrophoretic separation of C toxins (a) before and (b) after hydrolysis to the corresponding GTX toxins. Hydrolysis performed with 0.1 *M* HCl at 100°C for 10 min. Electrophoretic conditions as in Fig. 7.

Indirect determination of the C toxins by CE–UV can be achieved by measuring their hydrolytic products. Conversion of the C toxins into the corresponding GTX forms is achieved by briefly heating in 0.2 *M* hydrochloric acid [17]. This procedure is used in the standard AOAC mouse bioassay method [8], which converts the latent C toxins into the more toxic GTX forms. A pooled fraction containing mostly C toxins was divided into two equal samples, one of which was subsequently hydrolyzed with 0.1 *M* hydrochloric acid at 100°C for 10 min whereas the other was left intact. The CE–UV trace corresponding to the native C fraction is presented in Fig. 9a, and shows that no GTX toxins are observed at 16 min. However, this sample contained an unknown constituent at 11.28 min, which could be a degradation product of C toxins resulting in the formation of a dicationic product. In comparison, the hydrolyzed fraction of C toxins (Fig. 9b) clearly shows two peaks at 15.80 and 16.05 min, which correspond to GTX_2 and GTX_3, respectively. The ratio of the areas of these two peaks is 0.22. From the hydrolytic products of this reaction it was concluded that the pooled C fraction contained mainly C_1 and C_2 toxins.

The sample corresponding to the hydrolyzed C toxins was later subjected to the HPLC–FLD analysis to establish the toxin concentrations. This analysis confirmed the presence of GTX_2 and GTX_3 at concentrations of 29 and 70 μg/ml, respectively, a ratio of 0.41. Assuming that the concentrations of the GTX toxins are accurate, in the absence of pure standards the electropherogram in Fig. 9b corresponds to injections of *ca.* 290 and 700 pg of GTX_2 and GTX_3, respectively. For this analysis the signal-to-noise ratio observed with GTX_2 was *ca.* 8:1, indicating that the method appears to be less sensitive for the detection of GTX toxins than for NEO and STX. This observation is consistent with the sensitivity loss expected as the migration time is increased.

CONCLUSIONS

The work outlined above demonstrates the potential of CE combined with either UV or MS detection for the rapid and efficient determination of PSP toxins. Identification of the electrophoretic peak observed with UV detection was confirmed by MS using ionspray ionization. Although these compounds do not contain a chromophore with a significant molar absorptivity in the wavelength range 220–500 nm, the high sensitivity of the detector used permitted detection limits of 15 and 18 pg for underivatized STX and NEO, respectively, at 200 nm. Owing to the small volume of sample that can be injected onto the capillary (a few nanolitres), the concentration detection limit is of the order of 5 μ*M* for these two toxins. It is expected that improvements in sensitivity could be achieved by using pre- or post-column fluorescent derivatization.

Application of the CE–UV method has been demonstrated for the separation of PSP toxins and for the determination of STX and NEO found in plankton and in scallop liver extracts. In the presence of high salt concentrations in the sample (up to 0.3 *M* NaCl), the CE method reported here yielded reproducible results in terms of both migration times and sensitivity. Further investigations are in progress to evaluate the sample treatment required to analyse raw extracts from different sources and the results will be reported separately.

In comparison with existing techniques such as the mouse bioassay and HPLC

using fluorescence detection, the determination of PSP toxins by CE–UV holds promise for the routine screening of these compounds in natural extracts. Particularly attractive are its ease of operation, small sample consumption, speed of analysis, separation efficiency and the potential for automation.

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