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DEVELOPMENT OF HIGH-RESOLUTION SEPARATORY TECHNIQUES AND MASS SPECTROMETRIC METHODS FOR THE VERIFICATION OF SAXITOXIN AND TETRODOTOXIN IN WATER AND SOIL

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DEVELOPMENT OF HIGH-RESOLUTION SEPARATORY TECHNIQUES AND MASS SPECTROMETRIC METHODS FOR THE VERIFICATION OF SAXITOXIN AND TETRODOTOXIN IN WATER AND SOIL.

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A. BACKGROUND.

Nature produces many compounds that are highly toxic to humans. Among the most toxic are certain metabolites produced by marine organisms; these include saxitoxin (STX), a member of a related family of compounds[1] responsible for paralytic shellfish poisoning (PSP, the "red-tide" shellfish poisoning), and tetrodotoxin (TTX), the pufferfish toxin[2], (see Schemes I and II for structures).

These toxins have been recognized by the UN as potential chemical warfare agents. It is important therefore to have available analytical methods for verification procedures with respect to chemical warfare controls agreements. The extremely high toxicity of these marine toxins implies that such analytical methods must be capable of detecting, identifying, and if possible quantifying, the target compounds present at trace levels in complex matrices. (Symptoms of PSP are primarily neurological and can vary from facial paresthesis, nausea and vomiting, to death resulting from respiratory paralysis within 12 hours of ingestion; as little as 6-40 μg of PSP toxins per kg of body weight can cause illness[3,4]. No antidote is currently known).

The matrices appropriate to such chemical warfare verification procedures are fresh water and soil. These matrices are likely to be appreciably less complex than the shellfish tissues of interest in public health protection from PSP arising from consumption of cultured shellfish (Fisheries and Oceans Canada, and Health and Welfare Canada). Accordingly, the major initial thrust in the work conducted by the Institute for Marine Biosciences (IMB) of the National Research Council (NRC) has been to develop instrumental methods of analysis of appropriate extract solutions containing these toxins; development of appropriate extraction and concentration procedures, for the matrices of concern to the Department of National Defence (DND), will be undertaken in the current financial year (1991/92), the final year of the financial agreement between DND and IMB.

The present document thus represents an interim report on work done at IMB on instrumental techniques for trace analysis of these highly polar, thermally labile toxins. STX is the best known of the PSP toxin family, and is the only member of the family mentioned explicitly in the formal agreement with DND. However, it is the opinion at IMB that, since the PSP toxins are produced naturally as mixtures whose composition varies with the source, the interests of DND are best served by methods which can verify all of the PSP toxins as well as TTX. This approach coincides with that necessary for monitoring cultured shellfish, which become contaminated with PSP toxins *via* their consumption of the particular phytoplanton species which are the primary producers of the PSP toxins.

For purposes of discussion, this report is divided into three main sections, *viz.* preparation of highly purified toxin standards, development of analytical methods based upon relatively simple instrumentation, and finally techniques incorporating mass spectrometric detection as ultimate referee methods for verification.

B. PREPARATION OF HIGHLY PURIFIED TOXIN STANDARDS.

It is a truism in analytical chemistry that the reliability of both qualitative identifications and of quantitative analyses is contingent upon availability of highly purified standard samples of the target analytes. In the present context, the success thus far at IMB is due in no small degree to the achievement of Dr. M.V. Laycock of IMB in preparing pure samples, on the tens-of-milligrams scale, of STX, its N-hydroxy derivative neosaxitoxin (NEO, see Scheme I), and an equilibrium mixture of the sulfated forms GTX-2 plus GTX-3 (these latter two compounds are stereoisomers of one another (see Scheme I), and appear to be readily interconverted to an equilibrium mixture under conditions normally used to store the PSP toxins). Dr. Laycock's achievement depended, in turn, on successful culturing of the phytoplankton producers by Dalhousie University under contract to IMB. The planktonic biomass is considerably less complex, and thus easier to extract and purify, than the alternative source of these compounds, *viz.* naturally contaminated shellfish.

The PSP toxins (and TTX) are extremely hygroscopic, and it is unrealistic to supply them as analytical standards in the form of pure solids. IMB has initiated a study of the stability of pure PSP toxins in solution, as a function of solvent composition and pH, and of variables such as exposure to sunlight, temperature, *etc.* This stability study is the responsibility of Dr. P. Thibault of IMB, and is expected to continue until at least Fall, 1991. If a set of storage conditions can be found, which permit negligible decomposition of these toxins over periods of the order of one year or greater, it is planned to develop instrument calibration solutions for sale through NRC's Marine Analytical Chemistry Standards Program. (This program already offers a calibration solution of the unrelated marine toxin, domoic acid[5]). The availability of certified standards is of crucial importance for all analyses of these toxins, including those of direct interest to DND.

As a result of the availability of pure toxins, IMB has been able to evaluate relative molar responses of STX and NEO with respect to various detection techniques. This study was coordinated by Dr. M.A. Quilliam of IMB, and was complicated by the extreme hygroscopicity of these compounds which precluded accurate knowledge of quantities by weighing, despite the elaborate precautions taken in a series of weighing experiments devised by Dr. Quilliam. The very high purity of Dr. Laycock's preparations, however, permitted quantitation of STX and of NEO in their respective solutions by integration of NMR spectra (Dr. J.A. Walter and Mr. D. Leek, of IMB) with calibration of integrated proton absorption intensities in terms of those obseved for standard solutions of sucrose. These NMR experiments on concentrated stock solutions provided an adequate replacement for the preferred weighings as ultimate measurements of quantities of substance. In this way it was shown that, to within experimental uncertainty, the relative molar response of NEO to that of STX is unity ($\pm$ 0.022) for ionspray mass spectrometry (see Section D below), but has a value of 0.855 $\pm$ 0.037 for UV detection at 200 nm (see Section C below, under capillary electrophoresis methods) and a value of 0.267 $\pm$ 0.011 for the Sullivan HPLC method with fluorescence detection, as implemented at IMB (see also Section C). Extension of such measurements to other PSP toxins should be possible.

Unfortunately, IMB has no access to natural sources of TTX and is thus dependent for standard samples of this toxin upon the commercial supplier (SIGMA Chemical Corpn., St. Louis, MO). The ionspray mass spectrum of this standard, obtained by flow injection analysis (*i.e.* no separatory step) and published previously[23], showed mass peaks which were not present in either the tandem mass spectrum of the $(M+H)^+$ ion from TTX, nor in the corresponding spectrum of STX; this suggests that the TTX standard contains impurities which respond to ionspray ionization. Other evidence will be presented below (see Sections C and D) suggesting that this commercial TTX preparation is not pure. The commercially available STX standard (CALBIOCHEM Inc.) was shown to contain a significant impurity (see Section D below), and appears to be currently (April 1991) unavailable from the supplier.

C. INSTRUMENTAL ANALYTICAL METHODS WITHOUT MASS SPECTROMETRY.

Public health protection against PSP from contaminated shellfish is provided by the mouse bioassay procedure as prescribed by the Association of Official Analytical Chemists (AOAC)[6,7]. This procedure involves intraperitoneal injection of an HCl extract of shellfish tissue into a laboratory mouse of specified type and body weight; death from PSP then results with characteristic symptoms. Reasonably quantitative estimates of "total PSP toxicity" are thus obtainable from the observed time-of-death of the test animal. Shortcomings of this AOAC procedure, apart from its dependence upon use of lab animals, are its narrow dynamic range, limited sensitivity (about 40 μg per 100 g of shellfish tissue, compared with the permissible limit of 80 μg per 100 g for shellfish for human consumption), its lack of specificity, and variability due to effects of co-extracted salts and dependence on mouse strains. Nonetheless, the lack of specificity of the mouse bioassay has a positive aspect, in that it provides excellent protection against new or unexpected toxins. The method is currently regarded as essential to ensure public safety with respect to consumption of shellfish, although there is growing pressure in many European countries to discontinue the use of live animals for such monitoring programs.

Instrumental methods of analysis of PSP toxins with spectroscopic detection have been hampered by lack of a chromophore absorbing significantly at wavelengths greater than 220 nm or so, the usual range for UV-visible detectors. Neither the perhydropurine skeleton plus fused 5-membered ring, nor any of the functional groups in the PSP toxins (Scheme I), absorb significantly above 220 nm; the same is true of the TTX molecule (Scheme II). The highly polar, thermally labile nature of these compounds precludes analysis by gas chromatography (GC). Accordingly, analytical methods involving liquid-phase separations are required.

C(i). Liquid Chromatography.

The instrumental method most commonly used for routine determination of PSP toxins is

the Sullivan method[8-10], which involves high performance liquid chromatography (HPLC) using a reversed phase technique (mobile phase more polar than stationary phase) with a resin-based column. Due to the ionised state of PSP toxins (and TTX) in solution, good chromatographic behaviour on the resin column requires use of ion-pairing reagents (usually alkyl sulfonates). Detection of the toxins in the Sullivan method[8-10] involves post-column oxidation of the effluent, with fluorescence detection of the purine ring system thus produced. The Sullivan method provides adequate sensitivity and dynamic range for separation and detection of most PSP toxins. However, successful operation of a "Sullivan train" is highly specialised; the sensitivity is highly dependent upon the efficiency of the post-column chemistry *via* parameters such as reagent concentrations, reaction times, pH, and temperature. In addition, the different PSP toxins have widely different overall sensitivities (see *e.g.* the discussion, in Section B above, of the relative response factor for NEO relative to that of STX), and their variations with experimental parameters also vary. Further, TTX is not oxidised efficiently to give a fluorescent derivative under the Sullivan conditions, though it can be oxidized under more extreme conditions. **For these reasons, the Sullivan HPLC method[8-10] is not recommended for the purposes of occasional monitoring for these toxins in water and soil.** Successful operation of the Sullivan method requires meticulous attention to detail, including peculiarities of the particular instrumental components in use, on a continuing basis; however, when these conditions are met, it has been shown[11] by Dr. S. Ayer and Mr. J. Uher of IMB that reliable quantitative analyses of PSP toxins are possible provided that appropriate standards are available.

Nonetheless, in view of the widespread availability of HPLC equipment, an analytical method based upon this separatory technique is desirable. As described in Section C(ii) below, the native PSP toxins and TTX do exhibit adequate optical absorbance at wavelengths in the range 190-200 nm. Provided that a suitable mobile phase could be found which is transparent at these wavelengths (and this excludes the Sullivan chromatographic conditions[8-10]) optical detection of the toxins could be accomplished directly without the necessity for post-column oxidation to fluorescent products. Note, however, that almost every organic molecule absorbs at 200 nm so that such an HPLC detection technique would be highly non-specific. The reason for the success of the CE/UV technique for PSP toxins and TTX, described in Section C(ii), is that the necessary degree of selectivity is provided by the fact that these compounds exist as ions under the conditions used, and thus migrate efficiently under the applied electrophoretic field. (It is also possible to use an optically transparent buffer system in this CE method).

If fluorescence detection of the PSP toxins is to become more reliable, it will be necessary to exert better control over the oxidative chemistry. An alternative approach, which uses oxidation by peroxide or periodate under mildly alkaline conditions in a batch reactor under controlled conditions prior to injection, has been developed by Dr. J. F. Lawrence of Health Protection Branch, Health and Welfare Canada, Ottawa. The reaction products are aromatic with strong UV absorption at wavelengths >300 nm, and are strongly fluorescent. In addition, the chromatographic behaviour of the oxidation products is vastly improved relative to that of the native PSP compounds, and preliminary work at IMB suggests that there is considerable potential for LC/MS

analysis of the oxidised forms. Preliminary work[12] on pure standards, obtained from the U.S. Food and Drug Administration (Dr. S. Hall) demonstrated detection limits in the range of 20-50 pg for the non-N_1-hydroxylated toxins, and 100-500 pg for the N_1-hydroxy analogues. This work was later extended[13] to successful analysis of contaminated shellfish although chromatographic separation of all of the 10 toxins was not achieved; in addition, more than one oxidation product was observed for each toxin. Despite these difficulties, the authors concluded[13] that the new method performed about as well as the Sullivan post-column oxidation method[8-10] for those PSP toxins not subject to interferences and other ambiguities, but is much simpler to implement and is particularly suited to monitoring PSP toxins on an occasional basis. Work has been started by Dr. M.A. Quilliam at IMB to try to improve the chromatography and to use LC/MS/MS in an attempt to identify the reaction products; it will be necessary to investigate the nature of the chemistry involved in the oxidation reaction, in order to maximise yields, minimise side-reactions, *etc.* Figure 1 shows the result of HPLC analysis of the periodate reaction product of a pure saxitoxin standard, monitored by fluorescence detection (experimental details are included on the Figure itself); this chromatogram looks extremely promising, but the corresponding trace obtained using UV absorption detection showed an additional reaction product. Clearly, there are still considerable problems to be cleared up before this method[12,13] can be regarded as proven. Although work on this approach has only just started at IMB, in collaboration with Dr. Lawrence, it looks very promising for the PSP toxins. It is clear that the same oxidative conditions, as used[12,13] for the PSP toxins, can not produce a fluorescent product from TTX; much more extreme conditions, involving boiling with strongly alkaline periodate, are required. This aspect will also be investigated at IMB.

C(ii). Capillary Electrophoresis.

Electrophoresis is a general term applied to the phenomenon of migration of charged molecular species through a solution under the influence of an applied electric field. Several variants of this technique are in common use by biochemists, in order to separate polar biological molecules such as peptides and proteins. An early application of such classical biochemical techniques to PSP toxins involved separation of some of the toxins by cellulose acetate electrophoresis[14]. Work by Dr. Laycock of IMB, associated with his successful isolation of pure PSP toxins (see Section B above), used high-voltage paper electrophoresis to monitor relative amounts of classes of PSP toxins.

Capillary electrophoresis (CE) is a new approach to electrophoresis in which the traditional gel slabs , paper sheets, *etc.*, are replaced by narrow bore (typically 50-100 μm i.d., and approx. 360 μm o.d.) fused silica capillaries of length 50-150 cm. The capillaries are coated on the outside with a thin polyimide film in order to preserve flexibility and mechanical strength. CE accommodates very high voltages (up to 30 kV, giving field strengths up to 600V/cm) and current densities (up to 5A/cm^2, equivalent to currents of up to 300 μA depending on the nature of the supporting buffer) because of the efficient dissipation of Joule heat made possible by the large ratio of surface area to volume. In turn this efficient cooling results in minimal radial temperature gradients, thus

minimising problems associated with convection and variations in viscosity across the capillary cross section. This radial uniformity is the ultimate guarantor of the very high separation efficiencies (up to 10^6 theoretical plates in 20-25 min) achievable using CE. It is not appropriate here to describe in detail the physical principles underlying analytical CE; excellent expositions are available in the literature[15-17]. However, it is worthwhile for present purposes to emphasise that emergence of an analyte from one end of the capillary is the result of interplay of two different transport mechanisms. The first of these is the electrophoretic mobility of the charged analyte species through the supporting buffer solution, under the influence of the applied field; this mobility is a function of the size (and possibly shape) of the solvated species, and of its net charge. The second transport mechanism is electroendosmosis, which is the bulk flow of liquid resulting from the effect of the applied field on the electrical double layer adjacent to the capillary wall. Figure 2 illustrates these two effects as a function of pH, for the present case where the net charge on the silica internal wall is negative so that the adjacent double layer carries a net positive charge, so that this annulus of positive charge is drawn towards the negative electrode. The bulk liquid flow that results is characterised by its flat profile, in contrast to the parabolic profile typical of flow induced by pressure difference (Figure 3); thus the flow profile does not contribute to band broadening, as is the case for HPLC for example. The other feature of CE which will be important for Section D below is the very low volume flowrate (of the order of 100 nL/min) emerging from the capillary.

The first published application of CE to analysis of marine toxins was due to Wright *et al.*[18]. Fluorescence tagging agents were used to derivatize the primary amine ($-NH_2$) or imine ($=NH_2^+$) groups of STX, TTX, and a peptide marine toxin (microcystin toxin). Laser-induced fluorescence detection provided attomole detection limits of parts-per-trillion solutions, and excellent separations of the derivatized toxins obtained. Such a technique appears highly promising for toxins present in clean matrices such as fresh water, and the detection limits will be hard to match with any rival technique. Drawbacks of this approach include the non-specificity of the tagging reagents which could lead to considerable interferences in extracts of more complex matrices thus implying a requirement for extensive clean-up procedures, the difficulty of ensuring complete derivatization and the chemical instability of the derivatives used[18], and the highly specialised equipment required. In addition the method suffers from the drawback common to all procedures using a single parameter detector, *viz.* analyte identification is possible only *via* the migration time. No investigation of dynamic range was reported[17].

At IMB, an approach has been developed (Dr. P. Thibault) based on CE with UV detection at 200 nm. Although the detection limit thus obtained for STX (about 15 pg injected) is about 10^3 times greater than that achieved[18] using laser-induced fluorescence detection, this technique does have the advantages of a linear dynamic range of 2-3 orders of magnitude, of not requiring chemical derivatization and associated clean-up, and of considerably less specialised instrumentation requirements. The rather unusual UV detector, which is usable with good signal/noise ratios to wavelengths as low as 190 nm, is commercially available as the standard detector on the Applied Biosystems Inc. CE instrument used at IMB. This IMB work has been extremely successful, with proven

application to analysis of phytoplankton and scallop tissues for PSP toxins. The work has been accepted for publication[19], so no further experimental details are included within the present report.

Work on this CE/UV methodology is still in progress. Figure 4a shows an electropherogram of a solution containing 150 μg/mL of each of the commercial TTX standard and of the IMB STX standard (no detectable impurities in the latter); this electropherogram corresponds to 1.5 ng of each on-column. The objective of this experiment was not to determine detection limits, but to demonstrate separation of STX and TTX, and to investigate the purity of the latter; however, the response factors for TTX and STX are closely similar (Figure 4a), so the detection limits are presumably the same also. A partly-resolved peak is seen at a slightly shorter migration time than that of TTX. The characterization of this impurity (5-10% of the main TTX peak) is described in Section D(iii) below.

C(iii). Summary of Non-Mass Spectrometric Methods.

From the point of view of analyzing for PSP toxins and TTX, in soil and fresh water, for purposes of monitoring for chemical warfare, the two most promising approaches not involving mass spectrometry are currently the Lawrence pre-column oxidation HPLC method[12,13] with fluorescence detection (though unlikely to be applicable to TTX without considerable modification), and the CE method with UV detection at 200 nm[19] in which the necessary selectivity is provided by the separatory technique rather than by the detector. The Sullivan HPLC method[8-11] with post-column on-line oxidation to fluorescent products is too idiosyncratic to be used on an occasional basis. The extremely sensitive CE method[18] employing laser-induced fluorescence of pre-derivatized toxins suffers from several drawbacks, summarised in Section C(ii), for real-world samples, though it does work for TTX). However, in this regard the DND interest in monitoring fresh water could possibly be met by a version of this method[18] in which a less ambitious fluorescence detector is employed.

All of these methods, however, use non-specific detectors and thus rely on retention (or migration) times alone for analyte identification. For ultimate verification, it will be necessary to use mass spectrometric detection, and this aspect is considered in Section D.

D. MASS SPECTROMETRIC DETECTION OF PSP TOXINS AND TETRODOTOXIN.

These highly polar molecules are impossible to analyze using classical mass spectrometric techniques, such as electron ionization (EI) and chemical ionization (CI) in which vaporization of the analyte is a necessary prerequisite for ionization. However, they are ideal candidates for ionization techniques in which the sequence of events may be formally considered as pre-ionization in solution followed by sputtering or evaporation of

these ions from a liquid surface. Indeed, fast-atom bombardment (FAB) mass spectrometry of PSP toxins and of TTX has, in the batch (direct insertion probe) mode, been shown[20-22] to be a useful means of structural confirmation at moderate sensitivity. The direct insertion probe is not applicable to ionspray mass spectrometry; the relevant batch-mode technique is flow-injection analysis (FIA), in which an HPLC loop injector is used to inject 1-5 μL of solution of sample into an appropriate flow of mobile phase, but without passing through the HPLC column. (With care it is possible to arrange a post-column injector without introducing significant dead volume into the chromatographic train; such an arrangement facilitates optimization of the mass spectrometric parameters for the target analytes, under conditions simulating those of the actual LC/MS analysis). It was shown by Quilliam *et al.*[23] that FIA ionspray detection limits, using selected ion monitoring on the protonated molecules $(M+H)^+$ were 30 pg for STX and 200 pg for TTX, representing concentration detection limits of 0.1 μmol/L and 0.6 μmol/L, respectively (1 μL injection). These concentration detection limits may be compared with those for HPLC with post-column on-line reaction plus fluorescence detection, *viz.* 0.014 μmol/L with 20 μL injection for STX by the Sullivan method[8-11], and 0.3 μmol/L with 40 μL injection for TTX[24]. Acquisition of full-scan ionspray mass spectra, or of tandem mass spectra of the protonated species $(M+H)^+$, requires 100-1000 times greater quantity of sample injected[23]. Recently[25], thermospray ionization of STX was reported, though considerable ambiguities of interpretation were encountered.

However, the present problem is considerably more complicated than mass spectrometric analysis of highly purified compounds in batch mode. The likely occurrence of the target analytes in complex matrices implies that the mass spectrometer will have to be interfaced directly to high-resolution separatory techniques such as HPLC and CE. A brief general account of such interfacing techniques is attached as Appendix A of this report. The main thrust of the work in this area at IMB, for polar analytes including the marine toxins, has involved ionspray ionization; experience at IMB with continuous-flow FAB has been frustrating and time-consuming, with little reward for the considerable effort devoted to the experiment[26]. Practitioners who report success with continuous flow FAB appear to maintain an ongoing commitment to this technique, with at least one mass spectrometer devoted to it full time. In contrast, occasional set-up and optimisation of ionspray LC/MS experiments is probably no more difficult than for GC/MS. Accordingly, the only experiments described below involve ionspray as the LC/MS and CE/MS interface.

In the case of the LC/MS work, the mass spectrometry *per se* was not a problem; considerable effort was devoted to devising chromatographic conditions which were compatible with ionspray ionization. Thus, the ion pairing reagents used in the Sullivan HPLC method[8-11] suppressed ionization of the PSP toxins and TTX, so alternative mobile phases had to be investigated. The CE/MS work involved a more general problem of devising a rugged, reliable interface which yielded month-to-month reproducibility.

D(i). Ionspray Flow-Injection Analysis of Purified Toxins.

While the FIA technique can not be considered a serious candidate for analysis of real-world samples, due to possibilities of interferences and suppression of ionization by salts and other impurities, it can provide a valuable rapid screening method.

Examples are provided by observations made (Dr. S. Pleasance) while monitoring of fractions during the isolation and purification of PSP toxins at IMB. For example, Figure 5a shows a FIA ionspray spectrum of a mixture of STX plus NEO (m/z 300 and 316 for $(M+H)^+$ ions); the peak at m/z 338 is assigned as sodiated NEO, and that at m/z 282 represents loss of H_2O neutral from the STX protonated molecule. Following several separative steps, the two purified fractions gave the FIA spectra shown as Figures 5b and 5c. In both cases the doubly-protonated species $(M+2H)^{2+}$ are observed at low intensity (m/z values rounded up to integral values in annotation in Figures 5b and 5c). The impurity peak at m/z 268 in the STX fraction (Figure 5c) was observed in several isolates of PSP toxins prepared at IMB. Tandem mass spectrometry and comparisons with a standard quickly revealed this impurity to be the nucleoside, adenosine.

Occasional observation of an impurity peak at m/z 399 in some of Dr. Laycock's isolates led to full-scan examination of the commercially available STX standard (CalBiochem Biochemicals, San Diego, CA); a typical spectrum of a freshly opened vial is shown in Figure 5d, where the impurity at m/z 399 is seen to be the base peak with other intense ions at m/z 416, 430, 371 and 388. This observation has potentially serious implications, since the CalBiochem STX standard is currently used worldwide as the primary standard for the regulatory monitoring of PSP toxins; note, however, that nothing is known concerning the relative response factors for STX and these impurities *vis-a-vis* ionspray ionization. The suppliers of the standard indicate the purity of their product to be >90% as determined by mouse bioassay and by fluorescence, and >99% as determined "by HPLC". The same major impurity peak at m/z 399 was also observed by other workers using thermospray[25], and by FAB at IMB; tandem mass spectrometry experiments on this ion, produced by either ionspray or FAB ionization, revealed sequential losses of 100 Da to give fragment ions at m/z 299 and 199, and also a fragment at m/z 100. That this ion represents an impurity, and not merely a cluster ion of STX, was confirmed by LC/MS experiments (see below) which showed that m/z 300 (protonated STX) and m/z 399 had significantly different retention times. The workers who used thermospray ionization[25] suggested that this impurity might be a di-acetate salt of STX which has lost both a molecule of H_2 and of H_2O under thermospray conditions; this is difficult to reconcile with the tandem mass spectrometry result, and it is believed that this impurity probably represents a breakdown product from one of the columns used in the fractionation and purification procedures.

Tandem mass spectra of the $(M+H)^+$ ions of both STX and TTX were published previously by Quilliam *et al.*[23], using ionspray FIA. Further experiments have confirmed the non-specific nature of these low-energy collision-induced dissociations, some of which were reported previously[20] for high-energy collisions using the MIKES technique and FAB ionization. The fragment ion spectrum[23] of the $(M+H)^+$ ion of STX (m/z 310)

contains about 15 fragment ions of comparable intensities covering the entire mass range, plus numerous less intense ions; none of these fragments is easily interpretable, apart from that corresponding to expulsion of H_2O. This multitude of available fragmentation channels probably reflects the presence of seven non-equivalent basic nitrogen atoms distributed throughout the STX molecule (Scheme I), each of which is a potential site for protonation and thus for charge-site-initiated fragmentation. From a practical analytical point of view, this wide distribution of fragment ion intensity implies that the sensitivity of any analytical strategy for STX, based upon tandem mass spectrometry (*e.g.* the m/z 310 --> 204 transition), will be inherently limited. Figure 6 shows results of a calibration experiment using flow injection analysis of solutions of the CalBiochem STX standard, with selected ion monitoring; a detection limit of the order of 1 ng is indicated for this technique. In the case of TTX all 3 nitrogen atoms are situated close together at one end of the molecule (Scheme II), so that many fewer fragmentation channels are anticipated. In fact[23], the fragment ion at m/z 162 dominates the fragment ion spectrum of the TTX $(M+H)^+$ ion and, while not readily interpretable, the m/z 320 --> 162 transition could provide the basis for a sensitive and highly specific analytical technique for TTX.

D(ii). LC/MS Analysis of PSP Toxins and TTX.

The search for an HPLC mobile phase which permits adequate chromatographic resolution of the PSP toxins and TTX, but is in addition compatible with ionspray ionization, is currently only partly successful despite a strong effort (Dr. S. Pleasance). Before describing the remaining difficulties, however, it seems worthwhile to report that a specific goal of the DND financial arrangement with IMB has been successfully achieved, *viz.* an LC/MS method for analysis of a mixture of STX plus TTX. Figure 7 shows the result of an LC/MS analysis of an approximately equimolar mixture of STX and TTX standards (75 μg/mL, 1 μL injected) using full-scan mass spectrometric detection; the two traces represent reconstructed ion chromatograms for m/z 300 and 320 (the $(M+H)^+$ ions for STX and TTX, respectively). While the chromatographic peak shapes leave something to be desired, adequate resolution was obtained, with excellent signal/noise ratios. This analysis used a 1mm i.d. PRP-1 resin column, similar to that used in the Sullivan HPLC method[8-11]. However, the Sullivan mobile phase, containing 1.5 mmol/L of each of the ion pairing reagents sodium hexanesulfonate and heptanesulfonate, plus ammonium phosphate buffer, was found to completely suppress the ionization of STX and TTX. Instead a mobile phase consisting of a 10 mmol/L aqueous buffer solution of ammonium formate (pH 6.0), with 5% acetonitrile, was used at a flowrate of 50 μL/min. If the pH is lowered *via* addition of formic acid, the STX and TTX peaks merge together and elute much faster, as expected for species which are predominantly in the protonated form at lower pH values. The sensitivities also increase dramatically as the pH is lowered, and the conditions illustrated in Figure 7 represent a compromise between chromatographic performance and sensitivity.

These best-compromise HPLC conditions were arrived at as the result of an extensive series of trials using PRP-1 resin columns. The performance achievable for a suite of

PSP toxins is illustrated by Figure 8, obtained under similar chromatographic conditions except that an acetonitrile gradient and a 4.6 mm column were used. The mobile phase was based on an ammonium formate buffer (5 mmol/L), programmed from 1 to 3% acetonitrile over the first 5 min, from 3 to 10% over the next 5 min, followed by a hold at 10% for 5 min with an increase from 10 to 50% over the final 5 min of the program. Note that the sulfato-derivatives readily lose SO_3 (80 Da) from the $(M+H)^+$ ions, to give confirmatory peaks in the mass chromatograms for the analogues containing one fewer sulfate group. The chromatographic performance apparent in Figure 8 is adequate, although the isomeric disulfato-analogues C1 and C4 (Scheme I) are not resolved from one another, and indeed emerge with the solvent front. Thus, these compounds are unlikely to be analyzable by this method in real-world samples. Another problem with the PRP-1 resin columns, which is also found to occur to a lesser extent in their application to the Sullivan HPLC method[8-11], is that the performance deteriorates steadily with time until the column requires regeneration. In the case of experimental conditions exemplified by those used to obtain Figures 7 and 8, the chromatographic peaks for the more basic analytes (STX, NEO and TTX) are found at progressively longer retention times and peak widths with increased tailing, until they disappear into the baseline in extreme cases. The early-eluting components (GTX-2, GTX-3, C-1 and C-4) are much less affected by the deterioration of the column. This progressive deterioration in performance of PRP-1 columns represents a source of irreproducibility which must be taken into account, over a day's operation, by frequent injections of standards.

A complementary approach was then tried, using a 1mm bonded-C_8-amino column. It was hoped that the sulfate groups would interact with the bonded amino groups on the stationary phase. Indeed, as illustrated in Figure 9, the elution order was reversed relative to that observed in Figure 8, with the disulfated analogues (C- toxins, Scheme I) eluting last, the monosulfates (GTX group) eluting very early, and the basic toxins eluting with the solvent front. These conditions provide excellent separation for the C-toxins, but would not be useful for the other PSP toxins (nor for TTX).

An obvious next step is to try to combine the complementary properties of the two approaches (Figures 8 and 9), either by a mixed-packing column or by use of two sequential columns, or possibly by column-switching techniques. Some preliminary experiments using the sequential column approach have been tried. Dr. Pleasance will pursue these combined approaches over the next few months. Other packing materials and mobile phase compositions are also being considered.

In summary, although problems remain, considerable success has already been achieved in this difficult aspect of the research program. Chances are good that an LC/MS method, applicable to all of the native PSP toxins plus TTX, will emerge from this work. In addition, as mentioned in Section C(i) above, preliminary experiments on the oxidised forms of the PSP toxins[12,13] suggested that they are likely to be much more amenable to LC/MS analysis than are the native toxins themselves; however, the chemistry involved in the oxidation will need to be sorted out before any such technique can be validated.

D(iii). CE/MS Analysis of PSP Toxins and TTX.

As a necessary step in the development of the CE/UV method[18] for these toxins (see Section C(ii) above), the identities of the peaks in the electropherograms were confirmed by CE/MS. While the CE/MS data published[18] were entirely adequate for this purpose, the efforts required to obtain them (Drs. S. Pleasance and P. Thibault) may fairly be described as heroic. At the time when this early CE/MS work was done at IMB, the interface (Figure 10, based on the co-axial design of Smith *et al.*[27]) was extremely unreliable and temperamental. Other groups have published CE/MS data before and since that time[27-29], but there is not an overwhelming flow of published applications in the current literature. This could suggest that these other groups have also found it difficult to construct a robust, reliable CE/MS interface, capable of yielding data which are reproducible even when acquired on an occasional basis. A later example of the kind of performance obtainable using this "heroic" CE/MS interface is shown in Figure 11, which shows reconstructed ion chromatograms from a full-scan CE/MS analysis of a mixture of PSP toxins including decarbamoyl-STX; extracted mass spectra of each of the main components are shown in Figure 12. Figure 13 shows results of analysis of sonicated and filtered cells, with no other clean-up procedure, of *Alexandrium tamarensis*, using both CE/UV and full-scan CE/MS with the older interface. The native STX and NEO are readily identified in this electropherogram; the latter is readily resolved from the much larger peak due to arginine *via* appropriate reconstructed ion electropherograms (not shown).

However, in the past few months, Drs. Pleasance and Thibault have succeeded in learning from their earlier frustrations to the extent of developing a CE/MS interface, for the SCIEX API III ionspray mass spectrometer used at IMB, which is rugged and reproducible on a month-to-month basis. This greatly improved CE/MS interface is also based on the co-axial design of Smith *et al.*[27] (Figure 10), and will be the subject of a forthcoming publication. An example of results obtained using this new CE/MS interface is given by Figure 4b, which summarises the results of a full-scan CE/MS analysis of the same mixture of STX plus TTX as was used to obtain the CE/UV trace in Figure 4a. (Note that the electrophoresis conditions were very different for the two experiments summarised in Figure 4, so that no simple relationship exists between the two sets of migration times). However, reconstructed ion electropherograms from the CE/MS data confirm the ready separation of STX and TTX, and indicate that the impurity in the TTX has a molecular weight 16 Da less than that of TTX itself, suggesting that the impurity is a de-oxy form.

As an example of the flexibility of this vastly improved interface, an experiment was run in which PSP toxin analytes migrated past the UV detector into the zero-dead-volume T-junction (Figure 10), which is maintained at the potential of the ionspray needle by establishing a reliable electrical connection through the make-up flow of a suitably chosen buffer, and thence via the co-axial transfer line to the ionspray needle itself. It was thus possible to obtain a CE/UV analysis (200 nm) of the toxin mixture in the same experiment in which a CE/MS analysis was also obtained. The CE/MS peaks lagged behind their CE/UV counterparts by a fixed time of several minutes, corresponding to

the time required to traverse the capillary from the UV detector, through the T-junction, to the ionspray mass spectrometer; thus a direct correspondence can be established between the CE/UV peaks and those observed in the CE/MS analysis. Only slight degradation of separation efficiency was detected. This experiment is still in the process of optimization and evaluation, and the final conclusions and results will be communicated to DND when complete. This combined CE/UV/MS experiment, or even a CE/UV/MS/MS variant, has the potential to provide the ultimate degree of specificity possible for analysis of these toxins. As yet, no hard information is available on instrument detection limits or linear dynamic range for the CE/MS part of the combination.

D(iv). Summary of Methods Involving Mass Spectrometry.

As shown previously[23] for pure standards, ionspray is an ideal technique for the mass spectrometry of the highly polar PSP toxins and TTX. Use of flow injection analysis has been shown (Section D(i)) to be an excellent rapid screening technique for toxin preparations which are not heavily contaminated with salts and other potential interferences. The standard HPLC method for PSP toxins, incorporating ion-pairing chromatography, can be reliable but is difficult to implement on anything other than a meticulous, on-going basis, and is not compatible with ionspray. Pre-column oxidation[12,13] to fluorescent products offers the advantage of simpler HPLC conditions which are appreciably more compatible with ionspray mass spectrometry; however, considerable work on the oxidative chemistry involved is still necessary. LC/MS analysis of the native toxins (including TTX) is difficult, but a promising approach has been uncovered in which two complementary sets of chromatographic conditions, each compatible with ionspray ionization, will be combined. Finally, CE/MS analyses of PSP toxins have been demonstrated[18], and have since then been developed into a rugged, reproducible technique which can be combined with CE/UV analysis in the same experiment.

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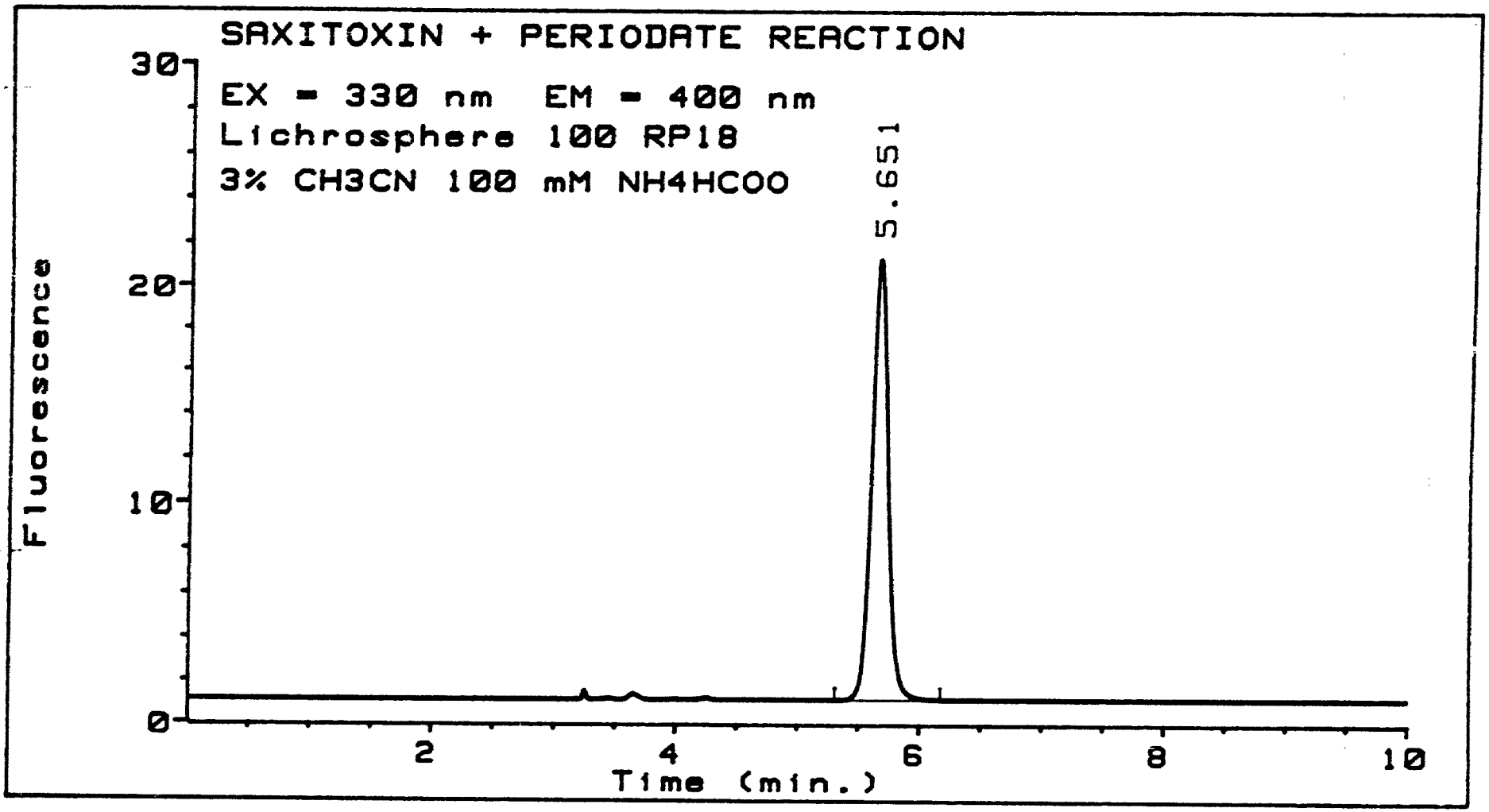


Figure 1. Fluorescence chromatogram of reaction products from mild alkaline periodate oxidation of a saxitoxin standard. Excitation and emission wavelengths 330 nm and 400 nm, respectively. Mobile phase 100 mmol/L ammonium formate aqueous buffer with 3% acetonitrile at 1 mL/min; Lichrosphere 100 RP18 column (250x4.6 mm).

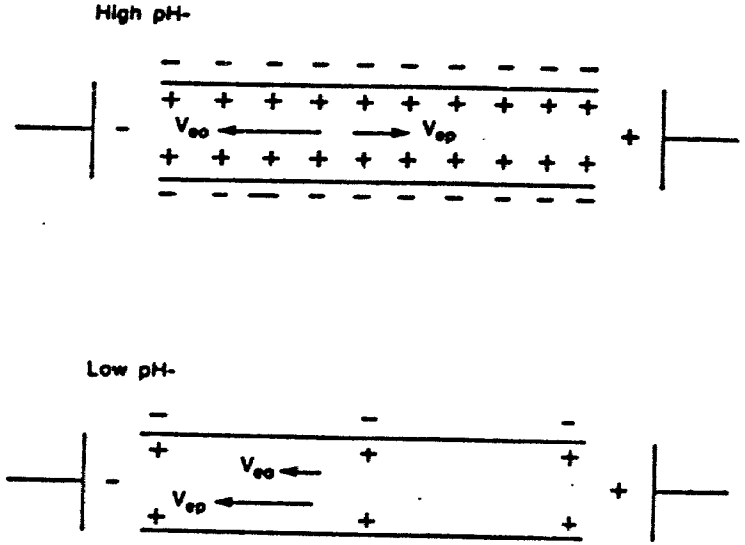


Figure 2. Diagram showing the mechanism of electroendosmosis, indicating the negative charge on the fused silica capillary wall, the positively charged double layer adjacent to the wall, the electroendosmotic velocity v_{eo}, and the electrophoretic velocity v_{ep}. At high pH the negatively charged analyte species undergo electrophoretic migration towards the positive electrode, but at sufficiently low pH they acquire a positive charge and the electrophoretic velocity is directed towards the negative electrode. The direction of the electroendosmotic flow is always towards the negative electrode, but the net concentration of positive charge in the double layer, and thus v_{eo}, varies with pH.

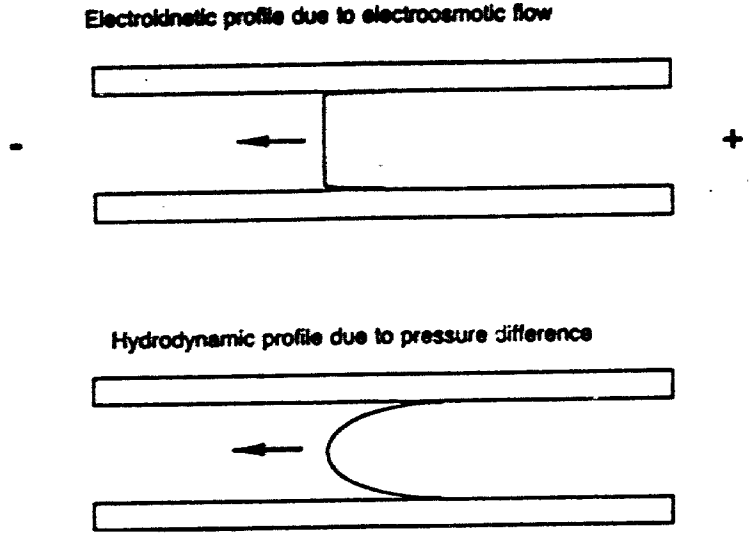


Figure 3. Comparison of the flow profiles characteristic of capillary electroendosmotic flow and of hydrodynamic flow.

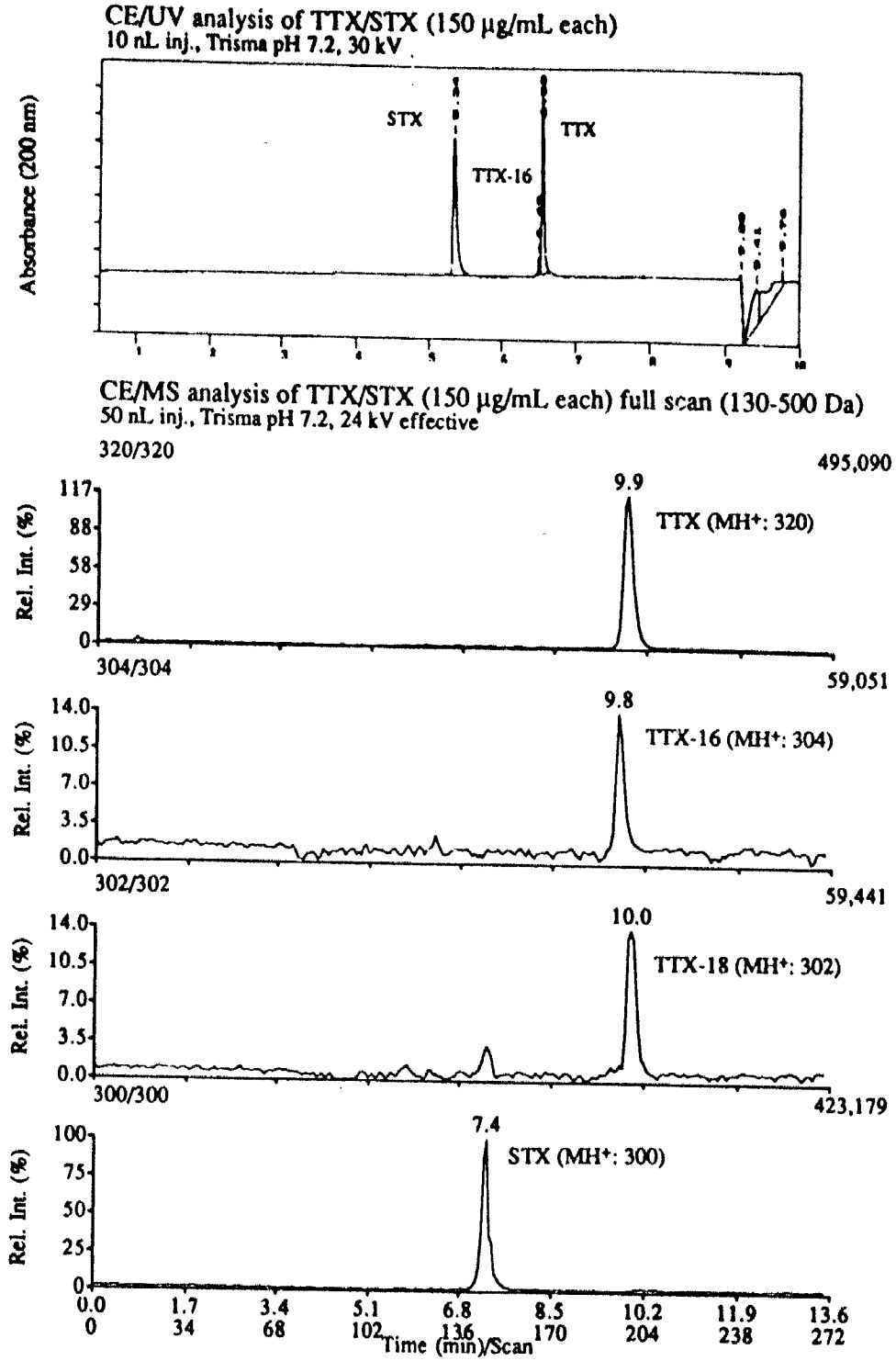


Figure 4. Electropherograms of marine toxin standards in Trisma buffer.
(a) CE/UV analysis (200 nm) of a mixture of STX and TTX standards, approximately 1.5 ng each on-column.
(b) CE/MS analysis of the same solution as that analyzed in (a), approximately 7.4 ng each on-column. Scan range m/z 130-500. Reconstructed ion electropherograms for the most intense ions observed. Note that the migration times are not related to those in Figure 4a.

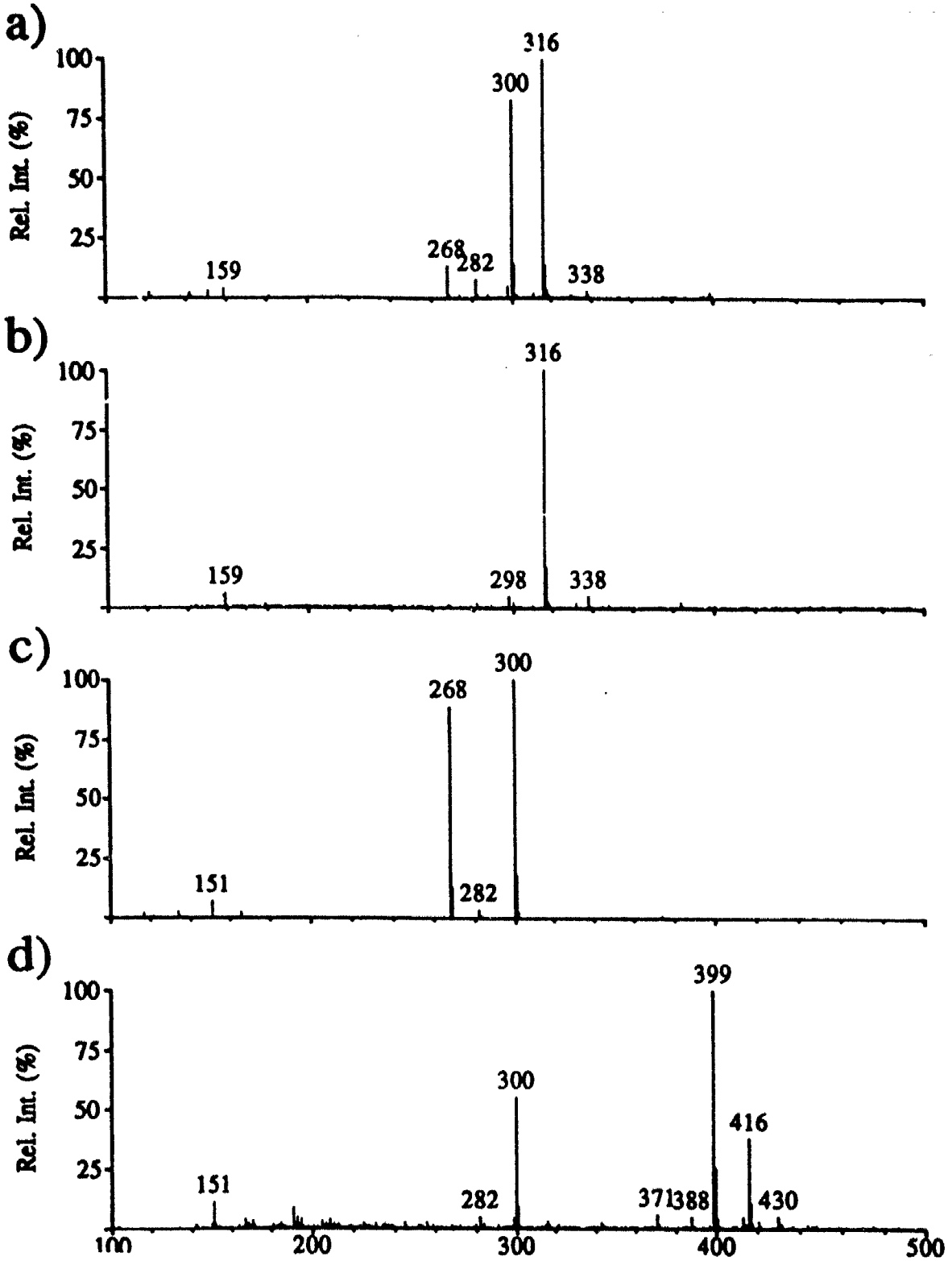


Figure 5. Ionspray mass spectra of marine toxin preparations, obtained by flow injection analysis. (a) Unseparated mixture of STX plus NEO. (b) Separated NEO fraction. (c) Separated STX fraction showing impurity peak at m/z 268, later shown to correspond to adenosine. (d) Freshly opened vial of STX standard purchased commercially (CalBiochem Biochemicals).

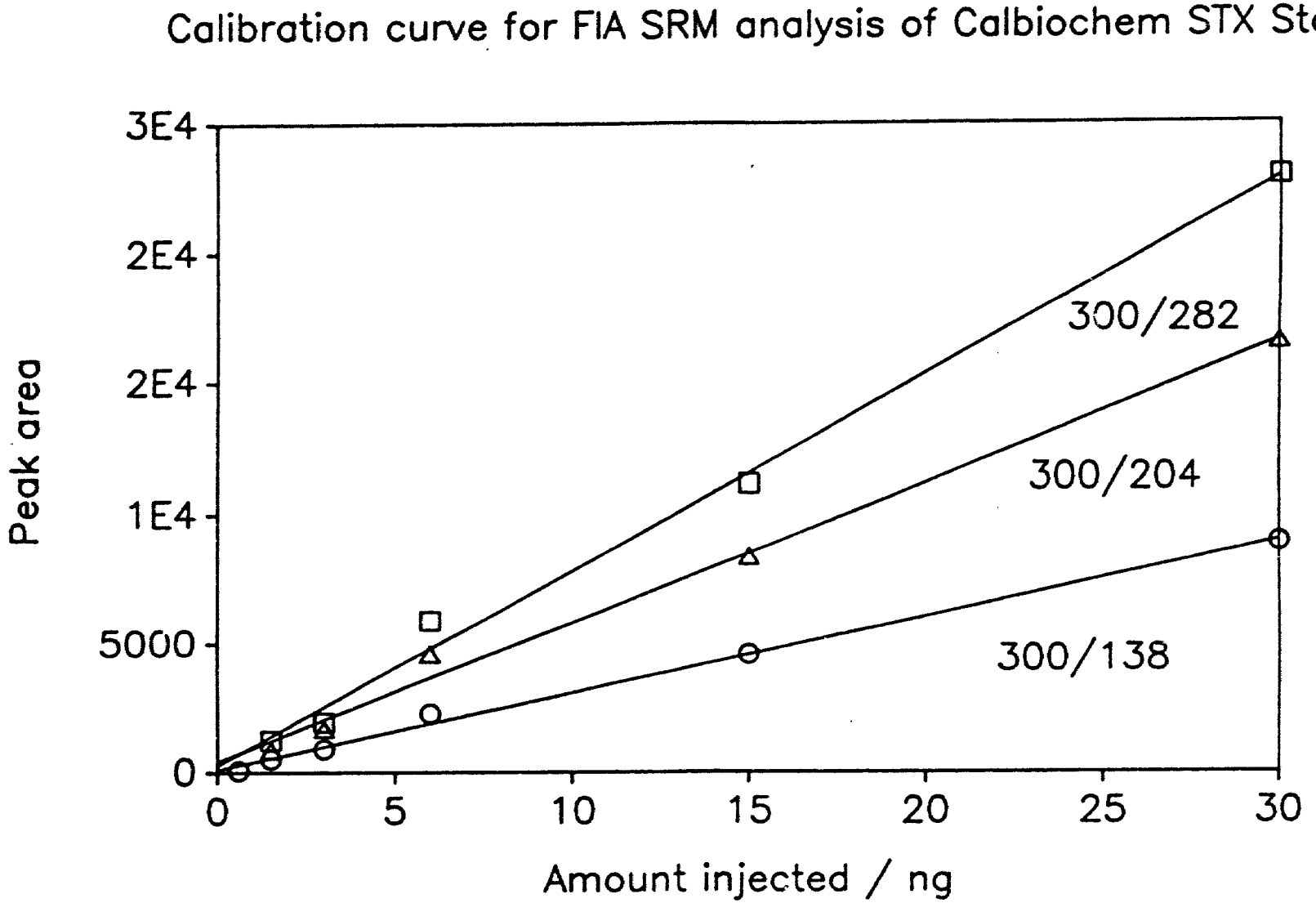


Figure 6. Calibration curves for STX (CalBiochem standard) obtained by flow injection analysis with selected reaction monitoring (SRM).

Extract

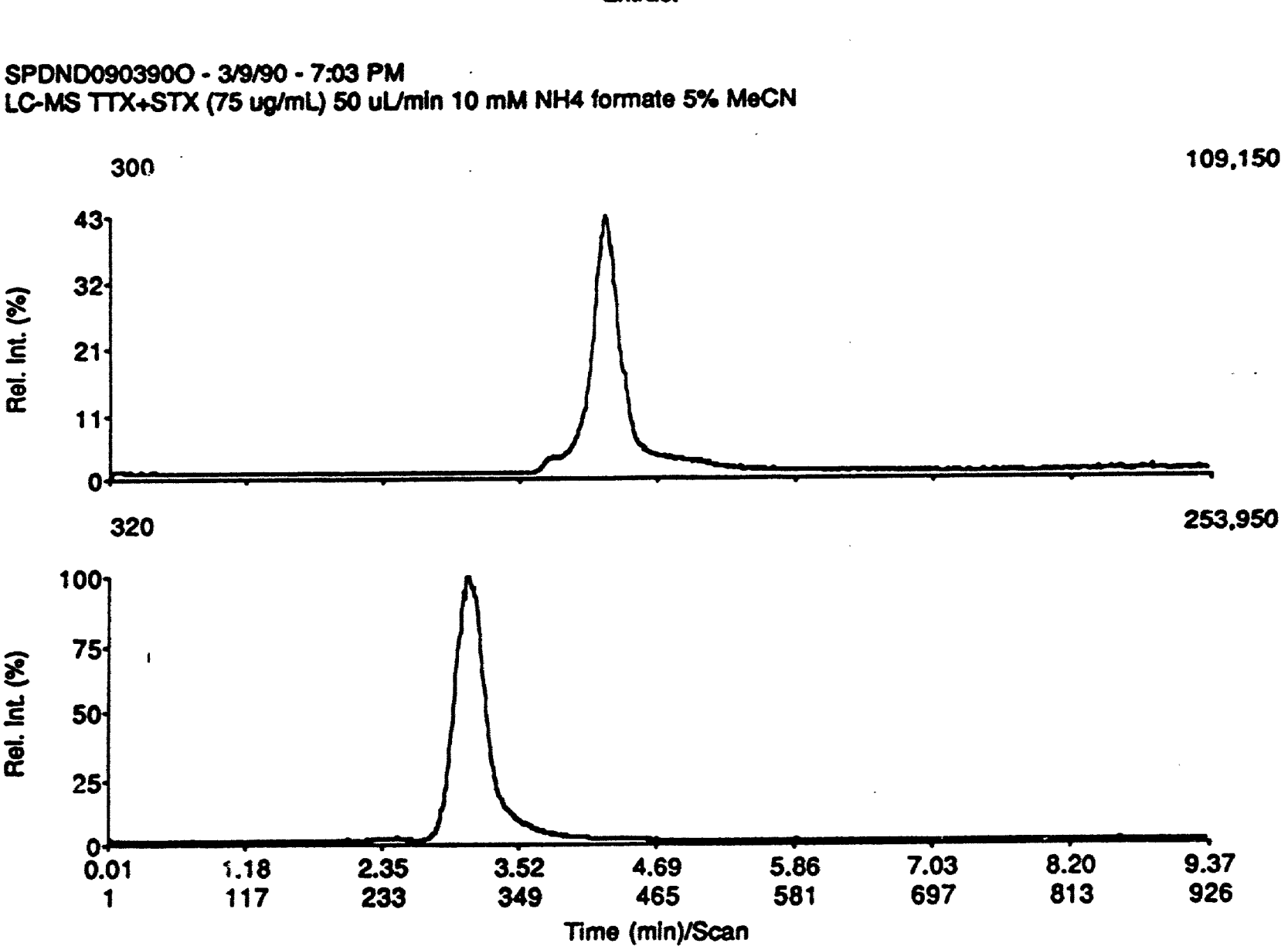


Figure 7. Reconstructed ion chromatograms from a full-scan LC/MS analysis of an approximately equimolar mixture of STX plus TTX, 75 μg/mL with 1 μL injected. Mobile phase aqueous ammonium formate (10 mmol/L) with 5% acetonitrile. Column was a 1 mm PRP-1 resin column, similar to those used for the Sullivan ion-pairing HPLC method[8-11].

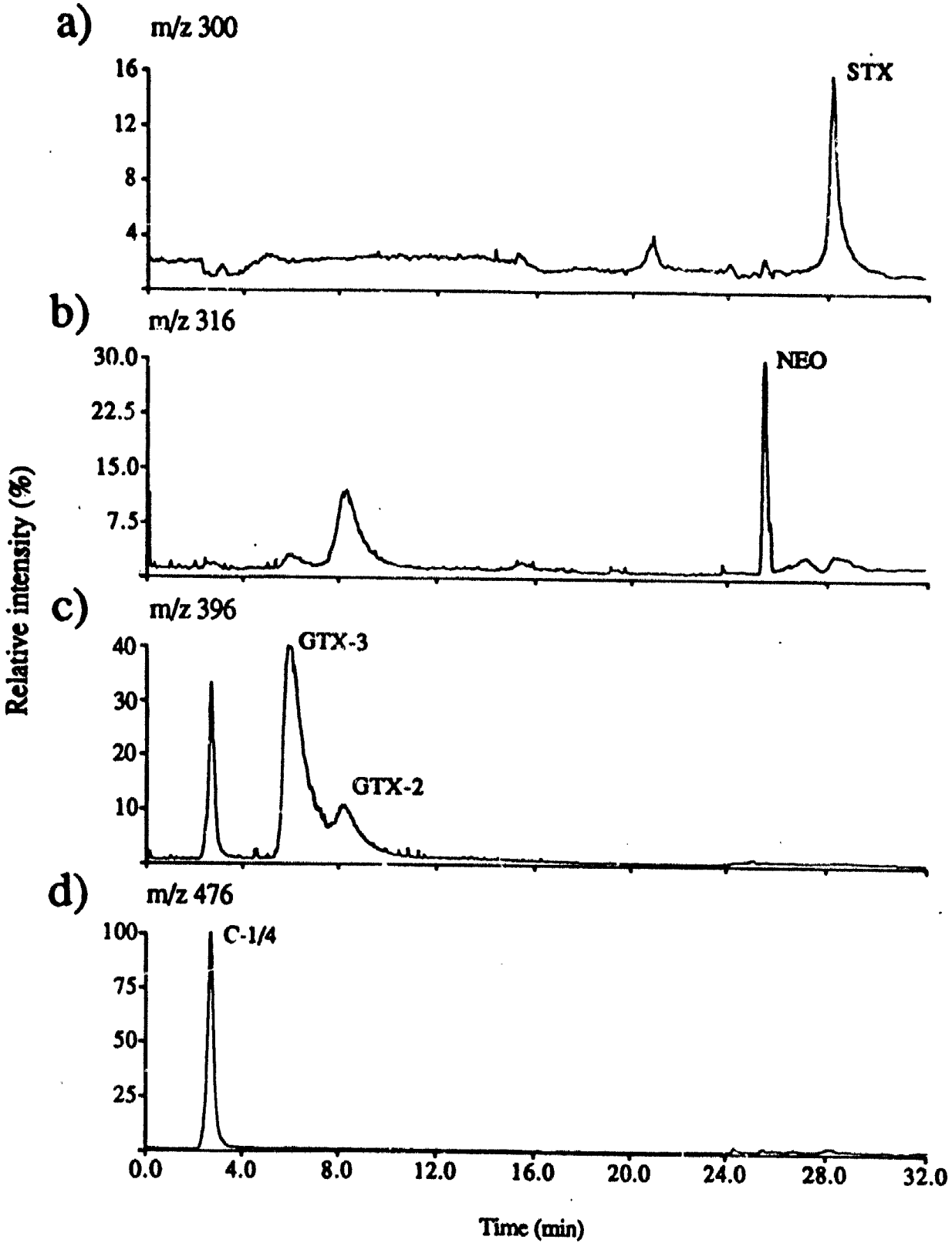


Figure 8. Mass chromatograms from LC/MS analysis (selected ion monitoring) of a mixture of PSP toxins. Chromatographic conditions were identical to those used to obtain Figure 6, except that a 4.6 mm PRP-1 column was used with a post-column split to the mass spectrometer.

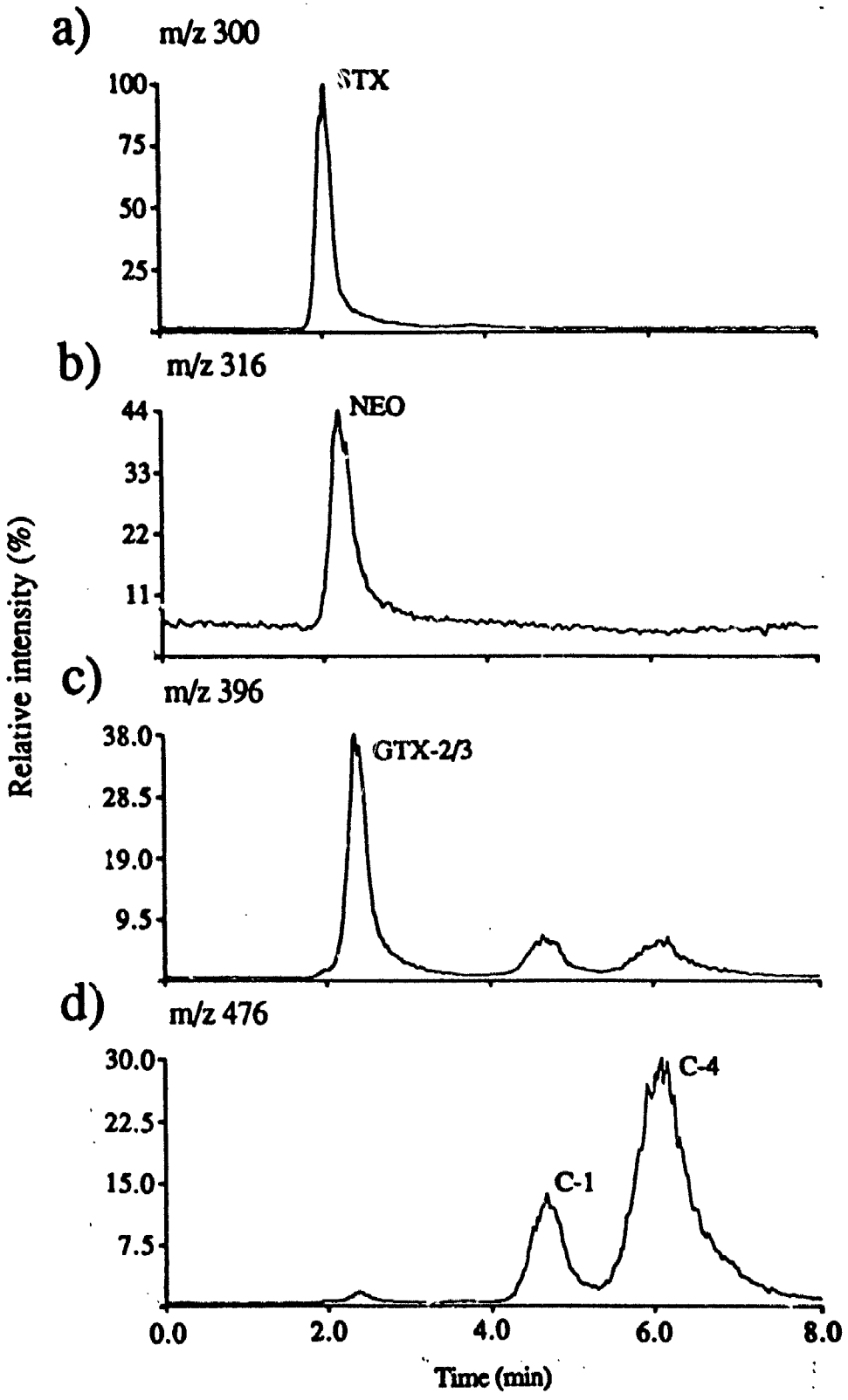
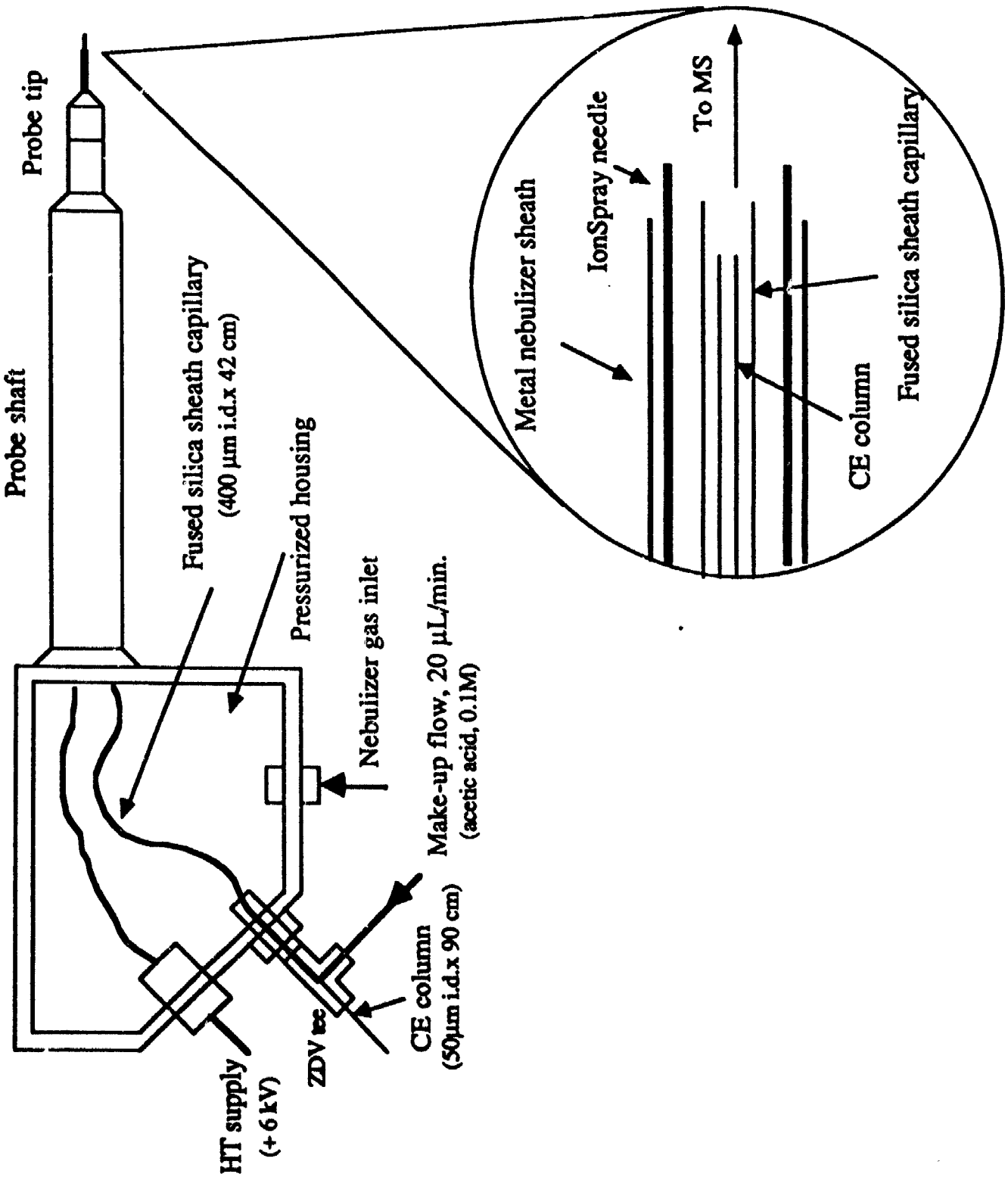


Figure 9. Mass chromatograms from LC/MS analysis (selected ion monitoring) of the same mixture of PSP toxins as was used in the experiment illustrated in Figure 7. Note the reversal of retention order, relative to that observed in Figure 7. The column used was a 1 mm bonded amino-phase column; other conditions as for Figure 7.

Figure 10 Diagrammatic representation of the CE/MS interface (taken from Ref.18). The ionspray needle is maintained at approximately +5 kV, and is electrically connected to the zero-dead-volume T-junction *via* the make-up flow through the sheath capillary; this make-up flow thus acts as the reservoir buffer at the negative CE electrode. The other end of the CE capillary (not shown) is immersed in a buffer solution maintained at +30 kV. The mass spectrometer orifice is maintained at a few volts above ground potential.



Analysis of PSP toxins by CE/MS

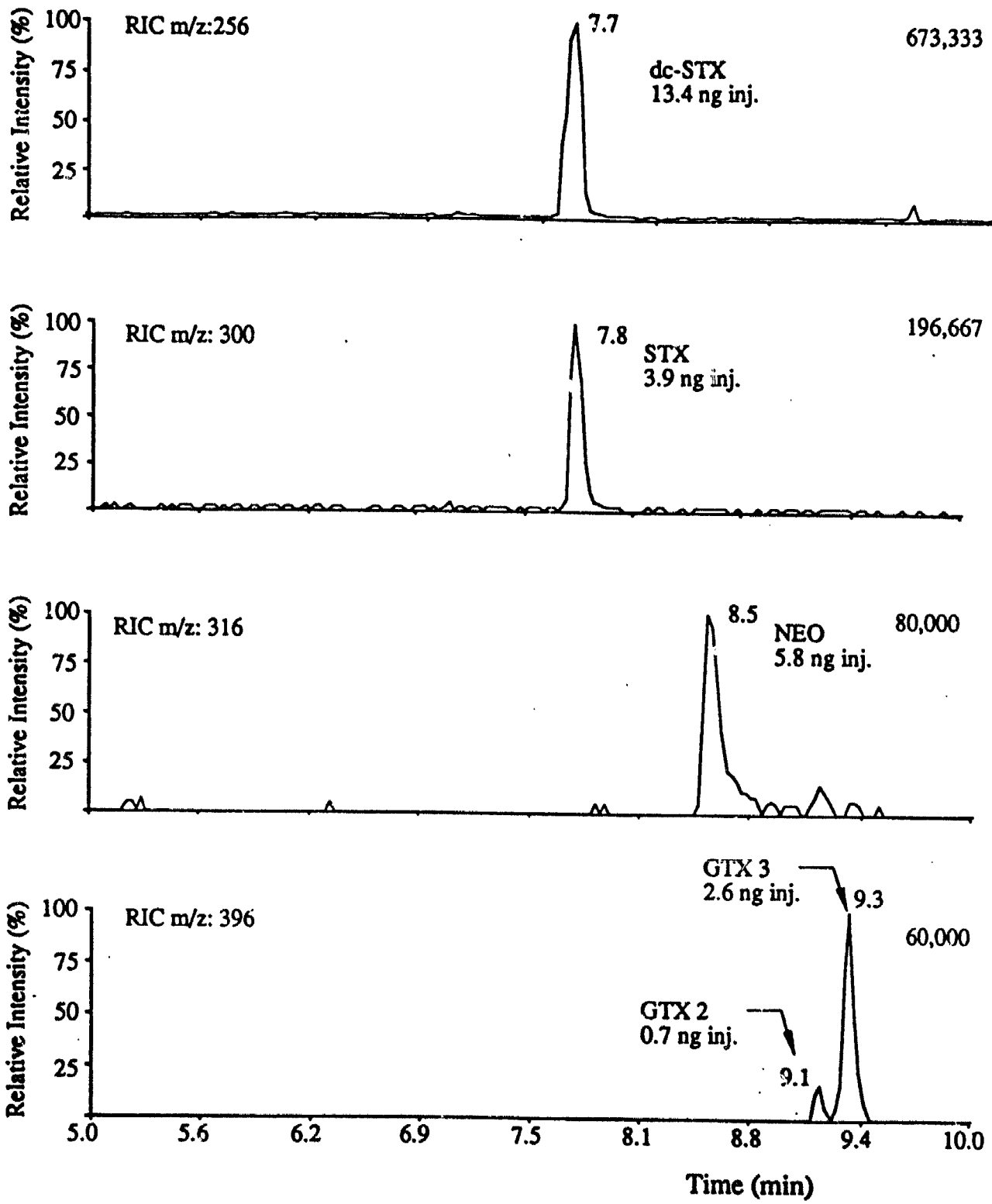


Figure 11 Reconstructed ion electropherograms (RIEs) from CE/MS analysis (scan range m/z 130-405) of a mixture of PSP toxins. Decarbamoyl saxitoxin (dc-STX) is a naturally occurring decomposition product of STX. Also shown are mass spectra, extracted from the CE/MS dataset, of each of the major components.

Extracted mass spectra from CE/MS analysis of PSP toxins

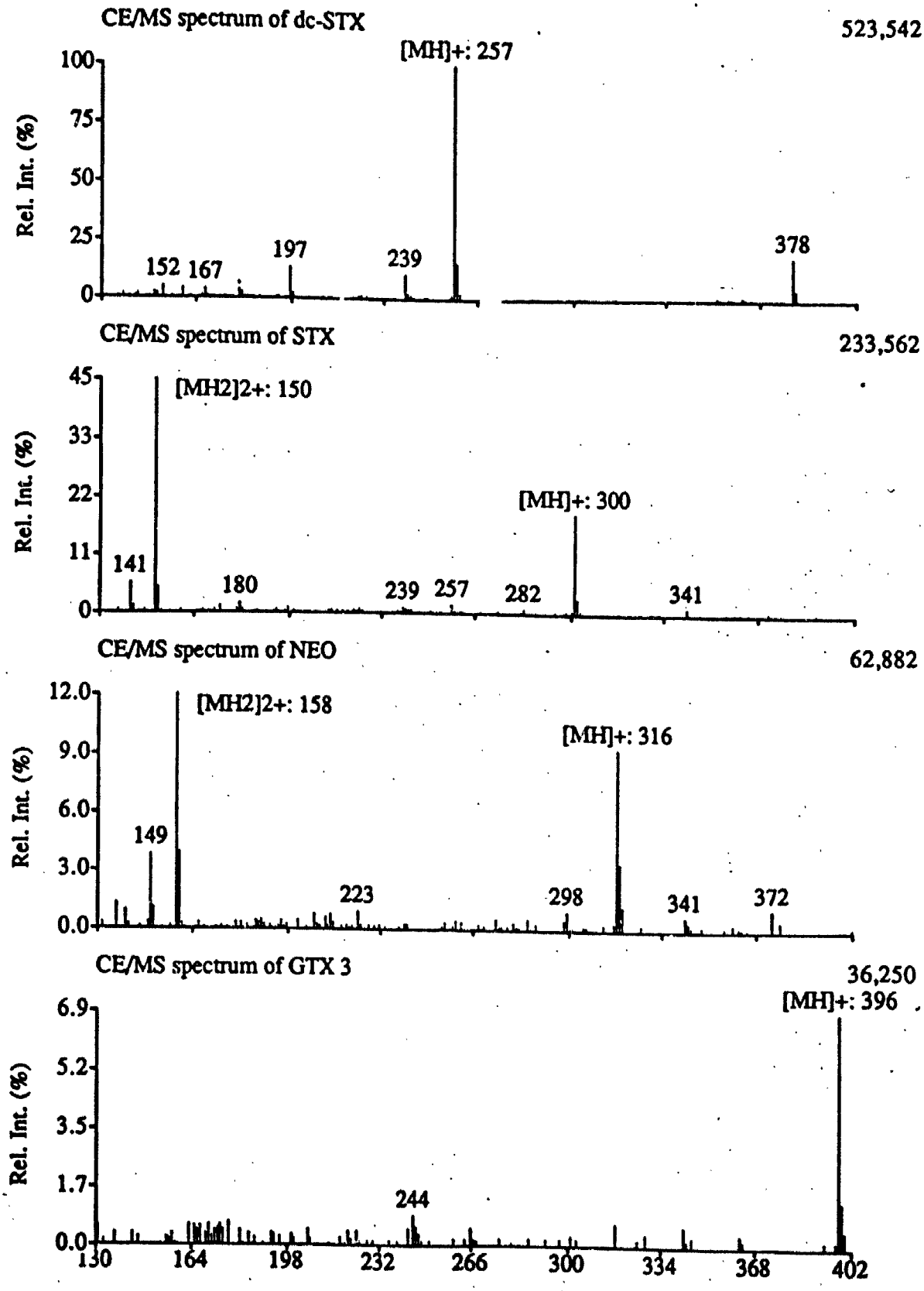
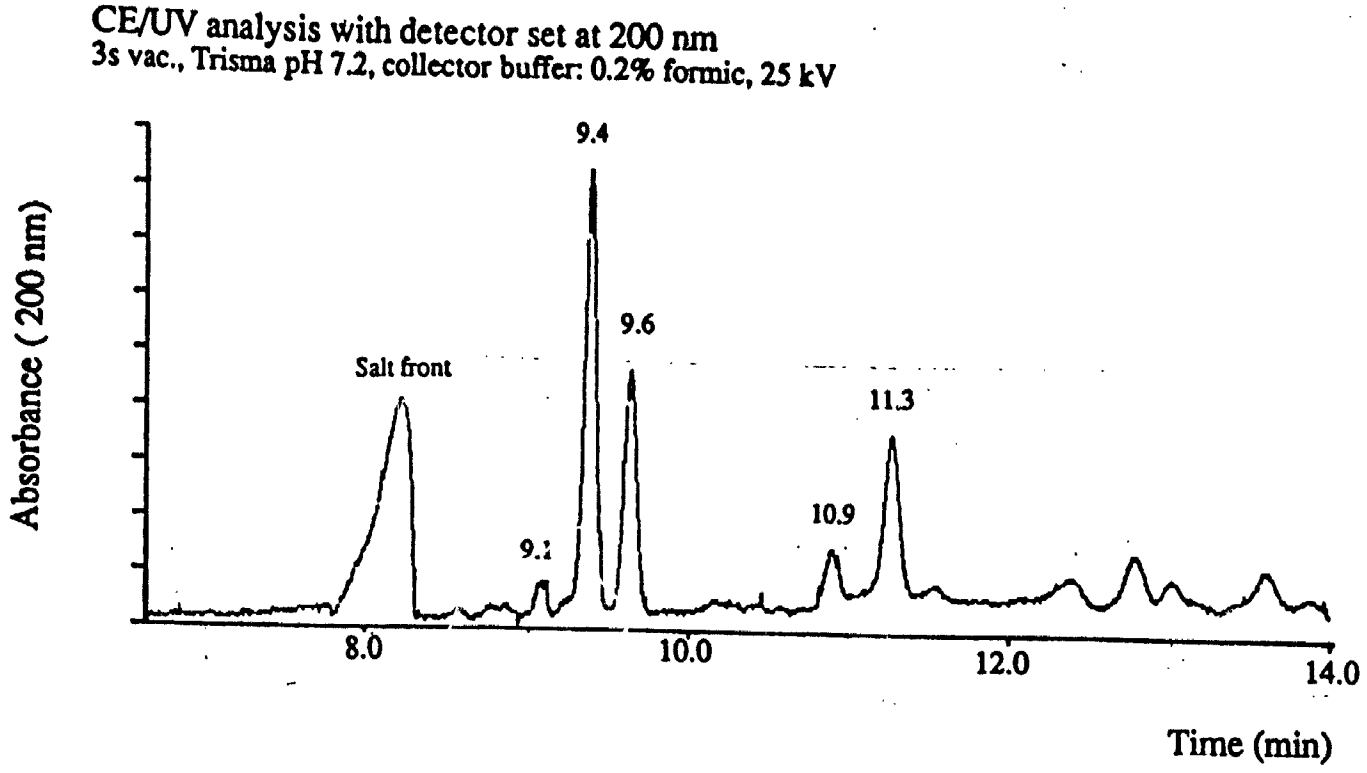


Figure 12 Full-scan mass spectra acquired at the maxima of each of the major peaks in the electropherograms shown in Figure 10.



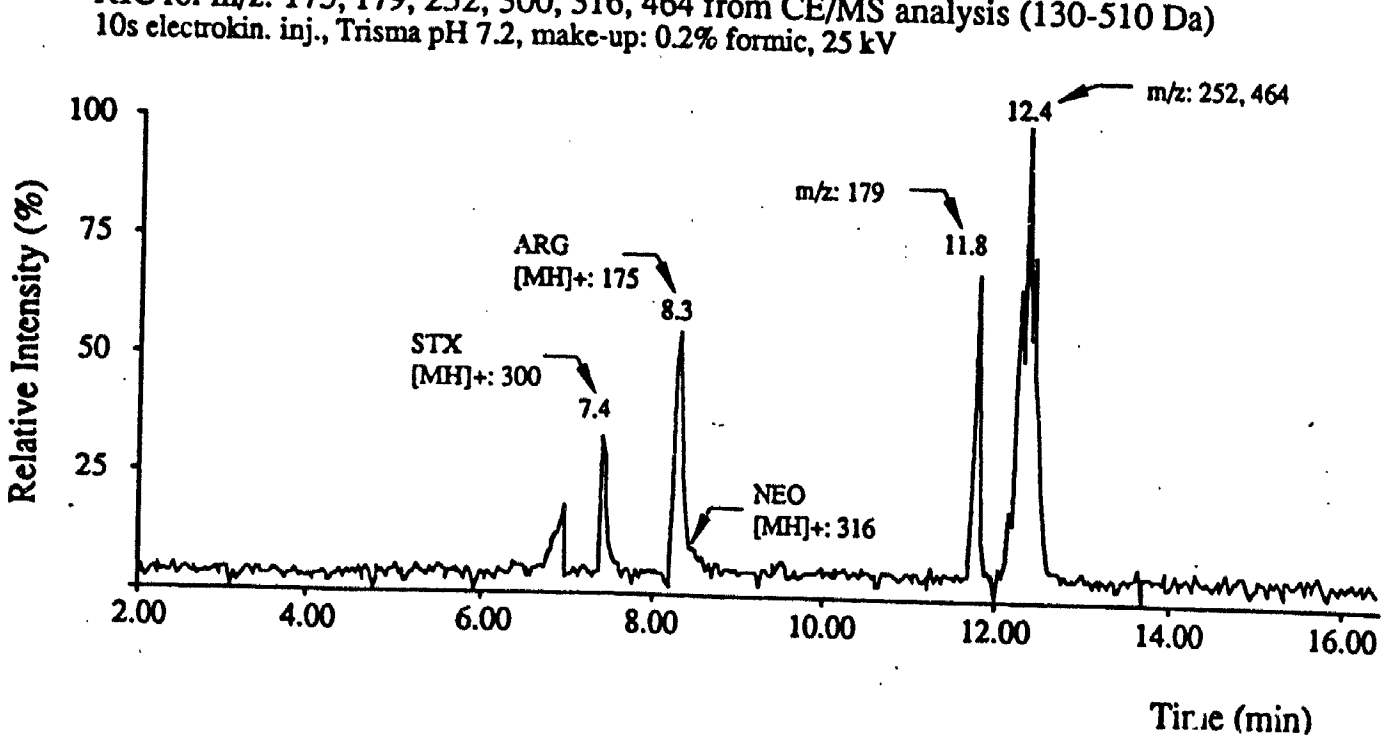
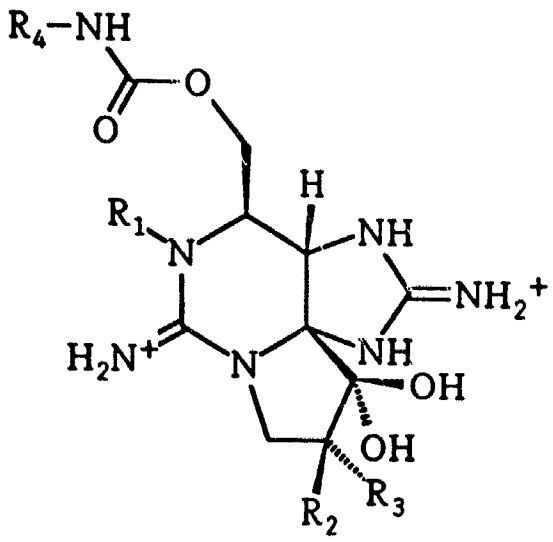


Figure 13 Analysis by (a) CE/UV (200 nm) and (b) by CE/MS (scan m/z 130-510, trace shown is a reconstructed ion electropherogram for sum of intensities of m/z 175, 179, 252, 300, 316 and 464) of an 0.03 mol/L acetic acid extract of 90 mg (wet weight) sonicated dinoflagellate cells *Alexandrium tamarensis*, filtered but with no other clean-up. The m/z values chosen for the RIE are those of the major components observed, and include those for protonated arginine (m/z 175), STX (m/z 300) and NEO (m/z 316).

Scheme I: Paralytic Shellfish Poisoning (PSP) Toxins

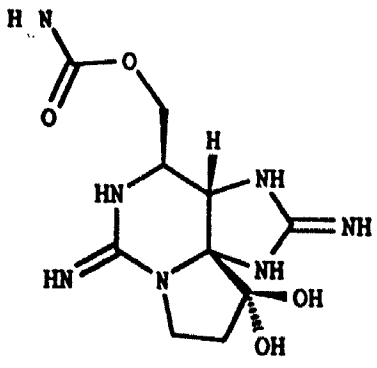


Saxitoxin (STX)

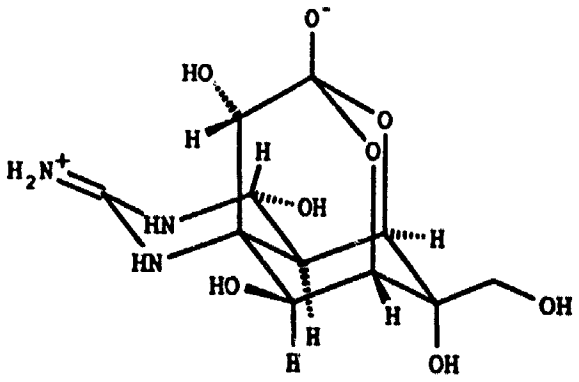
and derivatives

R_1	R_2	R_3	R_4	
H	H	H	H	STX
OH	H	H	H	NEO
H	H	H	SO_3^-	B1
OH	H	H	SO_3^-	B2
H	H	OSO_3^-	H	GTX2
H	OSO_3^-	H	H	GTX3
OH	H	OSO_3^-	H	GTX1
OH	OSO_3^-	H	H	GTX4
H	H	OSO_3^-	SO_3^-	C1
H	OSO_3^-	H	SO_3^-	C2
OH	H	OSO_3^-	SO_3^-	C3
OH	OSO_3^-	H	SO_3^-	C4

Scheme II



Saxitoxin (STX)



Tetrodotoxin (TTX)