Journal of Chromatography, 394 (1987) 155–170
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 283

DETERMINATION OF EICOSANOIDS, PHOSPHOLIPIDS AND RELATED COMPOUNDS BY THERMOSPRAY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

H. Y. KIM*, J. A. YERGEY and N. SALEM, Jr.
Section of Analytical Chemistry, Laboratory of Clinical Studies, DICBR, National Institute on Alcohol Abuse and Alcoholism, Building 10, Room 3C-218, 9000 Rockville Pike, Bethesda, MD 20892 (U.S.A.)

SUMMARY

Thermospray mass spectrometry has proven to be a useful technique for analyzing various biological compounds including eicosanoids and phospholipids. Molecular ions as well as fragment ions which reveal useful structural information are produced for underivatized eicosanoids and phospholipids using filament-off or filament-on thermospray mass spectrometry, respectively. In conjunction with on-line chromatographic separation, complex mixtures of biological samples can be rapidly analyzed with great reliability. Data will be presented concerning the analysis of prostaglandins, other eicosanoids and molecular species of phospholipids as well as the application of these methodologies to complex biological samples.

INTRODUCTION

Thermospray mass spectrometry (MS) has emerged as a powerful tool for analyzing biological samples since this technique permits ionization of non-volatile and/or thermally labile compounds without derivatization[1]. The thermospray technique usually requires an aqueous buffer containing volatile electrolytes such as ammonium acetate for ejecting ions from solution or for generating gas phase ions for neutral molecules[2]. With a sufficient concentration of such electrolytes, ionization occurs during the vaporization process without an external ionizing source. This so-called "thermospray ionization" usually produces abundant molecular ion species with minimal fragmentation and low background. When extremely non-polar samples are analyzed, or a mobile phase of high organic percentage is required to elute the samples from the column, an auxillary electron-emitting filament is turned on to produce ions. This "filament-on" ionization is very similar to chemical ionization. The thermospray interface is thus well suited to a wide range of sample types from polar peptides or metal complexes to non-polar fatty acids. It is important to keep in mind, however, that the sensitivity and extent of fragmentation varies for different classes of compounds. One further advantage of the thermospray ion source is its ability to handle liquid flows up to 2 ml/min, allowing practical on-line HPLC separation of a complex mixture prior to MS detection. The potential of this technique

0021-9673/87/$03.50

has been demonstrated for a great variety of applications including on-line peptide sequencing[3], analysis of glucuronides[4] and steroid conjugates[5].

In our laboratory the thermospray technique has been applied to the analysis of polyunsaturated fatty acid metabolites[6] and phospholipids[7,8]. Polyunsaturated fatty acids can be enzymatically oxygenated to form extremely potent bioactive compounds such as prostaglandins and leukotrienes. Alternatively, they may be esterified to phospholipids where they may modify the physical and chemical characteristics of cell membranes. For these reasons, it was important to develop analytical methods capable of evaluating both the composition of biological tissues with respect to phospholipid molecular species and also their metabolic capabilities with respect to both phospholipids and fatty acids. The thermospray, liquid chromatography–mass spectrometry (LC–MS) technique has proven very useful for the solution of these problems and several of the recent advances made in lipid chemistry are presented below.

EXPERIMENTAL

The thermospray system used for this research was a Vestec (Houston, TX, U.S.A.) interface mounted on an Extrel ELQ-400 quadrupole mass spectrometer (Pittsburgh, PA, U.S.A.). Solvents were delivered into the mass spectrometer by Beckman Model 114M pumps. The thermospray spectra for eicosanoid standards were obtained by injecting 200 ng of each compound into an Altex Ultrasphere-ODS column (3 μm, 7.5 cm $\times$ 4.6 mm I.D.). The mobile phase was 0.1 *M* ammonium acetate in water–acetonitrile (70:30). Derivatization of fatty acid metabolites was performed as described earlier[6,9]. For gas chromatography (GC)–MS analysis, a Kratos MS-80 (Manchester, U.K.) was used with a Carlo-Erba gas chromatograph equipped with a cold on-column injector. The MO-PFB-TMS derivative of 6-keto-$PGF_{1\alpha}$ was separated on a 30 m $\times$ 0.32 mm I.D. Supelco SPB-5 capillary column (Bellefonte, PA, U.S.A.) at 290°C (ramped ballistically after cold on-column injection at 110°C). The effluent was fed directly into the mass spectrometer, which was operated in selected-ion, negative chemical ionization mode. The procedures for extracting and separating docosahexaenoic acid metabolites were described in a previous publication[6]. All the eicosanoid standards were purchased from Sigma (St. Louis, MO, U.S.A.) except 5,12-dihydroxyeicosatetraenoic acid which was obtained from Oxford Biomedical Research (Oxford, MI, U.S.A.). Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL. U.S.A.) and mono- and digalactosyldiglycerides from Supelco. Plant lipids were extracted from purslane by the method of Bligh-Dyer[10]. Molecular species of phospholipids were separated by reversed-phase chromatography using an Altex Ultrasphere-ODS column (3 μm, 7.5 cm $\times$ 4.6 mm I.D.) and various methanol–hexane–0.1 *M* ammonium acetate in water mixtures. For separation of sugar containing diglycerides, a normal phase chromatographic system using an Altex Ultrasil-Si column (5 μm, 25 cm $\times$ 4.6 mm I.D.) was employed with hexane–isopropanol–methanol–0.1 *M* ammonium acetate in water (200:180:50:25) as mobile phase.

Eicosanoids and related oxygenated fatty acid metabolites

Among the polyunsaturated fatty acids, arachidonic acid (C20:4w6) has drawn much attention because of its metabolism to prostaglandins and lipoxygenase prod-

ucts such as 5-hydroxyeicosatetraenoic acid (5-HETE) and the leukotrienes. Since most of its metabolites have been well characterized and are commercially available, they can serve as model compounds for analyzing oxygenated metabolites of other polyunsaturates. In order to understand the pattern of fragmentation in thermospray spectra for these compounds, a series of prostanoids, leukotrienes and hydroxylated fatty acids were analyzed.

The standard eicosanoids such as PGE_2 and 5,12-dihydroxyeicosatetraenoic acid (5,12-diHETE) generated thermospray spectra which were dominated by the ammoniated molecule $(M + NH_4)^+$ and the losses of water from the protonated or ammoniated molecules, as shown in Fig. 1. In general, the number of water losses corresponds to the number of hydroxyl and/or ketone functions in the molecule, and

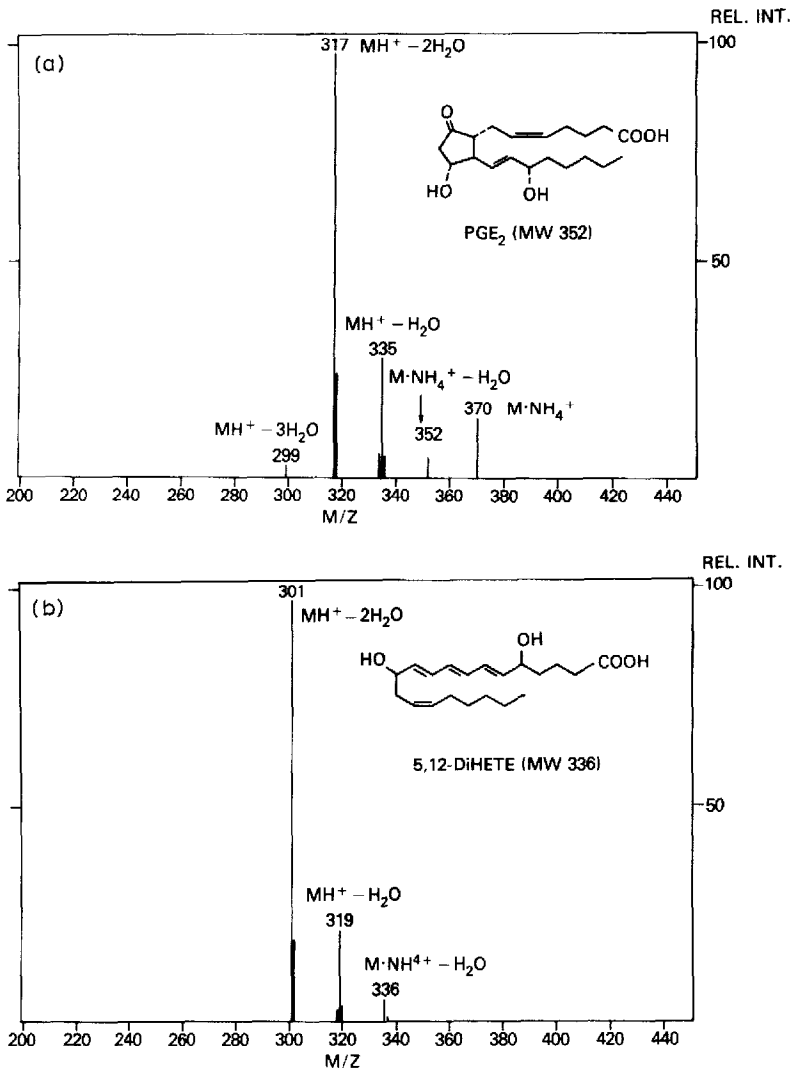

Fig. 1. Positive ion thermospray mass spectra for (a) PGE_2 and (b) 5,12-diHETE.

one of these ions is usually the base peak in the spectrum. Note that a peak at the molecular weight, corresponding to the $(M + NH_4^+ - H_2O)^+$ is usually observed. However, the protonated molecule itself (MH^+) is generally observed only for compounds containing α,β-unsaturated ketones such as 15-keto-$PGF_{2\alpha}$ (Fig. 2). For ether-containing hydroxylated eicosanoids such as prostacyclins or thromboxanes, the intensities of the MH^+ peak vary according to the environment near the ether linkage (Fig. 3). Thus, assignment of molecular weights for oxygenated fatty acids of unknown structure may be difficult if only the positive ion spectra are obtained.

Simple organic reactions in combination with LC–MS analysis can be very useful for confirming structural characteristics of oxygenated fatty acids, including molecular weights. For instance, methoximation of ketone-containing compounds produces the expected increase of 29 daltons in the positive ion thermospray spectrum, while the mass shift after hydrogenation allows confirmation of the number of double bonds. One particularly useful reaction is pentafluorobenzyl esterification, which permits unambiguous molecular weight assignment and also provides a significant sensitivity enhancement for fatty acid metabolites. Thermospray analysis in the negative ion mode and with the filament on produces spectra with the base peak corresponding to the parent acid anion, $[M - PFB]^-$. This derivative was explored because its use for GC–MS analysis of eicosanoids in negative ion chemical ionization (NICI) mode produces exclusively the $[M - PFB]^-$ ion by electron capture, yielding a significant sensitivity improvement. Typical limits of detection by GC–MS analysis of the methoxime, pentafluorobenzyl ester, trimethylsilyl ether derivative of a prostaglandin are in the range of hundreds of femtograms injected on-column (Fig. 4). The limits of detection by thermospray are improved by *ca.* 50 fold from the low nanogram range for underivatized eicosanoids analyzed by SIM in the positive ion mode to the 10–100 pg range for negative ion detection of the PFB ester. Although this is a significant improvement, NICI GC–MS is still needed for the analysis of eicosanoids in biological samples when they contain very low levels of these com-

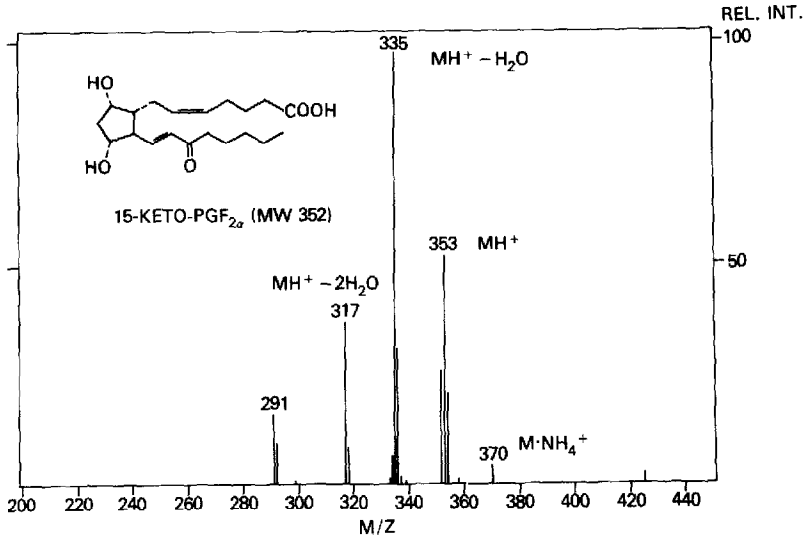

Fig. 2. Positive ion thermospray mass spectrum for 15-keto-$PGF_{2\alpha}$.

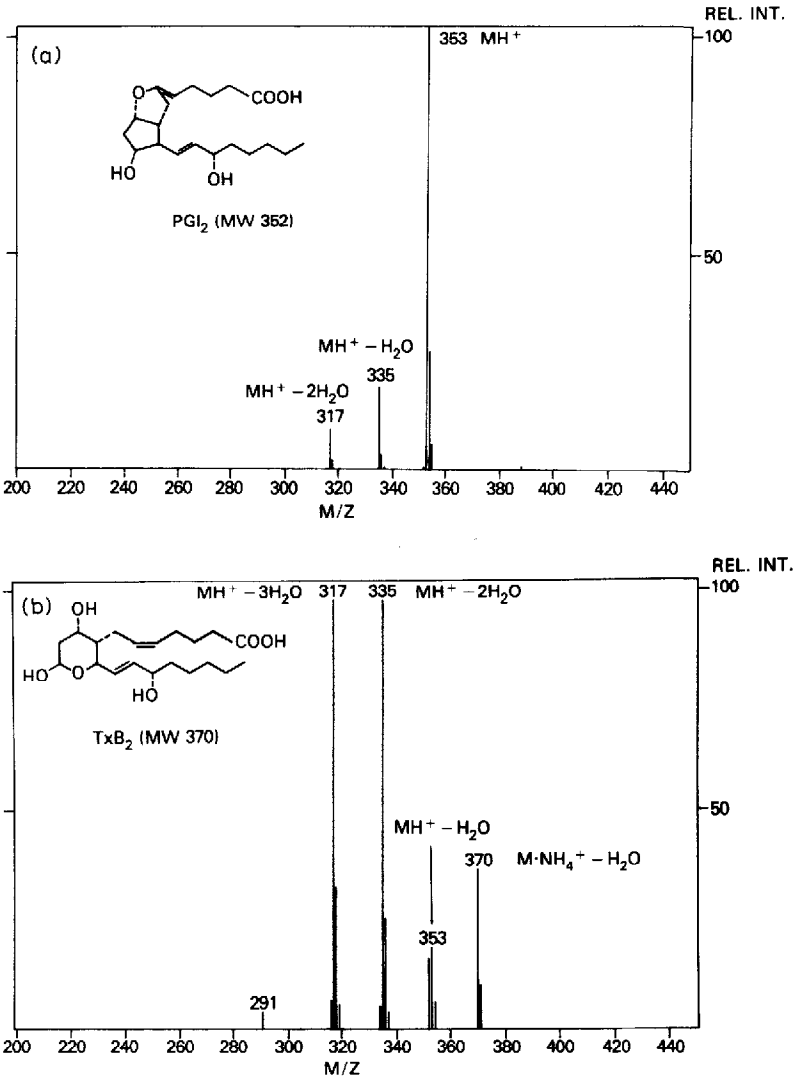

Fig. 3. Positive ion thermospray mass spectra for (a) PGI_2 and (b) thromboxane B_2.

pounds. However, when samples of unknown structure or composition are analyzed, the thermospray technique is particularly useful since it does not require derivatization steps and produces easily interpretable spectra. Nevertheless, as illustrated above, derivatization may be used in thermospray analysis for the purpose of gaining additional qualitative information about the compounds of interest.

This technique was applied to the analysis of docosahexaenoic acid (C22:6w3) metabolites. This fatty acid is of particular interest since it is found at high levels in metabolically active organs such as the brain, retina, heart and testes[11]. Its biological function is not well understood, but some studies have indicated an inhibitory action for prostaglandin synthetase and platelet aggregation and, possibly, a protective

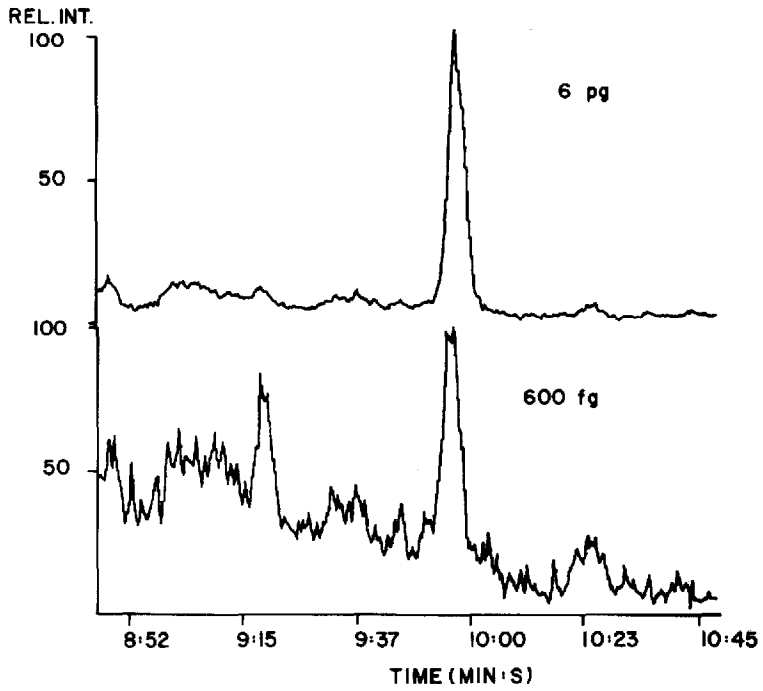

Fig. 4. NICI GC–MS analysis of 6-keto-PGF_{1a} by selected ion monitoring of $(M - PFB)^-$ after methoximation, pentafluorobenzyl esterification and trimethylsilyation.

effect on the cardiovascular system. It has also been shown that ethanol decreases the C22:6 level in cell membranes and this may be due to increased metabolism of the acid to oxygenated products. We have therefore sought to define the metabolites of docosahexaenoate by thermospray LC–MS and also to measure changes in the cellular pool which serves as the source of this fatty acid, namely polyunsaturated phospholipids.

Docosahexaenoic acid was incubated with brain homogenate, the metabolites were extracted with ethyl acetate from a C_{18} cartridge and an aliquot was injected into the thermospray LC–MS system. The total ion current (300–400 daltons) showed a complex trace consisting of many metabolites probably originating from both enzymatic and non-enzymatic processes (Fig. 5). Three groups of peaks were observed and assigned as mono-, di- and trihydroxy-C22:6 based upon interpretation of the spectra for the peaks in each group. The multiple chromatographic peaks in each group are postulated to be isomers since their thermospray spectra showed peaks at identical masses but with different intensities. An example is illustrated for the peaks eluting in the 21–27 min region. The positive ion spectra of these peaks contain peaks at *m/z* 327, 345, 363 and 380 (Fig. 6a). According to the peak pattern observed for the hydroxylated fatty acid standards (Figs. 1–3) the molecular weight of this metabolite must be either 362 or 380, corresponding to dihydroxy-C22:5 or trihydroxy-C22:4, respectively. The assignment of molecular weight was unambiguously made as 362 by analyzing the PFB derivative of this LC fraction as it contained a base peak at *m/z* 361 in its spectrum which was obtained by negative ion, filament-on

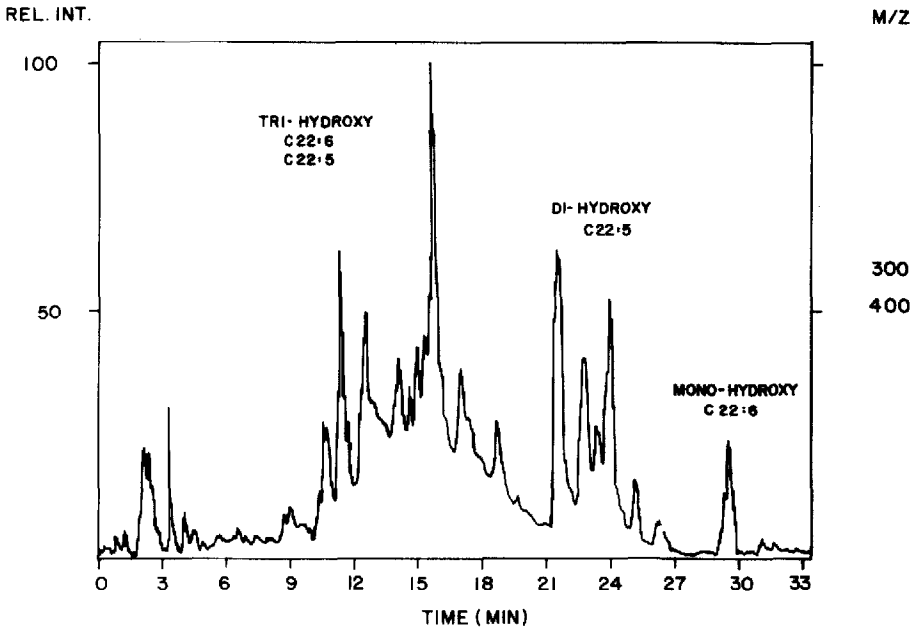

Fig. 5. Total ion chromatogram (mass range 300–400 daltons) obtained from an ethyl acetate fraction containing oxygenated metabolites of C22:6.

thermospray analysis (Fig. 6b). The structure was further confirmed by hydrogenation of this LC fraction and reinjection into the thermospray LC–MS system. An increase of 10 mass units was observed (Fig. 6c), indicating that five double bonds

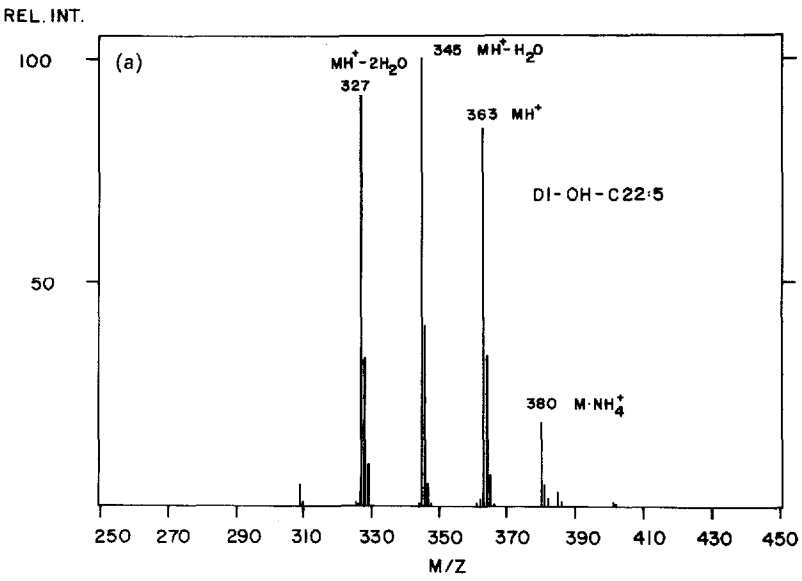

Fig. 6.

(Continued on p. 162)

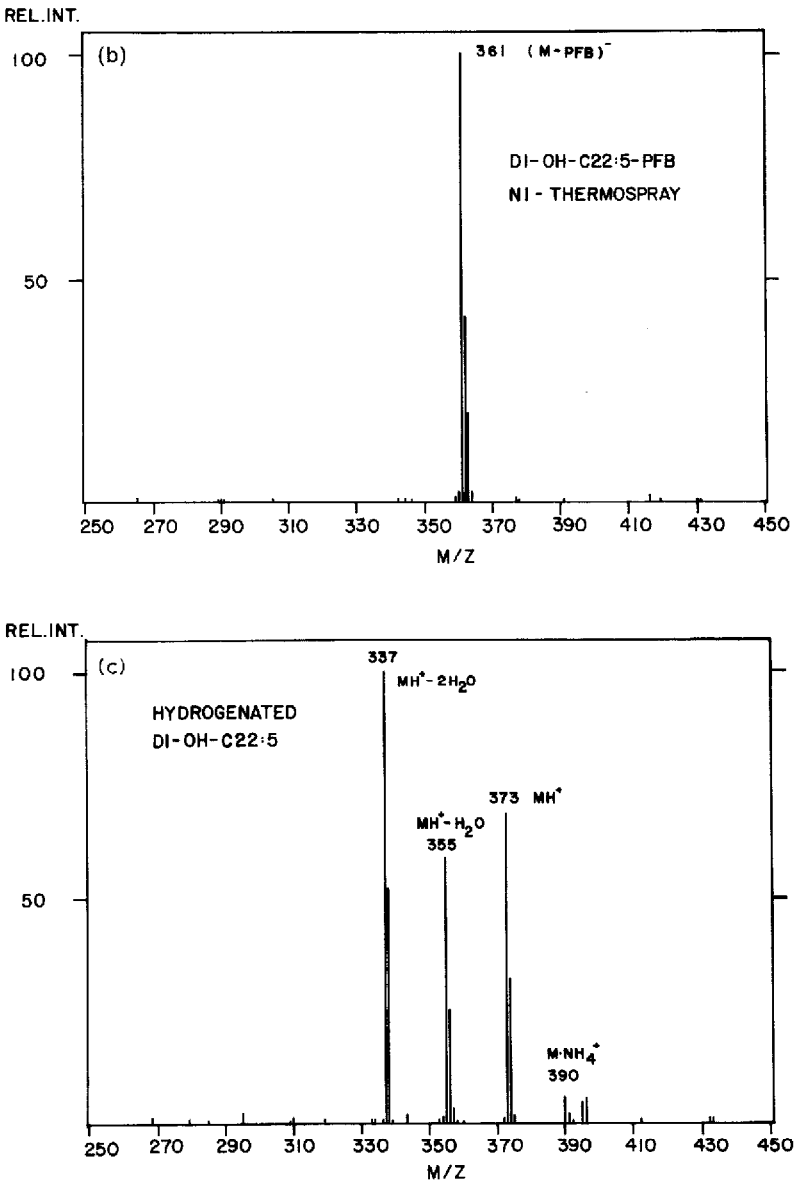

Fig. 6. Thermospray spectra of dioxygenated metabolite of C22:6: (a) positive ion spectrum, (b) negative ion spectrum of PFB derivative and (c) positive ion spectrum of hydrogenated derivative.

were present; thus this metabolite was a dioxygenated-C22:5. Other chromatographic peaks were assigned in a similar fashion.

Phospholipids

A second major application of thermospray LC–MS to biological samples developed in our laboratory involves phospholipids. Phospholipids are one of the prin-

cipal components of biological membranes. There are several classes of phospholipids and each of them consists of many and often characteristic molecular species which differ in fatty acyl composition. Since fatty acyl profiles in phospholipids are one of the critical determinants of membrane physical and chemical properties, techniques for analyzing the distribution of individual molecular species is of great value in lipid and membrane research.

We have recently demonstrated that thermospray LC–MS provides a rapid and sensitive technique for separation and identification of molecular species of most major lipid classes[7,8]. Since a mobile phase extremely high in organic content was required to dissolve and elute the phospholipids, sufficient ionization did not occur without an auxilliary ionizing source. Thus, the filament-on thermospray technique was employed for the analysis of these compounds. In this case, the thermospray technique generated fragments containing structural information which was unusually detailed. Typical thermospray spectra for phospholipids contain mono- and diglyceride ion peaks as well as peaks representing the polar head group and the intact molecule. The notation of "di-" and "monoglyceride" ions used in this paper indicates the ions resulting from the loss of head group and from the hydrolysis of either fatty acyl chain from the diglyceride ions, respectively. These are the ions which carry vital information concerning phospholipid fatty acyl composition. In conjunction with reversed-phase chromatography, mixtures of phospholipids were rapidly separated into several molecular species and the structures were simultaneously assigned by spectral analysis. Separations were based upon acyl chain length and the number of double bonds. Thus considerable savings in time and labor were made in analyzing complex biological samples by this approach. To demonstrate the power of the technique the data obtained from phosphatidylcholines and phosphatidylethanolamines will be presented.

Fig. 7 shows separation of molecular species of phosphatidylcholines from a bovine heart preparation. At least nine molecular species were separated and identified in 20 min using an Altex Ultraspere-ODS (3 μm, 7.5 cm × 4.6 mm I.D.) column. The mobile phase was methanol–hexane–0.1 *M* ammonium acetate in water (500:25:25) and the flow-rate was 1 ml/min. Since diglyceride fragments formed the base peak in these spectra, these ions for each species were plotted along with the total ion current to demonstrate the separation achieved. Assignment of each species was based upon examination of the entire mass spectrum, and an example is shown for the chromatographic peak assigned as a 16:0, 18:2 species (Fig. 8). The diglyceride ion is the base peak at *m/z* 576 and monoglyceride ions containing either a 16:0 or an 18:2 fatty acyl chain were detected at *m/z* 313 or 337, respectively. The ions from the head group were observed at *m/z* 184 and 142. The latter resulted from loss of trimethylamine from ammoniated phosphocholine as was reported previously in ammonia chemical ionization of phosphatidylcholines[12]. The intact molecule was also detected in the protonated or natriated form at *m/z* 759 and 781, respectively. Among the chromatographic peaks found in bovine heart phosphatidylcholine, we observed unusual species whose spectra showed molecular and diglyceride ions that were 16 daltons lower than expected but eluted later than the PC species of corresponding chain length. These compounds were phosphatidylcholines since the characteristic head group ions were detected at *m/z* 184 and 142. They appeared to be 1-alkenyl, 2-acyl phosphocholines (choline plasmalogens) and this was further confirmed by

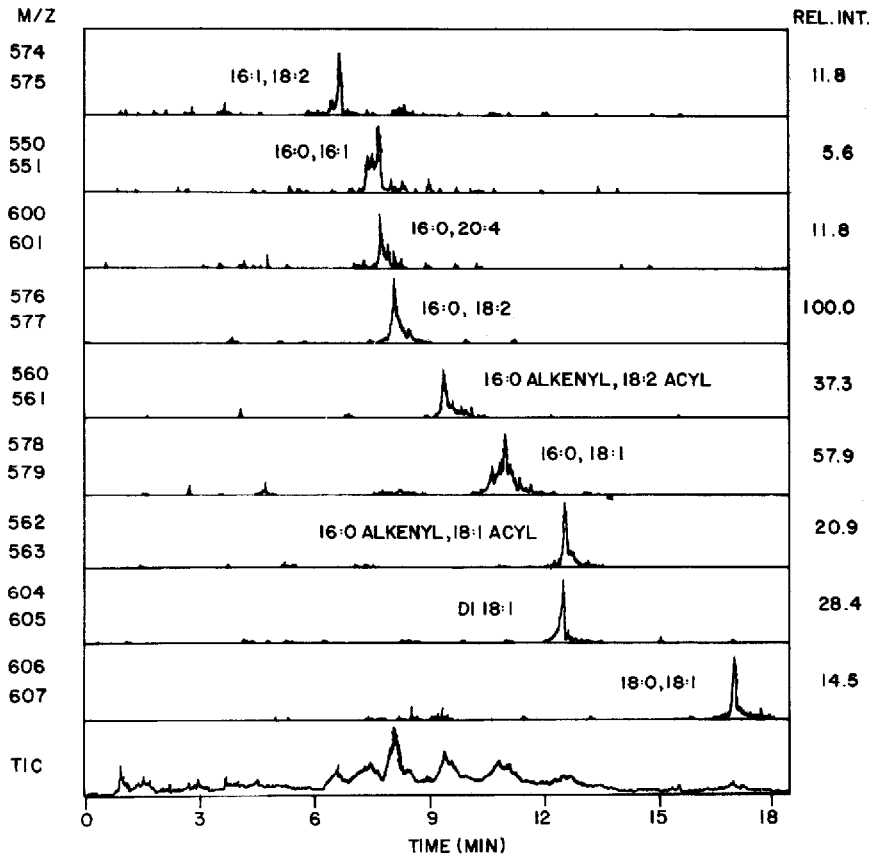

Fig. 7. HPLC separation of 50 μg of a natural phosphatidylcholine mixture from bovine heart. The reconstructed ion chromatograms of diglyceride ions were selected from data acquired by full mass scanning from 120 to 1020 daltons. The relative intensity is shown based on the peak height.

spectral analysis of standard plasmalogens. The spectrum shown in Fig. 8 was obtained from the chromatographic peak assigned as 16:0 alkenyl, 18:1-acyl phosphocholine in Fig. 7. In addition to the peaks from intact molecules and the head group, the diglyceryl fragment was detected at *m/z* 560. From the spectra of standard plasmalogens it was apparent that the ether linkage of plasmalogens is also hydrolyzed, in fact, more easily than the acyl linkage yielding the same type of monoglyceride ions as that of other phosphatidylcholines. Thus the peak at *m/z* 337 (Fig. 8) represents the monoglyceride ion containing an 18:2 acyl chain, and the intensity of this peak is higher than that of the monoglyceride ion peak containing the 16:0 alkenyl linkage (*m/z* 297). The peak at *m/z* 240 is believed to arise from the dehydrated ammonium adduct of the hydrolyzed alkenyl chain (OH–CH=CH–R).

Phosphatidylethanolamines produced the same type of thermospray spectra as phosphatidylcholines. Using reversed-phase chromatography as the sample inlet device, fast separation and identification of molecular species was achieved. Fig. 9 illustrates an example for a phosphatidylethanolamine preparation from soybean. The separation was performed using an Altex Ultrasphere-ODS column (3 μm, 7.5 cm

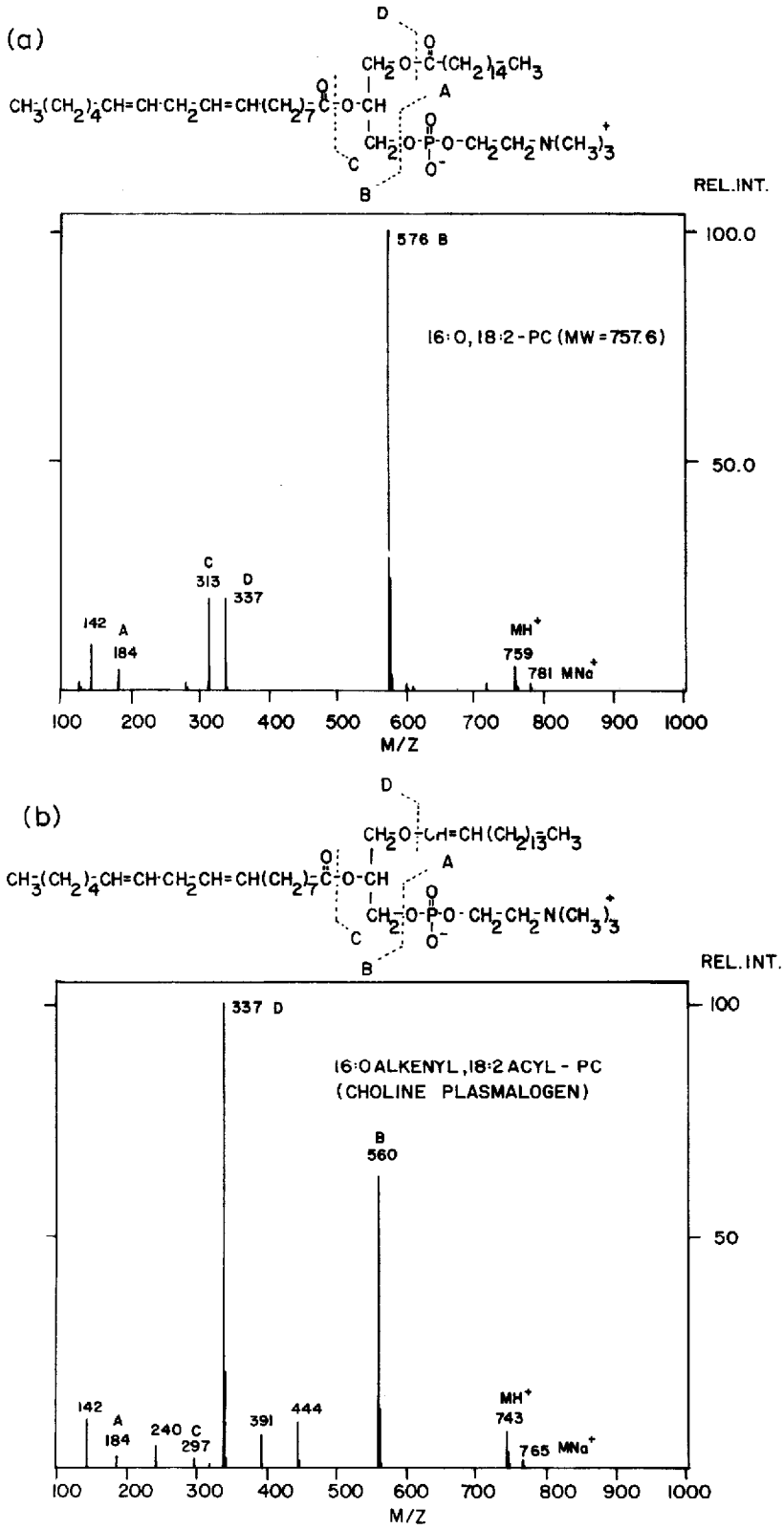

Fig. 8. Positive ion spectra obtained from the chromatographic peaks assigned as (a) 16:0, 18:2-phospha tidylcholine and (b) 16:0 alkenyl, 18:2 acyl-phosphocholine in Fig. 7.

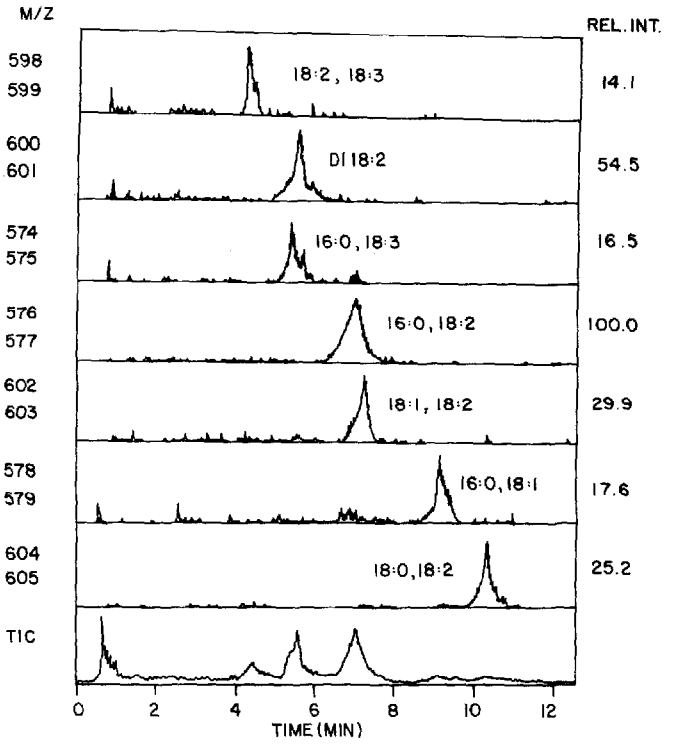

Fig. 9. Separation of 50 μg of a phosphatidylethanolamine mixture from soybean. The reconstructed ion chromatograms of the respective diglyceride ions are presented along with the total ion current (120–1020 daltons).

× 4.6 mm I.D.) and a mixture of methanol–hexane–0.1 *M* ammonium acetate (500:30:25) as mobile phase at a flow-rate of 1 ml/min. Six major molecular species were separated in 12 min according to chain length and the degree of unsaturation. Each chromatographic peak was readily identified by spectral analysis. As an example, the spectrum of a chromatographic peak assigned as a 16:0, 18:2 species is presented (Fig. 10). Diglyceride ions were detected at *m/z* 576 as the base peak along with monoglyceride ions containing either a 16:0 or an 18:2 fatty acyl chain at *m/z* 313 or 337, respectively. The peaks at *m/z* 141 and 124 represent the dehydrated forms of the ammoniated or protonated forms of phosphoethanolamine, respectively, and they are characteristic for compounds of this lipid class. The intact molecule is also detected in the protonated form or as a sodium adduct at *m/z* 717 and 739, respectively.

Similarly, structural information can be obtained for sugar-containing lipids such as mono- and digalactosyldiglycerides (Fig. 11). These spectra were obtained by injecting 20 μg of samples prepared from plants into a normal phase column (Altex Ultasil-Si, 5 μm, 25 cm × 4.6 mm I.D.) using a hexane–2-propanol–methanol–0.1 *M* ammonium acetate in water mixture (200:180:50:25) as mobile phase. Under these conditions the retention times of mono- and digalactosyldiglycerides were 2.5 and 3.1 min, respectively. Since the samples had been hydrogenated, simple spectra containing only saturated fatty acyl chains were produced. In both spectra, mono- and

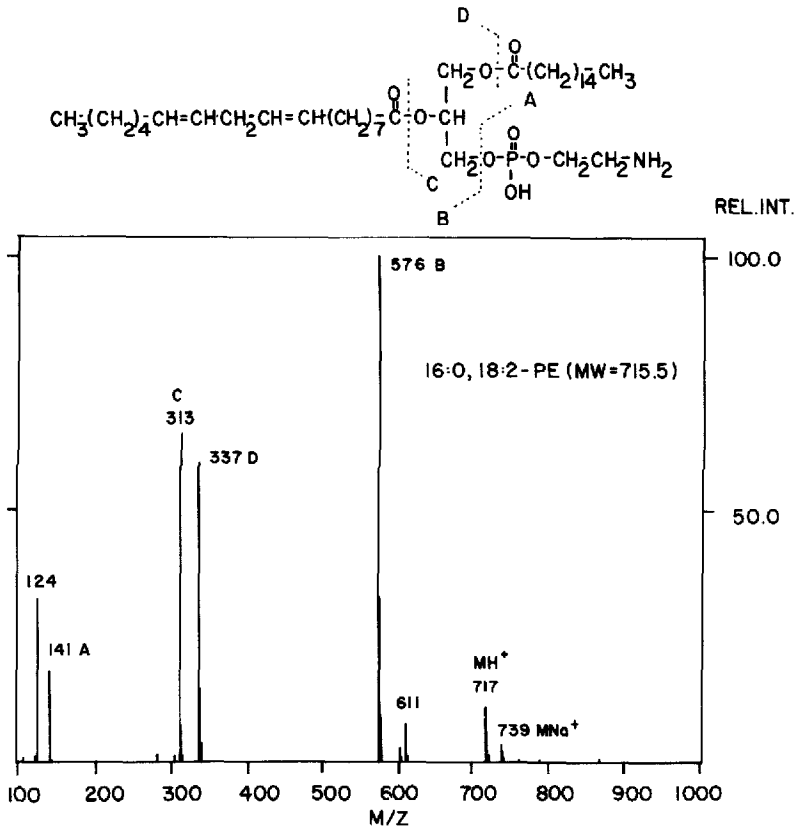

Fig. 10. Positive ion spectrum obtained from the chromatographic peak assigned as the 16:0, 18:2 species in Fig. 9.

diglyceride ions were detected along with the sodium adduct of the intact molecule.

These galactolipids are major components of plant cell membranes. Recently, it has been reported[13] that purslane, a common plant in the U.S.A. and Europe, contains high levels of linolenic acid (18:3w3). There is great interest in this fatty acid since it is the precursor of docosahexaenoic acid which is, in turn, an important component of cell membranes[11]. In addition, this family of fatty acids is considered by many to be important for human nutrition[14]. For these reasons, we sought to analyze the lipid composition of purslane in greater detail, *i.e.*, at the molecular species level. Thermospray analysis provides a good qualitative means for obtaining this information. As illustrated in Fig. 12, diglyceride ions from 16:0-18:3, 16:0-18:2, di-18:3, 18:2-18:3 and di-18:2 species are shown at *m/z* 574, 576, 596, 598 and 600, respectively. Monoglyceride ions containing either a 16:0, 18:3 or 18:2 fatty acyl chain are also observed at *m/z* 313, 335, and 337, respectively. The presence of the linolenic acid (18:3w3) in the galactolipids was confirmed by the consistency of the mass values of the natriated intact molecules and the expected diglyceride fragment ions.

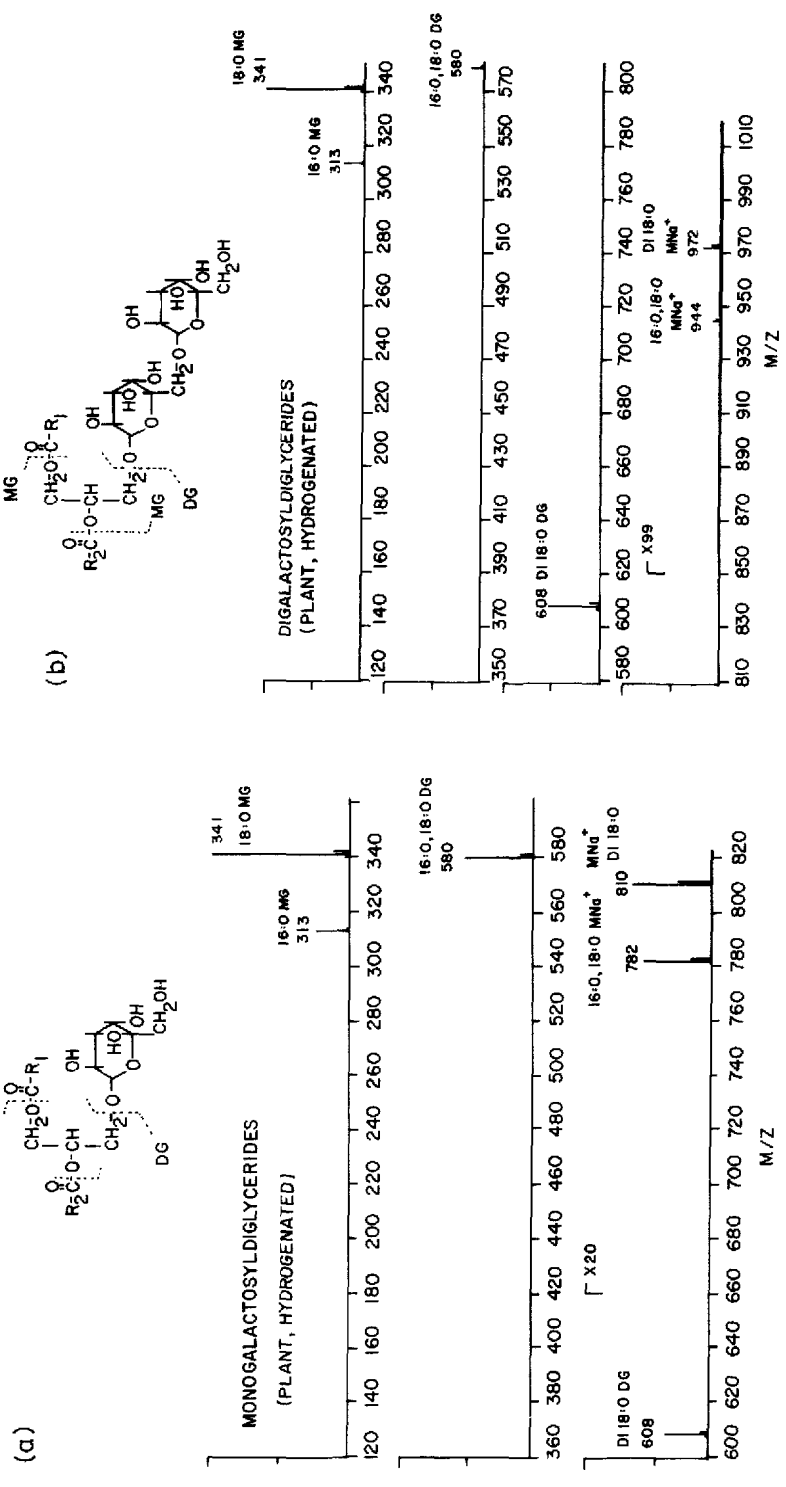

Fig. 11. Positive ion spectra obtained from 20 μg of (a) *mono-* and (b) digalactosyldiglyceride from a plant source.

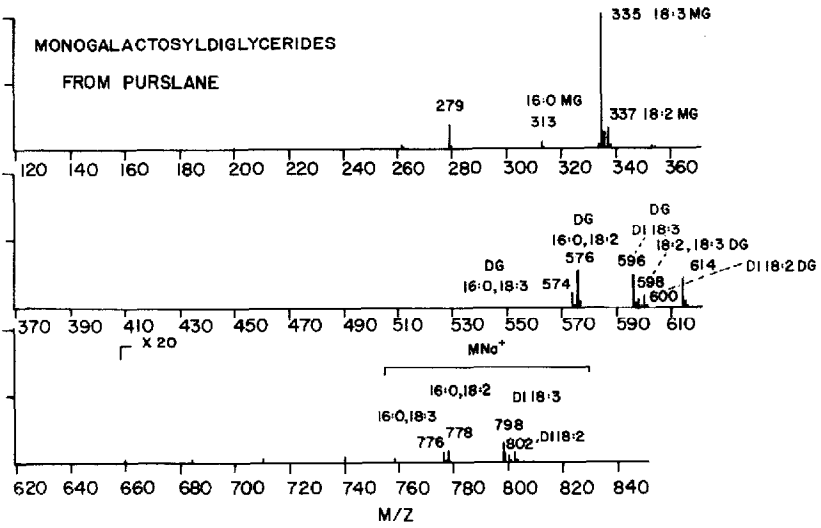

Fig. 12. Positive ion spectrum of a purslane extract containing monogalactosyldiglycerides.

CONCLUSION

The thermospray LC–MS technique was applied to the determination of eicosanoids and phospholipids and their patterns of fragmentation were described. Generally simple but informative spectra were obtained for these compounds. Pseudo-molecular ions along with losses of water were observed in eicosanoid spectra. Detailed structural information concerning both head group and fatty acyl composition of phospholipids was readily obtained by the filament-on thermospray technique. These methods were subsequently applied to the qualitative analysis of docosahexaenoate metabolites and galactolipids in biological samples. Simple derivatization reactions such as pentafluorobenzyl esterification, methoximation or hydrogenation were also shown to be useful for obtaining structural information in thermospray analysis.

ACKNOWLEDGEMENT

We thank Ms. Diamond Melville for preparing the purslane lipid extract.

REFERENCES

1 C. R. Blakley and M. L. Vestal, *Anal. Chem.*, 55 (1983) 750–752.
2 M. L. Vestal, *Int. J. Mass Spectrom. Ion Phys.*, 46 (1983) 193–196.
3 H. Y. Kim, D. Pilosof, D. F. Dyckes and M. L. Vestal, *J. Am. Chem. Soc.*, 106 (1984) 7304–7309.
4 D. L. Liberato, C. C. Fensleau, M. L. Vestal and A. L. Yergey, *Anal. Chem.*, 55 (1983) 1741–1745.
5 D. Watson, G. W. Taylor and S. Murray, *Biomed. Mass Spectrom.*, 12 (1985) 610–615.
6 J. A. Yergey, H. Y. Kim and N. Salem, Jr., *Anal. Chem.*, 58 (1985) 1344–1348.
7 H. Y. Kim and N. Salem, Jr., *Anal. Chem.*, 58 (1986) 9–14.
8 H. Y. Kim and N. Salem, Jr., *Anal. Chem.*, March (1987) in press.

9 K. A. Waddell, I. A. Blair and J. Wellby, *Biomed. Mass Spectrom.*, 10 (1983) 83–88.
10 E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Phys.*, 37 (1959) 911–917.
11 N. Salem, Jr., H. Y. Kim and J. A. Yergey, in A. P. Simopoulos, R. R. Kifer and R. E. Martin (Editors), *Health Effects of Polyunsaturated Fatty Acids in Seafoods*, Academic Press, New York, 1986, pp. 263–317.
12 C. G. Crawford and R. D. Plattner, *J. Lipid Res.*, 24 (1983) 456–460.
13 A. P. Simopoulos and N. Salem, Jr., *New Engl. J. Med.*, 315 (1968) 833.
14 A. P. Simopoulos, R. R. Kifer and R. E. Martin (Editors), *Health Effects of Polyunsaturated Fatty Acids in Seafoods*, Academic Press, New York, 1986.