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Journal of Chromatography A, 1000 (2003) 527–548
JOURNAL OF CHROMATOGRAPHY A
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Review

The role of chromatography in the hunt for red tide toxins

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Abstract

An overview is given of the different approaches that have been used to identify toxins responsible for seafood poisoning incidents, to investigate the origins of toxins, and to monitor seafood on a routine basis. It is shown that advancements in our knowledge of toxins and our ability to protect the public have often followed key developments in separation and analysis technologies. Specific examples of research in this field are presented to illustrate the significant role that chromatographic methods play. The presentation will be given in an order that reflects the typical sequence of investigations that follow a new toxin episode.

Keywords: Reviews; Detection, LC; Reference materials; Mass spectrometry; Marine toxins; Toxins

Contents

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0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00586-7

1. Introduction

When Captain Vancouver landed in British Columbia in 1793, tragedy struck when several of his crew suffered agonising deaths due to paralysis and asphyxiation after eating shellfish taken from an area now known as Poison Cove. The Captain later noted that the native Indians considered it dangerous to eat shellfish when the seawater was ‘‘coloured’’. This toxic event was one of the earliest recorded incidents of paralytic shellfish poisoning (PSP). Since then, many similar incidents have resulted throughout North America and world-wide. Even today, over 2000 cases of human poisoning (fatal in 15% of the cases) are reported annually on a global scale [1]. It was not until the late 1950s that the identities and origins of the toxic substances began to be known.

The culprits in these poisonings are phytoplankton, microscopic plants that live in the ocean and convert inorganic compounds into complex organic compounds. They are the foundation of the marine food chain. Among the thousands of dinoflagellates and diatom species, there are only a few dozen that produce toxic secondary metabolites called ‘‘phycotoxins’’. Toxic incidents can occur when ‘‘blooms’’ of such toxigenic species, often known as ‘‘red tides’’, appear unexpectedly in shellfish-producing regions [2–4]. The cell density can be high enough that waters become coloured, sometimes red (hence the term ‘‘red tide’’) but also green and brown. Filter-feeding bivalve molluscs such as mussels and scallops consume the plankton, thereby accumulating phycotoxins in their edible tissues. Herbivorous finfish can also accumulate toxins, which can then become part of the food chain to birds, marine mammals and man [5]. The frequency of occurrence, abundance and geographical range of many phycotoxins appear to be increasing worldwide. Although most plankton blooms are natural phenomena, their proliferation may be partly attributable to agricultural runoff and sewage in coastal waters, redistribution of microalgae via ship ballast water, and transfer of shellfish stocks. Red tides and phycotoxins present a serious threat to public health and have had a significant economic impact on fish and shellfish farming industries in much of the world.

Phycotoxins hold a particular fascination with chemists because of their unusual structures, toxic nature and history. As shown in Fig. 1, they have some of the most complex structures known in nature, ranging from low to high molecular masses and from very polar to highly lipophilic. They possess multi-functional characters and high degrees of chirality, and cause many different types of toxic effects. Phycotoxins present significant challenges to those interested in structure elucidation, synthesis and analysis.

The purpose of this overview is to acquaint the reader with some of the different approaches that have been used to identify toxins responsible for seafood poisoning incidents, to investigate the origins of toxins, and to monitor our seafood on a routine basis. It will be shown that advancements in our knowledge of toxins and our ability to protect the public have often followed key developments in separation and analysis technologies. It will not be possible to make this a comprehensive review of all the significant contributions of many other research groups. Instead, specific examples from the author’s personal experiences during the last 15 years of research in this field will be presented to illustrate the significant role that chromatographic methods play. The presentation will be given in an order that reflects the typical sequence of investigations that follow a new toxin episode, viz: (a) identifying the new toxin; (b) developing analytical methods; (c) detecting and confirming similar events; (d) searching for the source of the toxin; (e) searching for its analogues; (f) surveying different geographical areas; (g) implementing routine monitoring; and (h) developing calibration standards and reference materials.

2. Identifying new toxins

2.1. Preliminary considerations

The identification of a new toxin requires a systematic approach and a significant level of resources. When a toxic event occurs, one of the first steps is to determine if it was caused by a bacterial or viral contamination. Symptoms resulting from phycotoxin exposure usually develop more quickly than the symptoms of microorganism contamination,

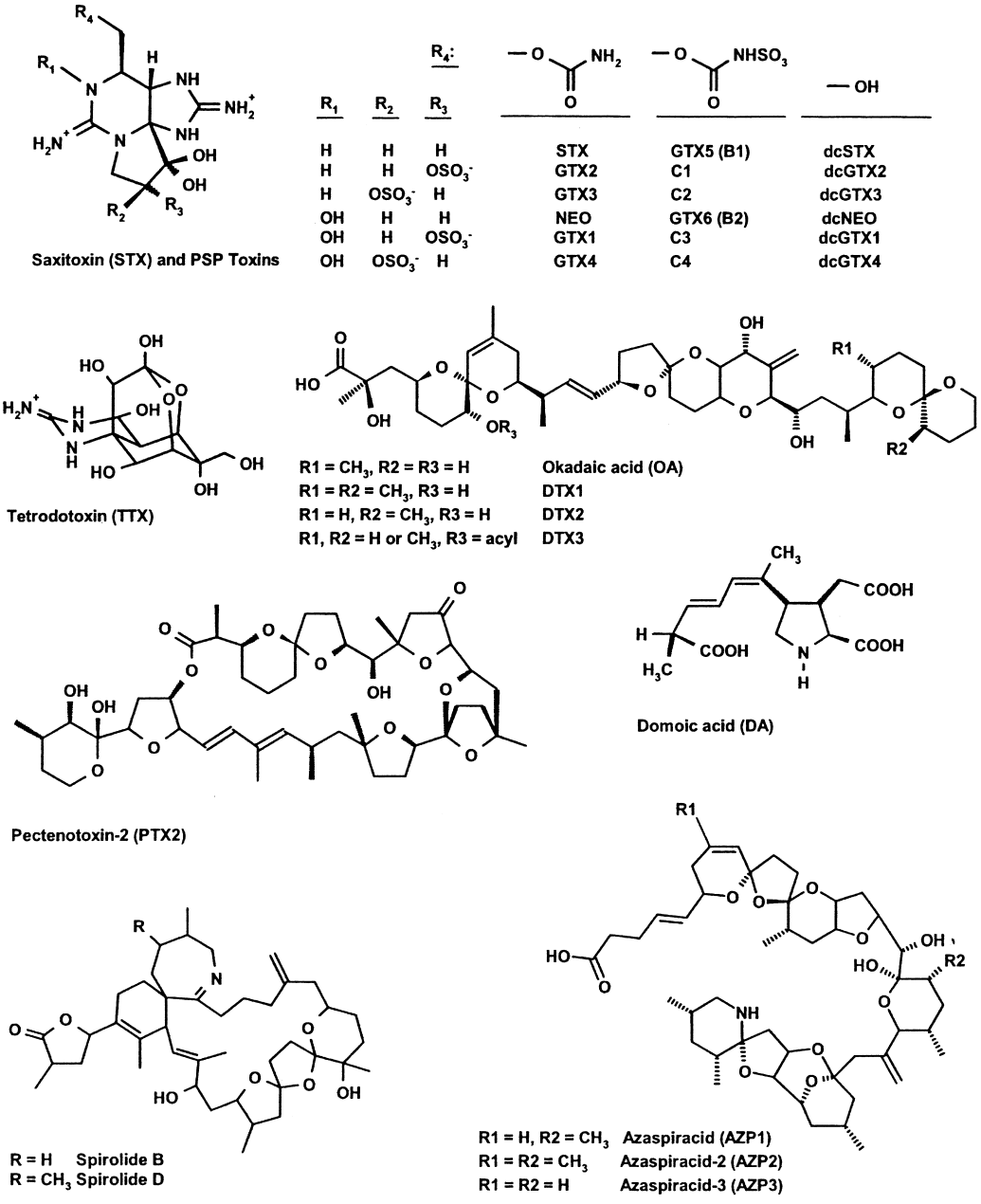


Fig. 1. Structures of various seafood toxins.

which typically take 12–24 h to manifest. The next step should be to examine the symptomology to see if a known toxin is responsible for the event. As shown in Section 4, chemical analysis may quickly establish that a known toxin is responsible for the observed toxicity. Acquiring a large amount of toxic sample is essential and this must be done quickly because shellfish left in the ocean can detoxify fairly quickly. It should be kept in mind that in many instances, very potent toxins are only found in very

low concentrations in tissue samples and therefore significant amounts of contaminated sample may be required to isolate the milligram-levels of compound required for structure elucidation.

An essential step in tracking down a new toxin is to establish a rapid, reliable assay system for detecting the toxin. The most common method is the mouse bioassay in which a sample extract is injected into the intraperitoneal cavity, followed by an observation period to determine symptoms and time-to-death, which usually correlates with the amount of toxin present. Other assay systems, based on organisms such as the brine shrimp [6] and the housefly [7], have been used in some studies but are somewhat more difficult to implement and not as representative of mammalian toxicity. In vitro cytotoxicity assays can also be useful in some cases [8–11]. A receptor or biochemical assay [11] may be available if the mechanism of action of the toxin is understood but this is rarely the case with a new toxin investigation.

Understanding the stability of the toxin may save a great deal of grief later in the process, since one does not want to have the toxin decompose during the separation process. A good approach is to start on a small scale and try to ensure that toxicity is not being lost in any isolation procedures being used, such as evaporation, solvent partitioning, pH adjustment or column chromatography.

2.2. Bioassay-directed fractionation

The general approach that has been used in many investigations of new toxins is termed ‘‘bioassay directed fractionation’’. The tactic is to take a sample extract through a series of preparative separation steps. After each fractionation, the toxicity assay is used to determine which fraction contains the toxin. This fraction is then taken to the next separation procedure. It is often best to start with a high capacity, low-resolution method and gradually move towards lower capacity, higher resolution methods. Ideally, each step should contribute a unique separation selectivity that is complementary to the other steps. One useful procedure to use at the start is liquid–liquid partitioning. With this procedure, it may be possible to isolate the toxin on a large scale on the basis of hydrophobicity or hydrophilicity, or adjustment of pH may allow a separation based on the toxin’s acidic or basic nature.

Molecular weight filters can be useful to determine if the toxin is low or high in molecular mass. Separation on a large size-exclusion chromatography column may be very useful as one of the early steps. Thereafter, the extract may be taken through a series of chromatography columns such as ion-exchange (anion or cation, weak or strong), normal-phase (silica, alumina, florisil) and reversed-phase (C_{18}-silica). A final step to purify the toxin for structure work by NMR spectroscopy is preparative HPLC typically using a C_{18}-silica column. Often several sequential procedures are required to isolate a toxin in a pure enough state for structure work. A typical investigation can sometimes take many weeks or months of dedicated effort.

There have been many examples of the application of these principles. One of the earliest was the investigation of the toxin responsible for paralytic shellfish poisoning (PSP). These very potent toxins act by binding to a voltage-gated sodium channel, thus inhibiting ion flux through these channels and rendering excitable tissues such as nerve and muscle nonfunctional. Death results from paralysis if significant levels of toxins have been consumed. In the early 1950s, before the identity of this toxin was known, the mouse bioassay was developed [12] to monitor shellfish on a routine basis. Efforts by E.J. Shantz and co-workers [13] using the mouse bioassay to direct the fractionation led to the isolation in 1960 of a compound called saxitoxin. The structure was not correctly established until 1971 by Wong et al. [14]. Subsequent work by Shimizu, Oshima and others has led to the isolation of over 20 other saxitoxin analogues [15,16] (Fig. 1). A similar toxin that also blocks sodium channels, is tetrodotoxin which is found in the skin and certain organs of the pufferfish and is believed to be produced by bacteria. The structure of tetrodotoxin was established in 1965 by Goto et al. [17]. Other examples of toxins identified using bioassay-directed fractionation include brevetoxins [18], okadaic acid [19], dinophysistoxin-1 [20], ciguatoxin [21], pectenotoxins [22], yessotoxin [23], spirolides [24], and azaspiracid [25].

2.3. Integration of bioassay-directed fractionation with analysis

A more sophisticated approach that allows a rapid tracking and identification of a toxic agent is based on an integration of the bioassay-directed fractionation approach with chemical analysis. The utility of this approach was demonstrated dramatically with the discovery of domoic acid [26,27].

In late 1987, a serious outbreak of food poisoning occurred in Canada. Symptoms of the poisoning included vomiting and diarrhea, followed in some cases by confusion, memory loss, disorientation, and coma. Three elderly patients died and other victims suffered long-term neurological problems. The term amnesic shellfish poisoning (ASP) has been proposed for this clinical syndrome [26]. The scientific detective story that unfolded was followed closely by a concerned Canadian public and made front-page newspaper headlines. For health, political, and economic reasons, scientists in Canadian government laboratories were eager to solve the mystery quickly.

Epidemiologists from Health Canada quickly linked the illnesses to restaurant meals of cultured mussels harvested from one area in Prince Edward Island, a place never before affected by toxic algae. Mouse bioassays on aqueous extracts of the suspect mussels caused death with some unusual neurotoxic symptoms very different from those of paralytic shellfish poison and other known toxins. On December 12, 1987, a team of scientists was assembled at the National Research Council laboratory in Halifax. This team developed a strategy based on an integration of bioassay-directed fractionation with chemical analysis. This efficient procedure led to the identification of the toxin on the afternoon of December 16, just 102 h after the start of the concerted investigation.

General principles of the integrated approach are summarized in Fig. 2. Extracts of both toxic and control mussels were taken in parallel through a series of preparative separation steps. After each fractionation, the mouse bioassay was used qualitatively and quantitatively to determine which fractions contained toxin. Various chromatographic and spectroscopic techniques were also used to profile toxic fractions and the corresponding fractions from control samples. The objectives were to determine possible differences between toxic and control fractions that might correspond to the toxin and to determine whether a fraction was simple enough to begin structure elucidation. Application of spectroscopic methods to the purified toxin then provided clues about its structure, and the chemical literature was searched to determine if it was a known compound. To avoid problems associated with sampling heterogeneity, all experiments were conducted

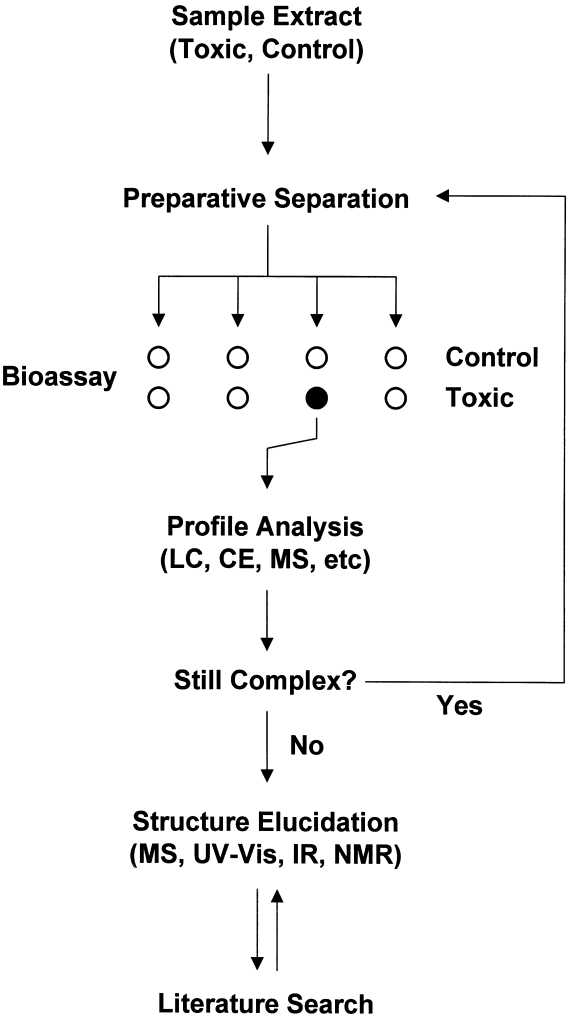


Fig. 2. Flow chart indicating the general principles of the toxin isolation strategy based on the integration of bioassay-directed fractionation with chemical analysis (from Ref. [26]).

using large portions of toxic and control tissue homogenates. Dose control in the bioassay was of crucial importance for effective tracking and for establishing a toxicity balance, so it was essential to establish a dose–response curve for the bioassay and to understand the dynamic range available in the assay.

A classical natural products mild extraction procedure using aqueous methanol at room temperature was selected for the initial extraction in case the toxin was labile. Partitioning between water and dichloromethane followed by XAD-2 column chromatography revealed that the toxin was water-soluble.

LC with UV diode array detection (DAD) was used as one of the profiling methods. LC conditions were selected on the premise that the unknown toxic substance was a polar, ionizable compound such as a peptide. Thus, gradient elution reversed-phase chromatography was used with an acidic acetonitrile/water mobile phase. The DAD was set to perform continuous scanning of spectra, as well as acquisition at 210±10 nm, a wavelength at which most compounds absorb to some extent.

A striking example of the LC–DAD profile analysis comparing XAD-2 fractions of toxic and control mussel extracts is shown in Fig. 3. Chromatograms for absorption at 210 nm indicated a peak at about 12 min, which occurred just after a tryptophan peak for toxic but not for control fractions. The complete three-dimensional representation of the LC–DAD data for the toxic sample at the appropriate time window is shown together with the UV spectrum taken at the peak maximum. The spectrum showed an absorption maximum at 242 nm (which immediately suggested a functional group such as a conjugated diene). Reconstructed chromatograms for absorption at this wavelength are also shown to accentuate the toxic/control dichotomy.

When these same XAD-2 fractions were analysed using high voltage paper electrophoresis (HVPE), a band running just behind glutamic acid, staining yellow rather than red with ninhydrin was observed in the toxic but not in the control extracts. The yellow stain suggested a proline derivative. Furthermore, the LC–DAD suspect peak when collected and analysed using HVPE gave the same yellow band; the converse crosscheck was also successful. Most important was the finding that these fractions, collected from the two complementary separation techniques operated on a preparative scale, were shown to account for all of the toxicity within the reproducibility of the dose–response curve.

While these highly encouraging results were being obtained, complementary profile analyses obtained for all toxic fractions by fast atom bombardment mass spectrometry (FAB-MS) showed that peaks at m/z 312 ($[M+H]^+$) in positive ion mode and at m/z 310 ($[M-H]^-$) in negative ion mode were increasing in prominence as the toxin was progressively purified. No significant corresponding signals arising from a compound of M_r 311 were evident in the control fractions. Accurate mass measurements revealed the compound's formula to be $C_{15}H_{21}NO_6$. A literature search on the accumulated data indicated that the isolated toxin was a known neurotoxic compound, domoic acid [28,29]. It was first identified in the Japanese seaweed, *Chondria armata*, but had never before been associated with human illness. Preparative isolation of the suspect compound using LC–DAD then provided sufficient material for confirmation of structure by MS–MS and NMR spectra.

3. Developing the tools

Methods for the determination of toxins may be divided into assay and chemical analytical methods. In assay methods, the measured signal is either a specific response to a single toxin structure or an integration of responses to several structures in a group. In order to use the assay result for evaluating seafood safety, it is most useful if the response correlates with overall toxicity. In a chemical analytical method, signals corresponding to individual toxin structures are measured. Most analytical methods are based on chromatography which allows the separation and detection of several toxins in one analysis. Calculation of individual toxin concentrations requires accurate standards to calibrate the responses and evaluation of seafood safety further requires specific toxicity data.

The most common assay is the mouse bioassay. For over 50 years, this assay has been used successfully in inspection programs to monitor for PSP toxins, which are easily extracted into an acidic

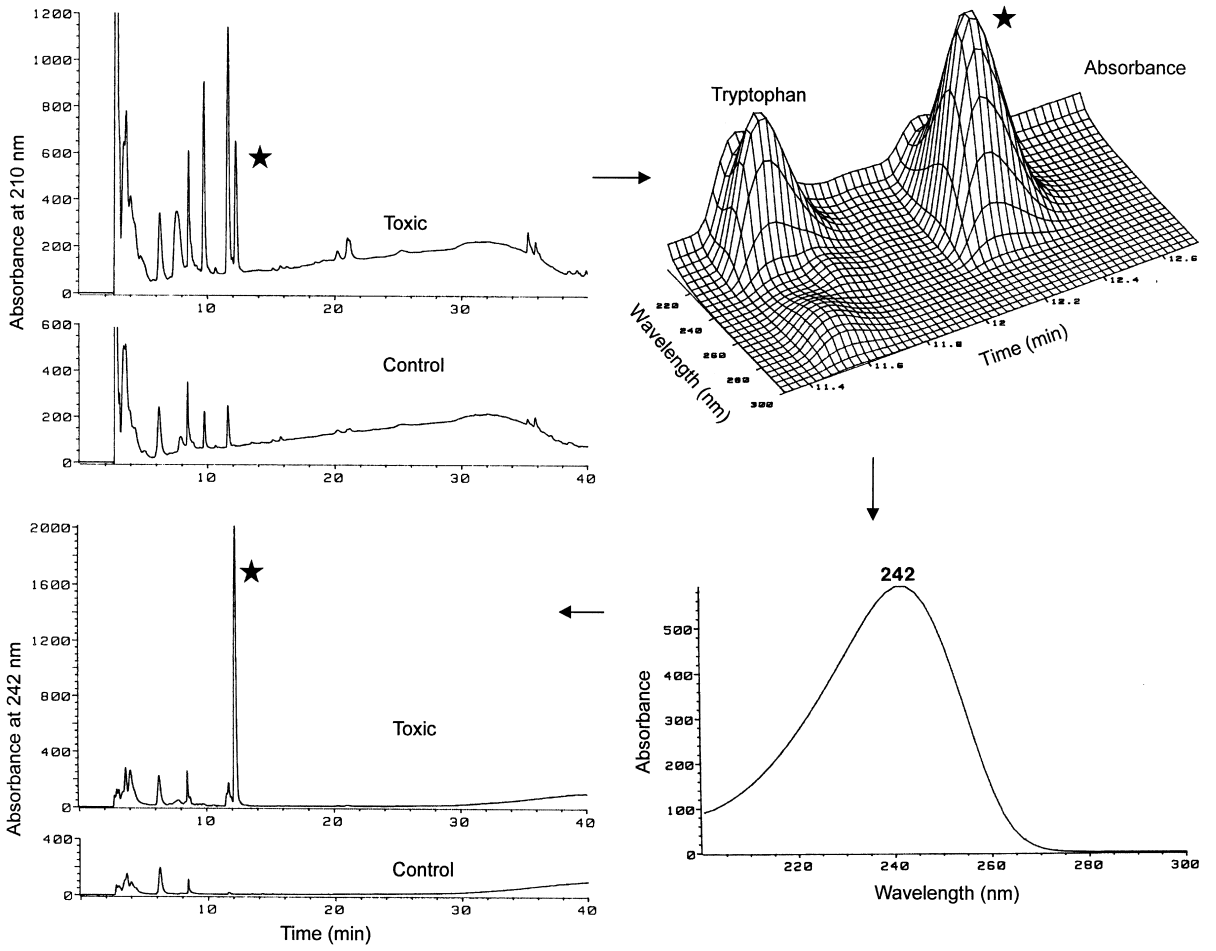


Fig. 3. LC–DAD profiles of corresponding XAD-2 fractions from toxic and control mussel tissues. The peak marked with a star was eventually identified as domoic acid, which shows a UV absorption maximum of 242 nm. Conditions: 25 cm×4.6 mm I.D. Vydac 201TP column with 1 ml/min acetonitrile/water/trifluoroacetic acid; gradient elution from 95.0:04.9:0.1 to 99.9:0:0.1 over 40 min (from Ref. [26]).

aqueous solution suitable for direct injection [12,30]. One of the problems with the method is that the inherent variability can exceed ±20%, compared to most chemical techniques that have uncertainties of less than 10%. For some toxins such as domoic acid, where the current legal limit (20 mg/kg in Canada) is less than the bioassay detection limit (40 mg/kg), it may not provide sufficient sensitivity to protect the public adequately. A similar problem occurs when ‘‘cryptic’’ toxins are present. Such toxins may not express toxicity until they are metabolised in the gastrointestinal tract. In addition, application to lipophilic toxins requires more complicated extraction methods and has been less successful due to severe matrix interferences that can give both false positives and false negatives. Finally, since several countries have banned animal bioassays due to protests by animal rights groups, there is considerable pressure to develop alternative methods.

A number of alternative assay methods have been developed [11]. These include in vitro cell toxicity assays, receptor protein assays, and immunological assays. Despite their potential for speed, high sensitivity and low cost, there are some limitations associated with such methods. Since receptor assays are usually based on radioactive tracers, they are

restricted to specially equipped laboratories. Immunoassays generally cannot be used for the precise quantitative analysis of samples containing families of toxins that have variable levels of individual toxins. Antibodies for such assays are initially developed for single toxins but can have varying degrees of cross-reactivity towards toxins of similar structure. Although assays are excellent for screening out negative samples, it is generally recognised that positive results should still be confirmed by chemical methods.

Chemical methods of analysis have the potential for sensitive, precise and fully automated quantitation of known toxins, as well as confirmation of identity. Methods based on chromatographic and spectroscopic techniques are particularly well suited for the identification of new toxins. It should be noted that there are many challenges to overcome before it is possible to fully implement any chemical method into comprehensive monitoring programs and research studies. In particular, some critical developments are required before complete success can be claimed.

All members of a toxin class must be well characterised in terms of structure. We have accumulated considerable information on the structures of toxins that can contaminate shellfish, but each year the situation becomes more complicated with the discovery of new toxin analogues and even new toxin classes. In addition, toxins produced by a particular microalgal species may be transformed in shellfish to metabolites, some toxic and others non-toxic. This is certainly a complicating factor in the development of methods and indicates a need to continue research on the identification of new toxins.

Accurate and readily available calibration standards are required for each of the various toxins. This has always been a major stumbling block in shellfish toxin research and monitoring, and much more effort has to be put into the development of reference materials and standards (see Section 8). The task is continually made more difficult by the growing list of toxins.

The specific toxicity of individual toxins must be determined to allow calculations of overall toxic potential of samples. Risk assessment studies also need to be conducted to establish allowable levels of toxins in seafood.

Finally, robust methods must be developed and validated for various shellfish tissues. They must provide adequate detection limits (μg/kg levels) and accurate quantitation of all individual toxin analogues within a toxin class. Many specific analytical methods have already been developed for individual toxins or toxin groups and some examples of the most useful chromatography-based ones are considered below.

3.1. Gas chromatography

Few toxins are sufficiently volatile for direct analysis by gas chromatography (GC). Chemical derivatisation to increase volatility for GC is possible for some toxins, such as domoic acid [31], but most toxins are too labile, both chemically and thermally.

3.2. Thin-layer chromatography

Thin-layer chromatography (TLC) is a technique that is widely used in the food analysis field. For example, many laboratories screen agricultural products for the presence of mycotoxins using TLC methods [32]. TLC offers several potential advantages for the screening of shellfish samples for marine toxins, namely: (a) simple methodology without the need for expensive equipment; (b) reasonably short analysis times; (c) simultaneous screening of multiple samples; and (d) the availability of colorimetric spray reagents for confirmation of toxin identity through selective chemical reactions. There are very few reported applications of TLC to marine toxins.

TLC has been used for the detection of PSP toxins [33] and tetrodotoxin [34]. A detailed investigation has been conducted on the analysis of PSP toxins using TLC with Chromarods-SIII and the Iatroscan (Mark-5) coupled with a flame thermionic detector (FTID) [35]. Polyether toxins such as okadaic acid can be detected by TLC methods after an SPE clean-up (unpublished results). A spray reagent such as vanillin in concentrated sulfuric acid–ethanol (5 *g*/l, 4:1) gives a pinkish-red stain when clean material is applied to a TLC plate, 1 μg of the toxin can be detected; with cruder fractions, 2–3 μg of toxin is required.

A method for domoic acid based on solid-phase

extraction (SPE) clean-up and silica gel TLC has been developed [36]. Domoic acid could be detected in shellfish tissue down to 10 mg/kg, half the regulatory level, using a simple hand-held short-wave UV lamp to detect fluorescence quenching. Confirmation is possible by spraying the plate with ninhydrin, which reacts with the secondary amine of domoic acid to give a distinctive yellow coloured product. Some degree of quantitation is even possible using an inexpensive scanner attached to a personal computer.

3.3. Liquid chromatography

Liquid chromatography has proven to be the most valuable instrumental analytical tool for toxins because it is so well suited to the analysis of polar, non-volatile compounds. It provides excellent quantitative precision and is easily automated. There are some difficulties with the technique, however, not the least of which is that most of the toxins do not possess a chromophore for sensitive UV absorbance or fluorescence detection. This has necessitated the use of either pre- or post-column derivatisation methods to allow detection and the development of many different methods designed for specific toxins or toxin groups. Just a few specific examples are given below.

PSP toxins are some of the most difficult compounds to analyse by LC due to their very polar, multi-functional nature. The most commonly used method is the combination of reversed-phase LC with on-line post-column oxidation and fluorescence detection (FLD) [37–39]. This approach evolved from earlier work by Bates and Rapoport [40], which showed that saxitoxin could be oxidised to a fluorescent purine [41] by an oxidising agent under alkaline conditions. Unfortunately, the set-up and operation of such equipment is complex and requires considerable daily maintenance. Analysis of the entire array of PSP toxins usually requires three separate isocratic elutions with both cationic and anionic ion pairing agents [39]. As shown in Fig. 4, it is possible to replace two of the runs with one gradient elution (unpublished results). A feature to note in this figure is the complex toxin profile typically present in an algal sample and the need to achieve good separations for quantitation of individual toxins.

An alternative method for PSP toxins is the pre-column oxidation approach developed by Lawrence et al. [42]. It does not require the complex post-column reaction system and can be fully automated [43]. Although it has proven to be a rapid, sensitive

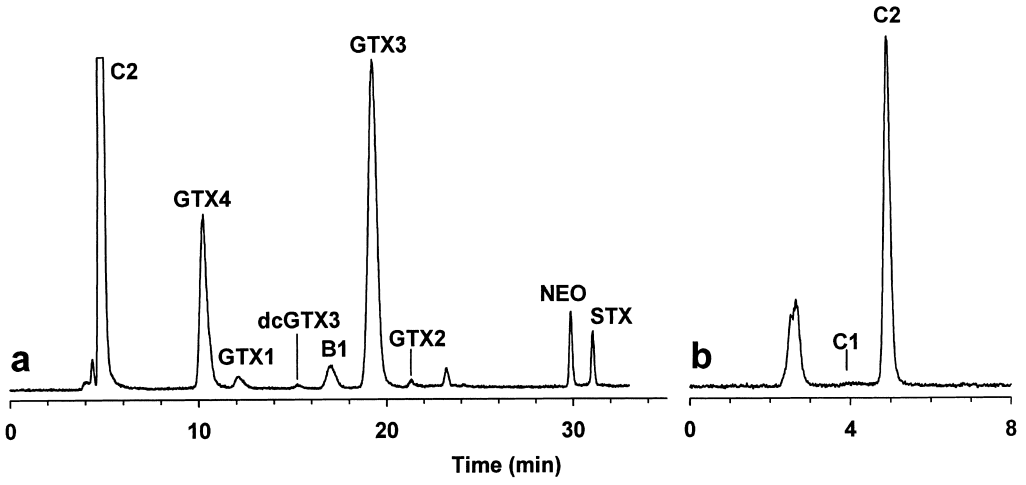


Fig. 4. Analysis of PSP toxins in a sample of the dinoflagellate, *Alexandrium tamarense*, using LC with post-column oxidation reaction and fluorescence detection with two different mobile phases: (a) heptanesulfonate ion-pairing agent, gradient elution from 0 to 20% CH_3CN; (b) tetrabutyl ammonium ion-pairing agent, with isocratic elution at 2% CH_3CN. See Fig. 1 for structures associated with various codes.

screening method, the interpretation of quantitative results is more complex because some toxins give the same oxidation product, while others give two or three products.

The diarrhetic shellfish poisoning (DSP) toxins, okadaic acid and dinophysistoxins, may be analysed by LC-FLD using derivatisation with the 9-anthryldiazomethane (ADAM) reagent, which reacts selectively with carboxyl functions [44–46]. Application of the method to standards, a mussel tissue reference material, and some mussel samples is shown in Fig. 5. The method is very labour-intensive because of the multiple clean-up steps required and low levels of toxins are difficult to measure reliably.

Domoic acid is analysed easily by LC because it has a strong chromophore allowing detection at 242 nm (as shown in Fig. 3) [47]. An SPE clean-up based on strong anion exchange can provide a high degree of selectivity to the analysis [48]. It is also possible to derivatise domoic acid with a fluorescent reagent, fluorenylmethoxycarboxyl (FMOC) [49], facilitating ppb detection limits in seawater and plankton.

3.4. Liquid chromatography–mass spectrometry

The combination of gas chromatography and mass spectrometry (GC–MS) became an invaluable tool for environmental analytical chemists in the 1970s. The mass spectrometer not only provided molecular mass and structural information, but could also act as a very sensitive and selective detector for quantitative analysis of complex mixtures. Through the 1980s, many efforts were made to achieve the same success with the combination of LC and MS. Early LC–MS interfaces such as thermospray ionisation and continuous-flow fast atom bombardment were only partially successful. The former was only

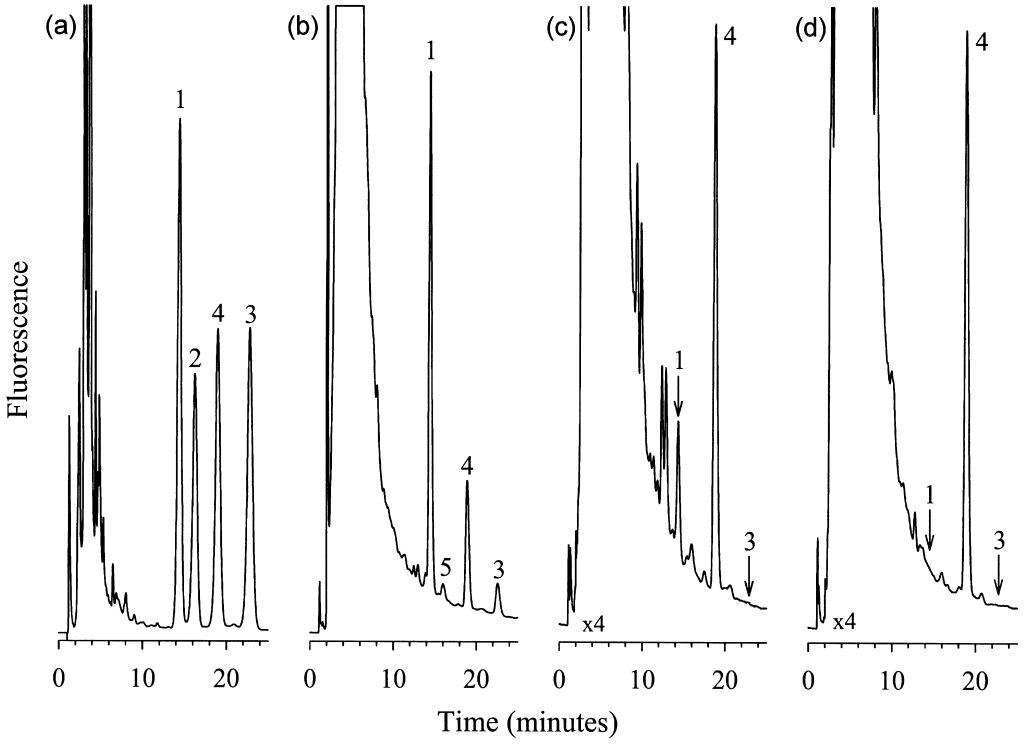


Fig. 5. Analysis of DSP toxins as ADAM derivatives using LC-FLD: (a) calibration standard mixture; (b) MUS-2 certified mussel tissue reference material (11 mg/kg OA, 0.9 mg/kg DTX1); (c) contaminated mussels (0.94 mg/kg OA); and (d) uncontaminated control mussels. Peak identities: 1 = OA; 2 = DTX2; 3 = DTX1; 4 = acetyl-OA (internal standard); 5 = isomer of OA. See Fig. 1 for structures associated with various codes (from Ref. [45]).

applicable to thermally stable molecules with medium polarity, while the latter was very difficult to implement on a routine basis.

In 1984, a breakthrough came with the development of electrospray ionisation (ESI) [50], which is well suited to compounds of widely ranging polarities and is easily implemented. Immediate success was achieved with the application of LC–ESI-MS to marine toxins using the first commercial mass spectrometer from SCIEX in 1989 [51]. Fig. 6 shows some of the data from that first paper for the analysis of domoic acid using a prototype instrument. It was possible to acquire both positive and negative ion mass spectra, as well as MS–MS spectra for detailed structural information. Fig. 6c shows an LC–MS analysis of a mussel tissue extract using selected ion monitoring. Due to its high sensitivity and selectivity, LC–MS quickly became the preferred method for the confirmatory analysis of toxins and methods have now been developed for all known toxins [52–54].

Recently, our research has been directed towards finding mobile phases and columns that would be suitable for the simultaneous separation and detection of a wide range of toxins, in both plankton and shellfish samples [54]. One of the most important factors for achieving success in electrospray LC–MS is the choice of mobile phase. Parameters that affect sensitivity include pH, ionic strength, type of buffer, and percentage of the organic solvent. A volatile buffer with a low ionic strength (ideally <10 m*M*) is desirable to prevent a build-up of salts on the sampling orifice of the MS. In addition, a higher percentage of organic solvent tends to give higher sensitivity with most compounds. Suitable mobile phases include aqueous methanol or acetonitrile doped with formic acid, acetic acid, trifluoroacetic acid (TFA), the ammonium salts of these acids, or ammonium hydroxide. TFA has been very popular because it is easy to use and produces a low pH that facilitates protonation of most toxins. The latter can be important for the chromatography of acidic and basic toxins on silica-based supports, as interactions with free silanol sites are minimized. However, it has been observed that TFA forms strong ion pairs with amines and this leads to suppression of ionization and reduced sensitivity. Also, TFA cannot be used if negative ion work is planned on the same day, as it gives a very strong signal due to the CF_3COO^- anion and this persists in the source for a long time.

We conducted an extensive survey of different mobile phases to see if it was possible to improve LC–MS sensitivity in both positive and negative ion modes and if it was possible to analyze a wide range of toxins in a single analysis. It was found that a mobile phase based on aqueous acetonitrile with 2 m*M* ammonium formate and 50 m*M* formic acid gave a 20- to 50-fold increase in sensitivity compared with TFA in the positive ion mode and also permitted the use of negative ion detection. Neutral and acidic compounds such as the okadaic acid family of toxins and pectenotoxins are ionized as ammonia adduct ions, $[M+NH_4]^+$, in the positive ion mode. Acidic toxins with sulfate or free carboxyl functions can also be detected as deprotonated molecules, $[M-H]^-$, in the negative ion mode. Basic compounds such as spirolides and azaspiracids give protonated molecules, $[M+H]^+$. A survey of different stationary phases and column dimensions revealed that short narrow bore columns (50 mm$\times$2 mm I.D.) packed with 3 μm Hypersil-BDS-C_8 phase are capable of separating a wide range of toxins using rapid gradients. High-resolution separations are possible as illustrated in Fig. 7 with an analysis of a blend of extracts of toxic shellfish samples. In the single analysis illustrated, domoic acid, spirolides, okadaic acid, dinophysistoxins, pectenotoxins, and azaspiracids were measured. This same system has also been successfully used to analyze gymnodimine, yessotoxins, brevetoxins and ciguatoxins.

Unfortunately, one toxin class was not amenable to analysis using this reversed-phase method. The PSP toxins, which are all based on the very polar saxitoxin structure, are not retained and elute at the solvent front. Attempts to retain these basic toxins on a reversed-phase column using a volatile ion-pairing agent such as heptafluorobutyric acid were successful [52], but the sensitivity was substantially reduced due to the ion suppression effect. In addition, C toxins are not retained and require another separation system.

Our more recent research has focused on the use of hydrophilic interaction LC (HILIC), which is well suited to the analysis of polar compounds [55]. The mechanism of separation is based on hydrophilic interaction of polar compounds with a stagnant layer

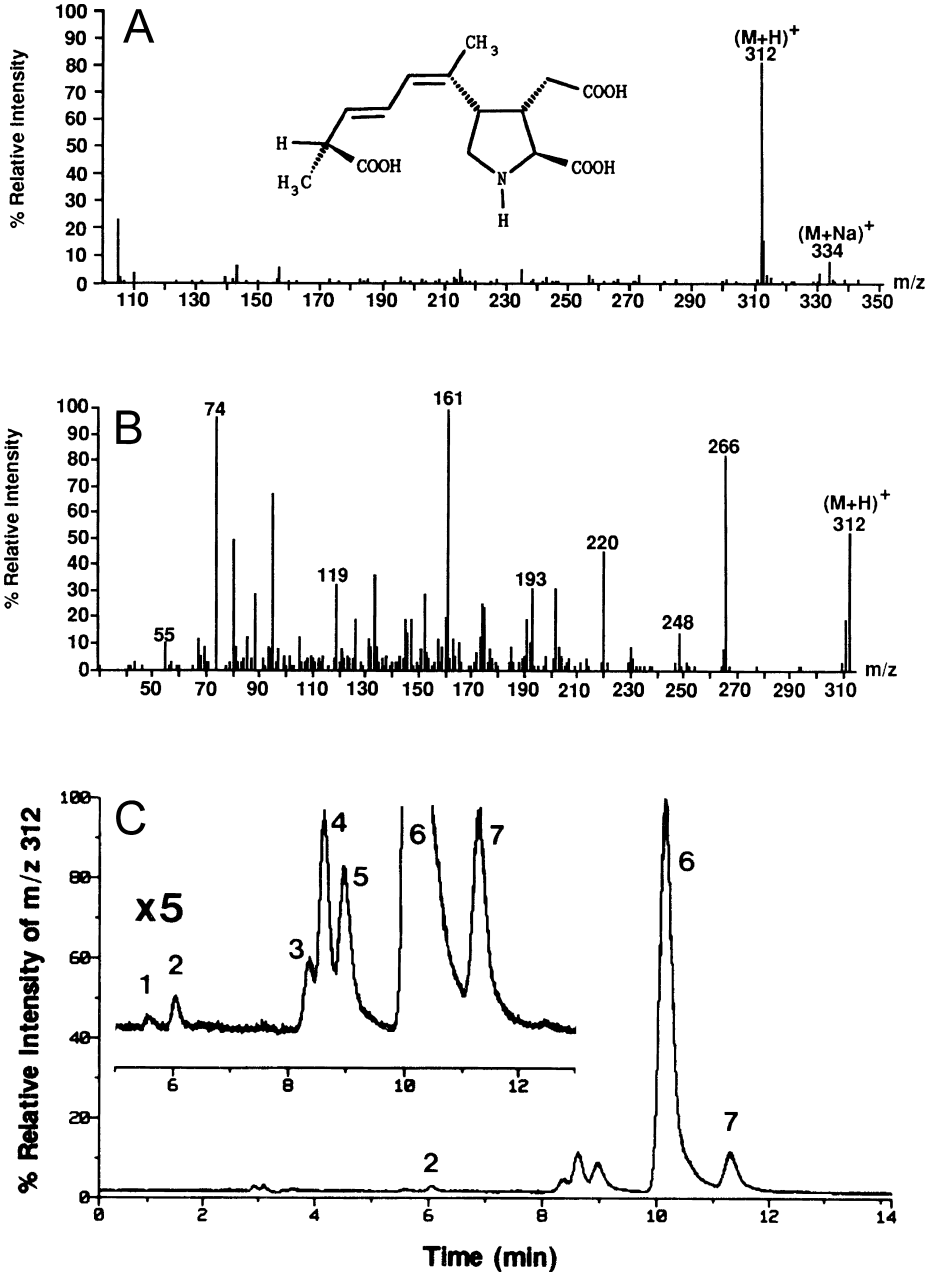


Fig. 6. First LC–MS analysis of a marine toxin using a prototype atmospheric pressure ionization mass spectrometer. The positive and negative electrospray mass spectra of domoic acid are shown in (a) and (b), respectively. The selected ion monitoring trace ($[M+H]^+$, m/z 312) trace in (c) shows the analysis of a contaminated mussel tissue. Peak 6 is domoic acid (74 ng injected), while the other peaks are various isomers. Conditions: 0.2 ml/min 10% acetonitrile with 0.1% formic acid; 25 cm×2 mm I.D. Vydac 201TP5 column (from Ref. [51]).

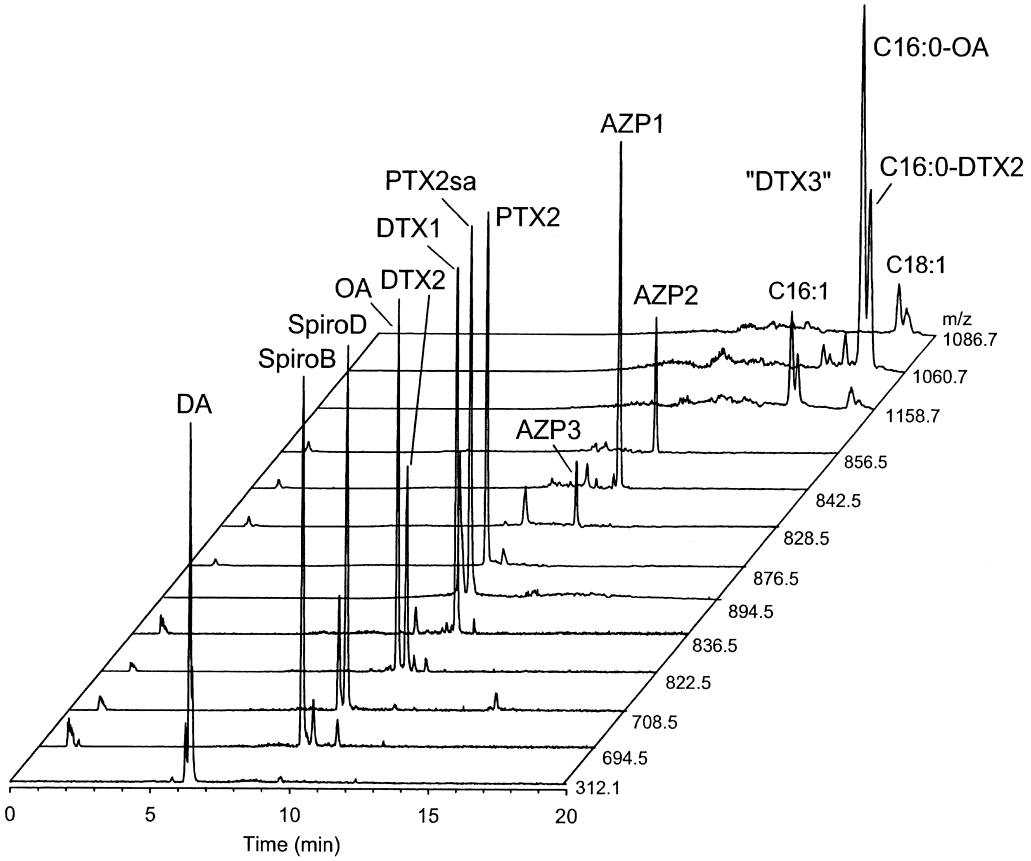


Fig. 7. Reversed-phase gradient elution LC–MS analysis of a range of toxins in a blend of contaminated mussel tissue extracts. Selected ion monitoring was carried out on either $[M+H]^+$ or $[M+NH_4]^+$ ions, which are displayed as individual mass chromatograms. The toxins present include domoic acid (DA), spirolides (SpiroB/D), okadaic acid (OA), dinophysistoxins (DTX1/2), pectenotoxins (PTX2 and PTX2sa), azaspiracids (AZA), and acyl esters of OA and DTX2 (''DTX3''). See Fig. 1 for structures associated with various codes (from Ref. [54]).

of water absorbed on a polar stationary phase (most commonly a silica-bonded phase with a free amide function), as well as some additional ion-exchange interactions. Application to the PSP toxins has been investigated [54]. An optimum separation was achieved with an isocratic separation, using 62% CH_3CN with 2 m*M* ammonium formate and 3.6 m*M* formic acid (pH 3.5). The mobile phase does not use ion pair agents, so ionization efficiency is not reduced, and is high in organic, so it actually enhances ionization yield. The technique provides high sensitivity with detection limits approaching those achieved with fluorescence detection.

Fig. 8 illustrates that HILIC-MS–MS allows the separation and selective detection of saxitoxin-related compounds in a plankton extract in a single 30-min analysis (analysis of this same sample by LC-FLD was shown in Fig. 4).

With these LC–MS methods, it should be possible to monitor plankton and shellfish samples for known phycotoxins. The remaining limitations of a multi-toxin approach lie not in the LC–MS system, but in sample preparation, i.e. finding a universal extraction solvent and clean-up scheme that give good recovery for all toxins.

LC–MS meets all the needs of laboratories involved in both monitoring and toxin research: universal detection capability, high sensitivity, high selectivity and specificity, minimal sample preparation, ability to deal with the structural diversity and

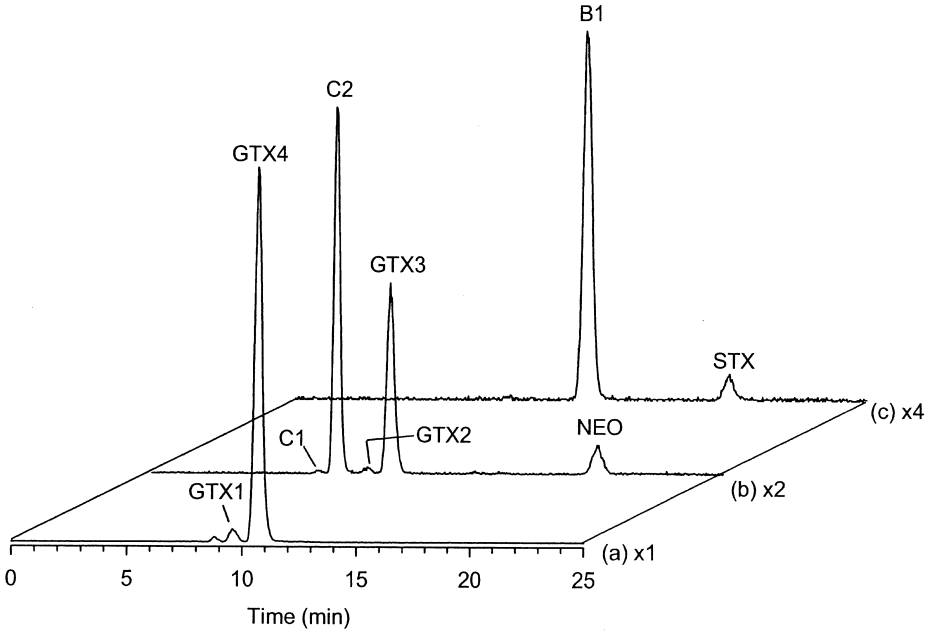


Fig. 8. Hydrophilic interaction LC–MS analysis of an extract of the same plankton extract analysed in Fig. 4. Selected reaction monitoring was carried out on a series of ion transitions, and signals common to groups of toxins were displayed as summed ion chromatograms: (a) m/z 412→332, 412→314; (b) m/z 396→316, 396→298, 316→298, 316→220; (c) m/z 380→300, 380→282, 300→282, 300→204. See Fig. 1 for structures associated with various codes.

labile nature of toxins, separation of complex mixtures of toxins, precise and accurate quantitation, wide linear range, automation, high throughput, rapid method development, legal acceptability for confirmation, and structural information for identification of new toxins, analogues and metabolites. One of the most appealing features of LC–MS to many laboratories is the possibility that a wide range of methods could be replaced by just one instrument.

3.5. Capillary electrophoresis

Capillary electrophoresis (CE) is a method that has considerable potential for marine toxins. Some preliminary CE methods have been developed for detection of maitotoxin [56], okadaic acid [57], palytoxin [58], tetrodotoxin [59], PSP toxins [60], and domoic acid [61]. A limitation of these methods is the use of the UV detector, which does not provide high sensitivity, except for the strongly absorbing domoic acid. The use of the very sensitive laser-induced fluorescence detector has been demonstrated recently with derivatised brevetoxins [62]. The combination of CE–MS has also been used for analysis of PSP toxins [63].

4. Following the symptoms

When a toxic event occurs, the symptoms that people or animals exhibit provide important clues to the nature of the toxin involved. If the symptoms are similar to those observed in previous events, it is possible to develop a hypothesis on the causative toxin. Analytical chemistry can either confirm such a hypothesis or reject it, which then leads to the search for a new toxin. As chemical analysis methods such as LC–MS were developed for various toxins, the task of confirmation started to become easier. For example, in the late 1980s, there were anecdotal reports of DSP incidents in North America but no confirmations due to the fact that few laboratories had established methods. In 1992, several people were stricken ill in Nova Scotia with symptoms typical of DSP. Within one day of receipt of samples, the presence of the DSP toxin DTX1 was confirmed

by a newly developed LC–MS method [64]. Subsequent monitoring of shellfish in the region showed that the toxin was localised to only one area and the level declined to non-toxic levels over the next 3 weeks, allowing a re-opening of the affected aquaculture farm.

That same year, a rat bioassay of mussels from Ireland suggested the presence of DSP toxins. In that case, an initial LC-FLD analysis indicated the presence of okadaic acid but its concentration did not account for all of the observed toxicity. When an analysis was carried out by LC–MS (Fig. 9), okadaic acid was easily confirmed by the presence of a peak in the m/z 805 mass chromatogram which had the correct retention time and gave the correct MS–MS spectrum. However, an additional peak was observed in the same mass chromatogram and its MS–MS spectrum was similar but slightly different from that of okadaic acid. This new compound was isolated and NMR revealed it to be DTX2, an isomer of okadaic acid [65].

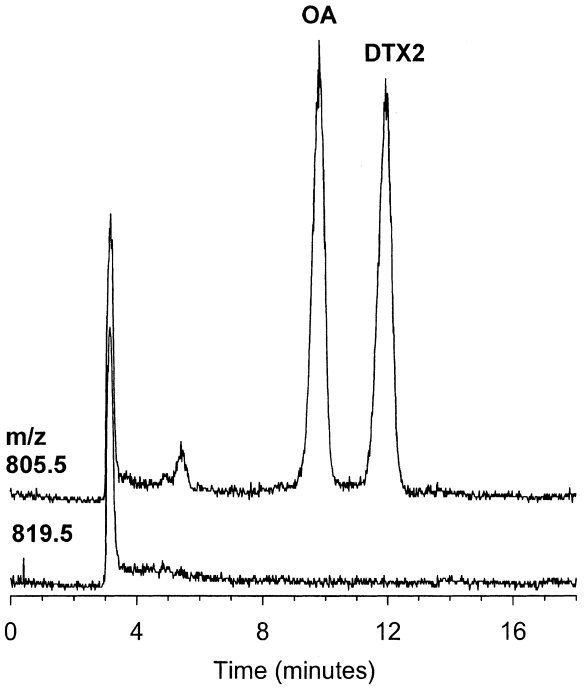


Fig. 9. Reversed-phase LC–MS analysis of DSP toxins in an Irish mussel digestive gland extract, showing the presence of okadaic acid and its isomer, DTX2. Conditions: electrospray ionisation and selected ion monitoring; 25 cm×4.6 mm I.D. column packed with Vydac 201TP; 0.3 ml/min 70% methanol with 0.1% trifluoroacetic acid.

In 1991, hundreds of sick and dying pelicans and cormorants were found on the beaches near Monterey, CA. The symptoms led veterinarian Thierry Work to suspect a natural toxin such as domoic acid. Samples were sent to our laboratory and LC–MS analysis quickly established the presence of domoic acid in the stomachs of the dead birds and in the anchovy that they had been eating [66,67]. Fig. 10 shows an LC–MS analysis of an anchovy extract. Several isomers and metabolites of domoic acid were also detected.

In a recent incident in 2002, a salmon farm in Nova Scotia experienced a dramatic kill of thousands of fish. From the symptoms observed and the type of

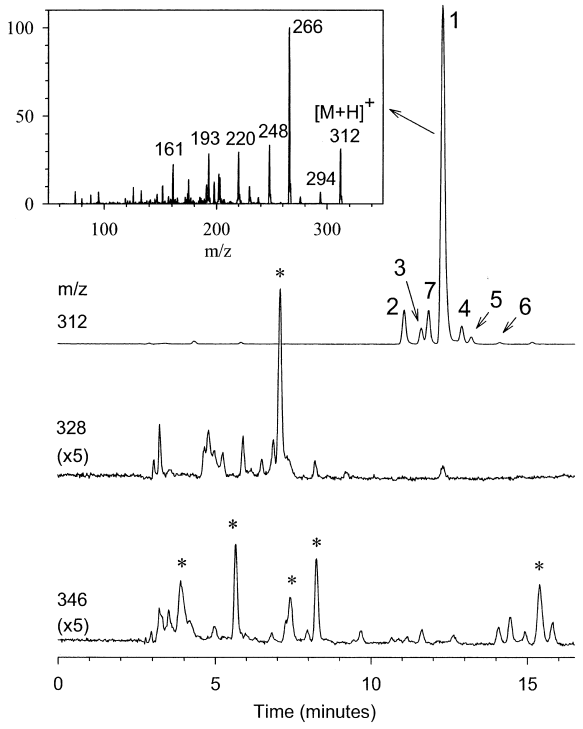


Fig. 10. LC–MS analysis of an extract of anchovy contaminated with domoic acid (75 mg/kg) using electrospray ionisation and selected ion monitoring. The inset shows the MS–MS product ion spectrum of the $[M+H]^+$ ion of domoic acid, m/z 312 (acquired in a separate analysis). Peak identities: 1=domoic acid; 2–7= isomers of domoic acid; other peaks are metabolites and/or degradation products of domoic acid. Same mobile phase and column as in Fig. 3, except gradient elution over 20 min from 5 to 25% acetonitrile (from Ref. [48]).

plankton in the waters, PSP toxins were suspected. When plankton samples were analysed by LC-FLD as well as LC–MS using the newly developed HILIC-MS method (Figs. 4 and 8), a complex array of PSP toxins was confirmed [68].

5. Tracking the source

Once a new toxin has been identified in seafood, it is important to determine its plankton source. Knowing the causative organism can allow plankton-monitoring programs to provide early warnings of possible shellfish toxification. Immediately after the 1987 ASP incident, an extensive investigation of the planktonic source, using the newly developed LC–UVD method and laboratory-grown cultures, revealed that a diatom, *Nitzschia pungens f. multiseries*, was the source [69]. This was the first time that a diatom had been implicated in a toxic event. After the 1992 Monterey incident, another diatom, *Pseudonitzschia australis*, was identified as the source of domoic acid using LC–MS [66].

Other examples include the identification of *Prorocentrum lima* as a source of okadaic acid [70], *Protoceratium reticulatum* as the source of yessotoxin [71], and *Alexandrium ostenfeldii* as the source of spirolides [72,73]. For the latter, a micro extraction procedure was developed that allowed the analysis of hand-picked cells from field samples. As few as 50 cells gave enough signal for confirmation of toxin by LC–MS [72].

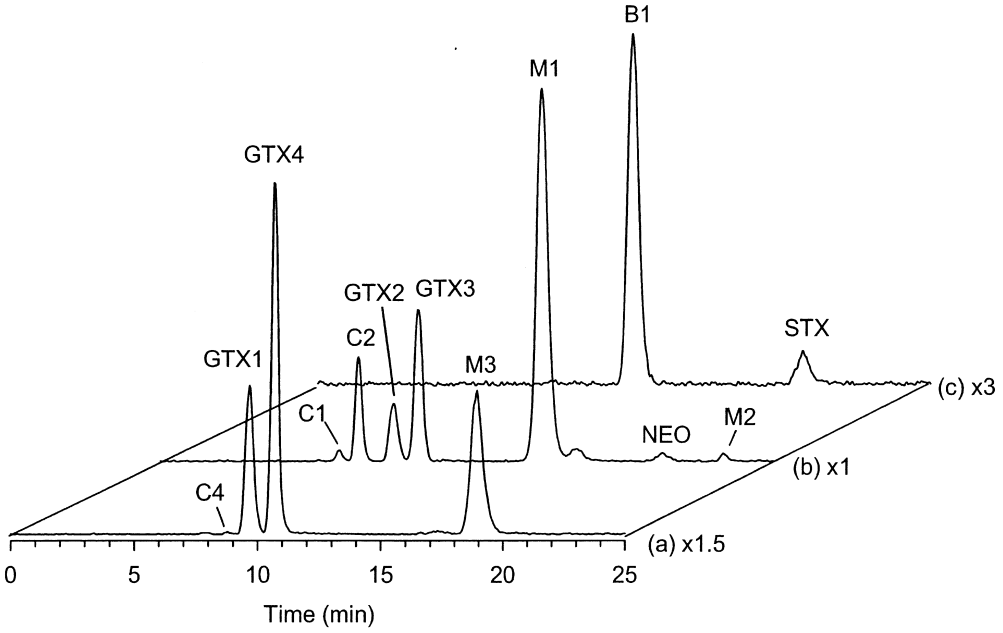
6. Prospecting for toxin analogues

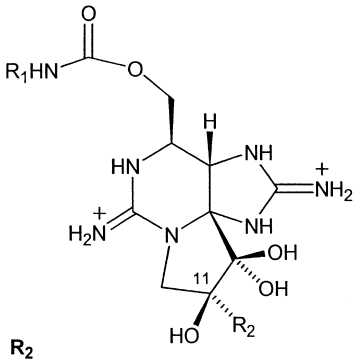
With the diversity of nature, when one toxin is found in an organism, there is a good chance that various structural analogues are also going to be synthesised by the same organism or in closely related species. In the ASP incident, it at first appeared that there was only one toxin, domoic acid. However, close examination of the LC–UVD and LC–MS chromatograms revealed several isomers and analogues (Figs. 6 and 10) [47,48], some of which have been isolated and identified as the isodomoic acids [74].

Once a toxin-producing phytoplankton is isolated and grown in a laboratory culture, it becomes much easier to search for related toxins. When various *Prorocentrum* spp. cultures were extracted to isolate okadaic acid, TLC monitoring of fractions from chromatographic columns indicated the presence of compounds related to okadaic acid. Isolation and structure elucidation revealed them to be okadaic acid with its carboxyl function esterified with a diol group [75]. Further studies led to the discovery of an unusual water-soluble derivative of okadaic acid, named DTX4 (Fig. 1), which has a diol group linked to a tri-sulfated aliphatic chain through another ester function [76]. In some *Prorocentrum* strains, it was found that all of the okadaic acid is produced in the form of DTX4 and that the diol esters and even okadaic acid are formed on sample handling when esterases in the plankton are released and hydrolyse the ester linkages in DTX4. If the plankton is heat-treated immediately after collection, the enzyme is destroyed. LC–MS analysis of the sample then revealed a complex mixture of DTX4 analogues [77].

LC–MS investigations of DSP-contaminated shellfish have also revealed a complex array of metabolites, called the ‘‘DTX3’’ complex, which is formed by fatty acid acylation of the 7-hydroxy function of okadaic acid, DTX1 and DTX2 [78,79]. These compounds are also important to measure when assessing a sample for toxicity as they can also cause diarrhetic symptoms. One procedure used to measure them is to perform a base hydrolysis of the acyl esters to release okadaic acid or the DTXs for analysis. They can also be measured directly by LC–MS [78,54] (Fig. 7).

The saxitoxins comprise one of the most complicated groups of toxins (Fig. 1), and new analogues are still being discovered. Recently, HILIC-MS analysis (Fig. 11) of mussel samples that had been exposed to toxic *Alexandrium tamarense* (the same one analyzed in Fig. 8) allowed the detection and identification of three new compounds, M1–M3 [80]. One of the compounds, M3, has a very unusual structure with a vicinal gem diol. These compounds were not detected previously because they have a very poor response in the post-column reaction LC-FLD system.





R_1	R_2		
SO_3^-	H	**M1**	11β-hydroxy-N21-sulfocarbamoyl-saxitoxin
H	H	**M2**	11β-hydroxy-saxitoxin
SO_3^-	OH	**M3**	11,11-dihydroxy-N21-sulfocarbamoyl-saxitoxin

Fig. 11. Hydrophilic interaction LC–MS analysis of an extract of mussels contaminated with PSP toxins feeding on toxic plankton (the same one shown in Fig. 8). Compounds M1, M2 and M3 are new toxins not present in the original plankton and that were formed as metabolites in the mussels. Conditions: same as in Fig. 8 (from Ref. [80]).

In many other studies, when analytical methods such as LC–MS are applied to samples, more and more toxin analogues and metabolites are revealed. Examples include ciguatoxins [81], pectenotoxins [82,83], spirolides [72], and azaspiracids [84].

7. Being proactive

7.1. Surveying for toxins

Although solving toxic episodes can be challeng-

ing and exciting, it is far better to be proactive rather than reactive. It is prudent to perform surveys of various shellfish-growing regions for new emerging toxins. Thus, various surveys for domoic acid contamination have detected its presence in plankton or in shellfish well before toxic levels were reached. Two examples include the US West Coast in 1994 [85], where razor clams and Dungeness crabs were contaminated, and the UK in 2001 [86], where scallops were affected.

The multi-toxin LC–MS screening method (Fig. 7) provides an excellent means for performing surveys. In 2000, in collaboration with the Canadian Food Inspection Agency, we used the method to survey mussels from various locations in eastern Canada. The analysis of one such mussel sample from Newfoundland revealed the simultaneous presence of three classes of toxins: DTX1, spirolides (including a new analogue, C2), and the pectenotoxins, PTX2 and PTX2 seco acids (Fig. 12). This was the first time that pectenotoxins had been detected in Canada (manuscript in preparation). Investigations of the source of the toxin determined that it was *Dinophysis acuminata*.

7.2. The future of monitoring?

What does the future hold for monitoring programs? There is general agreement that once a suitable alternative is available, the mouse bioassay should be eliminated. LC–MS holds great promise as a screening method. In fact, New Zealand is currently testing LC–MS for the monitoring of shellfish for ASP and all of the lipophilic toxins (P. Holland and P. McNabb, pers. commun.). Many other countries are adapting the technology to monitor several toxin classes. For some toxin classes, such as spirolides and azaspiracids, LC–MS is the only analytical method available. One limitation at present is the lack of standards for many of the toxins (see Section 8). In addition, an LC–MS system is best suited to a central laboratory where the necessary skilled personnel can attend to its operation and maintenance.

One approach that may be both cost-effective and comprehensive is the use of rapid assays for screening out the vast majority of negative samples. Any positive hits could be confirmed in a central laboratory using LC–MS. Many assay systems are now being developed for the different toxin groups [11]. Antibody methods are well recognized as specific, easy-to-use, reliable and inexpensive. One approach to implementing an immunoassay is to use the lateral flow immunochromatography (LFIC) technique. In such an assay, all the components are incorporated into a test strip, so that it is only necessary to add a sample extract to initiate the sequence of reactions. As a result, LFIC tests require no special expertise or laboratory equipment in their use.

However, the use of LFIC for PSP toxin detection presented some unique problems. Antibodies must recognize about 20 analogues of saxitoxin (STX) in a variety of complex matrices extracted from different shellfish species. Furthermore, in naturally contaminated shellfish, a wide range of concentrations may be encountered. An ideal assay should indicate a positive result at or above the regulatory limit of 800 μg STXeq per kg of tissue and a clear negative below this value.

Commercial LFIC assay kits, called MIST Alerts™, have now been developed for both PSP and ASP toxins by Jellett Biotek Ltd. in collaboration with the National Research Council of Canada [87]. Three validation trials have indicated good performance of these test kits in the field [87–89].

8. Standards and reference materials

The lack of accurate calibration standards for phycotoxins has been and still is a significant problem in the development and implementation of analytical methods for routine monitoring of seafood. In addition, regulatory laboratories now face the need to operate under GLP and ISO guidelines, which require validated methods, accurate calibration standards, and certified reference materials (CRMs). Very few of the shellfish toxins are available commercially, and for those that are available, the prices can be very high and the buyer must be cautious about putting too much faith in the stated quantity or purity of materials provided. In many cases, the materials are sold primarily for biological testing and may not be suitable for quantitative analysis work. Since many toxins are hygroscopic and difficult to isolate or crystallize in known anhydrous salt forms, quantitation by weighing is uncertain.

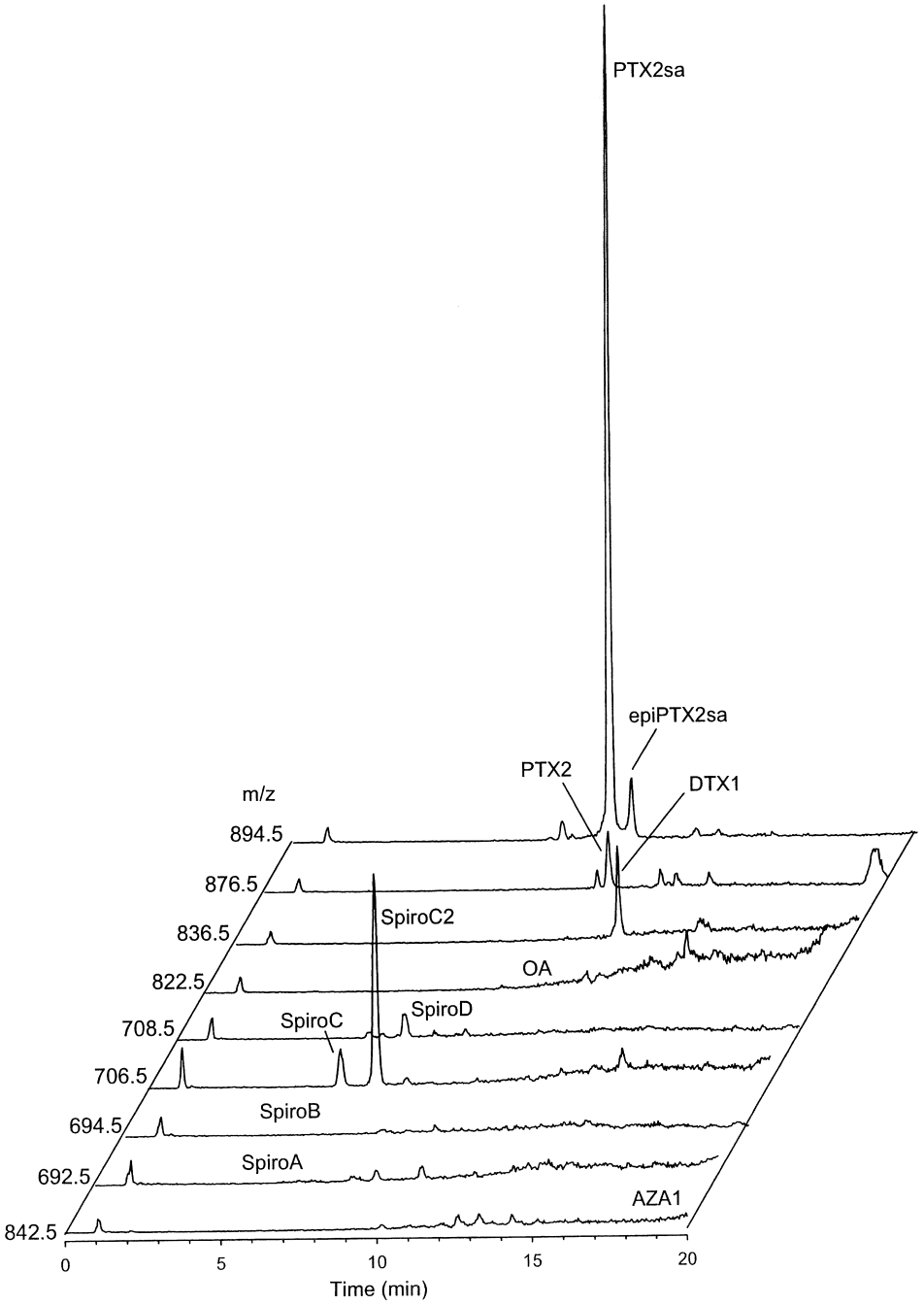


Fig. 12. Reversed-phase gradient elution LC–MS analysis of an extract of Newfoundland mussel digestive glands contaminated with three different toxin classes. Selected ion monitoring was carried out on either $[M+H]^+$ or $[M+NH_4]^+$ ions, which are displayed as individual mass chromatograms. See Fig. 1 for structures associated with various codes.

The National Research Council's Certified Reference Materials Program (CRMP) began producing a domoic acid certified reference material (CRM) in response to the 1987 ASP crisis [90]. The material was distributed as a solution of certified concentration in a flame-sealed ampoule, ready to use for instrument calibration. This CRM soon proved itself of great value when domoic acid was found on the US West Coast and facilitated the rapid implementation of monitoring programs. Since then, the program has expanded to include calibration solution CRMs and shellfish tissue CRMs for a variety of toxins of marine algal origin, including those responsible for amnesic, diarrhetic and paralytic shellfish poisoning. CRMs are currently in production for other toxins, such as pectenotoxins, yessotoxin, azaspiracid, spirolide and gymnodimine.

The preparation of calibration standards requires large-scale laboratory cultures of phytoplankton or highly contaminated shellfish tissues, as well as careful attention to degree of purity and stability of the toxins. Accurate quantitation of standards involves a cross-comparison of results from different procedures, including gravimetry, nuclear magnetic resonance (NMR), and separation methods, such as LC and CE coupled with diverse detection systems such as UVD, FLD, MS, etc. The production of a shellfish tissue CRM additionally requires the development of accurate and precise extraction/clean-up procedures coupled with instrumental analytical methods such as LC–MS and CE–MS. Periodic checks and eventual replenishment of CRMs are also necessary.

Recently, we acquired an Antek 8600R chemiluminescence nitrogen detector (CLND) for LC. This instrument can measure bound nitrogen with a high degree of specificity and allows the quantitation of nitrogen-containing compounds [91]. Since most compounds give equimolar responses proportional to the number of nitrogen atoms in the molecule, it is therefore possible to perform quantitation with a single nitrogen-containing standard such as caffeine. This technique has been very useful for the development of a series of new PSP toxin CRMs. The application of this technique to an NRC CRM-GTX2&3, a new CRM for toxins GTX2 and GTX3, is shown in Fig. 13. This solution contains an equilibrium mixture of the two epimeric toxins with concentrations now certified at 118±6 μ*M* for GTX2 and 39±2 μ*M* for GTX3, from the results of NMR and LC-CLND analyses.

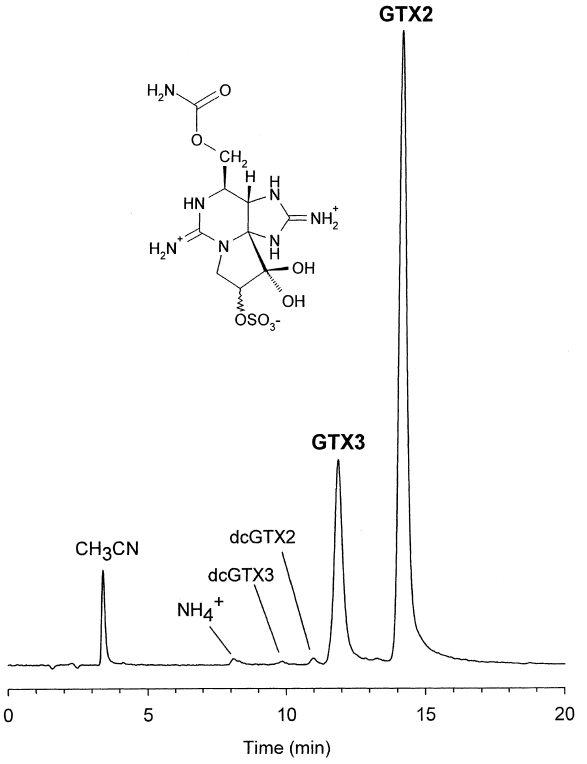


Fig. 13. Analysis of a calibration solution certified reference material (NRC CRM-GTX2&3) by liquid chromatography with the chemiluminescence nitrogen detection (LC-CLND). Conditions: column = Zorbax SBC8, 250 mm × 2 mm I.D. at 10 °C; flow = 0.2 ml/min; mobile phase = gradient from 5 to 15% methanol in water (with 2 m*M* heptanesulfonic acid) over 15 min; detection with an Antek 8060 CLND. Trace amounts of dcGTX2 and dcGTX3, CH_3CN and NH_4^+ were present as impurities.

9. Conclusions

Phycotoxins have presented significant challenges to chemists due to their complex diverse structures, high toxicity and unexpected occurrences. Chromatography has played an essential role in all phases of toxin investigations, including the identification of new toxins by bioassay-directed fractionation, the detection and quantitation of toxins in plankton and shellfish, the investigation of toxin production by

plankton, and the study of toxin metabolism in shellfish. The combination of liquid chromatography and mass spectrometry (LC–MS) has proven to be one of the most powerful tools. LC–MS in combination with rapid screening assays promises to greatly increase protection of public health through comprehensive monitoring of seafood for the presence of toxins.

Acknowledgements

The author thanks his colleagues at IMB and all his collaborators around the world for their significant contributions.

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