Journal of Chromatography, 434 (1988) 31-41
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4467

HIGHLY SENSITIVE DETERMINATION AND CHARACTERIZATION OF INTACT CELLULAR ESTER-LINKED PHOSPHOLIPIDS USING LIQUID CHROMATOGRAPHY–PLASMA SPRAY MASS SPECTROMETRY

GÖRAN ODHAM* and ANDERS VALEUR

Laboratory of Ecological Chemistry, Lund University, Ecology Building, Helgonavägen 5, S-223 62 Lund (Sweden)

PETER MICHELSEN and EVA ARONSSON

Division of Organic Chemistry 1, Chemical Centre, Lund University, P.O. Box 124, S-221 00 Lund (Sweden)

and

MARK McDOWALL

VG Masslab, Tudor Road, Altrincham, Cheshire WA14 5RZ (U.K.)

(First received June 10th, 1988; revised manuscript received September 5th, 1988)

SUMMARY

Liquid chromatographic class separations of common cellular phospholipids combined with plasma spray ionization of the effluents were investigated. Comparison with true thermospray ionization involving ammonium acetate buffering revealed a gain in total ionization in the plasma spray of a factor of approximately 10 using a cation-exchange column and a solvent mixture consisting of acetonitrile–methanol–water (400:100:15, v/v). Plasma spray ionization studies of bovine brain polyphosphoinositides interrelated by the phosphate content in the inositol moiety showed almost identical monoglyceride and diglyceride ion clusters, indicating possibilities of studying the biochemical turnover of such phospholipids. Plasma spray ionization liquid chromatography–mass spectrometry of bacterial membrane phospholipids (*Pseudomonas fluorescens*) revealed possibilities of obtaining indications of individual fatty acid compositions from the spectra of the phosphatidylinositol and phosphatidylethanolamine fractions present. Conventional gas chromatographic fatty acid analysis agreed with the direct mass spectrometric structure elucidations. Interestingly, the two phospholipid classes had different relative fatty acid compositions with a significantly higher degree of cyclic fatty acids in the phosphatidyl ethanolamines. Plasma spray ionization yielded linear dose–response curves for both the monoglyceride and diglyceride fragment signals in the selected-ion monitoring mode. The detection limit for the monoglyceride and diglyceride species of phosphatidylcholine under the chromatographic and mass spectrometric conditions used was found to be in the picogram range.

0378-4347/88/$03.50 © 1988 Elsevier Science Publishers B.V.

INTRODUCTION

It is now widely accepted that autonomous control of cell activity is mediated by membrane changes. Most membranes contain a complex mixture of lipids, the physicochemical properties of which influence the physical state and the physiological function of the membrane. In recent years, the basis has been laid for understanding the enormous diversity of membrane lipids. Low-abundance lipids have, for example, been shown to exhibit essential specialized functions, e.g., permitting communication between and within cells and allowing them to respond to their environment.

Increasing awareness of the polymorphic regulation of membrane lipid composition and the complex physical interactions between the lipid components has prompted a much more sensitive and detailed analysis of the phospholipid composition not only to the level of the bulk fatty acid composition of each individual phospholipid but to obtaining detailed information on the molecular species composition.

Two methods have hitherto been commonly employed for structural determination of phospholipid molecular species; (a) preparative argentation thin-layer chromatographic (TLC) separation and (b) a coupled capillary gas chromatographic–mass spectrometric (GC–MS) determination of the silylated diacylglycerols derived from the phospholipids. Both methods have their drawbacks. The former often gives unsatisfactory resolution and the latter is frequently impaired by degradation and incompleteness of the enzymatic prepreparation step.

To improve the methodology, much work is currently being devoted to the search for effective liquid chromatographic systems allowing separations of both phospholipid classes and species (e.g., ref. 1), and to more powerful techniques for the structure elucidations of the intact phospholipid fractions.

The full potential of high-performance liquid chromatography (HPLC) in phospholipid work has perhaps not been realized owing to a general lack of selective and sensitive detectors for lipids. The most useful of the common detectors for lipid analyses used hitherto is probably the mass (light scattering) detector as applied by, e.g., Christie [2]. The detector was found to work excellently at the 200–400 μg sample level with the capacity for a further increase in sensitivity by a factor of 10.

The mass spectrometer, particularly when adjusted for selected-ion monitoring (SIM), constitutes one of the most versatile, selective and sensitive detectors in chromatography. The recent introduction of interfaces allowing convenient ionization in HPLC–MS, in particular thermospray ionization, opens up new possibilities for studying phospholipid molecular species with high sensitivity. Kim and Salem [3] showed recently that such species could be ionized in a "filament-on" thermospray when a high percentage of organic solvent was present in the carrier solution. In late papers the same group [4,5] described detailed structural information obtained with the filament-on thermospray technique, including molecular mass and also diglyceride, monoglyceride and head-group portions of the molecule. A C_{18} silica column with 2-propanol–hexane–0.1 M ammonium acetate as the mobile phase served as the separating unit.

Recently, a novel discharge-assisted ionization technique, plasma spray, has been developed. Plasma spray enables samples, dissolved in either polar or non-polar solvents, to be ionized in a manner partially analogous to chemical ionization.

In our laboratory, considerable interest has recently arisen regarding rapid changes in membrane composition and functions in microbial and plant root cells [6] due to environmental stress and to the turnover of polyphosphoinositides in mammalian cells after stimulation by hormones and other factors [7,8]. Methods with which to quantify and at least partly characterize phospholipid species with ultimate sensitivity were therefore considered of great interest in our studies and it was thought that combined HPLC involving only class separation and plasma spray mass spectrometry would meet the requirements.

This paper describes the preliminary use of plasma spray ionization of phospholipids using full scan or SIM recordings in a non-polar solvent mixture suitable for HPLC class separation of the common ester-linked phosphatides. The findings were applied to studies of inositol phospholipids in an inositide-rich bovine brain extract and membrane lipids in a Gram-negative bacterium (*Pseudomonas* sp.).

EXPERIMENTAL

Chemicals and solvents

The phospholipids used as reference material were obtained from Serdary (Ontario, Canada) and stored in chloroform at −20°C. They were phosphatidylinositol (yeast) (PI), dioleyl-L-3-phosphatidylcholine and dipalmitoyl-L-3-phosphatidylcholine (PC's), dioleylphosphatidylethanolamine (PE), dipalmitoyl phosphatidic acid (PA), phosphatidylserine (pig brain) (PS) and cardiolipin (beef heart) (CL).

The solvents used for chromatography were obtained from Fisons (Loughborough, U.K.) and were of HPLC grade. Pentafluoropropionic anhydride (PFPA) and 2,3,4,5,6-pentafluorobenzyl bromide (PFB) were purchased from Fluka (Buchs, Switzerland) and the bacterial fatty acid reference mixture (Cat. No. 4-5436) from Supelco (Bellefonte, PA, U.S.A.). Solvents, chemicals and reagents were of analytical-reagent grade and were not purified prior to use. The bovine brain extract type used for preparation of the phosphoinositides was obtained from Sigma (St. Louis, MO, U.S.A.).

Fatty acids in this study are designated by the number of total carbon atoms: number of unsaturation. The symbol Δ is utilized to denote cyclopropane rings.

Bacterial samples

Pseudomonas fluorescens cells were grown at 20°C for 18 h in a 10-l aerated fermenter to stationary phase in a medium consisting of Nutrient Broth 8 g/l (Difco) supplemented with 5 g/l glucose. The cells were harvested by centrifugation at 10 000 *g* and washed twice carefully with 0.8% sodium chloride solution. The cell mass was freeze-dried and kept at −20°C prior to use.

Lipid extraction

A 75-mg amount of dry bacterial cells was extracted in 3 ml of a single-phase system (chloroform–methanol–water, 1:2:0.8, v/v) [9]. After 12 h, chloroform (1 ml) and distilled water (2 ml) were added to form two phases. The organic–aqueous phase system was centrifuged (6000 *g*), the aqueous phase discarded and the chloroform phase washed with a small portion of water. The solvent was removed at room temperature and the residue supplemented with 0.5 ml of dichloromethane and re-evaporated. The final residue contained 1.8 mg of crude phospholipids.

Fatty acid recovery and esterification

A 90-μg amount of the above phospholipids was supplemented with 19:0 (12 μg) acid and the mixture was subjected to mild alkaline hydrolysis [10] in a PTFE-lined screw-capped test-tube. After cooling, 2 ml of distilled water and 1 ml of hexane were added, the two-phase system was shaken vigorously and the organic phase was discarded. Dilute hydrochloric acid was added until the pH of the aqueous phase was below 2 and the free fatty acids were extracted with two 1-ml portions of dichloromethane. The combined organic phases were washed with distilled water and evaporated to dryness in a stream of nitrogen at 40°C.

The free acids were esterified with PFB and PFPA in acetonitrile in the presence of triethylamine according to the method of Sonesson et al. [11].

Preparation of phosphoinositides

Phosphatidylinositol phosphate (PIP) and PIP-2 (approximately 1 mg of each) were purified from a phosphoinositide-rich bovine brain extract (type I) by chromatography on immobilized neomycin [12].

Gas chromatography

GC studies of the derivatized fatty acids were performed using a Perkin-Elmer Model 900 (modified) instrument equipped with a 25 m × 0.2 mm I.D. fused-silica column coated with SE-54 with splitless injection and flame ionization detection.

Liquid chromatography

Class separation using a cation-exchange column. The separations were performed using a Varian Model 5000 HPLC instrument with a Varian Model UV100 variable-wavelength UV detector operating at 203 nm and with a 10-μl sample loop. The column was a 10 cm × 4.6 mm I.D. Nucleosil 100-5SA cation-exchange column (Macherey-Nagel, Düren, F.R.G.) and the solvent was acetonitrile–methanol–water (400:100:15, v/v) at a flow-rate of 1 ml/min [13]. The column was preconditioned with 100 ml of 0.1 *M* acetate–citrate buffer (pH 5.3). A Hewlett-Packard Model 3390 reporting integrator was used.

Class separation using a diol column. The separations were performed using an LDC Constametric III HPLC pump and a Shimadzu SPD-2AS UV spectrophotometric detector operating at 206 nm. The column used was Nucleosil 7 OH (diol) (25 cm × 4.0 mm I.D.) (Macherey-Nagel). The separations were per-

formed isocratically using 2-propanol–hexane–water (54:41:5, v/v) at a flow-rate of 1.2 ml/min [14].

Mass spectrometry

The HPLC effluent from the cation-exchange column (0.9 ml/min) was introduced into a VG Trio 3 quadrupole mass spectrometer via the standard LC–MS thermospray–plasma spray probe. The solvent mixture had the composition described. In the thermospray studies, post-column addition of ammonium acetate buffer (0.3 ml/min) was employed to give a final buffer concentration of approximately 0.1 *M*.

The vaporizer capillary had a temperature of 250°C, the ion source was at 240°C and, with plasma spray, the discharge current was 250 μA. A standard VG 11/253 data system configuration was employed to acquire mass spectral data.

RESULTS AND DISCUSSION

Plasma spray studies on reference material

The initial experiments consisted in studies of two HPLC systems involving a cation-exchange or a diol column [13,14] for phospholipid class separation. The classes of PI, PA, PE, PS, PC and CL were investigated chromatographically using conventional UV detection (203 nm). Both columns served well and exhibited maximum retention times (for PC) of approximately 15 min using the solvents indicated. Preliminary MS studies with plasma spray using loop injection revealed a five-fold excess of ions for the solvent acetonitrile–methanol–water (mixture I) compared with 2-propanol–hexane–water (mixture II). The cation-exchange column was consequently used in further studies.

A comparison between conventional thermospray involving 0.1 *M* ammonium acetate buffer in solvent mixture I and plasma spray using the same solvent but without buffer is shown in Fig. 1. The plasma spray spectrum (Fig. 1a) indicates intense fragmentation leading to diglyceride (m/z 551) and monoglyceride (m/z 313) positive ions, in agreement with the findings of Kim and Salem [3,4], who investigated filament-on thermospray. No molecular ion adducts (M+1) were observed but traces of very-low-intensity fragments corresponding to (M+23) (sodium) were detected. In contrast, thermospray yielded low-intensity molecular adduct ions of m/z 734 [(M+1), 2%] under the ion source conditions used (Fig. 1b). As in plasma spray ionization, fragment ions characteristic of both diglyceride and glyceride moieties were seen. Of importance is the finding that plasma spray ionization yielded a total ion current at least one order of magnitude higher than thermospray for PC.

Positive ion plasma spray spectra of the PI, PC, PE, PA, PS and CL phospholipid classes studied in this work all showed fragmentation patterns similar to those previously described for chemical ionization [15] and thermospray [3,4], with the characteristics that no or very low abundance molecular related ions are observed. The most dominant fragment ions were diglyceride and monoglyceride ion species, which are presumably obtained by loss of the head group from the molecule and additional loss of an acyl chain from the diglyceride ion, respec-

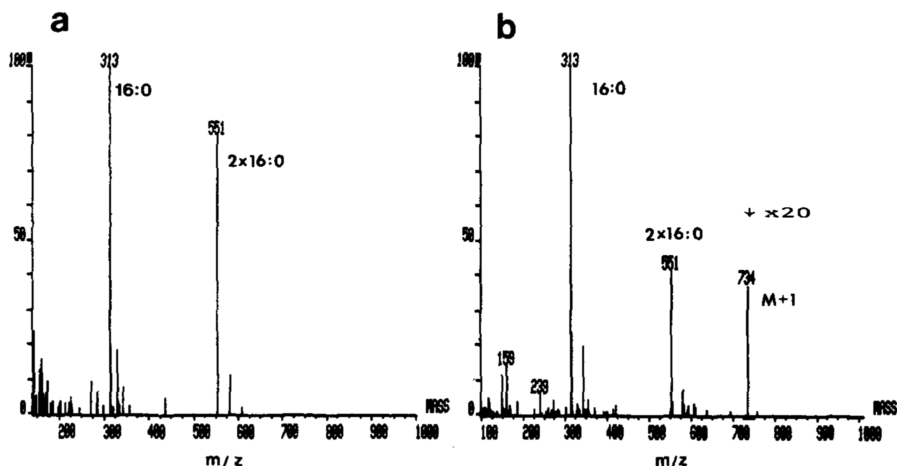

Fig. 1. Positive-ion mass spectra of dipalmitoyl-L-3-phosphatidylcholine. (a) Plasma spray ionization; (b) thermospray ionization with added ammonium acetate as buffer.

tively. However, as has been observed previously [3], if the diclyceride species contain a high degree of unsaturation, very abundant diglyceride adduct ions are obtained by the addition of a molecule of water (+18). This was especially pronounced for the fragmentation of PIP and PIP-2, which contain a high proportion of arachidonic acid (20:4) (see below).

Applications

As studies of stimuli turnover of phosphoinositides and possible accompanying changes in molecular fatty acid composition were considered of particular interest in our future work, a comparison between the mass spectra of PIP and PIP-2 derived from bovine brain extract was made. PIP-2 is considered to be derived from PIP simply by the phosphorylation of inositol in position 5 [8]. Fig. 2 shows plasma spray mass spectra using loop injection and using solvent mixture I.

Studies of the cluster of diglyceride ions indicated, for example, possible combinations of 16:0, 18:1 (or 16:1, 18:0) (*m/z* 577) and abundant 18:0, 20:4 (*m/z* 645) for both phosphoinositides. The diglyceride clusters for PIP and PIP-2 are clearly very similar, supporting the assumption that the two phosphoinositides are interrelated only by phosphorylations in the inositol moiety.

To study the accuracy in determining the fatty acid composition of phospholipid classes of a natural sample in more detail, a bacterial cell extract was examined by plasma spray LC–MS. Cells of the Gram-negative species of *P. fluorescens* were extracted for total lipids and the extract was subjected to analysis using the cation-exchange column (solvent mixture I). Fig. 3 (bottom trace) shows a mass chromatogram (positive total ion current) indicating at least four fractions (scan numbers 32, 68, 84 and 162). Reconstructed ion current profiles for a typical bacterial monoglyceride 18:0 (*m/z* 339) species and a likely diglyceride 16:0, 17:0Δ (*m/z* 563) are also shown (top and middle traces, respectively). Clearly the peaks at scan numbers around 32, 70 and 166 represent such fragments, although that at 70 should be suspected of being composed of additional hydrophobic material.

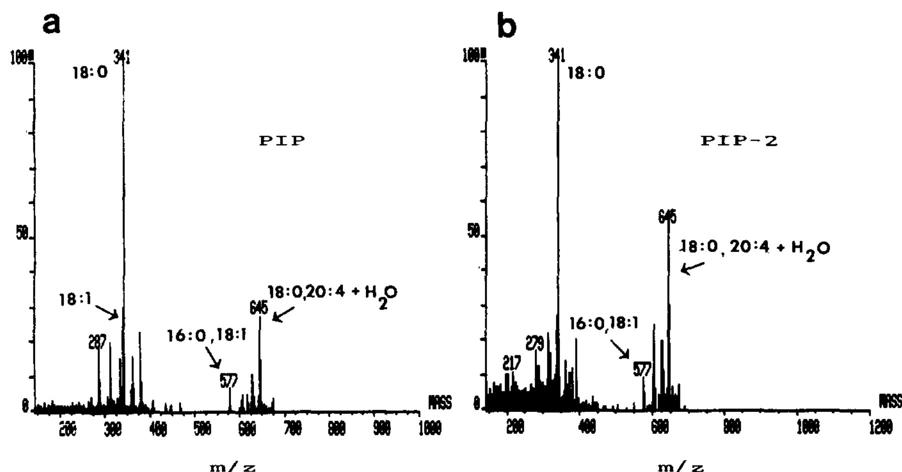

Fig. 2. Positive-ion plasma spray mass spectrum of (a) PIP and (b) PIP-2.

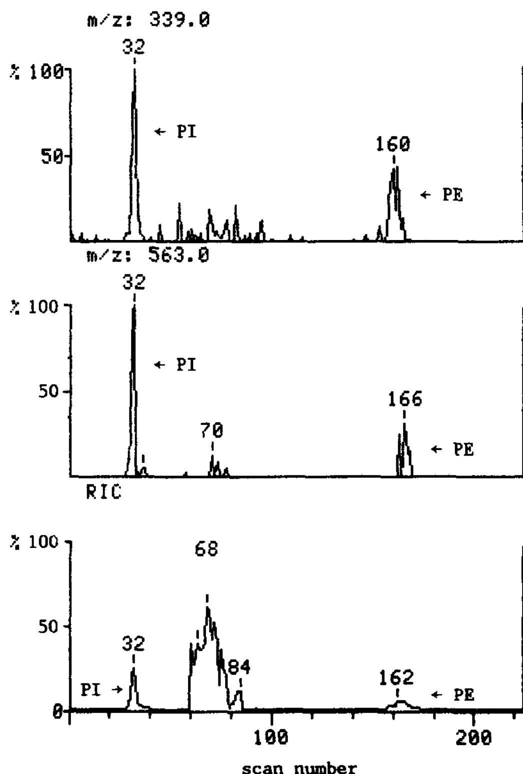

Fig. 3. Positive-ion plasma spray mass chromatogram (LC-MS) of lipid extract of *Pseudomonas fluorescens* cells. Top, extracted ion current profile for *m/z* 339 (monoglyceride fragment 18:1); middle, extracted ion current profile for *m/z* 563 (diglyceride fragment, 16:0, 17:0Δ); bottom, total ion current profile.

The assignment of the nature of the phospholipid classes corresponding to scans 32 and 162 was based on the chromatographic retention times in HPLC and TLC. The fraction designated 32 in Fig. 3 was thereby indentified as PI and that designated 162 as PE. Both phospholipid classes are known to be prominent membrane constituents of *Pseudomonas* species [16].

The plasma spray mass spectrum of the PE fraction (Fig. 3) averaged over the entire eluate and corrected for background signals is shown in Fig. 4. In addition to the prominent monoglyceride and diglyceride peak clusters, a series of very small peaks corresponding to molecular ion adducts with sodium (M+23) is observed. In Table I the monoglyceride ions from the plasma spray spectra of the *Pseudomonas* PE and PI are listed together with their relative MS abundances. The dominant fatty acid constituents in both phospholipid classes are 16:0, 16:1 and 17:0Δ. Interestingly, the spectra indicated that PI and PE have different

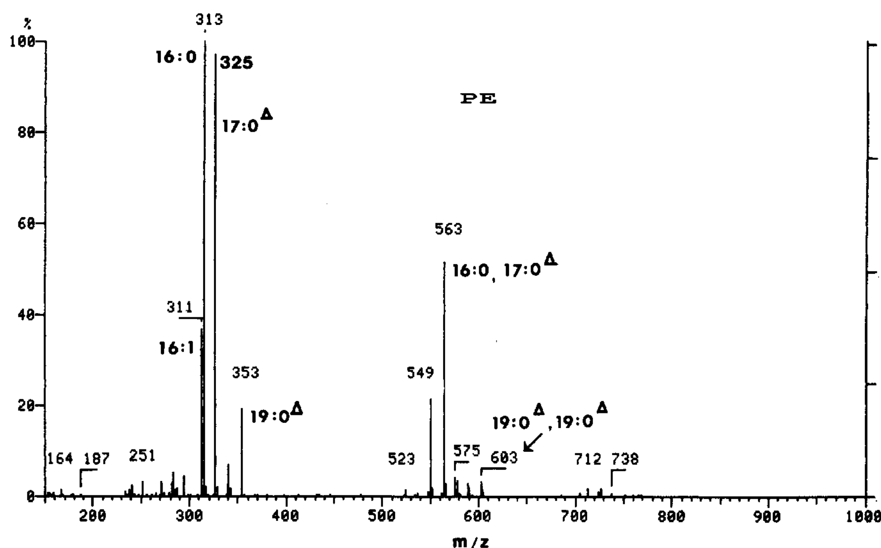

Fig. 4. Positive-ion plasma spray mass spectrum of phosphatidylethanolamines (PE) of *P. fluorescens* (LC-MS).

TABLE I

IONS INDICATING MONOGLYCERIDE FATTY ACID COMPOSITION

m/z	Fatty acid designation	Relative ion abundance (%)		Gas chromatography (PE) (%)
		PI	PE	
311	16:1	19.6	14.4	18.8
313	16:0	41.2	38.4	34.6
325	17:0Δ	24.9	36.9	34.3
339	18:1	11.7	–	1.0
341	18:0	1.7	2.9	4.2
353	19:0Δ	0.9	7.5	7.1

TABLE II

IONS INDICATING DIGLYCERIDE FATTY ACID COMPOSITION

m/z	Fatty acid designations		Relative abundance of diglyceride-related ions (%)	
			PI	PE
549	16:0	16:1	27.0	24.7
563	16:0	17:0Δ	34.0	55.9
575	16:1 17:0Δ	18:1 17:0Δ	21.6	5.4
577	16:0 16:1	18:1 18:0	10.8	5.3
589	16:1 18:1	19:0Δ 17:0Δ	3.2	4.3
603	19:0Δ	19:0Δ	3.4	4.4

relative fatty acid compositions with a significantly higher degree of unsaturation for PI and cyclic fatty acids for PE.

The bacterial PE fraction was hydrolysed and the fatty acid composition determined by capillary GC of the pentafluorobenzyl esters (Table I). The acids were then identified with respect to their chemical structures by comparison of the GC retention times with those of known reference compounds. Table I indicates agreement between the results from direct MS quantification and conventional GC fatty acid analysis.

Table II shows the relative abundances of the diglyceride fragments with the compositions indicated for the two phospholipid classes. The proportionally higher degree of unsaturation in PI compared with PE as observed from the monoglyceride clusters recurs in the diglyceride fatty acid composition. Interestingly, in both PE and PI, 17:0Δ appears, to a large extent, to be accompanied by 16:0 and the combination is particularly prominent in PE (63.1%).

Quantification

As studies of rapid changes in phospholipid fatty acid compositions in very small biological environments are considered very important in future work, plasma spray ionization was investigated with respect to sensitivity and quantification. SIM in the positive-ion mode of monoglyceride and diglyceride fragments was performed on the cation-exchange column eluate. In a typical study, dose–response curves obtained by monitoring dioleyl-L-3-phosphatidylcholine at m/z 339 (monoglyceride 18:1) and m/z 603 (diglyceride 2×18:1) were constructed (Fig. 5). Both curves show good linearity ($r=0.987$ for the monoglyceride and 0.993 for the diglyceride fragment signals), indicating straightforward potential for quantification in the nanogram range. The inset in Fig. 5 showing diglyceride signals of 10 and 5 ng of dioleylcholine further indicates detection limits in the high picogram range under the chromatographic and MS conditions used.

Plasma spray was significantly more sensitive than true thermospray using the

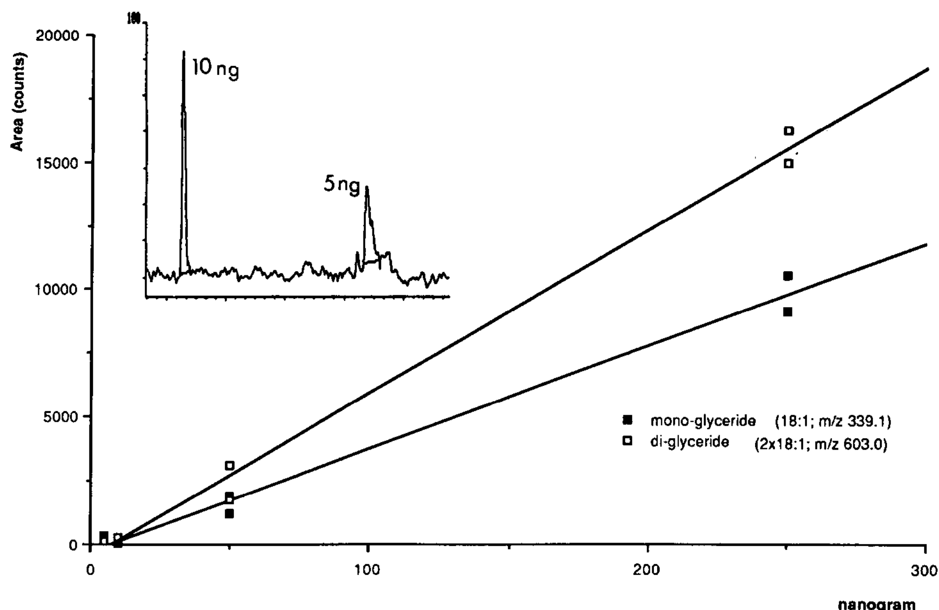

Fig. 5. Dose-response curves for dioleyl-L-3-phosphatidylcholine monitoring at monoglyceride and diglyceride fragments (m/z 339 and 603, respectively) in the positive-ion plasma spray (SIM) mode. Inset: signals from diglyceride ions (10 and 5 ng).

solvent mixture indicated, as judged from a comparison using PI. Kim and Salem [4] gave an average detection limit for phospholipids in the range of 10 ng using filament-on thermospray. Their findings may therefore suggest that plasma spray of phospholipids is superior to thermospray from a sensitivity point of view.

CONCLUSIONS

LC–MS involving plasma spray ionization with positive-ion recordings constitutes a highly sensitive technique in studies of biological phospholipid compositions and turnover. Combined with a cation-exchange LC column for class separation and a acetonitrile–methanol–water solvent mixture, the analytical system provides a means with which to obtain an indication of the relative proportions of the fatty acids in phospholipid classes with high sensitivity. Plasma spray ionization is particularly attractive compared with true thermospray in that it requires no addition of buffer prior to ionization and that the total ion current obtained is significantly higher.

Plasma spray of phospholipids is a more severe ionization technique than true thermospray. This is reflected by the fact that no or only extremely low abundance fragments related to the entire phospholipid molecule are observed in plasma spray ionization, whereas in thermospray such fragments (e.g., M+1) may account for several per cent, in the mass spectrum.

ACKNOWLEDGEMENTS

Much of the mass spectrometric work was performed in the application laboratory of VG Masslab (Altrincham, U.K.). For this and the valuable discussions with Dr. B. Jergil, Department of Biochemistry, Lund University (Lund, Sweden) and Dr. C. Liljenberg, Department of Plant Physiology, University of Göteborg (Göteborg, Sweden) the authors are greatly indebted.

REFERENCES

1 W.W. Christie, HPLC and Lipids; A Practical Guide, Pergamon Press, Oxford, 1987, pp. 87–132.
2 W.W. Christie, J. Lipid Res., 26 (1985) 507.
3 H.-Y. Kim and N. Salem, Jr., Anal. Chem., 58 (1986) 9.
4 H.-Y. Kim and N. Salem, Jr., Anal. Chem., 59 (1987) 722.
5 H.-Y. Kim and A. Yergey and N. Salem, Jr., J. Chromatogr., 394 (1987) 155.
6 C. Liljenberg and M. Kates, Can. J. Biochem. Cell Biol., 63 (1985) 77.
7 G.A. Lundberg, B. Jergil and R. Sundler, Biochim. Biophys. Acta, 846 (1985) 379.
8 A.A. Abdel-Latif, Pharmacol. Rev., 38 (1986) 227.
9 E.G. Bligh and U.J. Dyer, J. Biochem. Physiol., 37 (1959) 911.
10 C.W. Moss, M.A. Lambert and W. Merwin, Appl. Microbiol., 28 (1974) 80.
11 A. Sonesson, L. Larsson and J. Jimenez, J. Chromatogr., 417 (1987) 366.
12 J.D. Schacht, J. Lipid Res., 19 (1978) 1063.
13 R.W. Gross and B.E. Sobel, J. Chromatogr., 197 (1980) 79.
14 P. Juanéda and G. Rocquelin, Lipids, 21 (1980) 239.
15 P.E. Haroldsen and R.C. Murphy, Biomed. Environ. Mass Spectrom., 14 (1987) 573.
16 A.V. Few, Biochim. Biophys. Acta, 16 (1955) 137.