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DEVELOPMENT OF INSTRUMENTAL
ANALYTICAL METHODS FOR THE DETERMINATION OF
SAXITOXIN AND TETRODOTOXIN IN WATER AND SOIL.

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CONTENTS

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, (see "Report of the Ad Hoc Committee on Chemical Weapons to the United Nations Conference on Disarmament, United Nations Office, CD/1046, Genève, Switzerland, 1991). 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 the consumption of cultured shellfish; in Canada this is the joint responsibility of Fisheries and Oceans Canada and of 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.

The present document represents report on work done at IMB on techniques for trace analysis of these highly polar, thermally labile toxins, on the stability of STX and other PSP toxins in solution, and on experiments done on analysis of STX and TTX which had been spiked into soil samples. 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 phytoplankton species which are the primary producers of the PSP toxins.

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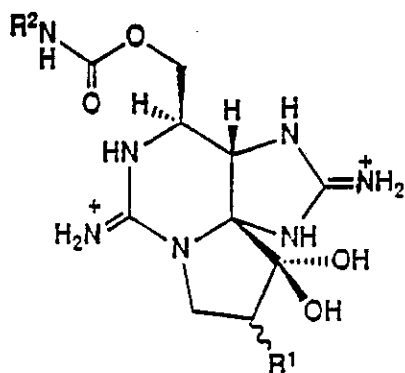
(STX) Saxitoxin $R^1 = H$, $R^2 = H$
(GTX-II) Gonyautoxin II $R^1 = \alpha - OSO_3^-$, $R^2 = H$
(GTX-III) Gonyautoxin III $R^1 = \beta - OSO_3^-$, $R^2 = H$
(B1) 21-Sulfosaxitoxin $R^1 = H$, $R^2 = SO_3^-$
(C1) 21-Sulfo-11α-hydroxysaxitoxin sulfate $R^1 = \alpha - OSO_3^-$, $R^2 = SO_3^-$
(C2) 21-Sulfo-11β-hydroxysaxitoxin sulfate $R^1 = \beta - OSO_3^-$, $R^2 = SO_3^-$

Scheme I: Paralytic Shellfish Poisoning (PSP) Toxins
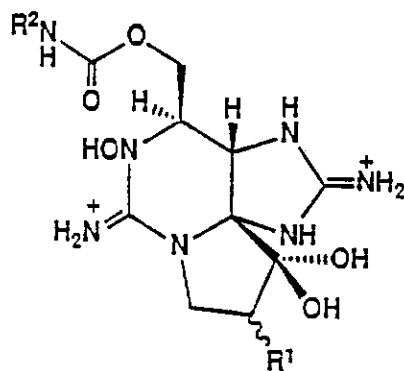
(NEO) Neosaxitoxin $R^1 = H$, $R^2 = H$
(GTX-I) Gonyautoxin I $R^1 = \alpha - OSO_3^-$, $R^2 = H$
(GTX-IV) Gonyautoxin IV $R^1 = \beta - OSO_3^-$, $R^2 = H$
(B2) 21-Sulfoneosaxitoxin $R^1 = H$, $R^2 = SO_3^-$
(C3) 21-Sulfo-11α-hydroxyneosaxitoxin sulfate $R^1 = \alpha - OSO_3^-$, $R^2 = SO_3^-$
(C4) 21-Sulfo-11β-hydroxyneosaxitoxin sulfate $R^1 = \beta - OSO_3^-$, $R^2 = SO_3^-$

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Scheme II
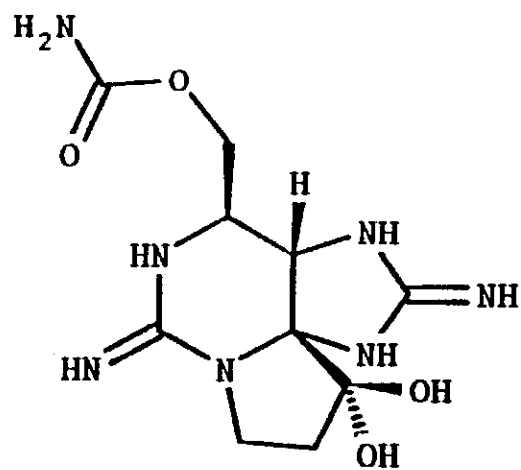
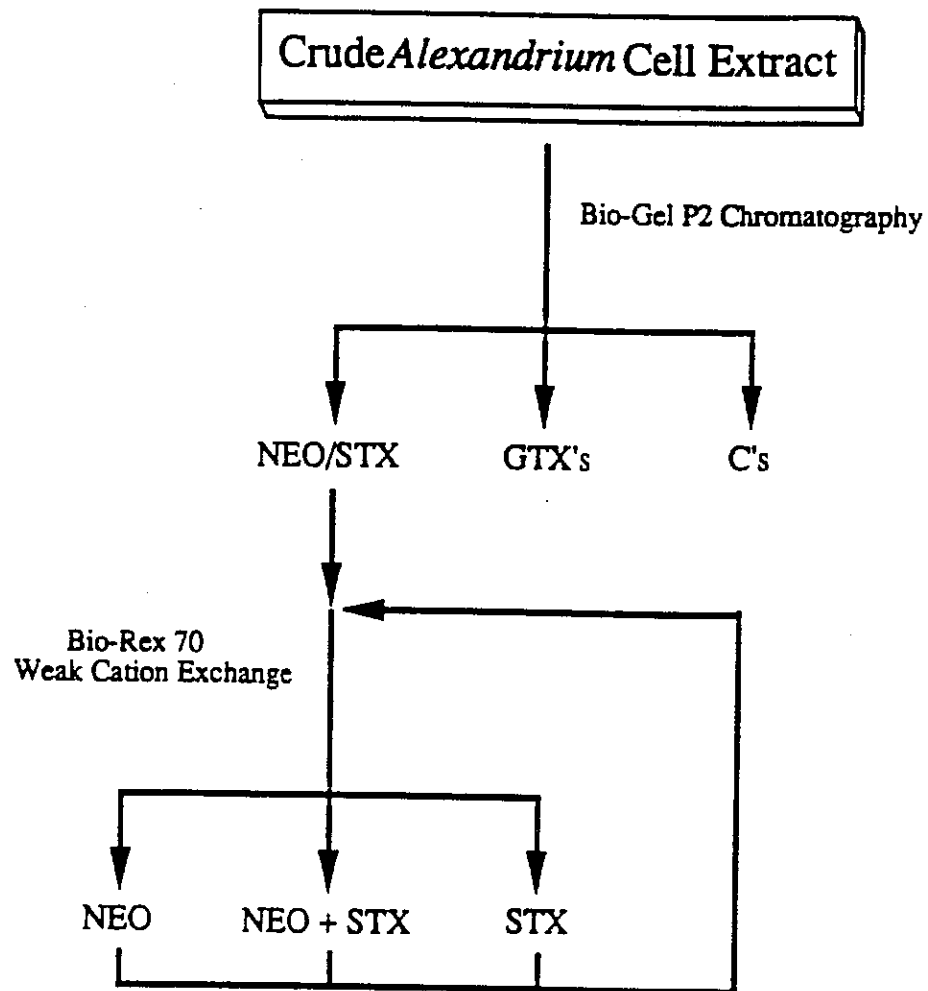

Saxitoxin (STX)
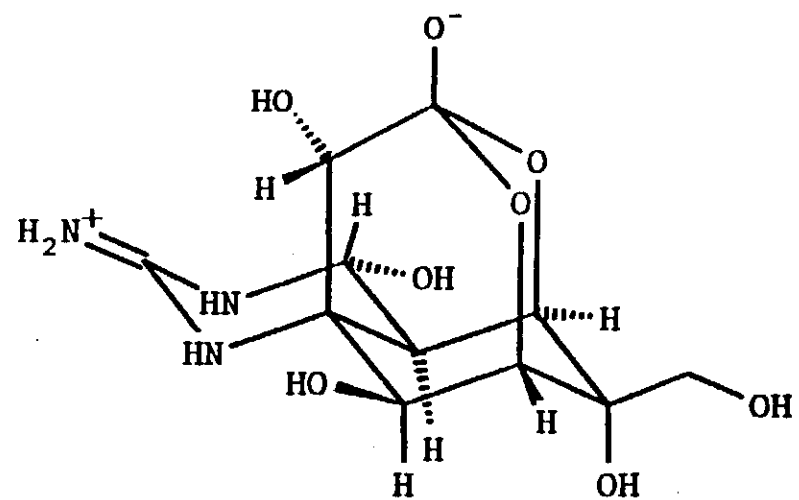
Tetrodotoxin (TTX)

B. AVAILABILITY OF 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. A summary of Dr. Laycock's method of extraction and purification of these PSP toxins has recently been published[5] together with details of some instrumental analytical methods developed at IMB. Scheme III is taken from this account[5], and further details may be found there.

The availability of certified standards is of crucial importance for all analyses of these toxins, including those of direct interest to DND. The PSP toxins are extremely hygroscopic, and it is unrealistic to supply them as analytical standards in the form of pure solids; TTX, however, is available commercially as a lyophilised powder. IMB has conducted 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 was the responsibility of Dr. P. Thibault of IMB, and a summary of his findings is included here as Section F. 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[6]).

A solution of STX is offered for sale by Calbiochem of San Diego, California. This solution has been shown by IMB (see Section D, below) to contain impurities which are either present in major amounts, or have much higher molar response factors in ionspray ionization than does STX itself. However, this item appears to be currently (April, 1992) unavailable from Calbiochem, although advertised in their current (1992) catalogue. Another company, Chiral Corporation of Miami, Florida, advertises a wide range of marine toxins including most of the more common PSP toxins. IMB has had an order placed with Chiral for all of their advertised toxins, for about 11 months now, but the only item delivered was a vial of STX. Finally, solutions of PSP toxins are available from Dr. Sherwood Hall, of the U.S. FDA in Washington, D.C.

SCHEME III. Isolation procedure used for the purification of PSP toxins from cultured dinoflagellates (from Ref.5).

Crude *Alexandrium* Cell Extract

Bio-Gel P2 Chromatography

NEO/STX   GTX's   C's

Bio-Rex 70
Weak Cation Exchange

NEO   NEO + STX   STX

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[7], 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 this TTX standard also contains impurities which respond to ionspray ionization. Other evidence will be presented below suggesting that this commercial TTX preparation is not pure.

It is necessary now to make some comments on a fundamental problem in the *quantitative* analysis of these toxins. Even though the PSP toxins prepared by Dr. Laycock at IMB are known to be of high purity[5] (see also below), it is not possible in this case to rely upon accurate weighing of these pure standards on an analytical balance, as the ultimate reference for quantitative analyses. This is due to the extremely hygroscopic nature of these compounds, and to the difficulty of removing acetate salts of the toxins. (The same is true of TTX, although here there is the additional problem[7] of chemical impurities). Dr. M. Quilliam of IMB devised a procedure whereby the amounts of toxins in Dr. Laycock's preparations could be measured with acceptable precision and accuracy. The very high purity of Dr. Laycock's preparations 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 observed 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 (iii) below), and a value of 0.267 $\pm$ 0.011 for the Sullivan HPLC method with fluorescence detection, as implemented at IMB (see Section C(ii)). Extension of this method to the GTX2/3 mixture has been accomplished by Dr. Thibault. These procedures and their quantitative results will be used in the certification of the MACSP standard solutions. For present purposes, it is important to realise that quantitation of these labile and hygroscopic substances involves problems not normally encountered for most analytes.

C. 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)[8,9]. 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.

C(i). Immunochemical Approaches.

An alternative to bioassays using live mammals is provided by immunochemical methods. These have the potential to be developed into kits for field use. As discussed briefly below, the main thrust thus far in this area has involved developments aimed at producing an early-warning screening technique for the shellfish industry.

PSP toxins are themselves not antigenic and therefore must be conjugated to a suitable carrier protein as the first step in the preparation of antibodies. An added complication is that the amount of conjugate that can be injected into an animal is small because the toxin is readily released. Although keyhole limpet hemocyanin (KLH)[10] and synthetic peptides such as polyalanine-lysine[11] have been used for this purpose, the most common method so far has been to use bovine serum albumin as the carrier protein, and formaldehyde or glutaraldehyde as the linking agent. Both monoclonal and polyclonal antibodies have been prepared using this method and shown to be highly specific for saxitoxin and its derivatives. It was found, however, that they did not cross-react well with neosaxitoxin in mixtures of PSP toxins, and only very weakly with the sulfocarbamoyl derivatives (B1, B2 and C1-4 Scheme I).

The most popular approach to the development of a rapid semi-quantitative immunochemical method for PSP toxin analysis is based on specific antibodies. Toxin concentration can be measured by conventional immunological methods incorporating

radioactivity (RIA) or via a colorogenic enzyme (ELISA). These are essentially laboratory methods because a scintillation counter is necessary for RIA and a colorimeter for ELISA. For the RIA method solutions of radioactively labelled (^{3}H) saxitoxin are necessary, thereby limiting the general use of the method and adding to its cost. The ELISA method offers the best opportunity for an early warning assay. Microtitre ELISA plates permit ninety-six assays at once, which means the cost per assay can be kept low. The microtitre plate method may still be practicable only in the laboratory because of the need for an ELISA reader. However, a small "in the field" colorimeter could be used for limited numbers of samples outside the laboratory.

Since marine samples often contain a mixture of toxins, with differing toxicities, the toxicity of a shellfish extract is due to the collective effect of all toxins present. Thus, application of an immunoassay for accurate detection of total toxicity is difficult. Most of the recently attempted assay techniques were developed specifically for STX, with cross-reactivity to other toxins being very poor[12]. Unfortunately, in most marine samples, STX represents a minor percentage of the overall toxicity. For the purposes of a field-test for early warning of STX itself as a chemical warfare agent, this cross-reactivity problem for the other PSP toxins would not be a problem. However, any large-scale preparation of STX for such a purpose would incorporate a range of PSP toxins derived from toxic shellfish. Further, the cross-reactivity to TTX, of the STX immunochemical assays developed thus far [10-12], is wholly unknown.

At present, IMB has little expertise in this area. However, it is hoped that a joint research program, involving IMB, Fenwick Laboratories, and Dalhousie University, will start within the next few months. The key personnel at IMB will be Dr. Laycock and Dr. A. Cembella, and DND may wish to be kept informed of the progress of this new initiative.

C(ii). Liquid Chromatography with Fluorescence Detection.

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 tetrahydropurine 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, with novel detection strategies, are required.

Figure 1. Diagrammatic representation of a Sullivan train[13-23] for analysis of PSP toxins by LC-FLD; adopted from Ref. 23
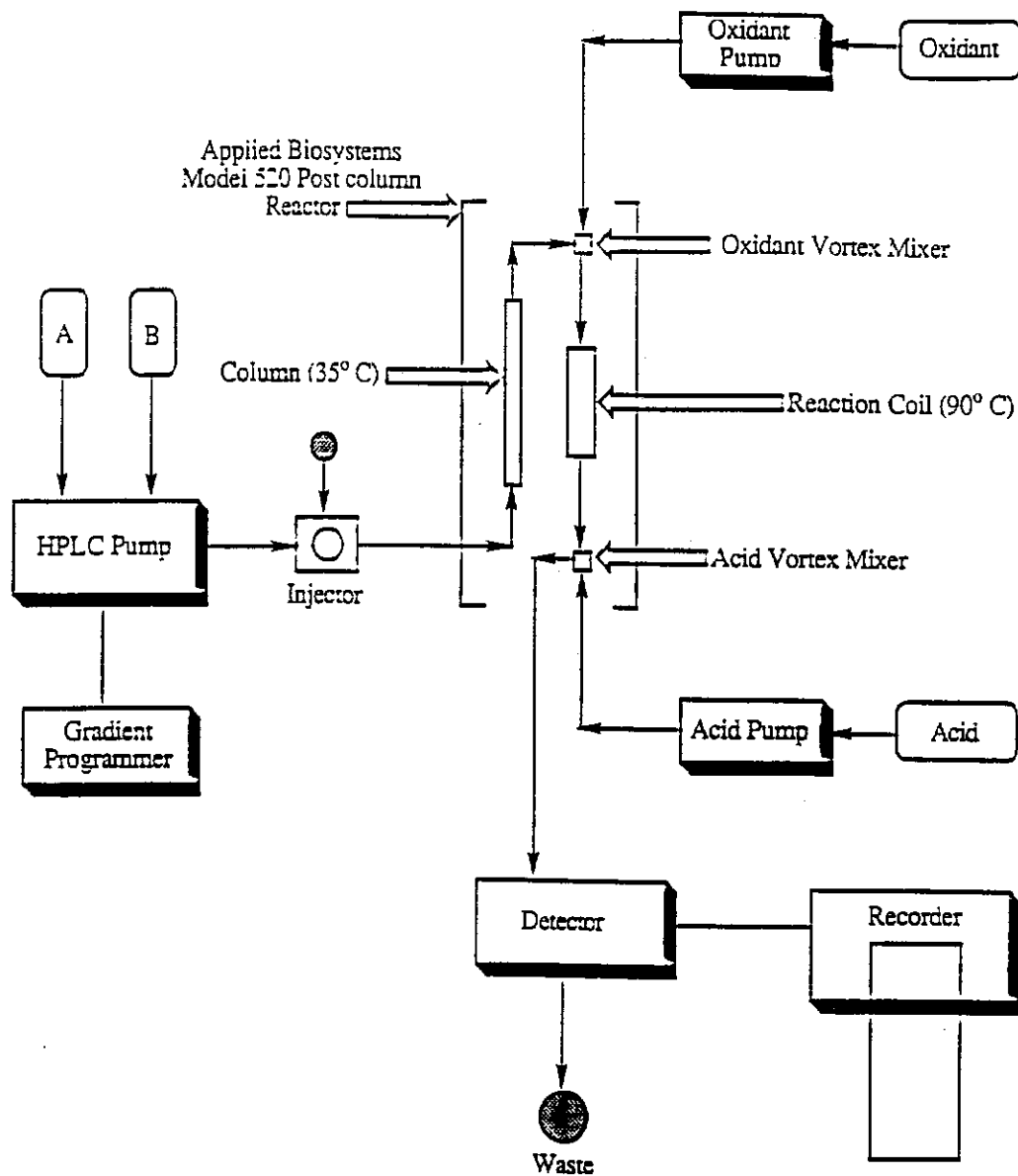
The instrumental method most commonly used for routine determination of PSP toxins is the Sullivan method[13-17] and its variants[18-22], all of which involve high performance liquid chromatography (HPLC, or LC) using a reversed phase technique (mobile phase more polar than stationary phase) with a resin-based 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[13-22] 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 (see Figure 1 for a diagrammatic representation); 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 alkaline conditions[19-22]. **For these reasons, the Sullivan HPLC method[13-22] is not recommended for the purposes of occasional monitoring for STX and TTX 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[23] 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. Dr. A.Cembella of IMB is also an expert on this technique.

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(iii) 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[13-23]), 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. (One reason for the success of the CE-UV technique for PSP toxins and TTX, described in Section C(iii), is that the necessary degree of selectivity is provided by the fact that these compounds exist as ions under the pH conditions used, and thus migrate efficiently under the applied electrophoretic field while likely contaminants do not do so. 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[24,25] 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. A silica-based C18 column was used to obtain better separation efficiencies compared to those obtainable using the PRP-1 column recommended in the post-column method[13-23]. Various mobile phases were investigated, and one containing aqueous ammonium formate provided the best peak shape and reproducibility. The composition of the reaction mixture for pre-column periodate oxidation was also carefully examined to improve fluorescence sensitivity. Detection limits are better than with the Sullivan LC system. One of the drawbacks to this approach is that some of the toxins produce the same oxidation products. This makes the method unsuitable for detailed analysis of toxin profiles in research work. It is valuable[25], however, for the rapid analysis of seafood products to detect PSP contamination. Another drawback of the method as described [24,25] is that the derivatization reaction is performed manually, and analyses must be performed immediately after the reaction to ensure quantitative determination. Demonstrated detection limits were in the range 20-50 pg for the non-N_1-hydroxylated toxins, and 100-500 pg for the N_1-hydroxy analogues[24]. This work was later extended[25] 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, and (as noted above) some of the different toxins produced the same set of oxidation products.

More recently, under contract with Dr. Lawrence of HWC, Dr. Quilliam and Dr. M. Janecek of IMB have undertaken further developments of Lawrence's prechromatographic oxidation approach[24,25]. Firstly, automated reactions were implemented using the Hewlett-Parkard HP1090M Automated Precolumn Sample Preparation System. This system is based around the powerful capabilities of the HP1090M/ChemStation combination, which enables the user to customize and automate various sample manipulations including precolumn derivatizations at ambient and elevated temperatures. Secondly, new mobile phases for the reversed-phase chromatography of the oxidized PSP toxins were investigated. Long-chain perfluorinated acids, when used as ion-pair agents, gave excellent chromatographic performance. This type of mobile phase is compatible with ionspray LC-MS (see below). Thirdly, the use of microcolumn technology was studied, with the intention of improved detection limits as well as compatibility with LC-MS systems. Interestingly, it was found that it is possible to use an HP1046A dual monochromator spectrofluorometric detector, equipped with a relatively large (4.5 µL) flowcell, without appreciable loss in column efficiency but with an enormous increase in sensitivity over the usual micro flowcell systems. In addition, by taking advantage of the high capacity factors provided by the combination of the perfluorinated ion-pair agents, gradient elution, and the low eluotropic strength of the sample reaction mixture, it was possible to increase the injection volume by 20-fold over the normal volume. All this results in a very sensitive, automated method for the monitoring of PSP toxins. The minimum detectable concentrations in sample extracts were estimated to be: 1 ppb (ng/mL) for STX; 0.7 ppb for GTX-2 and GTX-3; and 4 ppb for NEO. Figure 2 shows chromatograms for (a) a trace level mixture of PSP standards, and (b) an extract of shellfish tissue contaminated with PSP.

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Figure 2. Microcolumn LC-FLD analysis of the oxidation products of (a) a mixture of PSP toxin standards at 6 ng/mL, and (b) an extract of PSP-contaminated scallop liver homogenate. Column: 5 μm LichroSpher 100 RP18 microcolumn (250 x 1 mm id); mobile phase: 10 mM heptafluorobutyric acid, pH 4.4 (adjusted with NH_4OH) with a linear gradient from 0 to 20 % acetonitrile over 15 min; flow rate: 100 μL/min; 20 μL injection volume after reaction; FLD gain: (a) 18, (b) 14.
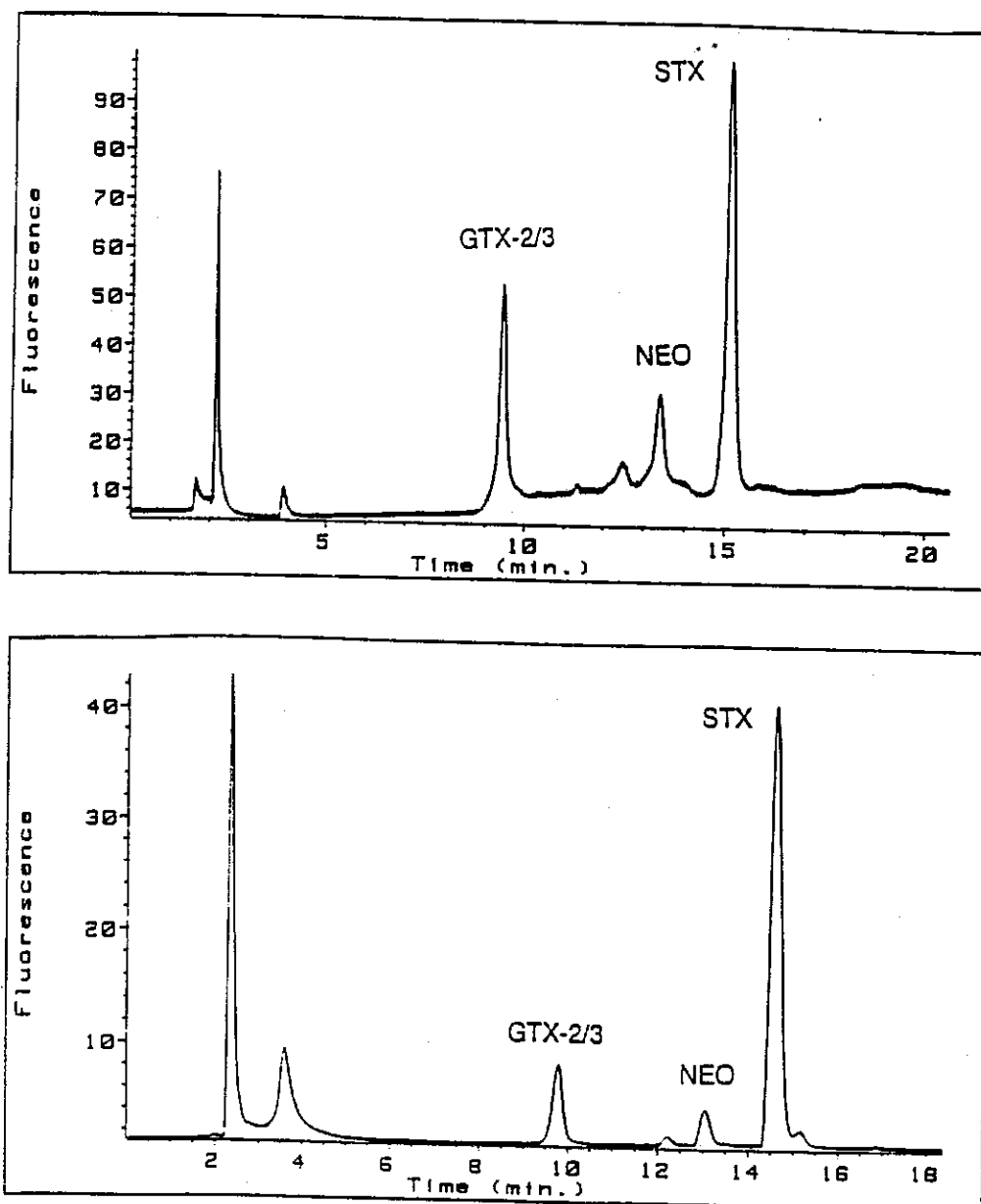
It was hoped that the oxidized PSP toxins would be suitable for ionspray LC-MS, as the chromatographic performance of these compounds is superior to that of the underivatized compounds. However, oxidization of the toxins led to almost a 10-fold decrease in ionspray sensitivity. This fact, coupled with the dilution imposed by the addition of reagents, limits the applicability of this approach to ionspray LC-MS analysis of trace levels of PSP toxins. It is likely, however, that the perfluorinated ion-pair agents could be used effectively for the LC-MS analysis of underivatized PSP toxins. This possibility will be pursued by Dr. Quilliam and Dr. Janecek in the near future.

This pre-column oxidation work will be written up for publication in the near future, once some final complications have been sorted out. From the point of view of DND, it could afford a confirmatory technique for STX with a moderate degree of selectivity if a dual-wavelength programmable fluorescence detector were used. Even better selectivity would be provided if it were possible to use on-line instrumentation to provide either excitation or emission spectra. Unfortunately, TTX was not efficiently oxidised under the reaction conditions found to be optimal for the PSP toxins. It may be possible to develop more fierce oxidising conditions, suitable for pre-column conversion of TTX to fluorescent reaction products, but as yet this has not been achieved at IMB.

C(iii). Capillary Electrophoresis with UV detection.

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[26]. Work by Dr. Laycock of IMB, associated with his successful isolation of pure PSP toxins[5] (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 ap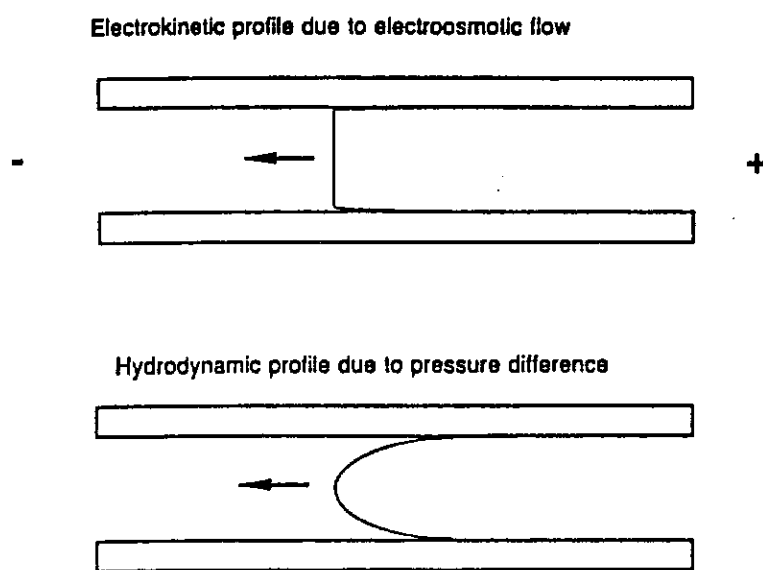propriate here to describe in detail the physical principles underlying analytical CE; excellent expositions are available in the literature[27-29]. However, it is worthwhile for present purposes to emphasise that emergence of an analyte from one end of the capillary is the result of interplay of two 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 3 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 viscous flow induced by pressure difference (Figure 4); 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 E (ii) below is the very low volume flowrate (of the order of 100 nL/min) emerging from the capillary. Incidentally, when desired it is possible to reverse the direction of the electroendosmotic flow from that (Figure 3) dictated by the usual negative charge on the surface of the untreated quartz capillary. This reversal can be achieved[30] by coating the internal wall with a suitable compound which presents a positively-charged surface layer to the running buffer solution within the capillary. This reversal has been shown[30] by Dr. Thibault and his collaborators at IMB to be beneficial for analysis of peptides and proteins by CE combined with mass spectrometry (Section E (ii)).

The first published application of CE to analysis of marine toxins was due to Wright *et al.*[31]. Fluorescence tagging agents were used to derivatize the primary amine ($-NH_2$) or imine ($=NH$) 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 were 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[31], 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[31].

Figure 3.   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 4. Comparison of the flow profiles characteristic of capillary electroendosmotic flow and of hydrodynamic flow.

Electrokinetic profile due to electroosmotic flow

Hydrodynamic profile due to pressure difference

At IMB, an approach has been developed[32] (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[31] 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 both the Applied Biosystems Inc. and the Beckman P/ACE CE instruments used at IMB. This IMB work has been extremely successful, with proven application to analysis of phytoplankton and scallop tissues for PSP toxins[32]. The keys to this success include the inherent selectivity of the CE process itself for highly polar compounds, the discovery of suitable running buffers (acetic acid or citrate buffers) which are sufficiently transparent at 200 nm that a usable absorption range remains for detection of the analytes, and the excellent properties of the UV detector itself. In addition, it was found[32] that use of these buffers makes the migration times of the toxins relatively insensitive to the details of the extraction procedure used. More recent developments (Dr Thibault and Dr. Pleasance) include development of alternative buffers permitting improved migration of the C-toxins and GTX-toxins (all PSP toxins), and of automation procedures incorporating both autoinjection and (if desired) fraction collection.

Work on this CE/UV methodology is still in progress. Figure 5a 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 5a), 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 E(ii) below.

C(iv). 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[24,25] with fluorescence detection (though unlikely to be applicable to TTX without considerable modification), and the CE method with UV detection at 200 nm[32] in which the necessary selectivity is provided by the separatory technique rather than by the detector. The Sullivan HPLC method[13-23], with post-column on-line oxidation to fluorescent products, can be operated reliably on a continuing basis[23] but is too idiosyncratic to be used on an occasional basis. The extremely sensitive CE method[31]

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<u>Figure 5.</u>   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 simply related to those in Figure
5a.

CE/UV analysis of TTX/STX (150 μg/mL each)
10 nL inj., Trisma pH 7.2, 30 kV

CE/MS analysis of TTX/STX (150 μg/mL each) full scan (130-500 Da)
50 nL inj., Trisma pH 7.2, 24 kV effective

20
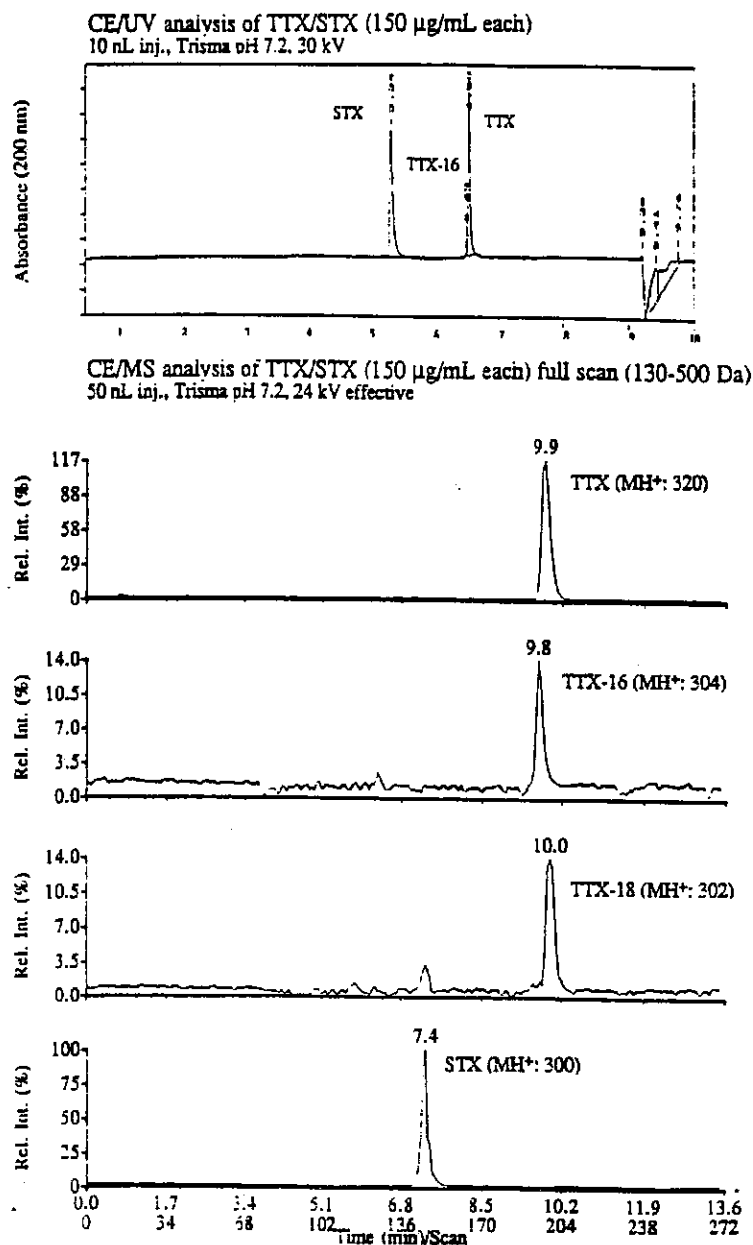
employing laser-induced fluorescence of pre-derivatized toxins suffers from several drawbacks, summarised above, for real-world samples (though it does work for TTX). However, in this regard the DND interest in monitoring fresh water could possibly be met by a version of this method[31] in which a less 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.

It is instructive to compare detection limits for STX, for different analytical methods discussed thus far. It is important in this context to carefully distinguish between *concentration* detection limits and *mass* detection limits. The parameter connecting the two is, of course, the injection volume. The summary in Table I below is adapted from that given as Table II in Ref. 32, plus the recent IMB data (see C(ii) above) on the HPLC-FLD method incorporating pre-column oxidation. Clearly, the HPLC-FLD method incorporating pre-column oxidation of STX to fluorescent products[24,25] shows promise, in Dr. Quilliam's hands, of being the most sensitive method available. However, other factors must be taken into consideration when deciding the most appropriate method for any particular purpose. Thus, the mouse bioassay is extremely insensitive, and also has a limited dynamic range, but is invaluable in screening any toxins of unknown identity. The CE-UV technique has excellent mass sensitivity and dynamic range but, due to the inherently very small injection volumes, has a large concentration detection limit; however, provided a CE instrument with an adequate UV detector is available, the CE-UV method has the advantage of speed and ease of use, and indeed the selectivity of the electrophoretic process itself appears[32] to make the anticipated problems associated with co-extractives, at the high concentrations used, less important than expected. Finally, the mass spectrometric detection limits are generally acceptable, but the most important feature of mass spectrometric detection lies in its specificity and thence the degree of confidence in the identity of the analyte detected. It is the combination of adequate sensitivity plus unequalled specificity which makes it so important to develop analytical methods based on high resolution separatory techniques combined with mass spectrometry. The flow-injection technique used[5] to obtain the mass spectrometric data in Table 1 is not applicable to real-world samples in which other compounds (including inorganic salts) compete with the toxin analytes for the ionspray ionisation current.

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Table I.   Detection Limits for STX Using Different Techniques

Technique	Concn.(ng/mL)	Inj.Volume(µL)	Mass(ng)	Reference
Mouse bioassay	150	1000	150	30
HPLC-FLD (post-column oxidation)	4	20	0.08	30
HPLC-FLD (pre-column oxidation)	1	4 •	0.004	M.A.Quilliam (pers.comm.)
CE-UV	1500	0.01	0.015	29
Ionspray MS (flow injection,SIM)	30	1	0.03	7
CE-MS (SIM)	2000	0.01	0.02	41
LC-MS (SIM)	1000	1	1	5

•   4µL of sample extract plus 16µL of reagent, for a total injected volume of 20µL.

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 (see Appendix). 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[34-36] to be a useful means of structural confirmation at moderate sensitivity. Very recently, Mirocha et al.[37] have examined the potential of continuous-flow FAB for analysis of STX in urine; fast clean-up on a weak cation-exchange column permitted detection limits in the 100-200 pg range, using flow-injection with a 200 nL injection volume. These workers[37] also observed methoxy derivatives resulting from reaction of hydroxyl groups in STX with methanol, as discussed previously[7] for ionspray ionization. Experiments on STX using continuous-flow FAB, conducted at IMB, have indicated a threshold level in the μg range for any mass spectrometric response to be obtained.

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.[7] that FIA ionspray detection limits, using selected ion monitoring on the protonated molecules $(M+H)^+$, were 30 pg for STX (see Table 1) 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[13-23], and 0.3 μmol/L with 40 μL injection for TTX[38]. 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[7]. Recently[39], thermospray ionization of STX was reported, though considerable ambiguities of interpretation were encountered, as discussed further below. Further, experiments conducted at IMB (by Dr. Quilliam) have shown that μg quantities of STX are required for any response to be obtained using TSP.

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 or CE. A brief general account of such interfacing

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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[37]. 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 (Section E) involve ionspray as the LC/MS and CE/MS interface.

In the case of the LC/MS and CE/MS work described in Section E below, 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[13-23] suppressed ionization of the PSP toxins and TTX, so alternative mobile phases had to be investigated. The novel ion-pairing reagents used by Dr. Quilliam at IMB, in his improvements on the pre-column oxidation approach to fluorescence detection (Section C (ii) above), may turn out to be compatible with ionspray. The CE/MS work involved a more general problem of devising a rugged, reliable interface which yielded month-to-month reproducibility.

D(i). Ionspray Mass Spectrometry 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. Some information thus obtained at IMB is included here, for the sake of completeness.

Examples are provided by observations made[5] while monitoring of fractions during the isolation and purification of PSP toxins at IMB. For example, Figure 6a 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 from the STX protonated molecule in the ionspray interface. Following several separative steps, the two purified fractions gave the FIA spectra shown as Figures 6b and 6c. 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 6b and 6c). The impurity peak at m/z 268 in the STX fraction (Figure 6c) 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.

Figure 6. 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).
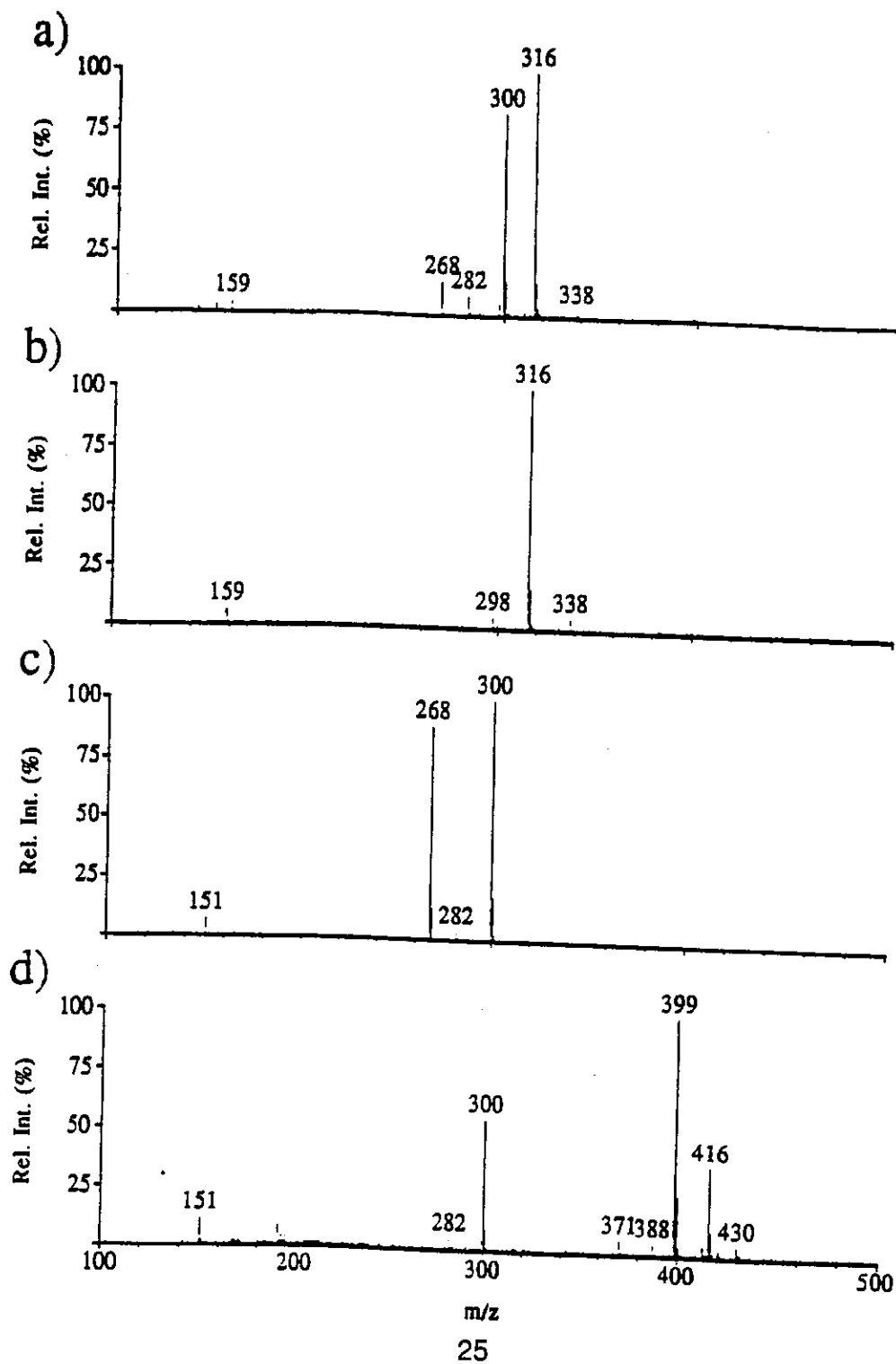
Occasional observation of an impurity peak at m/z 399 in some of Dr. Laycock's isolates led to full-scan examination of the commercially available STX standard (CalBiochem Biochemicals, San Diego, CA); a typical spectrum of a freshly opened vial is shown in Figure 6d, where the impurity at m/z 399 is seen to be the base peak with other intense ions at m/z 416, 430, 371 and 388. This observation has potentially serious implications; note, however, that nothing is known concerning the relative response factors for STX and these impurities vis-a-vis ionspray ionization. CalBiochem indicated the purity of their product to be >90% as determined by mouse bioassay and by fluorescence, and >99% as determined "by HPLC" (unspecified). The same major impurity peak at m/z 399 was also observed by other workers using thermospray[39], and by static 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[39] 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 data, 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.[7], using ionspray FIA. Further experiments[5] have confirmed the non-specific nature of these low-energy collision-induced dissociations, some of which were reported previously[34] for high-energy collisions using the MIKES technique and FAB ionization. The fragment ion spectrum[5,7] of the $(M+H)^+$ ion of STX (m/z 300) 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 7 shows results of a calibration experiment using flow injection analysis of solutions of the CalBiochem STX standard, with selected reaction monitoring; a disappointingly high 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[7], 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 reasonably sensitive and highly specific analytical technique for TTX.

Figure 7.

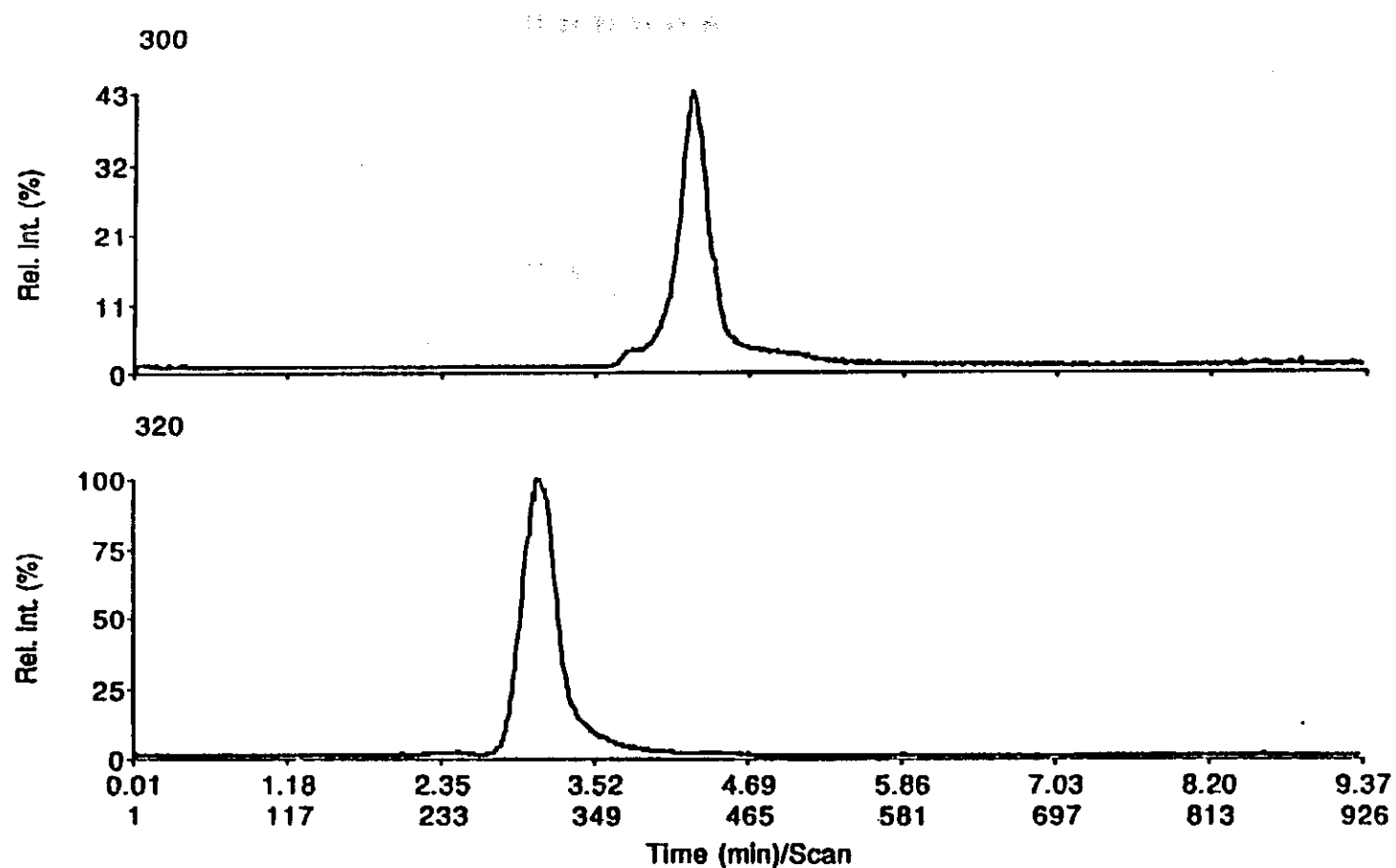# Calibration curve for FIA SRM analysis of Calbiochem STX Std
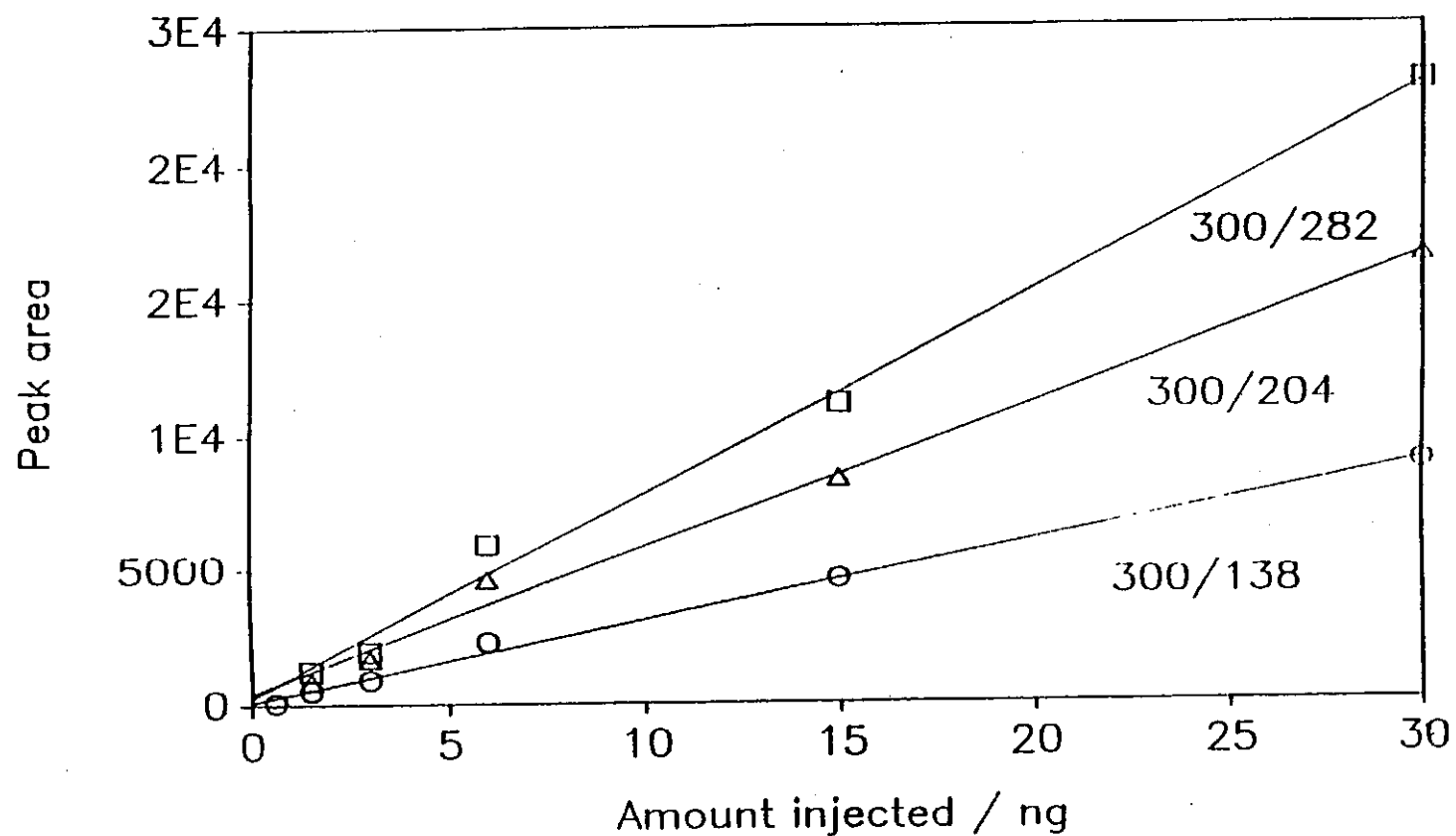
E. MASS SPECTROMETRY COUPLED TO HIGH-RESOLUTION SEPARATORY METHODS.

E(i). LC/MS Analysis of PSP Toxins and TTX.

The search for an HPLC mobile phase which permits adequate chromatographic resolution of the underivatized PSP toxins and TTX, but is in addition compatible with ionspray ionization, is currently only partly successful despite a strong effort. 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[5] due largely to the efforts of Dr. S. Pleasance, *viz.* an LC/MS method for analysis of a mixture of STX plus TTX. Figure 8 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[13-23]. 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 8 represent a compromise[5] between chromatographic performance and sensitivity (Table 1). The rather poor detection limits for this LC-MS approach could be improved by retaining a fairly high pH (5.6 or so) in order to optimise the chromatography, while using post-column addition of acidified mobile phase to increase the MS sensitivity to something approaching that achieved in the flow injection mode. While successful, this aspect of the IMB work is probably not adequate for DND purposes, since large-scale preparations of STX for chemical warfare purposes are likely to include many other PSP toxins. Accordingly, the extension of this LC-MS technique to other PSP toxins was investigated[5].

These best-compromise HPLC conditions (Figure 8) were arrived at as the result of an extensive series of trials using PRP-1 resin columns. The performance achievable using such a column for a suite of PSP toxins is illustrated by Figure 9, 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

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Figure 8. 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 (Refs. 13-23).

Figure 9. 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 8, except that a 4.6 mm PRP-1 column was used with a post-column split, and the acetonitrile content was prpgrammed from 1% to 3% over 5 min, from 3% to 10% over the next 5 min, a 5 min. hold at 10%, then programmed from 10% to 50% over the final 5 min. Flowrate was 1mL/min, with a 10 µL injection.

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 9 is adequate, although the isomeric disulfato-analogues C1 and C4 (Scheme I) are not resolved from one another, and indeed elute 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[13-23], 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 8 and 9, 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 of aged columns. 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[5], 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 10, the elution order was reversed relative to that observed in Figure 9, 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[5] provide excellent separation for the C-toxins, but would not be useful for the other PSP toxins (nor for TTX).

At present the approach to an LC-MS technique for the PSP toxins plus TTX, which appears to afford the most promise, involves the use of the perfluorinated ion-pairing reagents developed by Dr. Quilliam for the pre-oxidised toxins. Unfortunately, although the oxidised toxins show excellent chromatographic properties (Figure 2), the sensitivity of ionspray ionisation for these oxidised forms is disappointingly low (presumably reflecting the predicted drop in basicity of the nitrogen atoms upon aromatisation).

In summary, it has proved to be unusually difficult to devise robust LC-MS techniques for these highly polar toxins. The methodologies described here (Figures 8-10) have now been published[5]. They satisfy the basic requirements of DND, provided that their various limitations, described above, are kept in mind. Future LC-MS developments at IMB will centre on Dr. Quilliam's exploitation of the perfluorinated ion-pairing reagents.

Figure 10. 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 9. Note the reversal of retention order, relative to that observed in Figure 9. The column used was a 1 mm bonded amino-phase column; the mobile phase (50 µL/min, 1µL injection) contained 5 mmol/L of ammonium formate, with acetonitrile content programmed to vary from 1 to 10% in 5 min., and hold at 10% for a further 10 min.
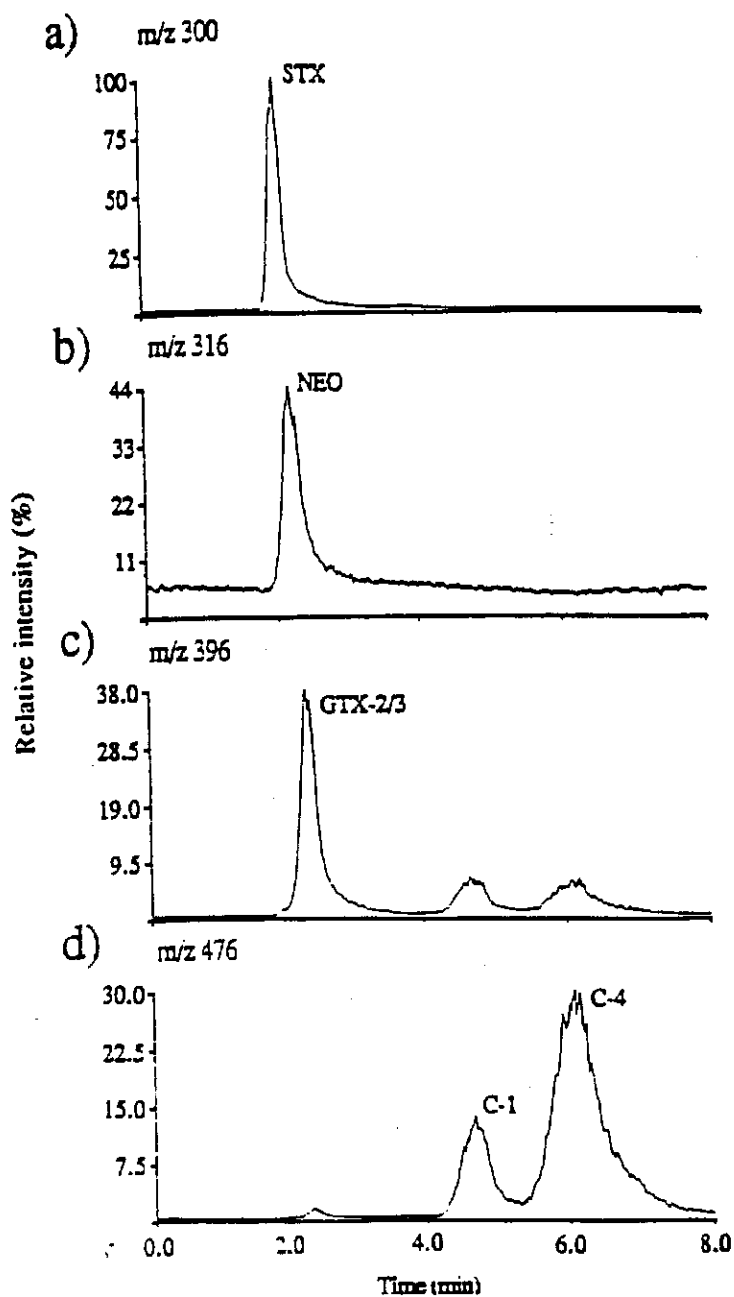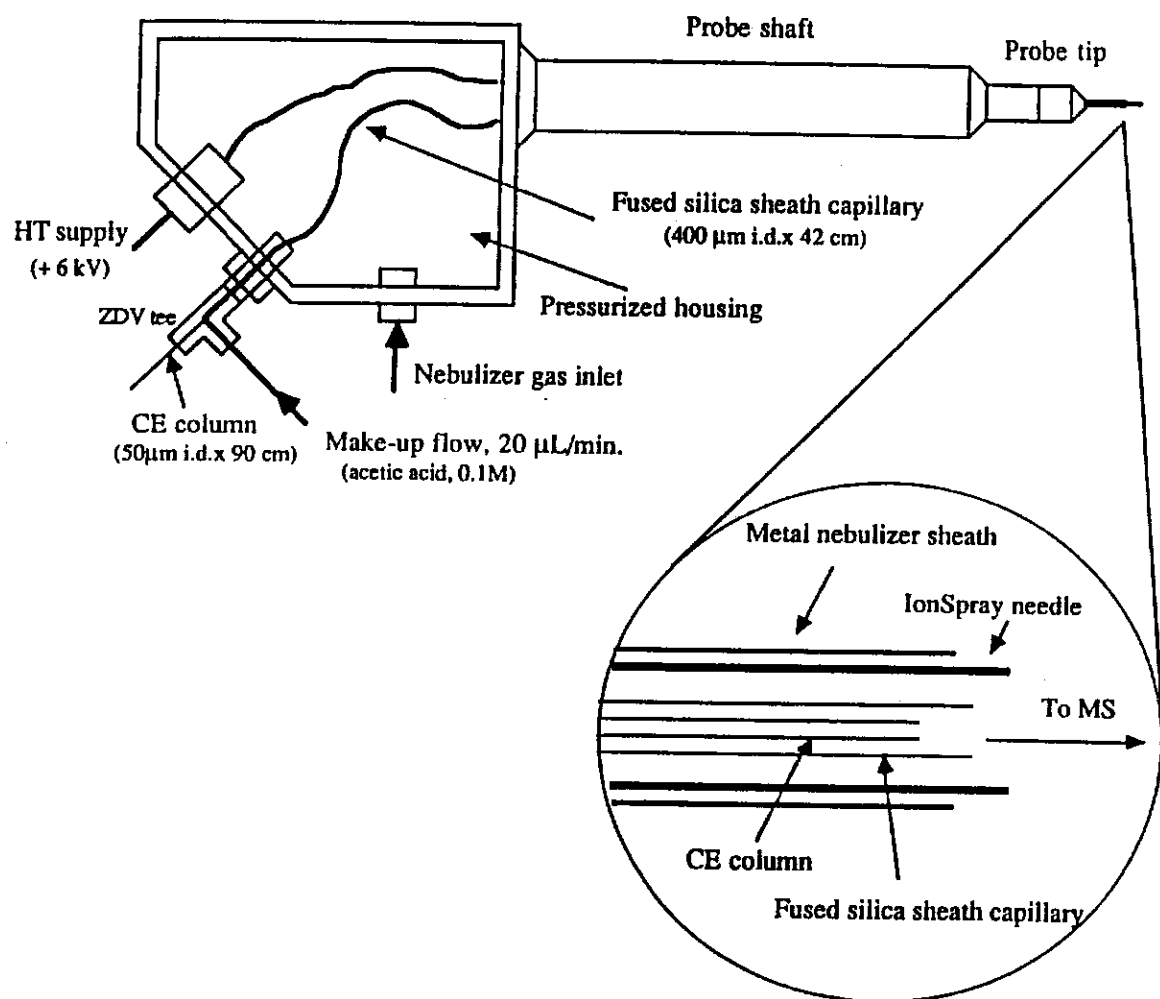
E(ii). CE/MS Analysis of PSP Toxins and TTX.

As a necessary step in the development of the CE/UV method[32] for these toxins (see Section C(iii) above), the identities of the peaks in the electropherograms were confirmed by CE/MS. While the CE/MS data published[32] 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 11, based on the co-axial design of Smith et al.[41]) was extremely unreliable and temperamental. Other groups have published CE/MS data before and since that time[41-43], 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 13, which shows reconstructed ion electropherograms 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 14. Figure 15 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).
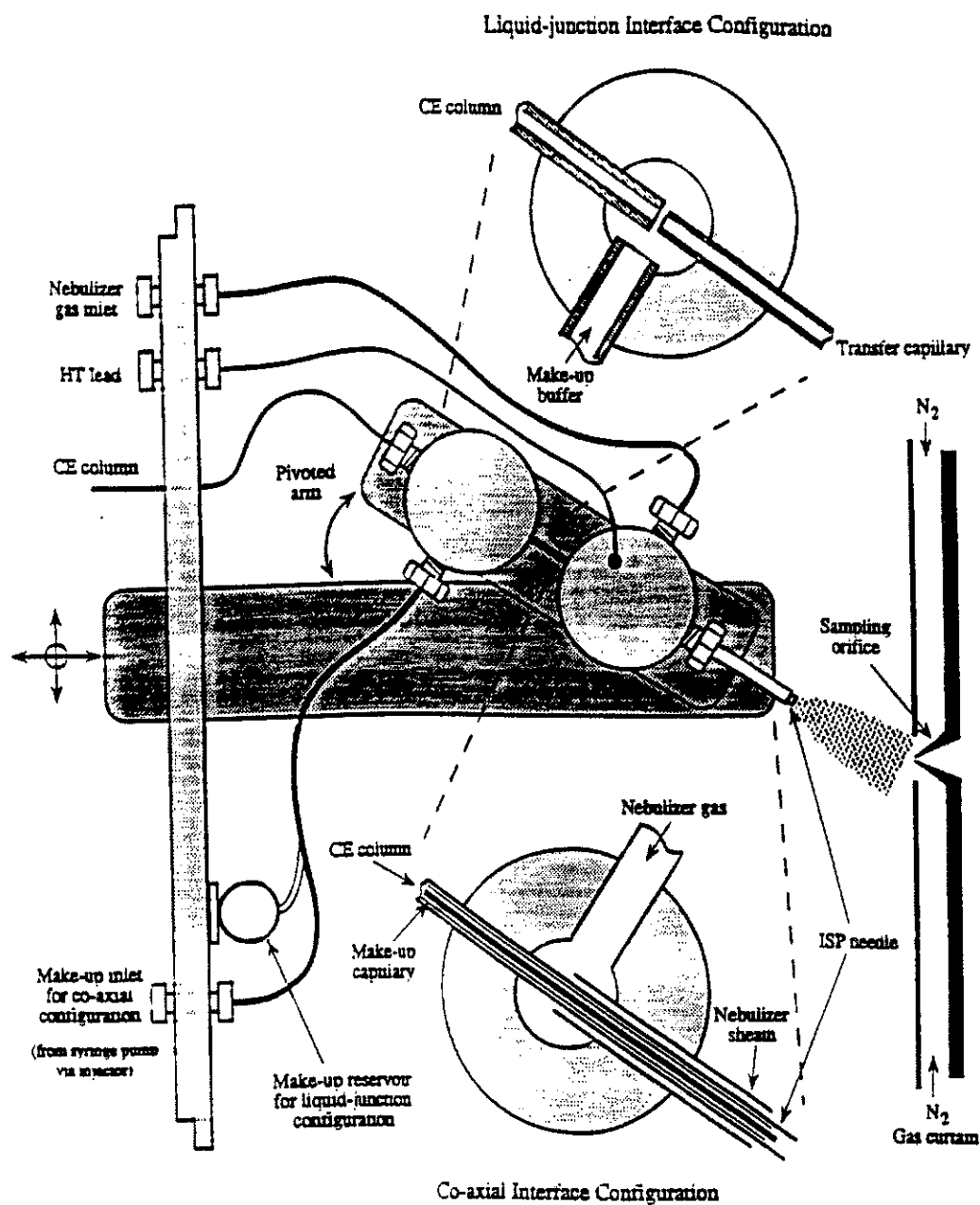
However, more recently Drs. Pleasance and Thibault have succeeded in developing a CE/MS interface, for the SCIEX API III ionspray mass spectrometer used at IMB, which is rugged and reproducible on a month-to-month basis. This greatly improved CE/MS interface is also based on the co-axial design of Smith et al.[41] and has been described in a recent publication[44]. This paper[44] also presented a detailed comparison of the preferred co-axial CE-MS interface[41] with so-called liquid-junction designs[42], as well as of various sample injection methods (see Figure 12 for a schematic of the arrangement used[44]). A CE-MS detection limit of about 20 pg was demonstrated[44] for pure STX standard (10nL injection volume). This sensitivity is about the same as for the CE-UV technique[32] (Table 1), and affords greatly improved confidence in the identity of the analyte detected. An example of results obtained using this new CE/MS interface is given in Figure 5b, 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 5a. (Note that the electrophoresis conditions were very different for the two experiments summarised in Figure 5, 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 impurities in the TTX have a molecular weights 16 and 18 Da less than that of TTX itself, suggesting that they correspond to deoxy and anhydro forms, respectively.

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Figure 11. Diagrammatic representation of the CE/MS interface (taken from Ref.32). 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.

Figure 12. Schematic diagram (from Ref. 44) of the SCIEX fully-articulated IonSpray interface, configured for both the liquid-junction and coaxial CE-MS interfaces.

Liquid-junction Interface Configuration

CE column

Nebulizer gas inlet

HT lead

CE column

Pivoted arm

Make-up buffer

Transfer capillary

N₂

Sampling orifice

Nebulizer gas

CE column

Make-up capillary

ISP needle

Make-up inlet for co-axial configuration

(from syringe pump via injector)

Make-up reservoir for liquid-junction configuration

Nebulizer sheath

N₂ Gas curtain

Co-axial Interface Configuration

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Figure 13. 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.

20s electrokin. inj., 25 kV, Trisma pH 7.2

Figure 14. Full-scan mass spectra acquired at the maxima of each of the major peaks in the electropherograms shown in Figure 13.

Figure 15. 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 extrac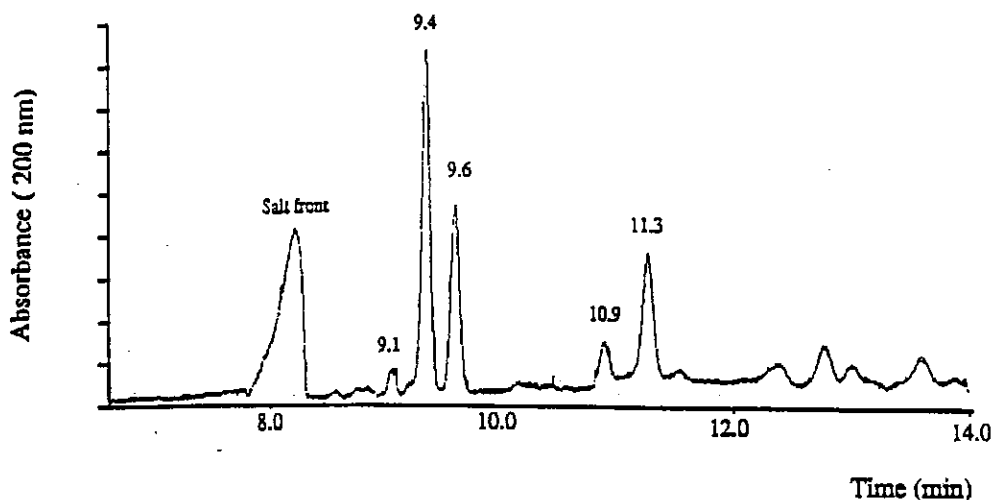t of 90 mg (wet weight) sonicated dinoflagellate cells *Alexandrium tamarensis*, filtered but with no other clean-up. The m/z values chosen for the RIE are those of the major components observed, and include those for protonated arginine (m/z 175), STX (m/z 300) and NEO (m/z 316).

CE/UV analysis with detector set at 200 nm
3s vac., Trisma pH 7.2, collector buffer: 0.2% formic, 25 kV

RIC for m/z: 175, 179, 252, 300, 316, 464 from CE/MS analysis (130-510 Da)
10s electrokin. inj., Trisma pH 7.2, make-up: 0.2% formic, 25 kV
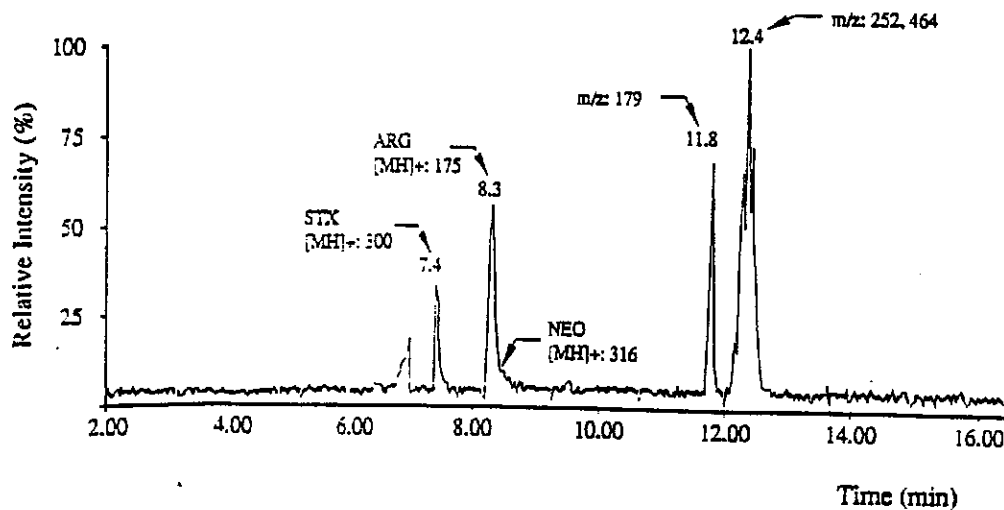
Figure 16. Analysis by CE-MS (most recently improved version of the IMB interface) of a mixture of PSP toxin standards. The (M + H)[+] ions for STX, NEO, B1, GTX 2/3 and GTX 1/4 are at m/z 300, 316, 380, 396 and 412, respectively, but some also form intense fragment ions which appear on other mass chromatograms.
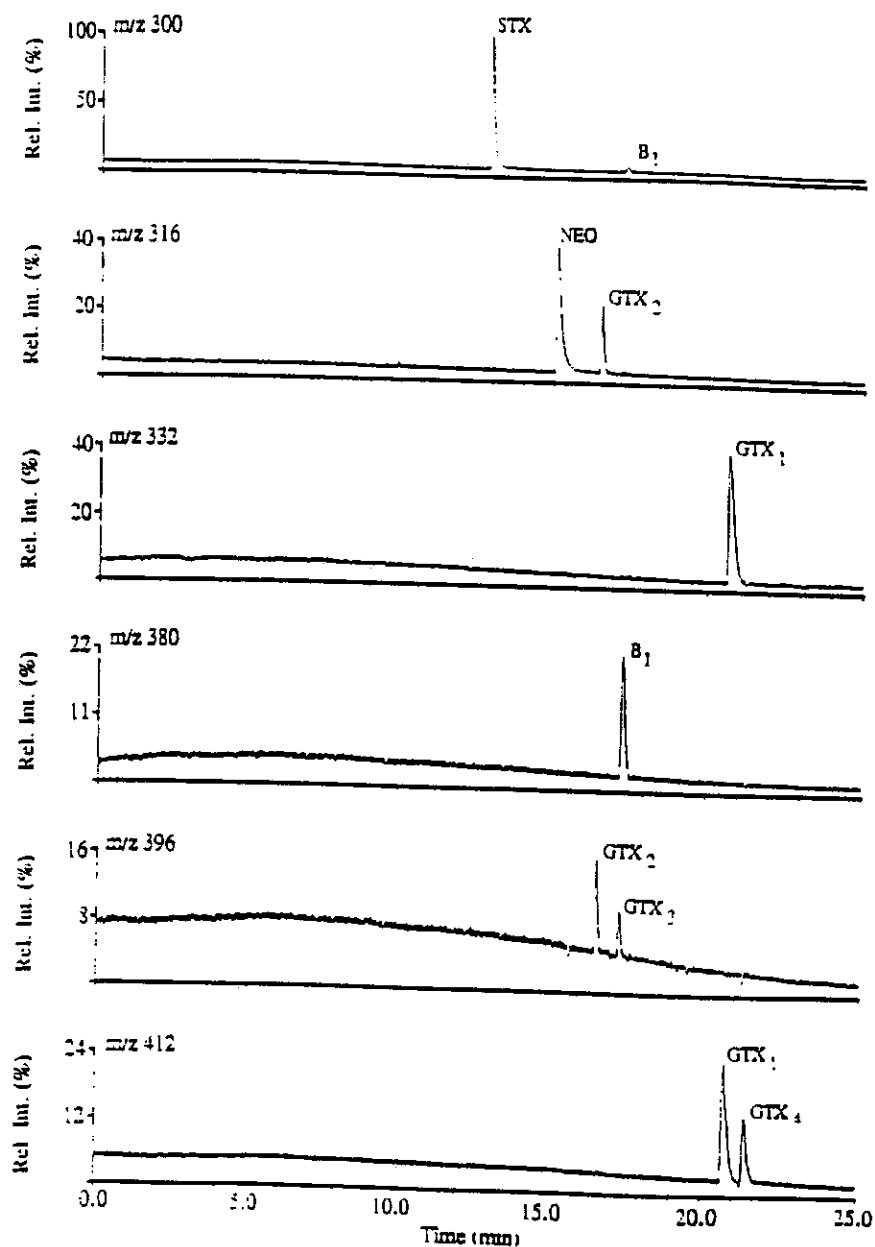
Even more recently, further improvements have been made to the CE-MS interface, and to the CE-MS methodology for PSP toxins and related compounds such as TTX. A different running buffer has permitted adequate resolution of the entire suite of PSP toxins in a single CE-MS experiment, and the method has been shown to be capable of application to extracts of contaminated scallops with only a simple clean-up step. In addition, it has been shown that CE autosampler technology works extremely well for these samples. This most recent work is currently being assessed by Dr. Thibault and Dr. Pleasance as a preliminary step to preparation for publication. Figure 16 is an example of the excellent performance, obtained for a mixture of PSP standards using this most recent version of the CE-MS interface at IMB.

E(iii). Summary.

As shown previously[7] 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[5] (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 LC-FLD method for PSP toxins[13-23], 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[24,25] to fluorescent products offers the advantage of simpler LC conditions which may turn out to be appreciably more compatible with ionspray mass spectrometry; however, considerable work on the oxidative chemistry involved is still necessary, and good progress on this approach is being made at IMB. LC/MS analysis of the native toxins (including TTX) is difficult, but a usable approach has been uncovered[5] in which two complementary sets of chromatographic conditions, each compatible with ionspray ionization, are combined. Finally, CE/MS analyses of PSP toxins have been demonstrated[32,44], and have been developed into a rugged, reproducible technique (which can be combined with CE/UV analysis in the same experiment).

It is important to set the competing analytical techniques in perspective, and Table 1 attempts to do this for the question of detection limits determined for standard solutions of pure STX. Other relevant criteria include:

(a)   susceptibility to interferences from co-extracted compounds;
(b)   dynamic range, and linear dynamic range;
(c)   reproducibility and repeatability;
(d)   ruggedness and ease of use of the technique once established in a
          laboratory;
(e)   cost and accessibility of equipment required;
(f)   degree of selectivity, and thus confidence in the interpretation of the
          measured response.

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While it is not possible to provide definitive comments for all of the techniques listed in Table 1 in the context of all of the above criteria, some more anecdotal discussion may be helpful to DND.

(a)   The analytical techniques most susceptible to interferences from co-extracted compounds are those which require large concentrations in the extract solution and which involve only minimal clean-up and separatory pre-treatment. The mouse bioassay clearly falls into this category, and indeed one potential shellfish poisoning scare in Atlantic Canada turned out, after investigation by IMB[45], to be a false alarm triggered by precisely this effect. The flow-injection mass spectrometry technique is also highly susceptible to such interferences. Both of the CE methods are potentially liable to such problems, due to the very high concentrations required in the final extract solution, but in practice this has turned out to be much less of a problem than anticipated even for matrices as complex as scallop tissue.

(b)   The dynamic range of the mouse bioassay is only about 30-40, and is linear over only part of this range. All other techniques listed in Table 1 do better. For example, the CE-UV technique has a demonstrated linear dynamic range of at least 200 for STX, while that for ionspray mass spectrometry with flow injection[5] is at least 10^3. The dynamic range of the LC-MS method developed[5] for the native toxins is poor due to the high detection limit, attributed[5] to the chromatographic requirement for a fairly high pH in the mobile phase; optimal sensitivity in ionspray mass spectrometry requires a high degree of liquid-phase ionization of the analyte, which in the case of STX and TTX implies a requirement for lower pH values. Use of Dr. Quilliam's new ion-pairing reagents may also result in a considerable improvement.

(c)   In the present context, "repeatability" is taken to refer to the short-term precision of the technique, e.g. as measured by the relative standard deviation (RSD) of the results from a series of replicate experiments all conducted within a short period of time, certainly all within the same operating day. The term "reproducibility" is frequently used synonymously with "repeatability" as defined above, but is here taken to refer to a longer-term comparison, e.g. between two such sets of replicate experiments, conducted several days or weeks apart.

The repeatability of the mouse bioassay, and of the Sullivan LC-FLD technique, have been given[33] by RSD values of ± 20% and ± 10% , respectively. The short-term RSD for the CE-UV technique[32] is better than ± 10% even at lower concentrations, while that for ionspray mass spectrometry using flow-injection[5] is ± 5-10%. No comparable information is yet available for the CE-MS technique, but preliminary indications suggest RSD values of a few per cent. In the case of the LC-FLD technique involving pre-column oxidation, current work at IMB[23] has achieved a RSD of ± 3% at the 300 ng/mL level for STX.

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The question of longer-term reproducibility is much more difficult. Any such problems can be greatly alleviated through use of internal standards, both surrogate (added to the raw sample prior to extraction and clean-up) and volumetric (added to the final extract solution). No hard information, on the extent of such problems for STX analyses, appears to be available.

(d)   The mouse bioassay[8,9] requires the maintenance of an appropriate animal colony, and a highly specialised skilled operator with an established reproducible technique for injecting the extracts into the animals and interpreting the consequent effects. Quite apart from the requirement for a skilled, experienced operator, the use of laboratory animals for such purposes seems likely to be prohibited in the future.

The Sullivan LC-FLD method[13-23] is complicated, requiring meticulous attention to detail if meaningful results are to be obtained. Nonetheless, it has proved capable of yielding excellent data on an ongoing basis, once the initial learning-curve has been traversed. Probably the most rugged and simple method, of those listed in Table 1, is the CE-UV technique developed at IMB[32]. Mass spectrometric methods are notoriously capricious, though the experience of ionspray technology at IMB over 2½ years has been that it is remarkably accessible to the inexperienced operator.

(e)   Sullivan and Wekell[16] have published an illuminating comparison of the mouse bioassay with their LC-FLD technique, with respect to several criteria including detection limits and repeatability. Also included were initial capital expense, the maximum number of assays possible per day, the cost (1986 dollars) of supplies per assay, and the total cost per assay excluding labor but including capital depreciation, maintenance costs, etc.

No such complete evaluation has been undertaken here. However, some orders-of-magnitude of capital costs can be given (Canadian dollars):

good quality CE, with 200nm UV detector;   **$40,000**

commercial LC-FLD system sold as carbamate analytical
train, adaptable to Sullivan method;   **$80,000**

good quality HPLC capable of binary solvent programming,
with fluorescence detector;   **$60,000**

ionspray mass spectrometer;   **$500,000**

The price of the mass spectrometer is likely to decrease in the near future (the price quoted is for the current "Cadillac version" with tandem mass spectrometry facilities). Also, it must be emphasised that such a mass

spectrometer is capable of application to a wide range of analytical problems, not only to STX and TTX. The same is true of the CE and LC systems.

(f)   The mass spectrometer offers a degree of chemical selectivity at high sensitivity which is unrivalled. The fluorescence methods are probably the best of the others in the present context.

F. STABILITY OF TOXINS IN SOLUTION.

The preparation of standard aqueous solutions of these toxins in water required careful handling not only in view of their relative potencies but also because of their labile and hygroscopic nature. Unlike tetrodotoxin, which is available commercially as a lyophilised powder, saxitoxin can be obtained commercially only in an acidic solution (≈pH 2) in order to avoid potential degradation in presence of oxygen or moist air.

The stability of saxitoxin (STX) in water has been investigated (Dr. P. Thibault) for a 6 months period, with the objective of eventually producing calibration solutions of selected PSP toxins. Standards of saxitoxin were obtained from Dr. M.V. Laycock as a 1mg/mL solution in 0.01 M acetic acid. An aliquot of 200 μL was taken from the stock solution and lyophilised overnight to remove the water and acetic acid, before a solution of saxitoxin could be prepared in distilled and deionised water (DIW). Because acetic acid cannot be removed completely, the resulting solution was slightly acidic (pH 5.5-6). Solutions of neosaxitoxin (NEO), and of a mixture of gonyautoxins 2 and 3 (GTX 2 and 3, Scheme I) were made up in a similar fashion. The test solution of tetrodotoxin was made up from the commercial lyophilised powder (SIGMA), without further treatment. Aliquots of these solutions were acidified with acetic acid, to various levels, for the stability tests.
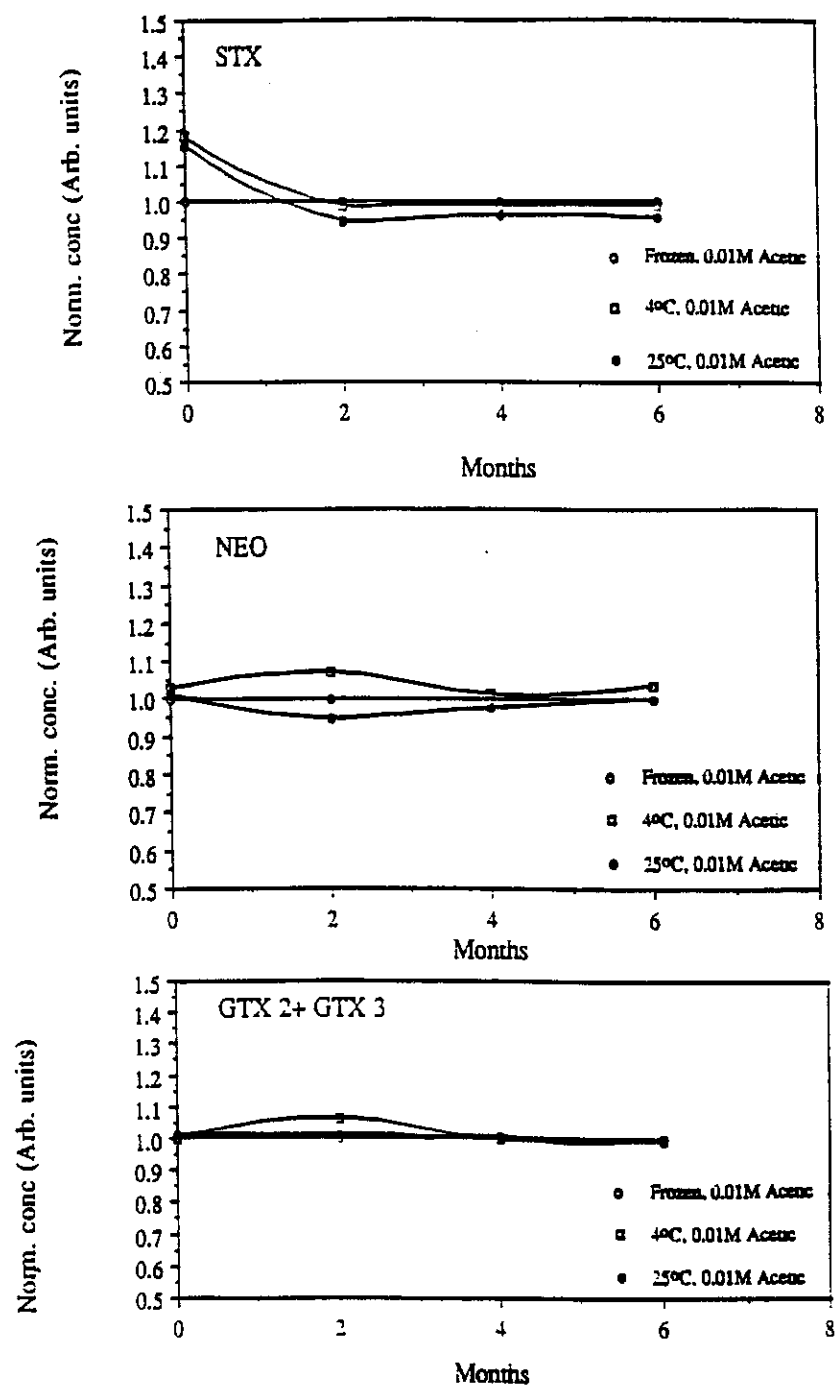
Although TTX stability in different solutions was not investigated as thoroughly as for STX, it can be stated that all the TTX solutions kept in DIW and in 0.01 M acetic acid were stable, over at least a month period, for the temperature range studied (frozen and 4°C). This general finding of relative stability of TTX is consistent with its availability as a lyophilised powder, and also with the results of the soil extraction experiments described in Section G, below.

Such a generalisation does not apply, however, to the STX solutions nor to any of the other PSP toxins tested in these experiments. A very large body of data was generated in this study, and the summary provided here represents only some of the more salient results. (It is significant that the experimental technique used to analyse the solutions in this study was CE-UV; this choice reflects the ease of use of this technique). A more complete account will be prepared for a publication concerning the instrument calibration solutions of the PSP toxins to be offered for sale by NRC's Marine Analytical Chemistry Standards Program (MACSP).

Acid solutions of all the toxins studied were found to undergo no detectable changes if maintained frozen. A comparison of the concentrations in acidified liquid solutions, maintained at 4°C and 25°C, compared with the same solutions kept frozen over a 6-month period, is shown in Figure 17. Within experimental uncertainty the liquid solutions in 0.01 molar acetic acid were as stable as the corresponding frozen solutions. (The zero-time values for NEO are almost certainly the result of some undetected aberration in the CE-UV apparatus used). Note also that, while the total

Figure 17.   Stability of solutions of saxitoxin (STX), neosaxitoxin (NEO) and a
mixture of isomeric gonyautoxins 2 and 3 (GTX), as a function of time
and storage temperature. All in solution in 0.01M acetic acid. All
analyses used the CE-UV (200 nm) technique[32], and concentrations are
referred to the values for the frozen solutions defined as 1.0 in all cases.
GTX concentrations are the sums of those for GTX2 plus GTX3.

<u>Figure 18.</u> Stability of a mixed solution of gonyautauxins GTX2 and GTX3 in 0.01M acetic acid, as a function of time and temperature. All analyses used the CE-UV (200 nm) technique[32], and concentrations are referred to the values for the frozen solutions defined as 1.0 in all cases. Differences between extinction coefficients for the two isomers are not known.

concentration of GTX-2 plus GTX-3 was unchanged over the 6-month test period, their ratio often varied reflecting the ready interconversion of these stereoisomers to an equilibrium mixture; this is illustrated in Figure 18 for the 25°C data.

The effect of acidity upon stability was negligible over the range 0.001 to 0.10 M acetic acid. Even as little as 0.001 molar acetic acid (pH 4 approx.) was sufficient to maintain the stability of these toxins (apart from equilibration of the GTX-2/3 mixture at 25°C) over 6 months. The solutions in de-ionised water (DIW, pH 5.5-6.0 due to residual acetic acid in the toxins themselves), on the other hand, showed erratic behaviour.

The situation of interest to DND concerns solutions of these toxins in a city reservoir, i.e. in aqueous (probably mildly acidic) solution, open to the air and daylight. The stability study conducted by Dr. Thibault and Mr. S. Locke at IMB used solutions sealed in glass ampoules. One set of solutions was thoroughly de-aerated with argon, and sealed under argon. The other set was similarly sealed under pure oxygen. No significant differences were detected between the stabilities of the two sets. Similarly, the studies conducted at 25°C (Figures 17 and 18) involved exposure to indirect sunlight, and results obtained suggest that the final concentration of STX and NEO after a 6 month storage are within 20% of their original values with no apparent negative consequences for stability. Only in the case of solutions stored in direct sunlight on a windowsill was there any evidence for deterioration, and this was most likely due to the consequent temperature rise rather than to photochemical effects (no UV absorption above about 210 nm).

In summary, the results of these small-scale laboratory tests suggest that TTX and the PSP toxins all have sufficient stability, in aqueous solution in glass ampoules, that they would persist for a considerable time if used to adulterate reservoirs. However, a city reservoir is not usually glass-lined. The effects of soil and sand on the stabilities of these toxins is described in the following section.

G. ANALYSIS OF SAXITOXIN AND TETRODOTOXIN SPIKED INTO SOIL.

No approved procedure currently exists for extracting saxitoxin and tetrodotoxin from contaminated soil samples. Extraction schemes were developed in the present work, based on known properties of these toxins and removal of potential interferences. In view of the likely degradation of saxitoxin in soil it was expected that recovery yields would be low irrespective of the extraction procedure used. Therefore the design of an analytical procedure required the use of a sensitive detection method such that saxitoxin would be detected even at low levels. For this reason it was decided to use HPLC-FLD (Section C (ii)) in the early development work since it yields good detection limits for saxitoxin (150 ng/mL) and is relatively insensitive to the medium in which the sample is present. However since the fluorescence yield for tetrodotoxin is much lower than that of saxitoxin, the HPLC-FLD method is quite insensitive to this compound. For this reason and also because of the limited amount of tetrodotoxin available at that time (1 mg, enough for only one 2 week spike experiment), it was decided to not include tetrodotoxin in the preliminary extraction experiments, and to focus initially on the recovery of saxitoxin only.

The extraction scheme used in the present investigation is described schematically in Scheme IV. One gram of each soil sample (both control and spiked) were left at room temperature (22°C) for periods extending from 1 hour to 2 weeks. The spike solution was made up to be 500 μg/mL saxitoxin in 0.01M acetic acid, and 100 or 200 μL of this solution was used to spike each of the three soil types. The different soils (sand, loamy sand, and sandy clay loam), were then sonicated twice at room temperature in 0.5 mL of an aqueous acetic acid solution for 10 minutes, centrifuged, and extracted twice with 1 mL of $CHC1_3$ to remove lipophilic material. The aqueous solution was filtered on a 0.22 μm filter and submitted for HPLC-FLD analysis. Results from these preliminary experiments are presented in Table 2 for two soils spiked with 50 μg of saxitoxin, using acidic solutions of different compositions for extraction.

It should be pointed out that no interference peaks were found in the extracts of any of the control soil samples submitted for HPLC-FLD analysis. These preliminary experiments indicate that, for the two soil samples chosen, the recovery yields are generally below 20% of the original amounts added to the soil. Recovery of saxitoxin from contaminated soils is better for extractions conducted 1 hour after spiking than it is for samples extracted 1 day later. As indicated in Table 2, recoveries of saxitoxin, from the two types of soil examined, were different. For example, extraction of saxitoxin from loamy sand using 2.5 M acetic acid was only 3% after 1 day whereas the corresponding yield for the sand samples was 11%. The recovery yields were also influenced by the acidity of the solution used for the extraction. For the loamy sand extracted with 1.7 M acetic acid (pH 2.5), 2.5 M acetic acid (pH 2.0) and 0.1 M HCl (pH 1.0), the recovery yields were 2, 3, and 7% respectively. Similar trends are observed for the sand samples. Progressive increase in the recovery of saxitoxin was generally obtained for extractions conducted with solutions of lower pH. However it is possible that the nature of the acid used (hydrochloric vs. acetic) would influence

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Scheme IV. Extraction scheme used for recovery of saxitoxin and tetrodotoxin from soils.

Soil samples, 1g each (spiked and control)

Sonication in 2 x 0.5 mL of 2.5 M
acetic acid for 10 min.

Centrifuge at 20,000g for 30 min.

Pellet   Supernatant

Extraction with 2 x
1 mL CHCl3

Organic phase   Aquous phase

Filtered,
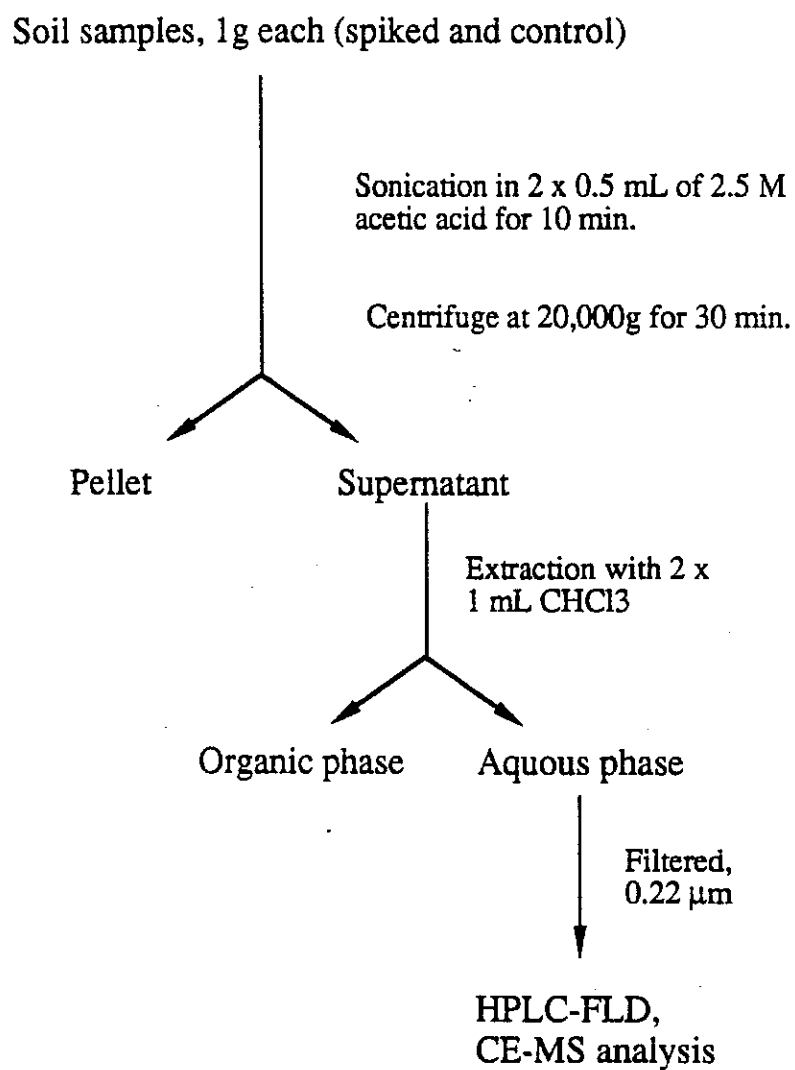0.22 μm

HPLC-FLD,
CE-MS analysis

Table 2. Preliminary experiments on extraction of Saxitoxin from soils, using HPLC-FLD technique for analysis.

Soil Type	Storage Period	Extraction Medium	Concentration (μg/mL)*	Recovery Yield**
Sand	1 hour	1.7 M CH_3COOH	10±2	20±4
	1 day	1.7 M CH_3OOH 2.5 M CH_3OOH 0.1M HCl	6*** 5.4±0.1 6.7±0.2	13% 10.8±0.2% 13.4±0.4%
Loamy Sand	1 hour	1.7 M CH_3OOH	2.3±0.3	4.6±0.6%
	1 day	1.7 M CH_3COOH 2.5 M CH_3COOH 0.1M HCl	0.8*** 1.6±0.1 3.3±0.2	2% 3.2±0.2% 6.6±0.4%

* Based on two measurements.

** 100% recovery would correspond to 50 μg/mL.

*** Only one value available.

these extraction yields. Availability of saxitoxin for extraction would be enhanced in presence of more acidic solutions if, for example, the toxin were covalently bonded to the soil. Under stronger hydrolytic conditions the toxin would be possibly more available for extraction. Although the best recoveries were obtained for 0.1 NHC1, it was presumed that such conditions might promote the degradation of tetrodotoxin. For this reason extraction with 2.5M acetic was preferred for the more extensive experiments.

A more extensive series of experiments was then undertaken. Recoveries of both STX and TTX, from three different soil types, were studied over storage periods of up to two weeks. Preliminary experiments had indicated some chemical transformation of TTX under these conditions, so analyses of soil extracts were conducted by CE-MS in Selected Ion Monitoring (SIM) and full-scan acquisition modes. Ions corresponding to the protonated molecules MH$^+$ of saxitoxin and tetrodotoxin at m/z 300 and 320, and their corresponding decomposition products at m/z 282 and 304, respectively, were selected for SIM experiments (dwell times of 100 msec/ion). Full-scan analyses were obtained by scanning the mass spectrometer from 150 to 500 Da in approximately 2 sec/scan. The experimental conditions were similar to those described in Figure 13. Details of the CE-MS interface design have been described (Section E(ii)). The CE-MS system used a P/ACE capillary electrophoresis unit (Beckman Canada, Toronto). The CE column (90 cm x 50 µm i.d.) was inserted in a sheath column used to provide a make-up solution (30% acetonitrile, 0.2% formic acid in water) at a flow rate of 15 µL/min. Separations were performed at 15 kV using a Tris-HCl (pH 6.0) buffer. After each analysis the capillary was washed with 1 N NaOH (1 column volume) followed by a rinse with the running buffer (6 column volumes). Pressure injection, corresponding to the introduction of approximately 30 nL of sample, was used throughout the investigation.

An example of a typical CE-MS electropherogram (SIM mode) obtained during this investigation is presented in Figure 19 for each of the three soil samples extracted after 2 weeks of storage. Good sensitivity was obtained with this CE-MS interface, providing detection of toxin even when present at levels below 1 µg/mL, such as the case of saxitoxin in the extract of sandy clay loam as will be described below. The electropherograms shown in Figure 19 represent the summed responses at m/z 320 and 300 (MH$^+$ ions of TTX and STX, respectively) and at m/z 304 and 282 (presumed to be MH$^+$ ions of deoxy-TTX and of dehydro-STX, respectively); these ions were prominent in the full-scan CE-MS analyses. The separate mass electropherograms for an extract of sand spiked with the STX/TTX solution are shown in Figure 20. The broad peak in the m/z 282 electropherogram is the salt front, which is also observable in the other electropherograms. The electropherogram for m/z 320 (MH$^+$ of TTX) shows two resolved peaks of which one corresponds to that observed for the TTX solution prior to spiking. This double peak is mirrored in the m/z 304 electropherogram, though at slightly shorter elution times. These observations are consistent with isomerisation of TTX, accompanied by reduction to corresponding deoxy-TTX isomers, catalysed by the sand particles.

<u>Figure 19.</u>   Analyses of extracts of three soil samples, spiked with a mixed solution
of saxitoxin (STX) plus tetrodotoxin (TTX). All three extractions were
performed 2 weeks after the original spiking. Analysis was by CE-MS
using selected ion monitoring at m/z 320, 304, 300 and 282; the
electropherograms shown represent summed intensities of these four
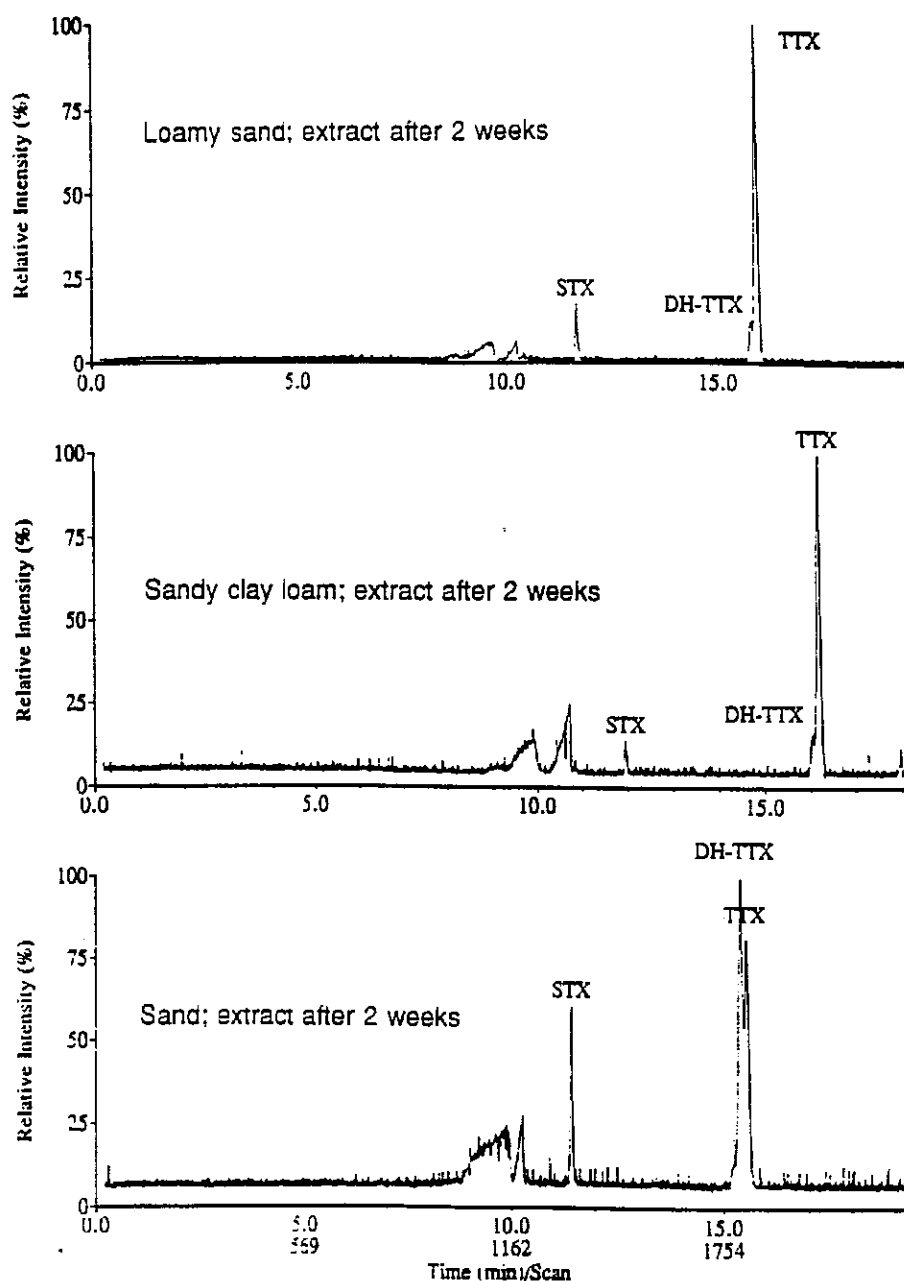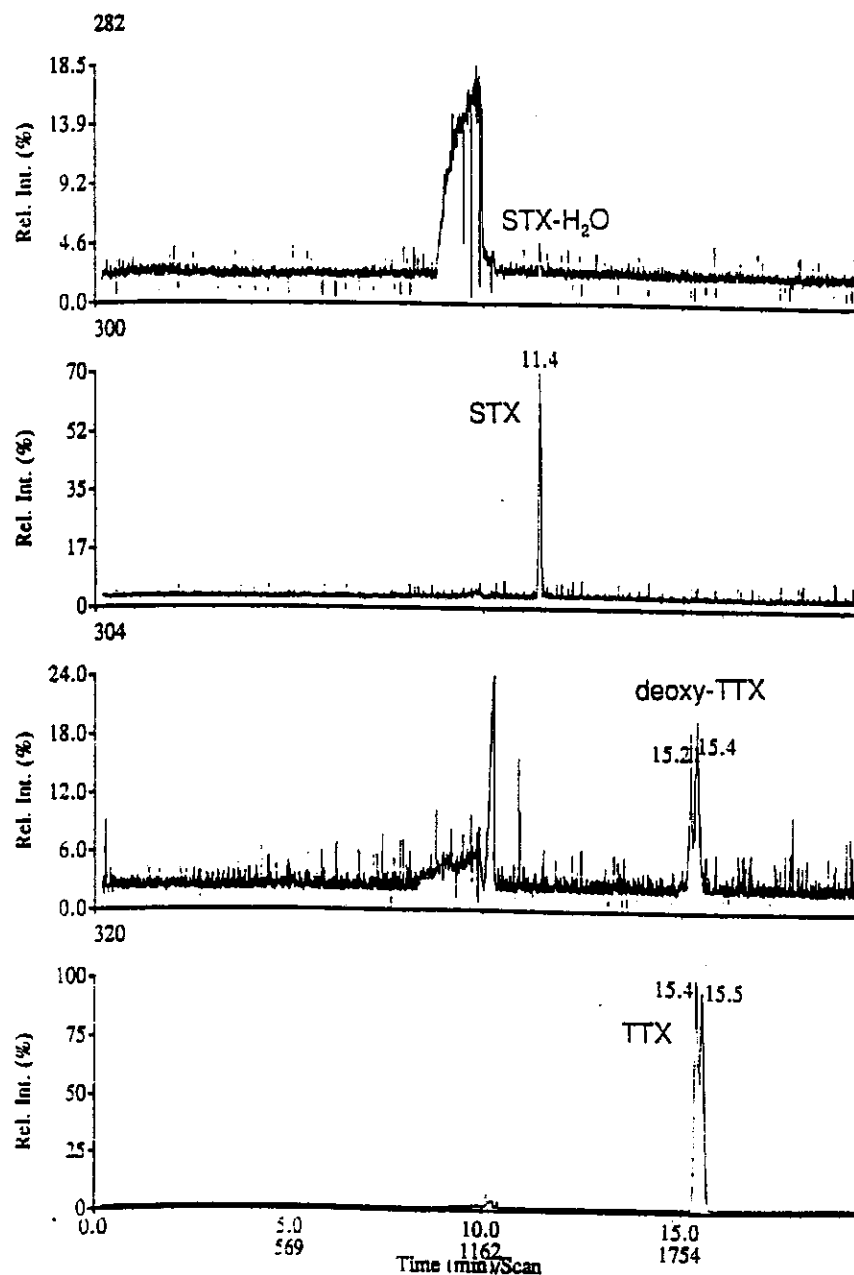ions.

Figure 20. Analysis by CE-MS of an extract of a sandy soil spiked 2 weeks previously with an equimolar solution of saxitoxin (STX) plus tetrodotoxin (TTX). Selected ion monitoring was at m/z 320, 304, 300 and 282, corresponding to MH⁺ ions of TTX, deoxy-TTX, STX and dehydro-STX, respectively.

The quantitative results for CE-MS analyses of extracts of 3 soil types, spiked with a mixture of STX and TTX, are shown in Table 3. Some of the observed trends are striking. Thus, the extraction yields of TTX are uniformly much higher than those for STX. The lowest yields for both toxins were obtained from the soil with a significant clay content; this was also the only one of the three soils tested which showed a statistically significant trend in STX recoveries with storage time. The highest STX yields were obtained from the soil sample which was essentially sand, although this was also the sample which most readily promoted isomerisation of TTX (Figure 20).

In summary, presence of clay particles in the soil greatly reduces the stability of both STX and TTX. It is not known whether this is due to irreversible adsorption, or chemical degradation, or both, of the toxins at the aluminosilicate surfaces. In all soils, STX is much less stable than TTX, a trend also observed (Section F) for these toxins in solution in glass ampoules.

Table 3.   Extraction yields of Saxitoxin (STX) and Tetrodotoxin (TTX) from different soil types, as a function of storage period. CE-MS analysis was used.

Soil	Duration	STX (μg/mL)*	Extr.yield**	TTX (μg/mL)*	Extr. yield**
Loamy Sand	2 Weeks	4.6±0.8	5±1%	92.0±6.6	92±7
	1 Day	6.5±0.6	7±1%		
	1 Hour	5.3±0.9	5±1%		
Sand	2 Weeks	13.5±1.5	14±2%	89.1±3.8	89±4
	1 Day	20.1±2.8	20±3%		
	1 Hour	17.1±4.2	17±4%		
Sandy Clay Loam	2 Weeks	0.6±0.4	0.6±0.4%	50±13	50±13
	1 Day	1.3±0.1	1.3±0.1%		
	1 Hour	2.2±0.1	2.2±0.1%		

*   Based on three independent measurements.

**   100% recovery would correspond to 100 μg/mL.

H.   SUMMARY.

The principal thrust of this work has been devoted to development and evaluation of instrumental methods of analysis of the highly polar PSP toxins and of tetrodotoxin (Schemes 1 and 2). Little attention was paid to methods of extraction and clean-up, since it was not expected that these would present serious problems in cases where the toxins are present in aqueous solution or in soil. Application of these instrumental techniques to analysis of toxins in shellfish, for example, will require that considerable effort be devoted to the extraction and clean-up steps.

Analysis of these highly polar compounds by liquid chromatography will always be difficult. The Sullivan method and its variants[13-23], incorporating ion-pairing reagents and detection by fluorescence of post-column oxidised analytes, is sensitive and reliable <u>provided</u> that the fairly complex apparatus (Figure 1) is maintained in good working condition by a skilled operator. It does not seem likely that this technique[13-23] could provide reliable data in a short period of time (emergency conditions) if it had to be started up following a considerable period since its last use. The LC-MS techniques developed here (Figures 8-10) are adequate, but require expensive specialised equipment and careful maintenance of the LC columns. Of the techniques described here, that most likely to provide a reliable results, on an "occasional - use" basis typical of chemical warfare requirements, is the CE-UV method[32] with confirmation by CE-MS[32,44].

ACKNOWLEDGEMENTS

REFERENCES.

1. Y. Shimizu, in *Handbook of Natural Toxins, Vol.3: Marine Toxins and Venoms*, A.T. Tu, Ed., Marcel Dekker Inc., New York, 1988, pp.63-85.

2. T. Goto, Y. Kishi, S. Takahashi and Y. Hirata, *Tetrahedron* **21**, 2059-2088 (1965).

3. R. Fortuine, *Alask. Med.* **17**, 71 (1975).

4. W.W. Carmichael, *Advan. Bot. Res.* **12**, 47 (1988).

5. S.Pleasance, S.W.Ayer, M.V.Laycock and P.Thibault, *Rapid Commun. Mass Spectrom.* **6**, 14 (1992).

6. W.R. Hardstaff, W.D. Jamieson, J.E. Milley, M.A. Quilliam and P.G. Sim, *Fresenius J. Anal. Chem.* **338**, 520-525 (1990).

7. M.A.Quilliam, B.A.Thomson, G.T.Scott and K.W.M.Siu, *Rapid Commun. Mass Spectrom.* **3**, 145 (1989).

8. *Official Methods of Analysis of the Association of Official Analytical Chemists*, Association of Official Analytical Chemists, Arlington, VA, 18.086-18.092.

9. *Recommended Procedures for Examination of Sea Water and Shellfish*, American Public Health Association, New York, 1970, p.61.

10. R.I.Huot, D.L.Armstrong and T.C.Charh, *J.Toxicol. Environ. Health* **27**, 381 (1989).

11. A.Cembella, Y.Parent, D.Jones and G.Lamoureux, in "Toxic Marine Phytoplankton", Elsevier, Amsterdam, 1990, pp. 339-344.

12. S.R.Davie, J.F.Hewtson and J.E.Beheler, in "Toxic Dinoflagellates", Elsevier, New York, 1985, pp. 343-348.

13. J.J. Sullivan and W.T. Iwaoka, *J. Assoc. Off. Anal. Chem.* **66**, 297 (1983).

14. J.J. Sullivan and M.M. Wekell, in *Seafood Toxins*, E.P. Ragelis, Ed., American Chemical Society, Washington, DC, 1984, p.197.

15. J.J. Sullivan, in *Marine Toxins: Origins, Structure, and Molecular Pharmacology*, S. Hall and G. Strichartz, Eds., American Chemical Society, Washington, DC, 1984, p.66.

16. J.J.Sullivan and M.M.Wekell, in *Seafood Quality Determination* , Devel. food Sce., vol. 15, D.E.Kramer and J.Liston, Eds., Elsevier, New York, 1987, p. 357.

17. J.J.Sullivan, J.Jonas-Davies and L.L.Kentala, in *Toxic Dinoflagellates*, Elsevier - North Holland, New York, 1985, p. 275.

18. Y.Oshima, M. Machida, K. Sasaki, Y. Tamaoki and T. Yasamoto, *Agric. Biol. Chem.* **48**, 1707 (1984).

19. Y. Nagashima, J. Maruyama, T. Noguchi and K. Hashimoto, Nippon Suisan Gakkawshia (*Bull. Japan Soc. Sci. Fish*) **53**, 819 (1987)

20. Y.Nagashima, J.Maruyama, T.Noguchi and K.Hashimoto, *Toxicon* **26**, 33 (1988)

21. D.F.Hwang, C.H.Chueh and S.S.Jeng, *Toxicon*, **28**, 21 (1990).

22. D.F.Hwang, K.P.Tai, C.H.Chueh, L.C.Lin and S.S.Jeng, *Toxicon*, **29**, 1019 (1991).

23. S.W.Ayer *et al.*, *Pre-collaborative Study of the High-Performance Liquid Chromatographic Method for Paralytic Shellfish Poisons*, National Research Council, Atlantic Research Laboratory Technical Report No. 60, Feb., 1990.

24. J.F. Lawrence, C. Ménard, C.F. Charbonneau and S. Hall, *J. Assoc. Off. Anal. Chem.* **74**, 404-409 (1991).

25. J.F. Lawrence and C. Ménard, *J. Assoc. Off. Anal. Chem.* **74**, 1006 (1991)

26. W.E.Fallon and Y.Shimizu, *J.Environ. Sci. Health* **A12**, 455 (1977)

27. J.W.Jorgenson, *Anal. Chem.* **58**, 743A-760A (1986)

28. A.G.Ewing, R.A.Wallingford and T.M.Olefirowicz, *Anal. Chem.* **61**, 292A-303A (1989)

29. P.D.Grossman *et al., American Biotechnology Laboratory*, Feb.1990, pp.35-43.

30. P.Thibault, C.Paris and S. Pleasance, *Rapid Commun. Mass Spectrom.* **5**, 484 (1991)

31. B.W.Wright, G.A.Ross and R.D.Smith, *J.Microcolumn Sepns.* **1**, 85 (1989)

32. P.Thibault, S.Pleasance and M.V. Laycock, *J.Chromatogr* **542**, 483 (1991).

33. J.J.Sullivan, M.M.Wekell and L.L.Kentala, *J.Food Sci.* **50**, 26 (1985)

34. K.D.White, J.A.Sphon and S.Hall, *Anal. Chem.* **58**, 562 (1986).

35. J.Maruyama, T. Noguchi, S.Matsunaga and K.Hashimoto, *Agric. Biol. Chem.* **48,** 2783 (1984).

36. Y.Nagashima, S. Nishio, T.Noguchi, O.Arakawa, S.Kanoh and K. Hashimoto, *Anal. Biochem.* **175,** 258 (1988).

37. C.J.Mirocha, W. Cheong, U.Mirza and Y.B.Kim, *Rapid Commun. Mass Spectrom.* **6,** 128 (1992).

38. T. Yasumoto and T. Michishita, *Agric. Biol. Chem.* **49,** 3077 (1985).

39. B. Luckas, G. Thielert and K. Peters, *Z. Lebensmittel-Unters. Forsch.* **190,** 491-495 (1990).

40. S. Pleasance, P. Thibault, M.A. Moseley, L.J. Deterding, K.B. Tomer and J.W. Jorgenson, *J. Amer. Soc. Mass Spectrom.* **1,** 312-319 (1990).

41. R.D. Smith, J.A. Loo, C.J. Barinaga, C.G. Edmonds and H.R. Udseth, *J. Chromatogr.* **480,** 211 (1989).

42. E.D. Lee, W. Much, J.D. Henion and T.R. Covey, *Biomed. Env. Mass Spectrom.* **18,** 844 (1989).

43. N.J. Reinhoud, W.M.A. Niessen, U.R. Tjaden, L.G. Gramberg, E.R. Verheij and J. van der Greef, *Rapid Commun. Mass Spectrom.* **3,** 348 (1989).

44. S.Pleasance, P.Thibault and J.Kelly, *J.Chromatogr.* **591,** 325 (1992).

45. A.W.McCulloch, R.K.Boyd, A.S.W.deFreitas R.A.Foxall, W.D.Jamieson, M.V.Laycock, M.A.Quilliam, J.L.C. Wright, V.J.Boyko, J.W.McLaren, M.R.Miedema, R.Pocklington, E.Arsenault and D.J.A.Richard, *J.Assoc. Official Anal. Chem.* **72,** 384 (1989).

Figure 1.   Diagrammatic representation of a Sullivan train[13-23] for analysis of PSP toxins by LC-FLD; adopted from Ref. 23

Figure 2.   Microcolumn LC-FLD analysis of the oxidation products of (a) a mixture of PSP toxin standards at 6 ng/mL, and (b) an extract of PSP-contaminated scallop liver homogenate. Column: 5 μm LichroSpher 100 RP18 microcolumn (250 x 1 mm id); mobile phase: 10 mM heptafluorobutyric acid, pH 4.4 (adjusted with NH_4OH) with a linear gradient from 0 to 20 % acetonitrile over 15 min; flow rate: 100 μL/min; 20 μL injection volume after reaction; FLD gain: (a) 18, (b) 14.

Figure 3.   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 4.   Comparison of the flow profiles characteristic of capillary electroendosmotic flow and of hydrodynamic flow.

Figure 5.   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 simply related to those in Figure 5a.

Figure 6.   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 7.   Calibration curves for STX (CalBiochem standard) obtained by flow
             injection analysis with selected reaction monitoring (SRM, m/z
             310 → 204).

Figure 8.   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 (Refs. 13-23).

Figure 9.   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 8, except that a 4.6 mm PRP-1 column was
             used with a post-column split, and the acetonitrile content was
             prpgrammed from 1% to 3% over 5 min, from 3% to 10% over the next 5
             min, a 5 min. hold at 10%, then programmed from 10% to 50% over the
             final 5 min. Flowrate was 1mL/min, with a 10 μL injection.

Figure 10.   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 9. Note the reversal of retention order, relative to that
             observed in Figure 9. The column used was a 1 mm bonded amino-
             phase column; the mobile phase (50 μL/min, 1μL injection) contained 5
             mmol/L of ammonium formate, with acetonitrile content programmed to
             vary from 1 to 10% in 5 min., and hold at 10% for a further 10 min.

Figure 11.   Diagrammatic representation of the CE/MS interface (taken from Ref.32).
             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.

Figure 12.   Schematic diagram (from Ref. 44) of the SCIEX fully-articulated IonSpray
             interface, configured for both the liquid-junction and coaxial CE-MS
             interfaces.

Figure 13.   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.

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Figure 14.   Full-scan mass spectra acquired at the maxima of each of the major peaks in the electropherograms shown in Figure 13.

Figure 15.   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).

Figure 16.   Analysis by CE-MS (most recently improved version of the IMB interface) of a mixture of PSP toxin standards. The (M + H)$^+$ ions for STX, NEO, B1, GTX 2/3 and GTX 1/4 are at m/z 300, 316, 380, 396 and 412, respectively, but some also form intense fragment ions which appear on other mass chromatograms.

Figure 17.   Stability of solutions of saxitoxin (STX), neosaxitoxin (NEO) and a mixture of isomeric gonyautoxins 2 and 3 (GTX), as a function of time and storage temperature. All in solution in 0.01M acetic acid. All analyses used the CE-UV (200 nm) technique[32], and concentrations are referred to the values for the frozen solutions defined as 1.0 in all cases. GTX concentrations are the sums of those for GTX2 plus GTX3.

Figure 18.   Stability of a mixed solution of gonyautauxins GTX2 and GTX3 in 0.01M acetic acid, as a function of time and temperature. All analyses used the CE-UV (200 nm) technique[32], and concentrations are referred to the values for the frozen solutions defined as 1.0 in all cases. Differences between extinction coefficients for the two isomers are not known.

Figure 19.   Analyses of extracts of three soil samples, spiked with a mixed solution of saxitoxin (STX) plus tetrodotoxin (TTX). All three extractions were performed 2 weeks after the original spiking. Analysis was by CE-MS using selected ion monitoring at m/z 320, 304, 300 and 282; the electropherograms shown represent summed intensities of these four ions.

APPENDIX

NOTES ON HIGH-RESOLUTION SEPARATORY TECHNIQUES
COUPLED TO MASS SPECTROMETRY

All chemical separatory techniques involve subjecting a chemical mixture to competing physical effects, so that the different components of the mixture reach different compromises between these effects. For example, classical chromatography involves competition between the tendency of the mobile phase to sweep the compounds with it out of the system, and that of the stationary phase to retain the compounds; the different compromises between these two effects result in separation of the different components in time (in the case of column chromatography) or in space (in the case of thin-layer chromatography). Other separatory methods, such as capillary electrophoresis, involve compromises between different competing physical effects, as discussed below.

The importance of analytical chromatography for the separation of complex mixtures (often including isomeric components) lies in its ability to simultaneously provide qualitative analyses (confirmation of compound identity *via* both retention time and signature from some selective detector, *vide infra*) and quantitative analyses (*via* appropriate comparisons with the response from some external or internal standard). **(See Figure A1).** A wide range of analyte types can be dealt with by the complementary methods of high-resolution separatory methods presently available, *viz.* gas chromatography (GC), high performance liquid chromatography (HPLC, or often simply LC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE, or HPCE, or sometimes CZE). Each of these separatory methods has its own unique combination of advantages and disadvantages, *e.g.* capillary column GC easily provides high resolution separations, but only for compounds which are sufficiently volatile and robust that they can withstand being held in the gas phase for times of up to 1 hour, at temperatures up to 250°C or greater.

Mass spectrometry (MS) currently provides the detection method of choice for analytical chromatography. **(See Figure A2).** Its combination of universal applicability at high sensitivity, together with the selectivity of detection implicit in its provision of both molecular weight information (including elemental composition if conducted at sufficiently high mass resolving power) and structural information (*via* characteristic fragmentations of the gaseous ions which are the sample-derived entities actually detected by MS), is unrivalled by any detector currently available. In order for the electromagnetic fields to separate the gaseous ions according to their mass-to-charge (m/z) ratios, the environment within the MS itself must be maintained at a sufficiently high vacuum that ion-neutral collisions do not perturb the field-induced motions of the ions which provide the basis for the m/z separation. For this reason it is not surprising that, historically, the first ionization methods, electron ionization (EI) and chemical

ionization (CI) **(see Figure A3)**, involved pre-volatilization of analytes prior to their injection into the MS vacuum chamber. For the same reasons, GC was the first chromatographic method to be successfully coupled to MS, and GC/MS is now a mature technique in wide use **(see Figure A4)**.

The limitation of GC/MS techniques to volatile, thermally robust compounds can be circumvented to some extent by chemical derivatisation, usually of functional groups involved in strong intermolecular hydrogen bonding. However, such methods are time consuming, and can introduce artifacts into the GC/MS datasets which can create confusion. Other shortcomings of GC methods in general, including GC/MS, include the low degree of repeatability of liquid injection volumes (analyte in liquid solution) into the gas stream mobile phase; this is frequently a limiting factor in the overall precision obtainable in quantitative analyses by GC/MS.

Many of these disadvantages of GC do not exist for HPLC **(see Figure A5)**. Thus, separations are conducted at or near room temperature in a liquid mobile phase, so that lack of volatility and thermal fragility are no longer a problem. The repeatability of liquid injection volumes into the liquid mobile phase, using modern injection loop technology, is sufficiently high that this is seldom (if ever) the limiting factor in quantitative analyses. Other advantages of HPLC over GC include its amenability to preparative fraction collection for off-line characterization. These advantages come at the price of lower column efficiency than that obtainable using GC, and difficulties in interfacing to MS.

It is possible to use off-line MS analysis of fractions separated by HPLC, but on-line LC/MS is preferable for several reasons **(see Figure A6)** including possibilities for computer-assisted analyses of complex mixtures not fully resolvable by HPLC, quantitative analyses, and savings in time and effort. However, until recently, fundamental problems in interfacing HPLC to MS made combined LC/MS a specialised analytical technique. The most obvious problem **(see Figure A7)** involves the mismatch between the HPLC mobile phase and the vacuum pumping capability of even the best modern MS vacuum systems. The sheer volume of gas generated from the liquid mobile phase (up to 1200 mL/min of gas, measured at atmospheric pressure, from 1 mL/min of liquid) is some 200 times too great for the MS pumping system, and this problem can be exacerbated by the changing mobile phase composition characteristic of gradient elution HPLC. In addition, many mobile phase modifiers (buffers, ion-pairing regents, *etc.*) are insufficiently volatile to be pumped away by vacuum pumps. A less obvious problem involves the wide range of analyte types which are amenable to HPLC methods, which in turn implies a requirement for a wide range of ionization methods in the mass spectrometric stage of LC/MS analyses; for example, EI or CI are desirable ionization methods for thermally robust analytes such as polycyclic aromatic compounds (PACs), but for fragile analytes such as peptides and proteins, or nucleosides and nucleotides, methods such as fast-atom bombardment (FAB) or its variants are required. All of these problems must be solved with no deterioration of chromatographic performance, a daunting task.

Successful methods of interfacing HPLC to MS can be categorized as involving mobile-phase removal, reduction of flow-rate into the mass spectrometer, or special methods combining the interfacing of a liquid effluent to a vacuum chamber with the ionization process itself **(see Figure A8)**. The most common method based on mobile phase removal is that provided by the moving belt interface **(see Figure A9)**, which can be used successfully with El, Cl or FAB ionization and is thus applicable to a wide range of compound types; however, the use of FAB ionization with this interface has proved, in practice, to be difficult and far from routine. The particle-beam interface, also involving solvent removal, has been recently introduced for use with El or Cl, but its range of applicability to real-world problems has yet to be established. An alternative approach to the problem of overloading the MS vacuum system, is to reduce the mobile phase flow-rate to values compatible with the pumping capacity. This can be done either by splitting the HPLC effluent from standard analytical HPLC columns (i.d. values 1 - 4.6 mm), diverting the majority of the flow to a complementary detector (*e.g.* UV-visible) or fraction collector, or by using micro-LC columns (i.d. less than 300 microns) with no split. The use of a large split ratio, or of low sample loadings characteristic of micro-LC columns, leads to reduced sensitivity for minor components. Ionization methods used in conjunction with each of these flow-rate reduction strategies include Cl with the mobile phase acting as Cl reagent gas (direct liquid introduction), and continuous-flow FAB **(see Figure A10)**; both methods have considerable problems in practice.

The special combined techniques, in which the transformation of an atmospheric pressure liquid into gas phase ions is accomplished within a single device, include thermospray (TSP) and its variants, and various methods of atmospheric pressure ionization (API). The TSP method **(see Figure A11)** involves heating the liquid effluent just before its introduction to the MS vacuum chamber; the mobile phase thus protects the analyte by absorbing most of the thermal shock, and the rapid expansion of the partially vaporized effluent into the vacuum chamber provides expansion-cooling together with analyte ionization *via* mechanisms which are probably related to the ion-evaporation process discussed below. In practice TSP is a reliable technique for LC/MS, though its efficiency is highly compound-dependent (probably reflecting some residual susceptibility to thermal degradation) and the derived ion currents are highly irreproducible. More recently API methods for LC/MS have been introduced, and these show considerable promise for making LC/MS analyses almost as routine as the well-established GC/MS techniques. The heated pneumatic nebulizer (HPN) bears a superficial resemblance to TSP; however, the heating is much more gentle (about 100°C maximum) and is applied at atmospheric pressure. The nebulised droplets thus drift through a hot atmosphere, permitting rapid evaporation of mobile phase leaving extremely small micro-particles of analyte whose vapour pressure is many times larger than that of the bulk material. The vaporized analyte is then subjected to soft chemical ionization initiated by a corona discharge at atmospheric pressure, and the ions are sampled through a micron-sized orifice, protected by a counter-current of dry nitrogen, into the MS vacuum chamber. This HPN technique can be highly efficient for analytes of moderate volatility and thermal stability, but is not applicable to very large and/or polar analytes.

Fortunately, a complementary API technique is particularly sensitive for just those highly polar analytes unsuited to the HPN interface. This technique presently exists as two variants, electrospray (ESP) and ionspray (ISP); the latter is essentially identical to the former, but with the addition of pneumatic nebulization of the LC effluent. The essential features of both ESP and ISP include operation at room temperature and pressure, and no subsidiary ionization step such as atmospheric pressure CI initiated by corona discharge. The effluent is pumped through a stainless steel needle maintained at a potential of several kV, and of polarity the same as that of the desired ions (see Figure A12). Without pneumatic assistance, electrostatic instabilities at the needle can effect efficient nebulization at flow-rates of up to 5-10 µL/min, but this upper limit can be increased by factors of up to 20 with pneumatic assistance. The nebulised droplets emerging from the charged needle are themselves charged, and drift towards the orifice leading to the MS vacuum under the influence of the resulting Coulombic repulsion. During this drift time the mobile phase is progressively removed into the ambient atmosphere, until the droplets have shrunk to a small enough size that the charge density at the surface renders the droplets electrically unstable. The electrostatic stress is relieved by expelling pre-formed ions from the liquid surface into the surrounding atmosphere. These ions possess much higher mobilities than do the droplets from which they originated, and are accelerated towards the orifice under the influence of the applied fields. The MS is protected from droplets and most of the ambient atmosphere by a counter-current flow of dry nitrogen (gas curtain); even so, extremely efficient vacuum pumps are required, usually cryopumps operating at liquid helium temperatures.

The LC/ISP/MS combination is an extremely powerful tool for analysis of polar compounds which readily produce pre-formed ions in the liquid phase. Although flow-rates of up to 200 µL/min can be accomodated, optimum efficiency generally requires a flow-rate of 50 µL/min or less. This can be achieved using split ratios of about 20:1 and 4:1 for 4.6 mm and 2.1 mm i.d. columns, respectively, or direct connection of 1 mm or 320 µm i.d. columns, under typical operating conditions (see Figure A13). The LC/ISP/MS interface introduces no deleterious effects on chromatographic performance relative to that obtainable using a modern UV detector. In positive ion mode the mass spectra are typically dominated by protonated molecules MH^+, with little evidence of fragmentation (see Figure A14). While this concentration of ion current, in just one ionic species carrying molecular weight information, is of value for high sensitivity quantitative analyses of target analytes by selected ion monitoring (SIM) techniques, no structural information is available. Use of tandem mass spectrometry (MS/MS) techniques (see Figure A15) to fragment these MH^+ ions can, however, fill this gap if required (see Figure A14c). Quantitation using ionspray ionization is excellent, even though some slight curvature is typically observed in the calibration curves over several orders of magnitude (see Figure A16). For analytes amenable to the technique, LC/ISP/MS provides an analytical method with excellent sensitivity, selectivity and reproducibility.

This discussion thus far has concentrated on the well-established high-resoltuion separatory techniques, GC and LC. The more recently developed technique of capillary electrophoresis will now be considered. 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. 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, (*J.W.Jorgenson, Anal.Chem. 58, 743A-760A (1986); A.G.Ewing, R.A.Wallingford and T.M.Olefirowicz, Anal. Chem,. 61, 292A-303A (1989); P.D. Grossman, Amer. Biotech. Lab., Feb. 1990, pp.35-43*). However, it is worthwhile for present purposes to emphasise that emergence of an analyte from one end of the capillary is the result of the interplay of two competing 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 A17 illustrates these two effects as a function of pH, for the present case where the net charge on the untreated silica internal wall is negative so that the adjacent double layer carries a net positive charge; this annulus of positive charge is thus drawn towards the negative electrode. The bulk liquid flow that results is the electroendosmotic flow, and is characterised by its flat profile, in contrast to the parabolic profile typical of flow induced by pressure difference (Figure A18); thus the flow profile does not contribute to band broadening, as is the case for HPLC for example. The other important feature of CE is the very low volume flowrate (of the order of 100 nL/min) emerging from the capillary. These facts have implications for development of CE-MS technologies. The narrow peak widths (1-4 sec typical) imply that mass spectrometers must be scanned with scan cycles of a fraction of a second, if severe spectral distortion is not to result. In turn this has implications for mass spectrometric sensitivity, at least for scanning spectrometers. Sensitivity is also an issue in the context of the low sample volumes (a few nL) which can be used with CE. Use of selected ion monitoring for target analytes (possibly identified by a preliminary rapid-scan CEMS survey experiment) can greatly alleviate these problems.

Figure A1. Schematic representation of essential features of analytical chromatography.

Figure A2. Schematic representation of essential features of analytical mass spectrometry.

Figure A3. Ionization techniques for mass spectrometry of pre-volatilised compounds.

Figure A4. Some important considerations for gas chromatography with mass spectrometry (GC-MS).

Figure A5. Some considerations for analytical high-performance liquid chromatography (HPLC).

Figure A6. Justification for on-line HPLC with mass spectrometry.

Figure A7. Fundamental problems in LC-MS interfacing.

Figure A8. Current methods for LC-MS interfacing.

Figure A9. Schematic diagram of the moving-belt LC-MS interface.

Figure A10. Schematic diagram of the continuous-flow fast atom bombardment (CF-FAB) interface for LC-MS.

Figure A11. Schematic representation of the thermospray interface for LC-MS.

Figure A12. Schematic representation of the ionspray interface for LC-MS.

Figure A13. Compromises recommended for ionspray LC-MS operation with LC columns of different diameters, and thus different mobile phase flowrates.

Figure A14. Typical results obtained by ionspray LC-MS analysis of mussel tissue contaminated by domoic acid.

(a)   Reconstructed ion chromatogram for m/z 312, which corresponds to the protonated molecule $(M + H)^+$ of domoic acid.

(b)   Mass spectrum acquired at the crest of peak 6 in (a).

(c)   Tandem mass spectrum of fragment ions of m/z 312, acquired at the crest of peak 6 in (a)

Figure A15. Schematic representation of principles of tandem mass spectrometry using a triple quadrupole instrument.

Figure A16. Calibration curves for quantitation of the cardiac drug diltiazem (DTZ), and one of its metabolites, using ionspray LC-MS by monitoring the respective protonated molecules $(M + H)^+$.

Figure A17. 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 toward the negative electrode, for <u>untreated</u> silica capillaries, but the net concentration of positive charge in the double layer, and thus V_{eo}, varies with pH.

Figure A18. Comparison of the flow profiles characteristic of capillary electroendosmotic flow and of the hydrodynamic flow typical of pressure-induced HPLC flows.

Figure A1. Schematic representation of essential features of analytical chromatography.

Analytical Chromatography
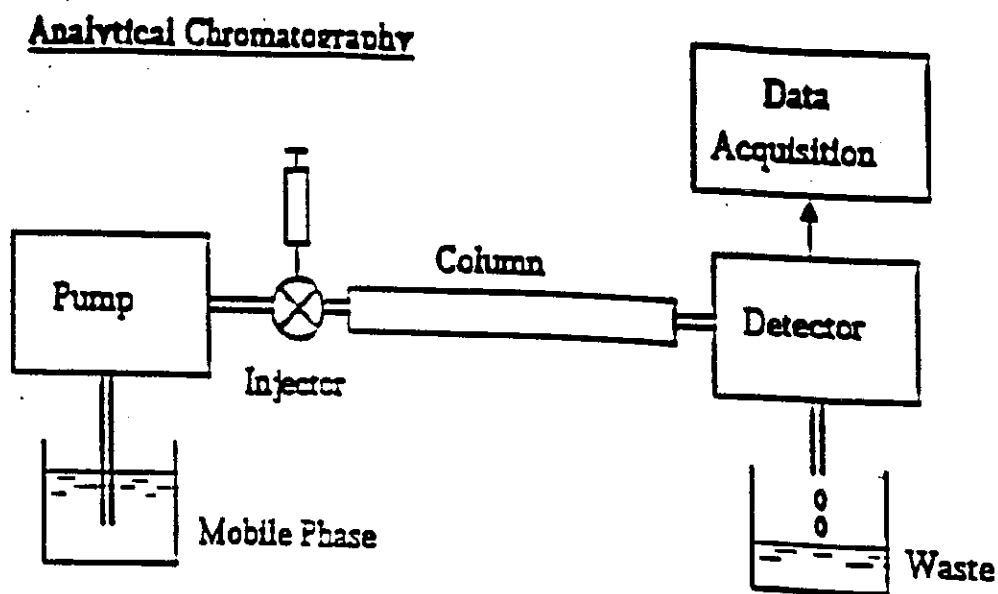
Importance:

* Separation of complex mixtures

* Separation of isomers

* Routine quantitative analysis

* Confirmation of compound identity
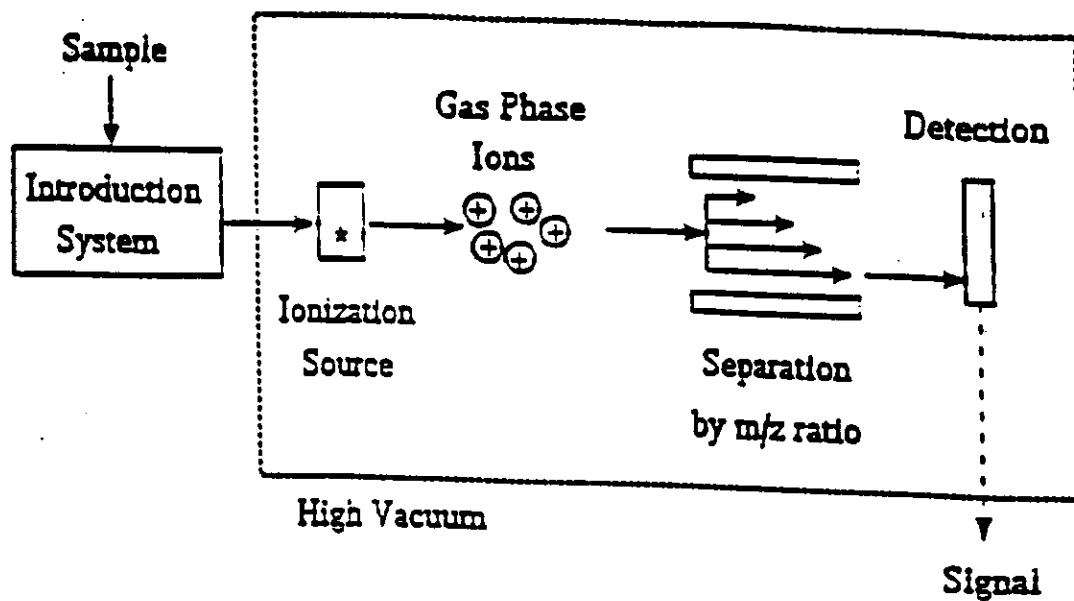   (retention time and selective detectors)

Types:

* Gas chromatography (GC)

* Liquid chromatography (LC, HPLC)

* Supercritical fluid chromatography (SFC)

* Capillary electrophoresis (CE, HPCE, CZE)

Figure A2. Schematic representation of essential features of analytical mass spectrometry.
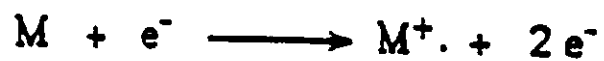
Mass Spectrometry

Sample

Introduction System

Ionization Source

Gas Phase Ions

Detection

Separation by m/z ratio

High Vacuum

Signal

Importance:

* Molecular weight

* Elemental composition

* Structure information (fragmentation, MS/MS)

* High sensitivity

* Universal detection

* Selective detection

Figure A3. Ionization techniques for mass spectrometry of pre-volatilised compounds.

<u>Electron Ionization</u>

$$M + e^- \longrightarrow M^+\cdot + 2e^-$$

$$\downarrow$$

Fragment Ions and Neutrals

<u>Chemical Ionization</u>

$$M + BH^+ \longrightarrow MH^+ + B$$

Figure A4. Some important considerations for gas chromatography with mass spectrometry (GC-MS).

Gas Chromatography - Mass Spectrometry (GC-MS)

Interfaces:

* Effusion separator

* Membrane separator

* Jet separator

* Direct connection of capillary columns

Advantages:

* Mature, easy-to-use technique

* High resolution separations

* Very sensitive (100 pg full scan)

* Numerous detectors for GC prior to GC-MS

Limitations:

* Not suitable for high MW compounds

* Not suitable for thermally labile/low volatility compounds
      (derivatization may be used to enhance volatility)

* Poor injection volume precision
      (internal standards required for quantitation)

Figure A5. Some considerations for analytical high-performance liquid chromatography (HPLC).

<u>Liquid Chromatography (LC, HPLC)</u>

<u>Advantages:</u>

* Adjustable separation selectivity

    (mobile and stationary phases, column temp)

* Suitable for high MW compounds

* Suitable for thermally labile compounds

* Excellent for quantitative analysis

* Preparative capabilities (NMR, bioassay, LSC, etc.)

* On-line acquisition of optical spectra

* Several sensitive and selective detectors

    (UV, fluorescence, electrochemical, etc.)

<u>Limitations:</u>

* Lower column efficiency than GC

* Lacks a universal sensitive detector

Figure A6. Justification for on-line HPLC with mass spectrometry.

Combined LC-MS ?

Why not off-line?

* LC has good preparative capabilities

* Independent optimization and scheduling of instruments

* Choice of ionization methods

Why on-line LC-MS?

* Universal yet selective and sensitive detection

* Complex mixture analysis

* Quantitative analysis

* Search for new compounds

* Special ionization methods

Figure A7. Fundamental problems in LC-MS interfacing.

Fundamental Problems in LC-MS Interfacing

1. The mobile phase:

   a) flow rate mismatch problem

   > MS pumping speed = 5 mL/min gas max.
   > LC at 1 mL/min = 1200 mL/min gas (water)

   b) mobile phase modifiers

   > (acids, buffers, ion-pair reagents, etc.)

   c) gradient elution

   > (changing mobile phase composition)

2. Wide range of analyte types:

   a) stable to thermally labile

   b) non-polar to polar, including salts

   c) volatile to non-volatile

   d) low MW to high MW

3. Which ionization method to use?

   > (EI, CI, FAB, etc.)

4. Chromatographic performance must be maintained

Figure A8. Current methods for LC-MS interfacing.

LC-MS Interfacing Methods

Mobile phase removal	Ionization
* Moving belt interface	EI, CI, FAB
* Particle beam interface (MAGIC)	EI, CI

Flow rate reduction (splitting or micro-LC)

* Direct liquid injection	CI
* Continuous flow FAB	FAB

Special ionization/interface methods

* Thermospray	TSP
* Atmospheric pressure ionization	API
- Heated pneumatic nebulizer	APCI
- Electrospray	ESP
- Ion-spray	ISP

Figure A9. Schematic diagram of the moving-belt LC-MS interface.

Moving Belt LC-MS Interface
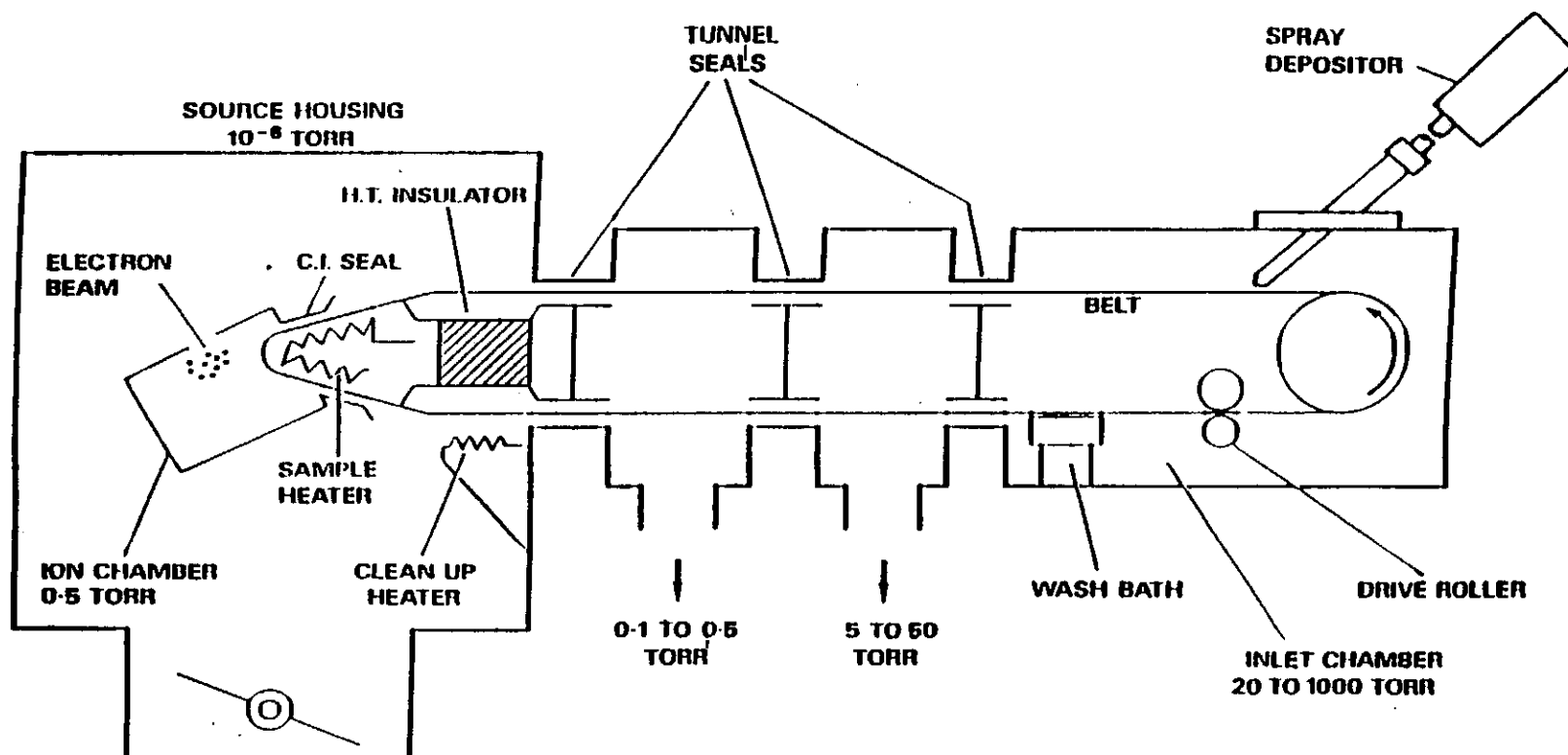
TUNNEL SEALS

SPRAY DEPOSITOR

SOURCE HOUSING
10^{-6} TORR

H.T. INSULATOR

ELECTRON BEAM

C.I. SEAL

BELT

SAMPLE HEATER

ION CHAMBER
0·5 TORR

CLEAN UP HEATER

0·1 TO 0·5 TORR

5 TO 50 TORR

WASH BATH

DRIVE ROLLER

INLET CHAMBER
20 TO 1000 TORR

79

Figure A10. Schematic diagram of the continuous-flow fast atom bombardment (CF-FAB) interface for LC-MS.

Continuous Flow HPLC/FAB-MS
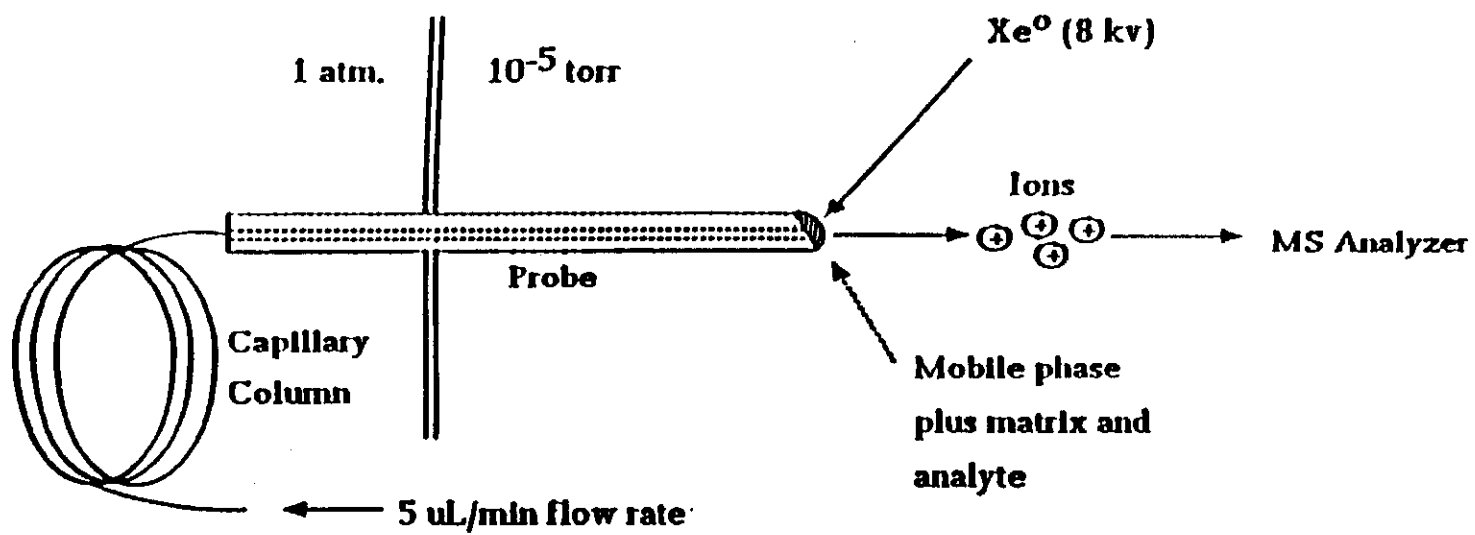
Figure A11. Schematic representation of the thermospray interface for LC-MS.
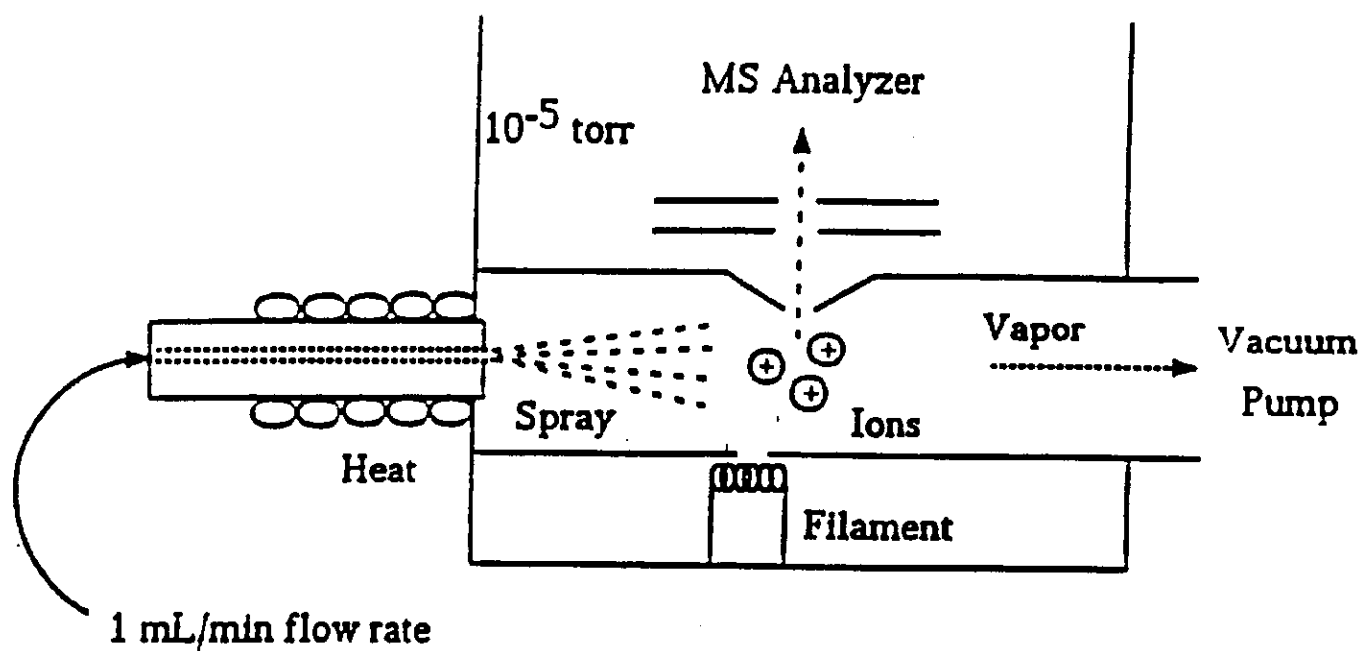
Thermospray LC-MS Interface

IonSpray Mass Spectrometry

o Atmospheric pressure ionization method.

o Mechanism: "Ion evaporation".

Droplet formation

Solvent evaporation and ions to surface

Ion emission from surface

Involatile residue

MS
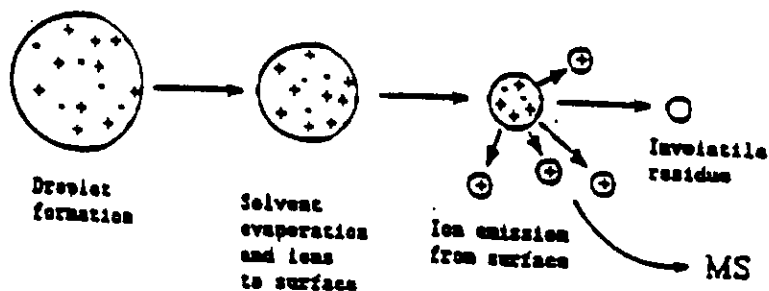
o High sensitivity for polar compounds.

o Flow rates: 1 to 200 uL/min.

o Very easy to interface to HPLC.

A.P. Bruins, T.R. Covey and J.D. Henion, Anal. Chem. 59, 2642 (1987).

The IonSpray API interface for combined liquid chromatography-mass spectrometry
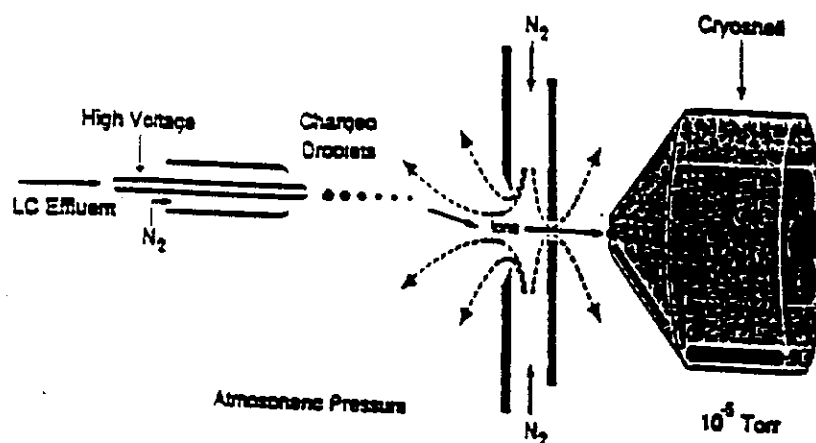
High Voltage

Charged Droplets

N_2

Cryopanel

LC Effluent

N_2

Ions

Atmospheric Pressure

N_2

10^{-5} Torr

Figure A13. Compromises recommended for ionspray LC-MS operation with LC columns of different diameters, and thus different mobile phase flowrates.

LC Configurations for combined LC-ISP-MS
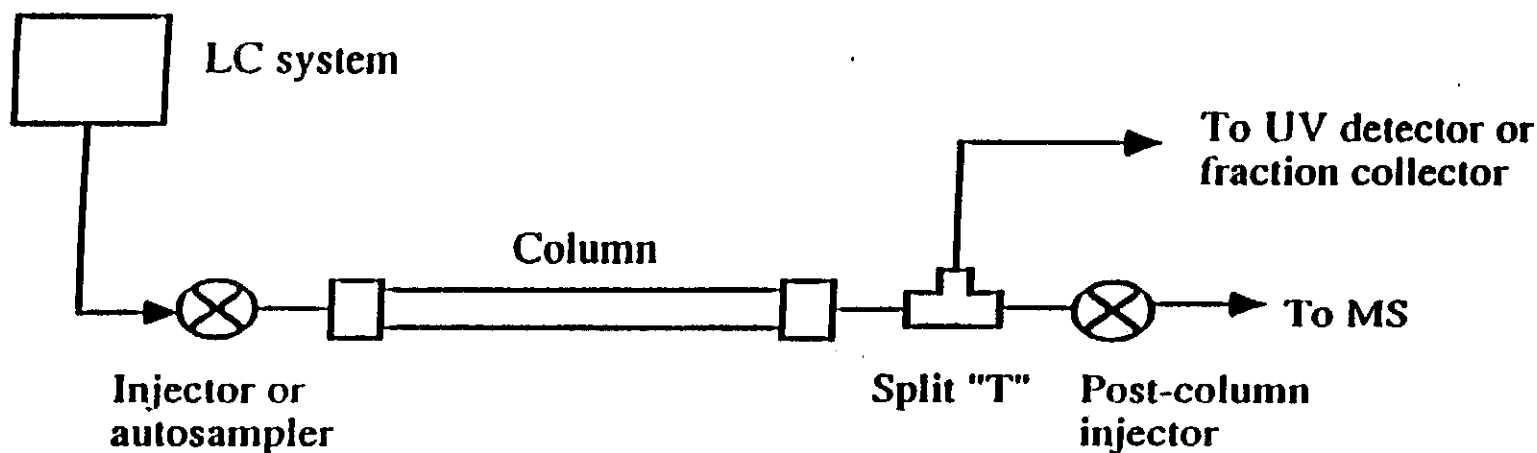
Flow rate (μL/min)	Injection volume (μL)	Column I.D. (mm)	Split	Post column injection vol. (μL)	Mode
1000	25.0	4.6	20	5.0	Semi. Prep Minor peaks
200	5.0	2.1	4 (direct)	1.0	Method dvlp. Analytical (DAD)
50	1.0	1.0	direct	0.1	Analytical
5	0.1	0.32	direct	0.06	Sample limited

Figure A14. Typical results obtained by ionspray LC-MS analysis of mussel tissue contaminated by domoic acid.

(a) Reconstructed ion chromatogram for m/z 312, which corresponds to the protonated molecule (M + H)[+] of domoic acid.

(b) Mass spectrum acquired at the crest of peak 6 in (a).

(c) Tandem mass spectrum of fragment ions of m/z 312, acquired at the crest of peak 6 in (a)

(a) HPLC/IonSpray-MS

(b) Positive IonSpray Mass Spectrum
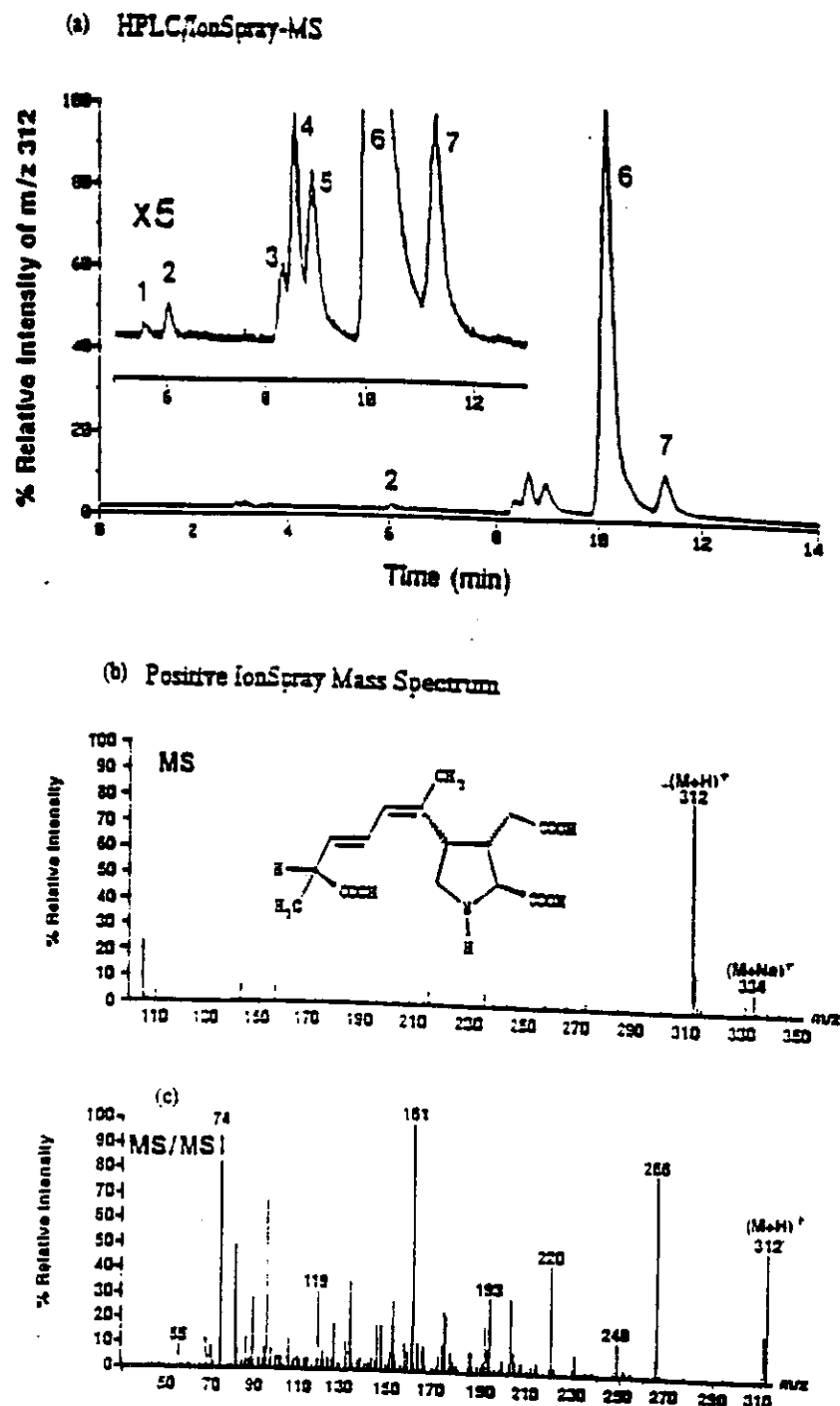
Figure A15. Schematic representation of principles of tandem mass spectrometry using a triple quadrupole instrument.
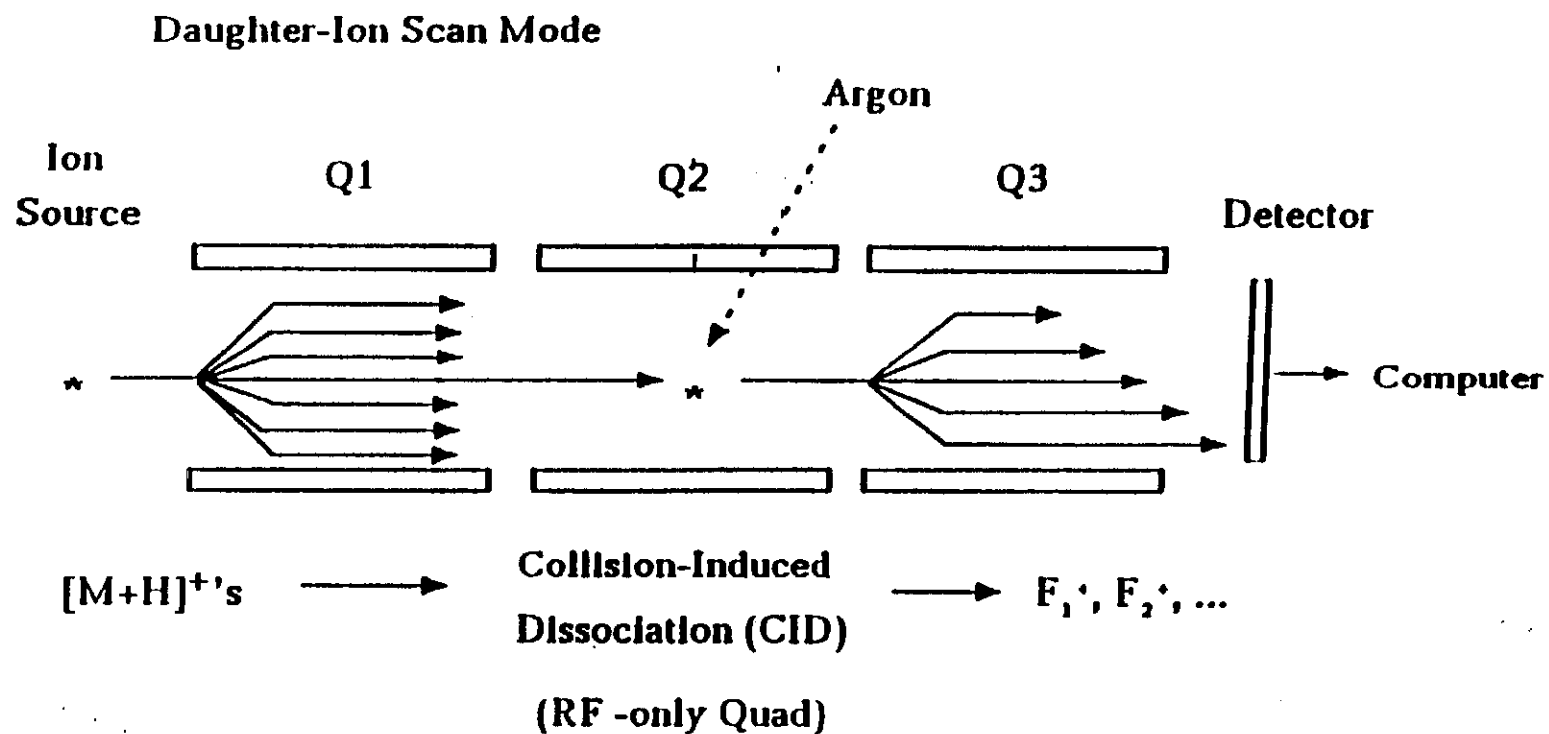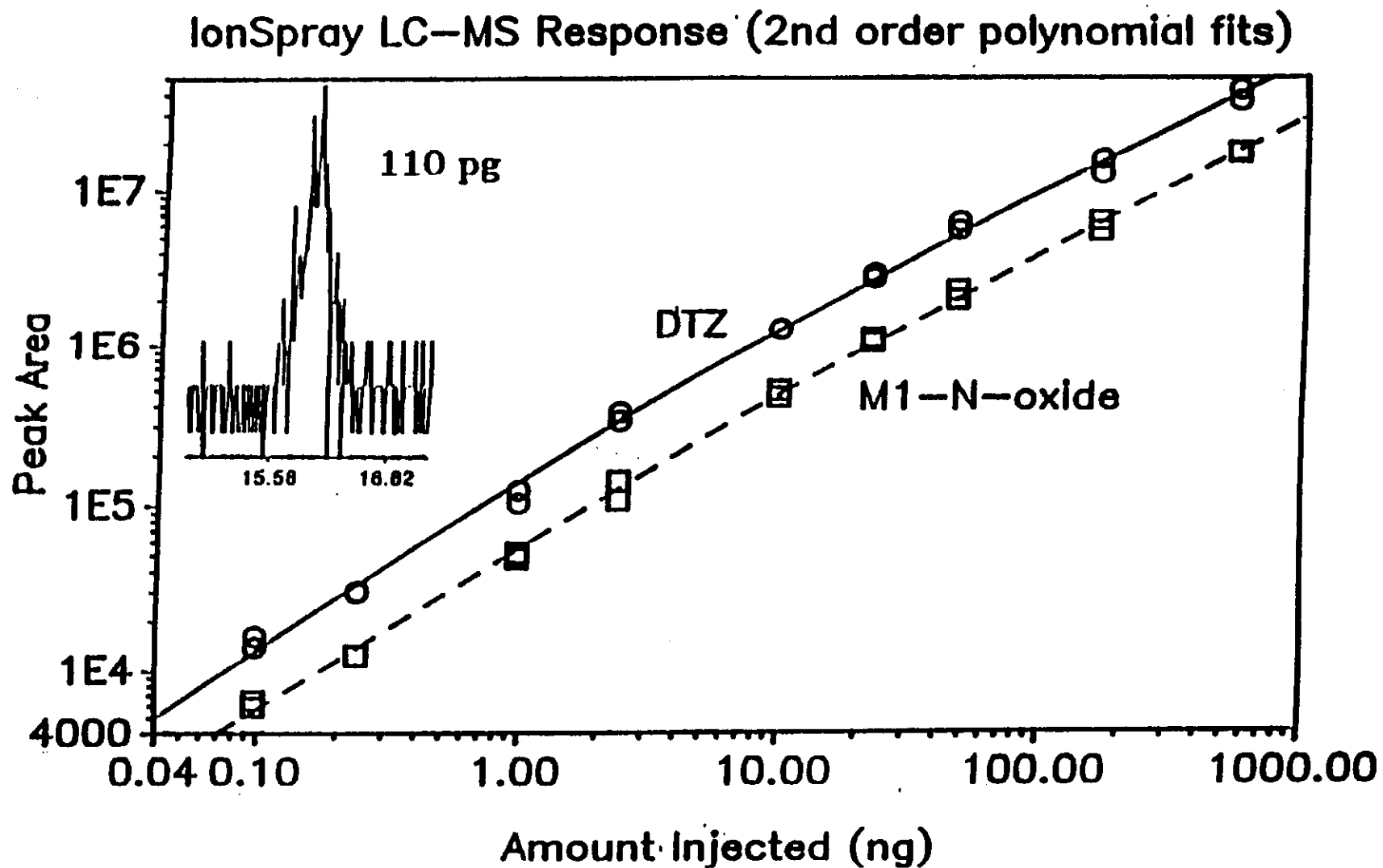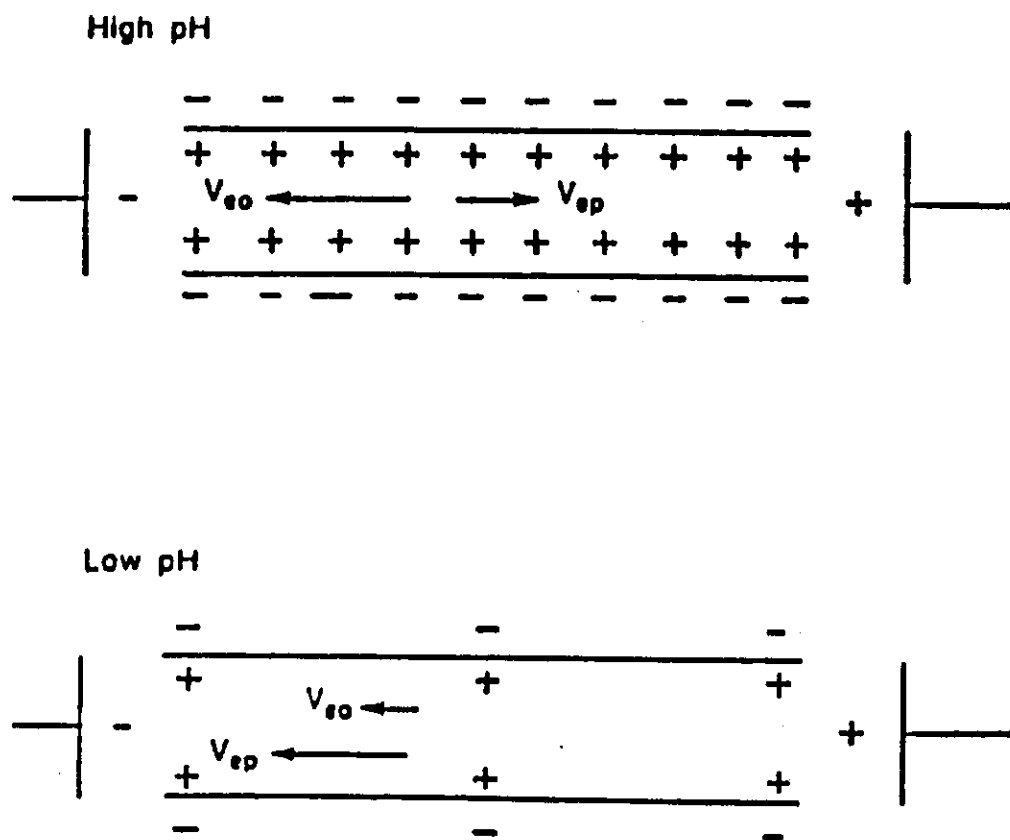
<u>Tandem Mass Spectrometry with the Triple Quadrupole</u>

Daughter-Ion Scan Mode

$[M+H]^+$'s $\longrightarrow$ **Collision-Induced Dissociation (CID)** $\longrightarrow$ $F_1^{\cdot}, F_2^{\cdot}, ...$

(RF -only Quad)

Figure A16. Calibration curves for quantitation of the cardiac drug diltiazem (DTZ), and one of its metabolites, using ionspray LC-MS by monitoring the respective protonated molecules $(M + H)^+$.
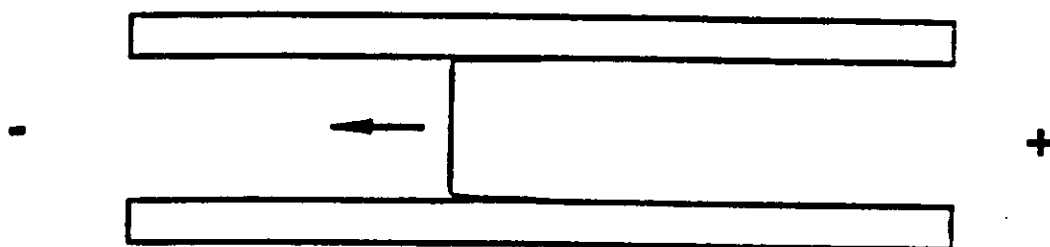
Figure A17. Diagram showing the mechanism of electroendosmosis, indicating the negative charge on the fused silica capillary wall, the positively charged double layer adjacent to the wall, the electroendosmotic velocity V_{eo}, and the electrophoretic velocity V_{ep}. At high pH the negatively charged 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 toward the negative electrode, for <u>untreated</u> silica capillaries, but the net concentration of positive charge in the double layer, and thus V_{eo}, varies with pH.

High pH

Low pH

Figure A18. Comparison of the flow profiles characteristic of capillary electroendosmotic flow and of the hydrodynamic flow typical of pressure-induced HPLC flows.

Electrokinetic profile due to electroosmotic flow

Hydrodynamic profile due to pressure difference