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LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY FOR THE ANALYSIS OF MARINE TOXINS*

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RESUME

L'avènement récent de techniques d'ionisation telles que "Ion-spray", opérant à pression atmosphérique, a permis le couplage direct du chromatographe liquide au spectromètre de masse (CL/MS). Cette technique d'ionisation s'est avérée particulièrement efficace pour l'analyse de composés polaires présents à l'état ionique en solution. Dans le cadre de notre programme d'analyse de toxines marines, nous avons évalué la technique combinée de CL/MS pour l'identification de composés associés aux cas d'intoxication amnésiante, paralysante et diarrhéique. Les spectres de masse de ces toxines sont relativement simples et exempts de fragments abondants, permettant ainsi d'obtenir des limites de détection se situant sous le nanogramme.

La technique CL/MS a également été utilisée pour l'analyse d'extraits biologiques issus de planctons marins ou de mollusques contaminés par des toxines marines. Ces dernières études ont non seulement permis d'établir la concentration de toxines connues à l'intérieur d'échantillons marins, mais elle a aussi permis de mettre en valeur le potentiel de la technique "Ion-spray' CL/MS pour l'identification et la caractérisation de toxines inconnues.

ABSTRACT

Ion-spray is a recently developed atmospheric pressure ionization method for combined liquid chromatography/mass spectrometry (LC/MS) that is highly sensitive for polar compounds that can be ionized in solution. We have investigated the suitability of this technique for the analysis of marine toxins responsible for incidents of amnesic, paralytic and diarrhetic shellfish poisoning. All of the toxins studied gave very simple spectra with sub-nanogram sensitivity.

Ion-spray LC/MS has been used to analyze plankton and shellfish extracts for the quantitative analysis of known toxins and for the identification of new toxins.

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1. INTRODUCTION

Marine toxins pose a significant challenge to the analytical chemist (1). They range from the very polar saxitoxin-based compounds responsible for paralytic shellfish poisoning (PSP) to the high molecular weight lipophilic toxins such as okadaic acid responsible for diarrhetic shellfish poisoning (DSP). The potent toxicities of these substances demand that analytical methods provide high sensitivity, while complex shellfish tissue matrices require high selectivity of separation and/or detection. Although routine regulatory work generally uses inexpensive, high speed methods such as animal biossays and immunoassays, it is important that instrumental methods be available for the confirmation of toxin identity and for the identification of new toxins.

Mass spectrometry (MS) is a powerful tool that has an important future for the analysis of marine toxins. In addition to being very sensitive and selective, MS can provide molecular weight and structural information. Unfortunately, highly polar compounds such as toxins are not amenable to direct analysis by classical mass spectrometry techniques, such as electron ionization and chemical ionization, in which vaporization of the analyte is a necessary prerequisite for ionization. However, toxins are ideally suited to ionization techniques in which the sequence of events may be considered as pre-ionization in solution followed by sputtering or evaporation of these ions from a liquid surface. Indeed, fast atom bombardment (FAB) MS has been investigated for PSP toxins (2, 3), DSP toxins (4-6) and amnesic shellfish poisoning (ASP) toxins (7), and has proved useful as a means of structural confirmation at moderate sensitivity.

The greatest potential of MS for the analysis of real-world samples lies in its combination with liquid chromatography (i.e., LC/MS) (8). LC/MS with continuous flow FAB has been demonstrated for okadaic acid (4), but we have found this technique to be very difficult to use on a routine basis. Recently, we have shown that Ion-spray LC-MS is an excellent technique for the analysis of marine toxins (6, 9). Ion-spray is an atmospheric pressure ionization technique that is very sensitive for polar compounds that can carry a charge in aqueous solution (10). The ion formation mechanism is based upon the ion evaporation phenomenon (field assisted desorption of pre-formed solute ions from micro-droplest of solution) (11). The technique can be used with flow rates from a few µL/min up to 0,2 mL/min and thus can be used on-line with HPLC as well as for direct sample introduction.

EXPERIMENTAL

All toxins were prepared by researchers at the Institute for Marine Biosciences from cultured plankton or shellfish tissue. Many of these are or will soon be available as certified instrument calibration solutions from the NRC'S Marine Analytical Chemistry Standards Program (MACSP, Halifax). The MUS-1 mussel tissue reference material for domoic acid (12, 13) is already available from MACSP.

Measurements were perfomed on a SCIEX API-III triple quadrupole mass spectrometer equipped with an Ion-spray source (SCIEX, Thornhill, Ontario, Canada). The mobile phase flow rate was controlled between 5 and 200 µL/min with an HP 1090 HPLC with a DR5 pumping system.

RESULTS

Ion-spray provides very simple spectra dominated by the protonated molecule, $(M + H)^+$, with very little fragmentation. This is apparent in Figure 1a wich shows the positive ion spectrum of domoic acid (DA), the toxin responsible for ASP (12). This spectrum was acquired by injection of 70 ng of DA into a mobile phase flowing at 0.1 mL/min directly to the ion-spray probe. This mode of operation, called flow injection analysis, can be very useful for rapid analysis of samples. However, extracts must first be taken through an extensive clean-up to prevent interference from salts and other substances which can create excessive background or even suppress the ionization of the analyte. As little as 1 ng of DA is required to produce a recognizable spectrum. A limitation of "soft" ionization methods such as ion-spray is the lack of fragment ions for structural information. However, it is possible to use the tandem mass spectrometry (MS/MS) capability of the SCIEX triple quadrupole instrument to generate fragment ion spectra as shown in Figure 1b for DA.

Combining LC with the ion-spray MS is very easily accomplished and has proved very reliable in routine work. Figure 1c shows the results of a combined LC-MS analysis of an extract of the mussel tissue reference material, MUS-1 (certified for DA at 126 µg/g). In addition to observing a strong peak for DA at the correct retention time, it was also possible to detect several isomers of DA. Detection limits in the order of 50 to 100 pg injected on-column were possible with selected ion monitoring and excellent quantitative measurements have been demonstrated.

PSP toxins are also well suited to ion-spray MS as demonstrated by the spectrum of GTX-2 in Figure 2. Again, an abundant $(M + H)^+$ was observed, but this time some fragmentation due to loss of SO_3 also occured. Combined LC/MS of PSP toxins has proved more challenging, unfortunately. Alkysulfonates used as ion-pair reagents in published separation methods (1) interfered with ion-spray. Initial tests with ammonium acetate buffer have been very promising, however, as shown in Figure 3. We are currently investigating other mobile and stationary phases to further improve separations.

A good example of the power of ion-spray LC/MS was provided by a recent incident in Nova Scotia in which diarrhetic shellfish poisoning was suspected. In early August 1990, at least 16 people developed symptoms of nausea, vomiting and diarrhea after eating cultured mussels from the Mahone Bay area. Extracts of the suspect mussels were found to be toxic to mice (IP injection). As shown in Figure 4 analyses of whole mussel tissue extracts by combined LC-MS confirmed the presence of the DSP toxin DTX-1 within hours of receipt of the samples.

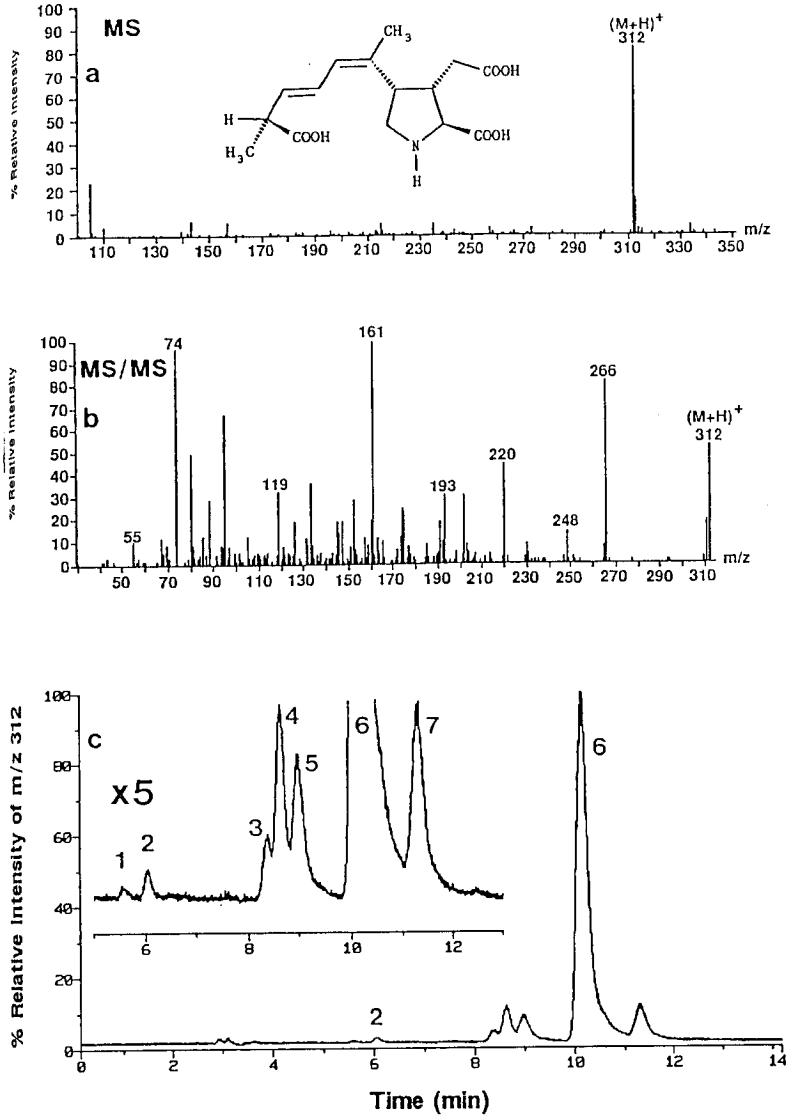
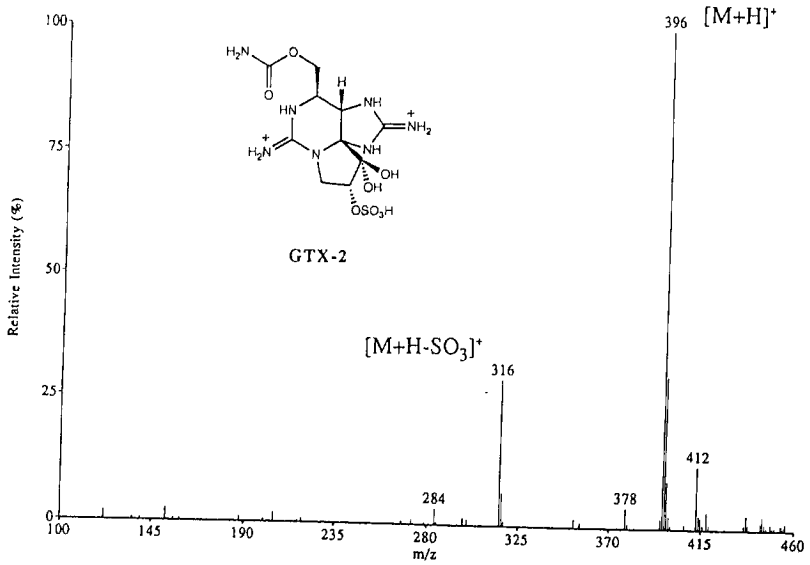


FIGURE 1 : Ion-spray mass spectrum of 70 ng domoic acid (a) and ion-spray MS/MS spectrum of the protonated molecule (m/z 312) of domoic acid (b) acquired by flow injection analysis in the positive ion mode (mobile phase 0.05 mL/min 10% acetronitrile in water with 0.1% formic acid). The bottom trace (c) shows the ion-spray LC/MS analysis of an extract of the mussel tissue reference material, MUS-1 (13), after clean-up with C18 solid-phase extraction (column : 5 µm Vydac 201 TP column (25 x 0.21 cm); mobile phase : as above, except 0.2 mL/min). Peak 6 is due to domoic acid while the other numbered peaks represent isomers of domoic acid present in the mussel tissue.



igure 2 :
on-spray mass spectrum of 100 ng of the PSP
oxin GTX-2 in the positive ion mode.

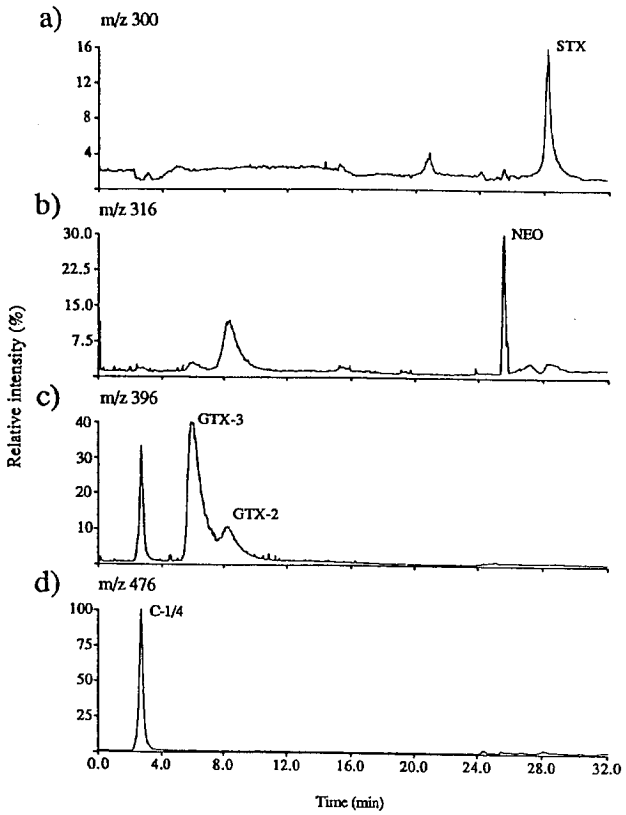
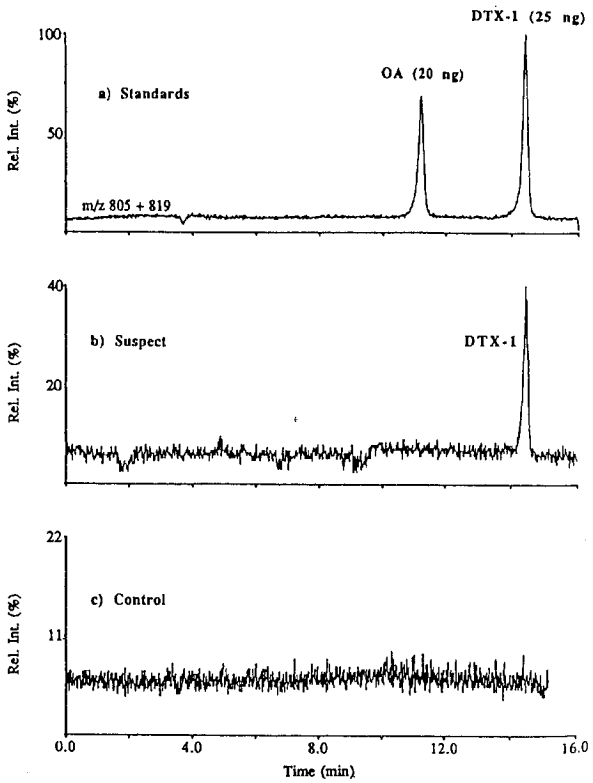


FIGURE 3 : Mass chromatograms from the ion-spray LC/MS analysis of a mixture of PSP toxins. **Conditions :** PRP-1 resin column (25 x 0.46 cm), 1 mL/min acetonitrile/10 mM ammonium formate, gradient from 0% to 20% acetonitrile over 30 min.

Figure 4 :
Ion-spray LC/MS analysis of a standard mixture of okadaic acid (OA) and DTX-1 (top), an extract of toxic mussels suspected to contain DSP toxins (middle), and an extract of non-toxic control mussels. **Conditions :** 5 µm Vydac 201 TP reversed phase column (25 x 0.1 cm), 50 µL/min acetonitrile/water with 0.1% trifluoroacetic acid, gradient from 40% to 100%.



Quantitative analyses of survey samples showed that the incident was highly localized to one mussel growing lease and that by the end of August, the mussels had depurated all of their toxin load.

CONCLUSIONS

Although HPLC-MS equipment is expensive, possibilities of automation can begin to justify such an investment if large numbers of samples can be analyzed quickly with high speed methods. For research studies, of course, the amount of information provided by an HPLC-MS analysis is unsurpassed and will facilitate a greater understanding of the chemistry and biochemistry of marine toxins.

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ACTES DU COLLOQUE

SUR LES BIOTOXINES MARINES

PROCEEDINGS OF SYMPOSIUM

ON MARINE BIOTOXINS

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