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LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY OF POLAR LIPIDS

COMPARISON OF ON-LINE METHOD USING DIRECT LIQUID INTRODUCTION INTERFACE WITH OFF-LINE METHOD USING DESORPTION/CHEMICAL IONIZATION

FRANCOIS R. SUGNAUX* and CARL DJERASSI
Department of Chemistry, Stanford University, Stanford, CA 94305 (U.S.A.)

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.. INTRODUCTION

The classical methods of analysis for molecular species of polar lipids involve many cumbersome separation and chemical reaction steps, and yet are relatively poor in sensitivity and precision[1,2]. These methods consist of a combination of column chromatography with thin-layer chromatography and degradation reactions, so that the final analysis is concerned with only relatively simple compounds such as fatty acids or simple sterols. These compounds can then be easily separated and characterized by combined gas chromatography–mass spectrometry (GC–MS).

In our current research into lipids in marine organisms, a group of polar sterols, which eventually were recognized as sterol peroxides, had to be derivatized and degraded into acetates of known sterols in order to be able to undergo GC–MS[3]. Similarly, some novel phospholipids from sponges had first to be degraded into their fatty acids and phosphoglycerol before GC–MS analysis[4]. The drawback of these procedures is that the actual structure of these polar lipids can be assessed only indirectly after a chemical degradation which does not preclude possible artefact production.

A substantial improvement in the separation of these compounds was made possible by the advent of high-performance liquid chromatography (HPLC)[5]. Nonetheless, this technique suffered from the lack of a detector more sensitive than the differential refractometer. To overcome this problem, different solutions have been

* Visiting Research Associate on sabbatical leave from University of Geneva, Switzerland.

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proposed, which include the moving-wire detector[6] with flame ionization detection[7], a pyrolytic moving-belt liquid chromatographic (LC)–MS detector[8], the use of non-UV-absorbent solvents and photometric detection in the region of 200 nm[9,10], the liquid scintillation detection of phosphorus-32-labeled phospholipids[11], an automatic colorimetric phosphorus analysis[12] and the derivatization of the lipids into the photo-absorbing *p*-nitrobenzoates[13] or the fluorescent Dns derivatives[14]. Most of these techniques of detection, however, have not found wide application and offer only a low specificity.

We have therefore pursued the most specific and promising approach for the analysis of polar lipids, the LC–MS method proposed by Privett and Erdahl[8]. In the present paper, two alternative approaches are described, *i.e.* on-line LC–MS using direct liquid introduction (DLI) interface, and off-line LC–MS using desorption/chemical ionization (DCI), and their relative merits are compared.

2. EXPERIMENTAL

2.1. *Materials*

Mixtures containing only epidioxysterols were obtained after extraction and silica gel column chromatography purification from the tunicate *Ascidia nigra*, the sea hare *Aplysia dactylomela* and the soft coral *Dendrogyrus cylindrus*[15]. All the phospholipids, either natural mixtures or synthetic molecular species, were purchased from Sigma (St. Louis, MO, U.S.A.). The analytical grade solvents were supplied by J. T. Baker (Phillipsburg, NJ, U.S.A.)

2.2. *Equipment*

The mass spectrometer used in this study was a Ribermag (Nermag, Rueil-Malmaison, France) R-10-10-C quadrupole, equipped with a SADR data system. Desorption/electron impact (DEI) and DCI mass spectra were obtained with the standard fast-heated wire probe[16], with a linear current program from 0 to 450 mA at a rate of 5 mA/sec. The source temperature was kept at 120 C and for DCI the reagent gas pressure in the source was $1.5 \cdot 10^{-1}$ Torr. The reagent gas transfer line temperature was 290°C.

For LC–MS, the flow of solvent was introduced into the mass spectrometer by means of the standard Ribermag DLI interface[17]. A schematic diagram of the set-up used in these experiments is shown in Fig. 1.

The interface design is based on the principles originally developed by Baldwin and McLafferty[18]. In this method, the highest fraction of the LC flow which can be handled by the vacuum pumps is jetted into the mass spectrometer through a 1–3 μm pinhole in a diaphragm. The amount of liquid introduced is controlled by the pressure of the liquid in front of the diaphragm. This pressure results from either the partially closed needle valve splitter built in the interface or from the diaphragm flow restriction only, when the needle valve is completely closed.

The complete DLI/LC–MS apparatus comprised a Beckman (Fullerton, CA, U.S.A.) model 100A HPLC pump, a Rheodyne (Cotati, CA, U.S.A.) 20 μl loop injector, a 150 × 4.6 mm I.D., 5 μm particle diameter, RP-18 column (Ultrasphere-ODS, Altex, Berkeley, CA, U.S.A.), a 0.5-μm line filter, and the previously described DLI interface–mass spectrometer–computer assembly. For DLI without separation,

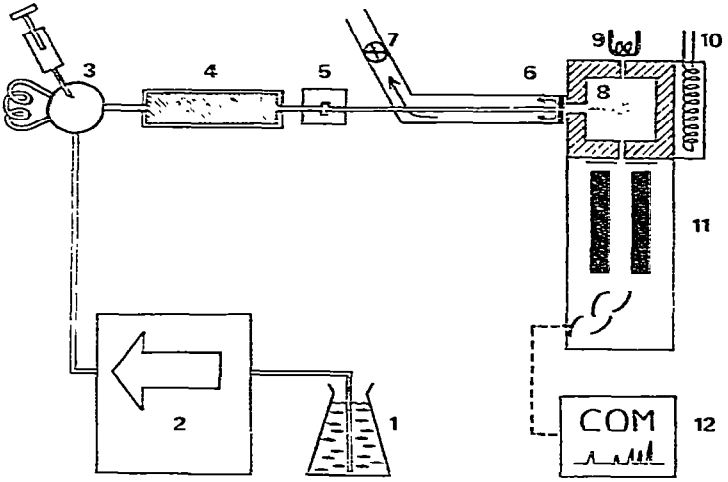

Fig. 1. Schematic diagram of the DLI/LC–MS instrument. 1 = Solvent reservoir; 2 = liquid pump; 3 = sample loop injector; 4 = HPLC column; 5 = line filter; 6 = DLI interface; 7 = fine-metering needle valve; 8 = droplet spray in the source block; 9 = filament (actually located at the back face of source) and make-up reagent gas inlet; 10 = heater block; 11 = quadrupole mass filter and electron multiplier; 12 = computer.

only the column was removed while the rest of the set-up remained the same.

The amount of solvent taken into the mass spectrometer was adjusted to produce a chemical ionization (CI) reagent gas pressure of 0.15 Torr in the source. Alternatively, for introduction without separation, the needle valve was completely closed and the solvent admission was controlled by the HPLC pump working in a constant pressure mode instead of a constant flow. In this case, the selected source pressure was also 0.15 Torr. At this source pressure, the corresponding vacuum in the source envelope was *ca.* 6 times higher than the 10^{-4} Torr safety limit set for the proper functions of the mass filter and the electron multiplier of the quadrupole. The addition of a cryopump to supplement pumping by the 1000 l/sec diffusion pump, as proposed for the prototypes Lucie and Carole[19], was therefore not necessary.

The source temperature was carefully selected, since sufficient energy must be brought by convection from the source walls and optionally from a heated make-up reagent gas to dissociate solvent–solute association and allow the vaporization of single molecules. This temperature must not be so high, however, as to cause the pyrolysis of fragile molecules.

Heating of the source assembly was provided by both a heater block and the electron-producing filament. The source temperature selected, from 100 to 260°C, was that of the heater block, but the actual surface temperature in the source was lower, due to the thermal energy supplied by convection to the droplets of solvent. The data acquisition was started at mass 150 to avoid a high background, originating in solvent CI clusters and impurities. The mass range 150–500 for the epidioxysterols and 150–850 for the phospholipids was scanned continuously at an average speed of 3 msec/a.m.u.

3. RESULTS

The two classes of polar lipids considered in this study, the epidioxysterols and the phospholipids, were treated separately, due to their large differences in proton affinity and volatility. With the epidioxysterols, HPLC separation was achieved on the reversed-phase column with methanol as solvent. Fifteen different sterol peroxides, for which structures are given in Fig. 2, were analyzed in this study. Beside the basic four rings of the steroids and the saturated or unsaturated side-chain, the epidioxysterols so far discovered have a 5α,8α-epidioxy group which forms a supplementary cyclic system[15]. This group, together with the 3β-hydroxy substituent, renders these compounds more polar and heat sensitive than the regular sterols. This explains why the sterol peroxides decompose rapidly during a GC analysis. Two different types of ring unsaturation were encountered: type A, with one Δ^6 double bond; and type B, with two double bonds $\Delta^{6,9(11)}$.

The EI mass spectra of these compounds gave very weak molecular ions. An example of the EI mass spectrum of 7-dehydrocholesterol peroxide is given in Fig. 3. Even with the soft in-beam desorption/electron impact (DEI) vaporization technique, the molecular ion intensity is very low and the base peak corresponds to the loss of a

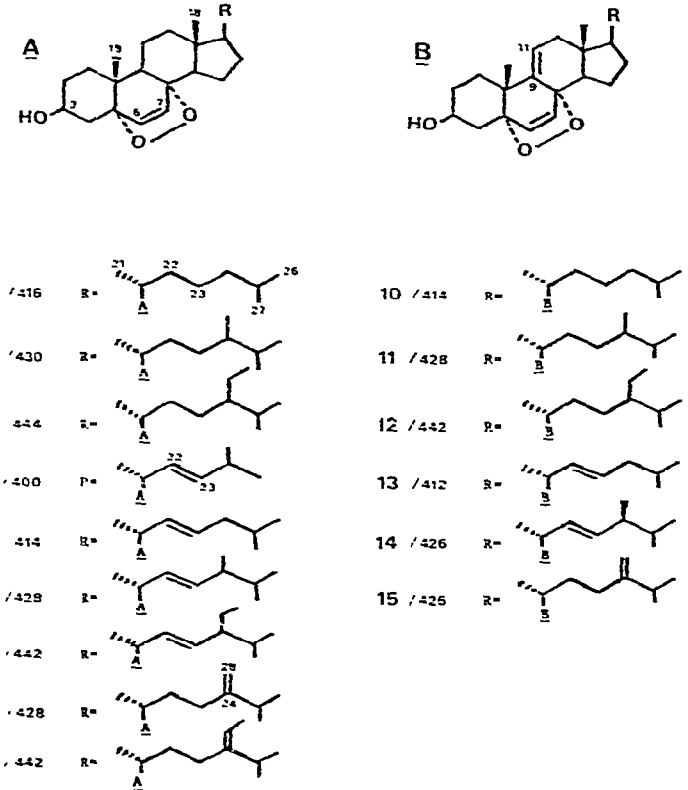

Fig. 2. Chemical structures of epidioxysterols 1–15/molecular weight. A, Δ^6 ring unsaturation; B, $\Delta^{6,9(11)}$ ring unsaturation.

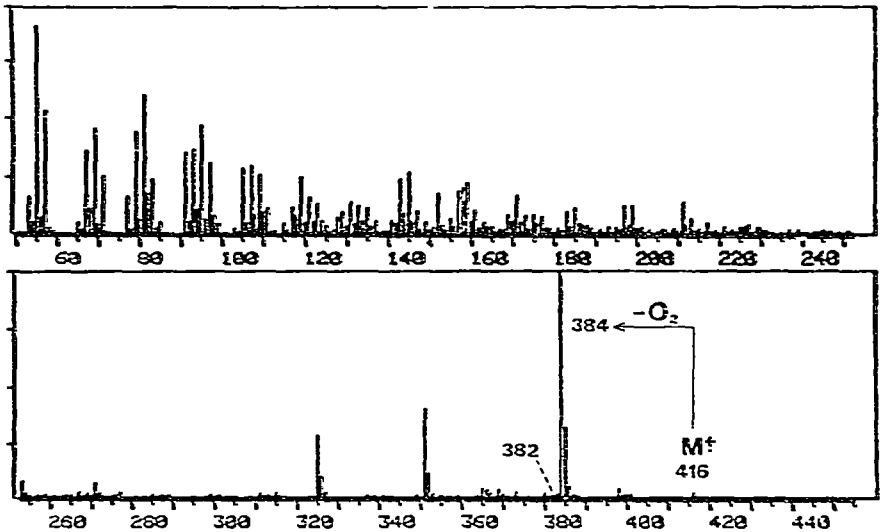

Fig. 3. DEI mass spectrum of compound 1 (7-dehydrocholesterol peroxide).

molecule of oxygen. A minor fragment of mass 382, which plays a major role in the CI of these peroxides, is due to the loss of H_2O_2. The elementary composition of all these ions was confirmed by high-resolution MS with a Varian 711 instrument. EI was therefore not an appropriate technique for obtaining information on minor unknown peroxides in mixtures when they were not extensively purified.

Therefore we considered LC–MS for the separation and analysis of sterol peroxides.

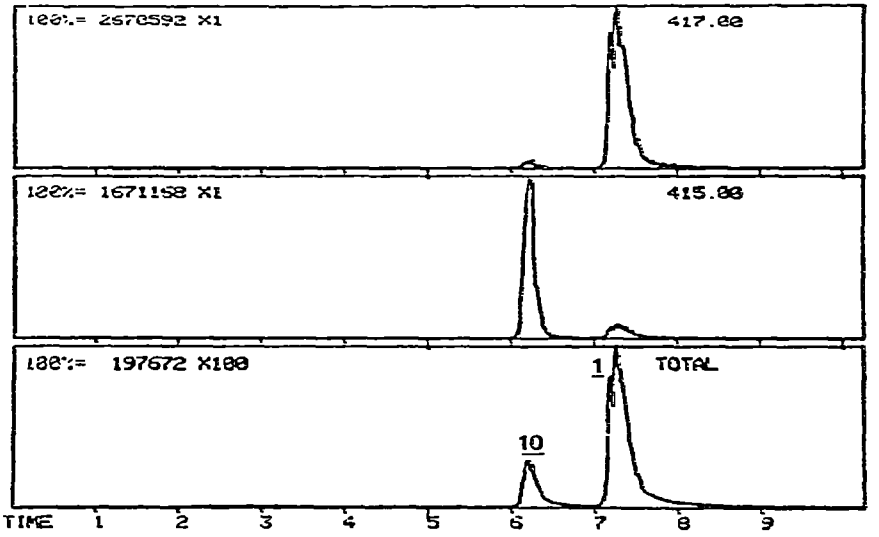

Fig. 4. DLI/LC–MS analysis of epidioxysterols of *Aplysia dactylomela:* extracted ion-current profile for ions at m/z = 417 and 415, and TIC. HPLC: C_{18}-bonded silica column, eluted with methanol–water (99:1) at 1 ml/min, room temperature.

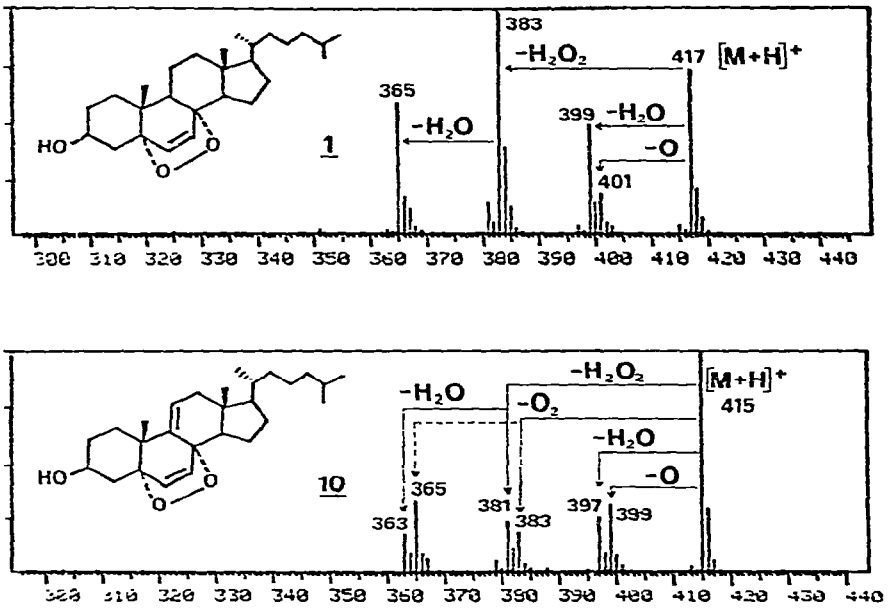

Fig. 5. DLI LC–MS mass spectra of epidioxysterols 1 and 10. Reacting ion species are from methanol (LC solvent). HPLC conditions as in Fig. 4.

A first example of DLI/LC–MS separation is given in Fig. 4. This chromatogram was obtained at a source temperature of 106°C, sufficient to vaporize completely the solvent, but not so high as to pyrolyse the peroxides. From the constancy of the pressure in the source, it appeared that the diaphragm was sufficiently cooled by the splitted excess of HPLC solvent and needed no additional temperature control.

A 10-μg portion of the sterol peroxides fraction of *Aplysia dactylomela* was injected into the HPLC column. That corresponded to an effective injection of *ca.* 100 ng of the mixture in the mass spectrometer source, at the estimated 99:1 splitting ratio. Accordingly, a flow of *ca.* 10 μl/min into the mass spectrometer corresponded to the 1 ml/min flow-rate of methanol pumped through the HPLC column. The peaks were 21 sec wide at the base, equivalent to a solvent volume of *ca.* 3.5 μl. In Fig. 4, the top two boxes show the reconstructed ion chromatograms the protonated molecular ions, while the total ion current is displayed in the lower box.

The DLI/CI-MS spectra of the two epidioxysterols 1 and 10 are given in Figs. 5a and 5b. The ratio of the relative intensities of the ions in the 20 spectra recorded for each peak remained constant.

Compound 1 (Fig. 5a), having an m/z 417 protonated molecular ion and a retention time of 7.3 min, was found to be 7-dehydrocholesterol peroxide. The LC–MS mass spectrum and retention time of the synthetic reference compound, matching those of compound 1, further confirmed this structure. This compound readily lost both one oxygen atom and one molecule of water, and gave as base peak an ion resulting from the loss of H_2O_2 from the $(M+H)^+$ ion. A subsequent loss of H_2O gave the completely deoxygenated ion of mass 365.

In Fig. 5b, the mass spectrum of compound 10 indicates that this chromatographical-

ly more polar compound contained two hydrogens fewer than 7-dehydrocholesterol peroxide. Also, the intensity ratio of the $(M+H)^+$ ion to the fragment ions was higher than that of compound 1. In addition to the previously observed losses of H_2O and O, there was also a loss of one molecule of oxygen from $(M+H)^+$, competing with the loss of H_2O_2, which indicates some lack of availability of hydrogen for the leaving oxygen molecule. This observation, together with the better stabilization of the $(M+H)^+$ ion, strongly suggested conjugated double bonds in $\Delta^{9(11)}$ and Δ^6. This was confirmed by the perfect matching of all spectroscopic and chromatographic characteristics of the natural and synthetic compound 10.

Another example of the DLI/LC–MS analysis of sterol peroxides, those of *Ascidia nigra*, is given in Fig. 6. Instrumental settings similar to those described for the previously mentioned epidioxysterol mixture were used. From the peak ratios of

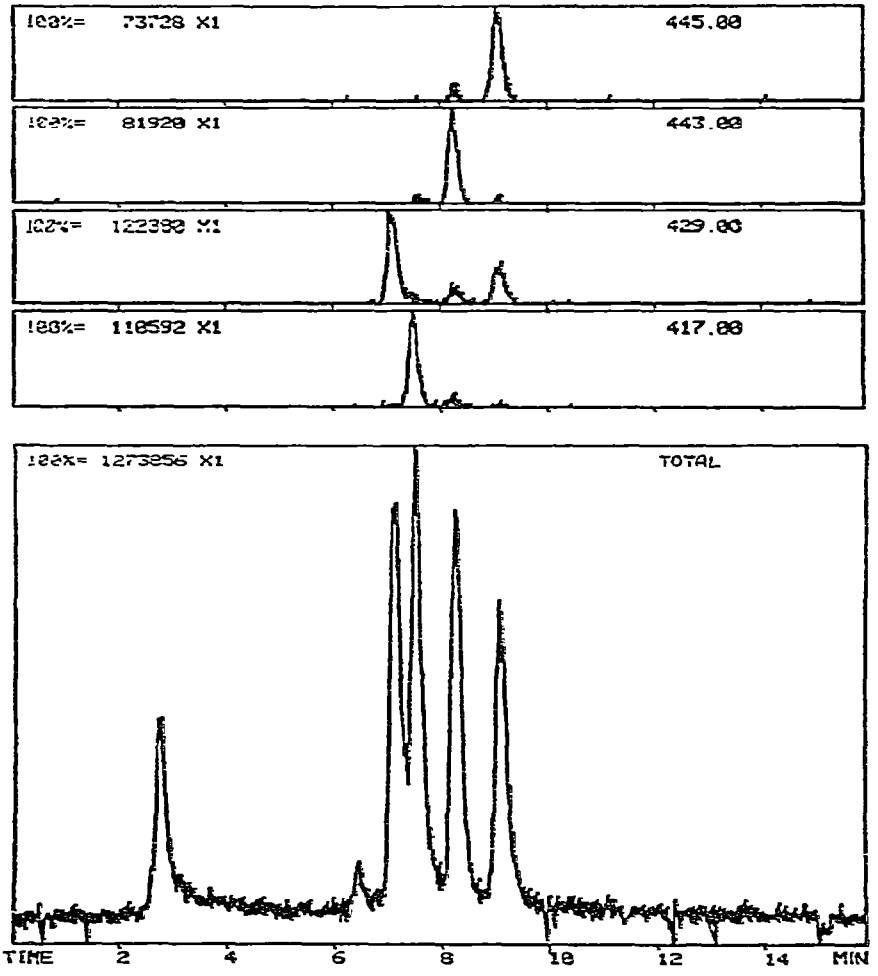

Fig. 6. DLI/LC–MS analysis of epidioxysterols of *Ascidia nigra*: extracted ion-current profile for ions at m/z = 445, 443, 429, 417, and TIC.

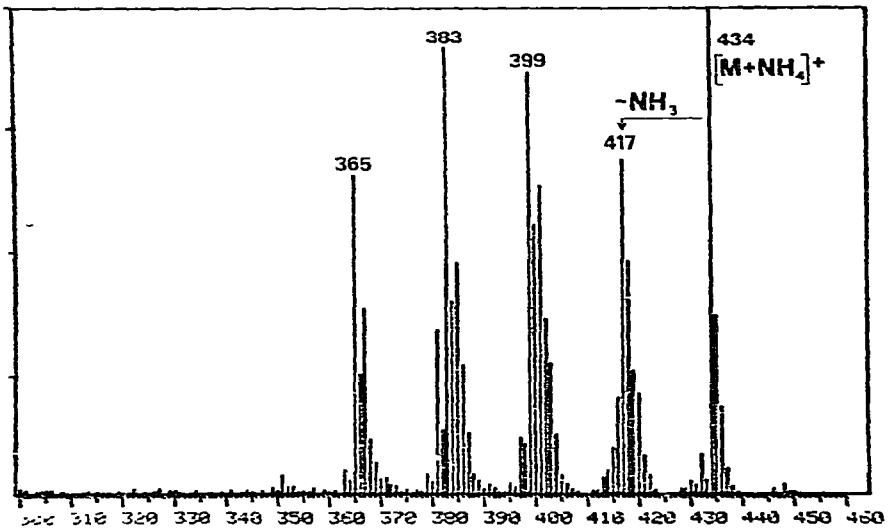

Fig. 7. DLI/LC–MS mass spectrum of compound 1 (7-dehydrocholesterol peroxide). Reacting ion species are from methanol (LC solvent) and ammonia (make-up reagent gas). Temperature of ion source block, 108°C.

the mass spectra, it was also possible to attribute either type A or type B ring structure to the separated compounds. The molecular weight and relative chromatographic retention permitted the deduction of the structure of the separated compounds shown in the mass fragmentogram in Fig. 6.

In the source, the addition of hot ammonia as make-up reagent gas to methanol permitted control of the chemical ionization due to the higher proton affinity of ammonia. The predominant formation of the adduct ion $(M+NH_4)^+$ is shown in Fig. 7 for 7-dehydrocholesterol peroxide.

DLI–NH_3/LC–MS revealed the active hydrogen of the epidioxysterols and allowed a fragmentation independent of the solvent composition used for HPLC. This would be particularly useful with gradient elution[17].

When compared to DLI/LC–MS, DCI mass spectra did not show striking differences in fragmentation. For example, the methane and ammonia DCI "best spectra" of ergosterol peroxide (6) are shown in Figs. 8a and 8b. The more acidic methane provided the protonated ion $(M+H)^+$ along with a few significant fragments, while NH_3 gave the adduct ion $(M+NH_4)^+$, the protonated ion $(M+H)^+$ and the same fragments as CH_4.

A problem encountered with the DCI technique is the time dependence of the molecular ion[20]. A typical illustration of a DCI run is shown in Fig. 9. When the vaporization temperature is reached, desorption of intact neutral molecules takes place for a short period, immediately followed by desorption of decomposition products formed on the surface. If a large scan range and a fast heating rate are needed, even the scan considered the "best spectrum" is biased and not reproducible, because the relative ratio of the ions changes dramatically during that scan. Therefore, the constant and more reproducible vaporization conditions of on-line DLI/LC–MS

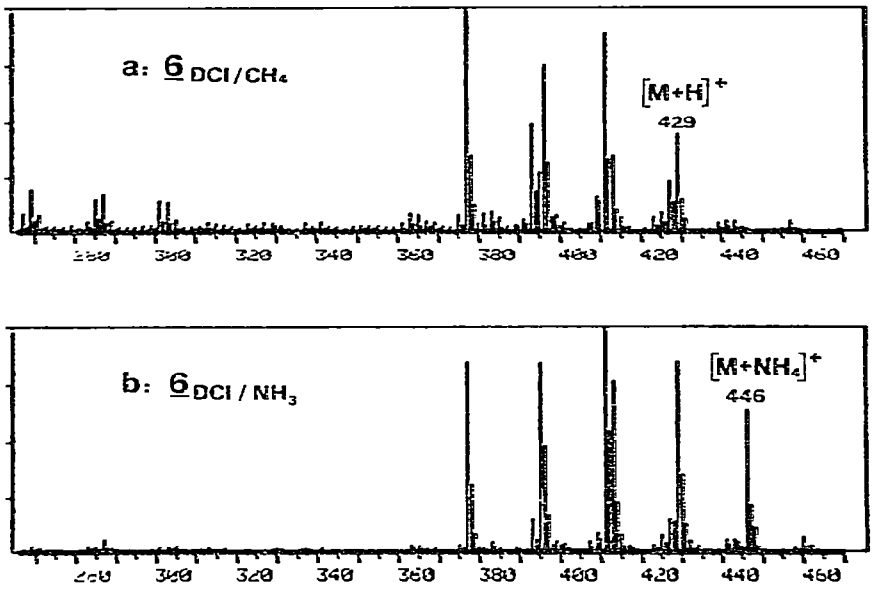

Fig. 8. Desorption/chemical ionization (DCI) mass spectra of compound 6 (ergosterol peroxide). (a) Methane CI. (b) Ammonia CI.

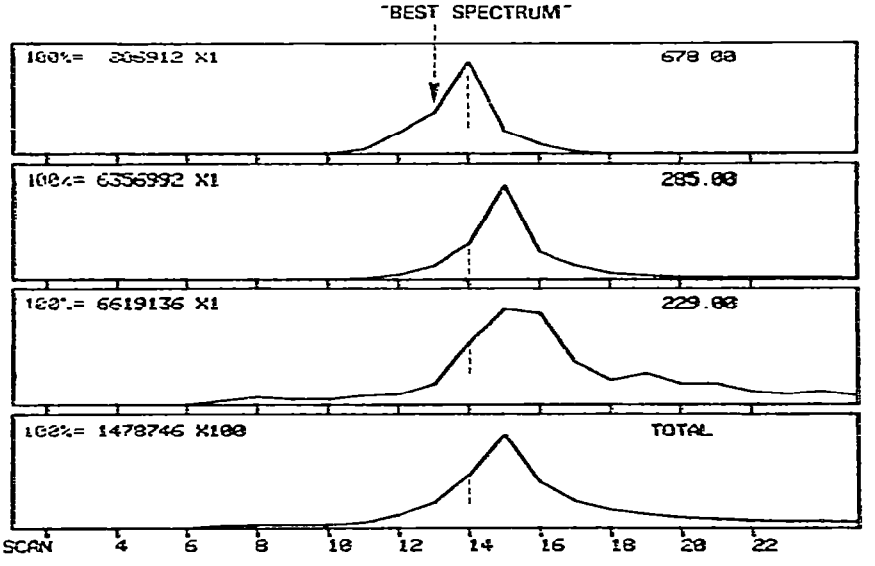

Fig. 9. DCI (ammonia) evaporation profile of dimyristoyl-L-α-phosphatidyl choline: extracted ion-current profile for ions at m/z = 678, 285, 229, and TIC.

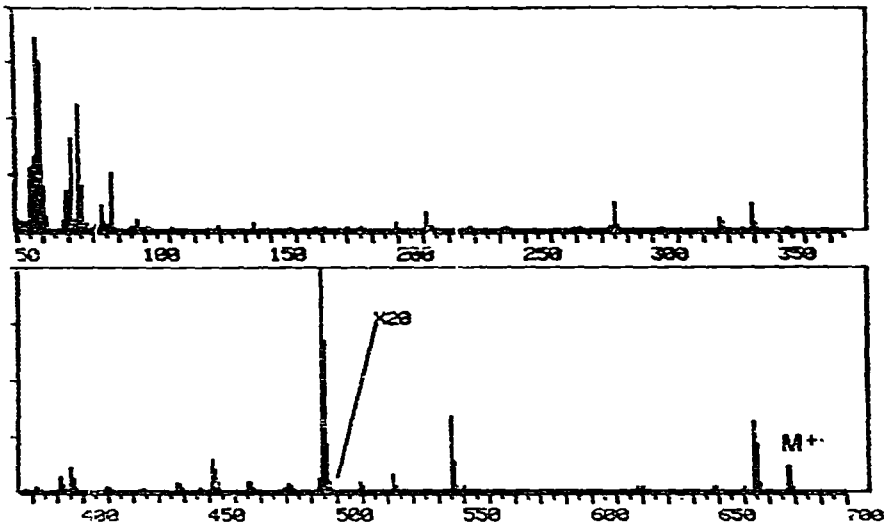

Fig. 10. DEI (in-beam, 70 eV) mass spectrum of dimyristoyl-L-α-phosphatidyl choline.

coupling strongly favor this technique for the purpose of quantitation, over off-line LC–DCI/MS coupling.

Vaporization of the phospholipids requires a temperature higher than that needed for the epidioxysterols, because these are more polar and non-volatile. The smaller difference of energy between intramolecular and intermolecular bonds renders the MS analysis of these molecules in the intact, underivatized state rather difficult[21].

An example of the in-beam DEI mass spectrum of dimyristoyl-L-α-phosphatidyl choline (Myr-PC) is given in Fig. 10. This spectrum shows a molecular ion of very low abundance (0.6% of base peak) and an m/z 494 base peak, resulting from the loss of the phosphoryl choline moiety.

A substantial increase of the molecular ion intensity was obtained with chemical ionization. The methane and ammonia DCI mass spectra of Myr-PC are presented in Figs. 11a and 11b. Along with the $(M+H)^+$ ion, many fragments can be observed. With methane CI, the acidic reagent gas favors the loss of phosphoryl choline from the protonated molecular ion ($MH^+ - 183$), while with ammonia CI, exhibiting a higher reagent gas proton affinity, the trimethylamine group in the PC was substituted by ammonia ($MH^- - 42$)*.

A study of DLI/LC–MS of phospholipids in flow injection, without separation, was conducted to determine the proper MS instrumental conditions for this class of lipids.

Various HPLC methods for the separation of phospholipids on normal and reversed phases have been reported[6–12], so the composition of the eluent used for LC is not unique. We have used ethanol and a solution of dimyristoyl-L-α-phosphatidyl choline (Myr-PC) with ammonia make-up reagent gas to obtain the DLI/LC–MS spectrum shown in Fig. 12. The temperature of the source had to be raised to 260°C

* Evidence for this process will be reported in a future communication from this laboratory.

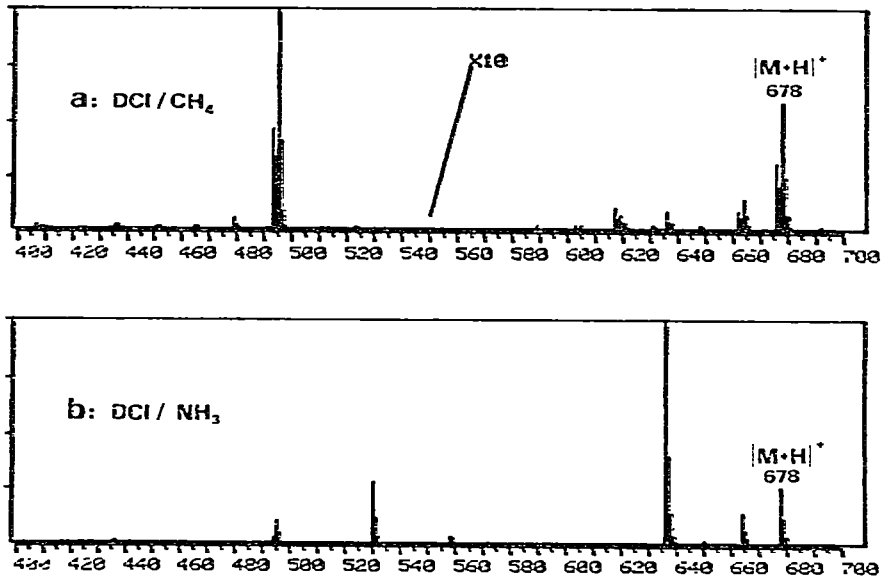

Fig. 11. DCI mass spectra of dimyristoyl-L-α-phosphatidyl choline. (a) Methane CI. (b) Ammonia CI.

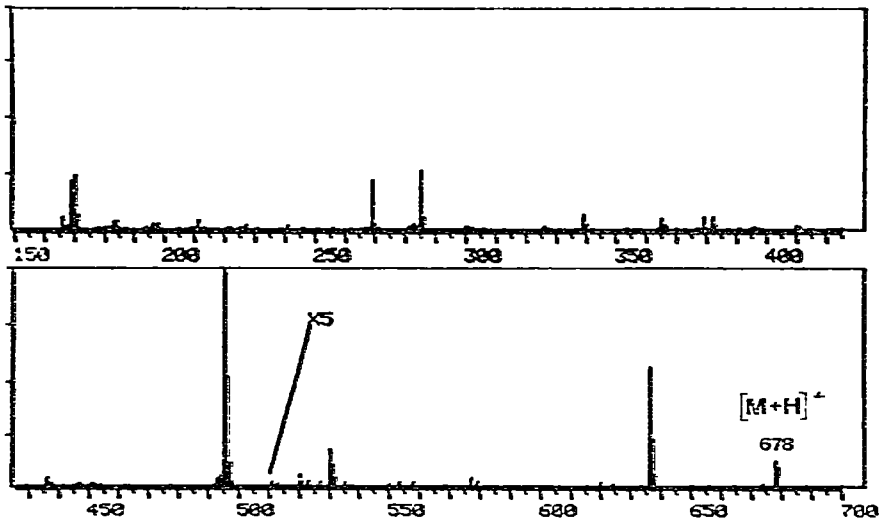

Fig. 12. DLI (flow injection)/LC–MS mass spectrum of dimyristoyl-L-α-phosphatidyl choline. Reacting ion species are from ethanol (LC solvent) and ammonia (make-up reagent gas). Temperature of ion source block, 260°C. LC solvent: ethanol (100%) at *ca.* 10 μl/min. room temperature.

to sustain the vaporization of the LC solvent and the solute. This mass spectrum had some of the characteristics of both methane and ammonia DCI: mainly the loss of phosphoryl choline, but also the substitution of trimethylamine by ammonia. Without ammonia as make-up gas, the ethanol DLI/LC–MS spectrum of this lecithin provided only barely detectable $(M+H)^+$ peak at m/z 678.

4. DISCUSSION

LC–MS has been shown to be feasible with two different families of polar lipids, either on-line with DLI/LC–MS, or off-line with DCI.

DCI is now a routine technique, while DLI/LC–MS in our laboratory is still under development. With regard to convenience, DCI has the advantage of being rapidly operational, with a minimum of MS equipment and no sample separation needed at the same time as the analysis, while the LC separations problems can be solved elsewhere, independently of the MS instrumental requirements.

Once the on-line DLI/LC–MS has been started and the column and source temperatures and pressures have been equilibrated, however, the instrument can effortlessly make many separations and hundreds of data acquisitions. Therefore, when only very small amounts of sample are available, on-line DLI/LC–MS would be the method of choice.

When the amount of sample is large enough to permit LC separation with refractive index detection, the polar lipids we have studied can be also handled off-line with DCI, although at a higher time cost. When other spectroscopic methods for the identification of the purified sample are required anyhow, especially the sample-consuming NMR, off-line DCI/LC–MS still seems to be more suitable for routine application.

An alternative possibility, the flow injection of HPLC purified compounds directly into the DLI interface, without a column, would combine the qualities of speed and softness of DLI with the convenience of off-line LC separation.

The CI spectra observed in DCI and DLI for the studied polar lipids always comprised many informative fragments, in contrast with the corresponding EI spectra where the molecular ion was difficult to detect for low sample amounts. The soft ionization provided by CI was therefore needed for all the fragile polar lipids, but not at the cost of a loss of structural information, often mentioned for CI in comparison to EI.

The use of ammonia CI in DCI or as make-up reagent gas to the LC vaporized solvent produced the $(M+NH_4)^+$ adduct ion with the sterol peroxides, but did not provide more useful mass spectra than with methane or only vaporized methanol. With phospholipids, however, ammonia CI gave protonated molecular ions five times more intense than those observed with methane CI, and even a higher increase of intensity in DLI.

The use of a reagent gas with high thermal energy and high proton affinity, such as ammonia, would therefore offer for polar lipids, as for saccharides[17], a solution to the problem of the dependence of the DLI/LC–MS on the CI reactant species imposed by HPLC solvents.

5. ACKNOWLEDGEMENTS

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6. SUMMARY

The sterol peroxides and phospholipids found in marine organisms are polar and heat sensitive. Therefore they cannot be analyzed underivatized by gas chromatography–mass spectrometry. Furthermore, even in the soft in-beam desorption/electron impact mode, they yielded mass spectra with very weak molecular ions. Consequently these polar lipids were good candidates for low-temperature liquid chromatography–mass spectrometry. The Ribermag R-10-10-C quadrupole mass spectrometer equipped with the direct liquid introduction interface permitted to perform liquid chromatographic introduction followed by on-line chemical ionization detection of the separated lipids. Alternatively, off-line liquid chromatography–mass spectrometry using manual transfer of the compounds separated by high-performance liquid chromatography to a desorption/chemical ionization probe was also studied, and its merits were compared to those of the former method. Both methods gave chemical ionization spectra with informative fragments, and the use of ammonia as desorption/chemical ionization reagent gas or as chemical ionization make-up gas to the vaporized liquid chromatography solvent enhanced the intensity of the pseudo-molecular ions of phospholipids.

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