Molecular species composition of rat liver phospholipids by ESI-MS/MS: the effect of chromatography

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Abstract **Using electrospray ionization tandem mass spectrometry (ESI-MS/MS) this study shows that the loss of glycerophospholipid (GPL) after chromatography was unevenly distributed across the GPL molecular species. Both TLC and HPLC caused a preferential loss of GPL with 0 to 3 double bonds: 20% and 7.2% for choline glycerophosphates (PC) and 19.7% and 7.5% for ethanolamine glycerophosphates (PE), respectively. A consequence of these losses was that GPLs containing fatty acids with four or more double bonds had a greater contribution to the total after chromatography. ESI-MS/MS analysis also showed that PC molecular species with four or more double bonds migrated at the front of the TLC band of PCs. GPLs extracted from TLC plates occasionally contained PCs that were smaller than those in the original extract. These low molecular mass PCs were easily reduced to alcohols and formed derivatives with 2,4-dinitrophenylhydrazine, suggesting that aldehydes were generated by the oxidation of unsaturated fatty acids. Directly analyzing lipid extracts by ESI-MS/MS without preliminary chromatographic separation gives an accurate distribution of GPL molecular species in lipid mixtures. However, the ionization of the phospholipids in the electrospray jet maximized at relatively low concentrations of GPL. There was a linear response between phospholipid mass and ion intensity for concentrations around 1–2 nmol/ml for both PC and PE. The total ion intensity continued to increase with concentrations above 1–2 nmol/ml, but the response was non-linear.**—DeLong, C. J., P. R. S. Baker, M. Samuel, Z. Cui, and M. J. Thomas. **Molecular species composition of rat liver phospholipids by ESI-MS/MS: the effect of chromatography.** ***J. Lipid Res.*** **2001.** 42: **1959–1968.**

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Glycerophospholipids (GPLs) are the basic building blocks for cellular membranes and define cellular and subcellular structures. In addition to being critical components of cellular membranes, GPLs interact with all membrane proteins and many non-membrane proteins as well as mediate signal transduction (1). GPLs contain five structural moieties, including polar head group, phosphoryl group, glycerol backbone, and either a fatty ether or acyl side chains at the *sn*-1 position and fatty acyl chains at the *sn*-2 position. The numerous combinations of chain lengths, double bonds, linkages to the glycerol backbone of the side chains, and different head groups enable the formation of an immense number of molecular species. Many of the functional aspects of GPL depend upon these structural subtleties. Given their structural and functional roles in mammalian cells, the understanding of GPL composition, metabolism, and regulation at the level of molecular species has become increasingly important.

Conventional strategies for quantitation of GPL molecular species require many steps. The first step is to separate total lipid extracts into lipid subfractions by either TLC or HPLC. Subsequent steps include the removal of the GPL head-group, derivatization of the *sn*-3 position, and then separation by normal and reverse-phase HPLC. Individual molecular species are collected from the HPLC, then saponified and esterified to produce fatty acid methyl esters and dimethylacetals that are analyzed by gas chromatography (2–5). These prior separations and manipulations are labor-intensive, time-consuming, and may suffer from reduced recovery and selective loss of certain molecular species.

Analysis of the unprocessed total lipid extract by electrospray ionization tandem mass spectrometry (ESI-MS/MS) bypasses many of these problems. ESI-MS/MS is a powerful tool for the study of phospholipids because it: *1)* requires minute amounts of sample, *2)* employs a "soft" ionization procedure that produces mostly singly charged GPL ions (6–8), *3)* can process a sample containing a mixture of different GPLs, *4)* can distinguish GPL classes and identify individual molecular species via unique collision-induced decomposition pathways (9–12), and *5)* is very fast. Thus, ESI-MS/MS methods provide unparalleled speed and precision for the rapid quantitation of GPL mixtures.

Abbreviations: ESI-MS/MS, electrospray ionization tandem mass spectrometry; GPL, glycerophospholipid; MS, mass spectrometry; MS/MS, triple quadrupole mass spectrometry; MS1, first quadrupole of a triple quadrupole MS; PC, choline glycerophosphate; PE, ethanolamine glycerphosphate; PS, serine glycerophosphate.

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In this study, we have assessed the effects of TLC and HPLC separation on GPL composition using ESI-MS/MS. This is the first study to provide experimental proof of the changes in molecular species composition that occurs during chromatography. We provide additional detail on sample and instrument parameters that are important for the use of ESI-MS/MS for quantitative analysis of total cellular GPL. Several studies have focused on quantitation of GPL (6, 8, 13–21), but these reports have not addressed the changes in GPL molecular species composition that take place during chromatography.

MATERIALS AND METHODS

Materials

Phospholipid standards were from Avanti Polar Lipids. Solvents (HPLC or Optima grade) were from Fisher Scientific. Silica gel H TLC plates were from Analtech, Inc. The Supelcosil LC-Si HPLC column, 4.6 mm diameter by 250 mm length packed with 5 micron particles, was from Rainin Instrument, Inc. MDA-231 cells were from ATCC. All other reagents were the highest commercial grade available from Fisher Scientific.

Analysis of TLC and HPLC recoveries

Lipid extraction. Lipids were extracted from rat liver by the method of Bligh and Dyer (22). Total phospholipid content of the extracts was determined using the lipid phosphorus assay of Rouser et al. (23).

Phospholipid recovery. Ten aliquots of total rat liver lipid extract were prepared in methylene chloride at 150 nmol phospholipid/sample. Three aliquots were analyzed by MS/MS without manipulation, three separated by TLC before analysis, and three by HPLC. For analysis, each sample was reconstituted in a solution of methylene chloride–methanol–water 45:45:10 (v/v/v) containing 1% formic acid.

Thin layer chromatography. Three 150 nmol aliquots of lipid extract were separated by TLC on silica gel H plates that were dried in an oven at 110°C for 2 h. The loaded plates were developed in a solvent system of chloroform–methanol–ammonium hydroxide 65:35:8 (v/v/v). The silica from the entire length of each TLC lane was scraped into a glass tube and extracted twice with 2 ml of chloroform–methanol–water 2.5:2:1 (v/v/v) followed by two Bligh and Dyer extractions. The combined extracts were centrifuged to remove residual silica, transferred to clean glass tubes, and dried under nitrogen. If choline glycerophosphate (PC) samples showed the presence of sodium adducts, the sodium was removed by treating samples dissolved in 100 μl of methylene chloride with 10 μl of formic acid for 5 min. An equal volume of water was added and the mixture vigorously vortexed for 1–2 min and allowed to stand 5 min. The organic layer was removed and diluted 1:2 with methanol plus 5 μl formic acid. The sample was immediately dried in a stream of nitrogen and dissolved in a solution of methylene chloride–methanol–water 45:45:10 (v/v/v) containing 1% formic acid.

HPLC. Three 150 nmol aliquots of lipid extract were dried down, resuspended in hexane–isopropanol–water 2:2.7:0.15 (v/v/v), and then separated into phospholipid subclasses by normal phase HPLC as described by Surette et al. (24) with a 4.6 mm × 250 mm Supelcosil LC-Si HPLC column. The eluant was 2-propanol–ethanol–phosphate buffer-pH 7.4–hexane–acetic acid in the following compositions: 367:100:30:490:0.2 (v/v/v/v/v) (Solvent A) and 367:100:50:490:0.6 (Solvent B) at a flow rate of 1 ml/min. GPLs were eluted with a gradient of 0% B (5 min), 0–100% B (10 min), and 100% B (75 min). The elution of GPL classes was monitored at 203 nm. The neutral lipid, ethanolamine glycerophosphate (PE), serine glycerophosphate (PS), inositol glycerophosphate, and PC fractions were collected, the individual components pooled, and the solvent removed in a stream of nitrogen. The combined fractions for each run were dried down, then dissolved in methylene chloride methanol and stored at −70°C.

Analysis of phospholipid decomposition products

TLC separation. GPLs were extracted from ~1.0×10^7 MDA-231 cells by an acidified Bligh and Dyer extraction (22) containing 0.7% acetic acid. The lipid extract was divided into several aliquots, then separated by TLC using chloroform–methanol–ammonium hydroxide 65:35:8 (v/v/v) as the mobile phase. In some studies, methylene chloride was used in place of chloroform. After removing the plates from the development tank, the plates were allowed to stand for various lengths of time before scraping PC fractions from each lane. The silica gel was mixed with 1 ml of 1 M NaCl followed by Bligh and Dyer extraction (22) to recover lipids. Mass spectrometry (MS) samples were prepared by dissolving the isolated PC in 500 μl methylene chloride–methanol–water 45:45:10 (v/v/v). Residual particulates were removed with a 2 μm stainless steel filter.

Derivatives: sodium borohydride reduction and dinitrophenylhydrazone formation. Developed TLC plates were dried in a fume hood for 12 h. The region corresponding to PC was scraped and extracted as described above. Approximately 1 mg PC was dissolved in 400 μl of 95% ethanol, 100 μl of 0.1 M $NaBH_4$ was added, and the mixture incubated at room temperature for 30 min. An additional 100 μl 0.1 M $NaBH_4$ was added and the reaction continued for 30 min. For derivatization by DNPH, the lipids were extracted by the method of Bligh and Dyer (22) followed by ESI-MS/MS analysis. Approximately 1 mg of lipid was dissolved in 100 μl Tris-HCl (0.2 M, pH 7.5) containing 2 mg/ml fatty acid free BSA and added to 500 μl 1.8 mM DNPH in 1 N HCl. The reaction proceeded for 2 h at room temperature, after which the lipids were extracted by the method of Bligh and Dyer (22).

Electrospray ionization tandem mass spectrometry. GPLs were analyzed on a Micromass Quattro II triple quadrupole mass spectrometer. Data were acquired using MassLynx NT software. All analyses were performed at a flow rate of 5 μl/min provided by a Harvard Apparatus model 55-2111 syringe pump, an argon pressure of 1.8×10^{-3} mBar, and a source temperature of 200°C. Typical values for capillary and cone voltages were 4.5 kV and 85 V, respectively. Data were recorded at 16 points/Da with a scan time of 1.00 s and a scan delay of 0.10 s. The GPL classes PC, PE, and PS were analyzed in the positive ion mode using collision energies ranging from 15 to 40 V, respectively. PC-containing species were discriminated by measuring the precursors of *m/z* 184. PE-containing species were detected by scanning for a neutral loss of 141 Da. PS-containing species were detected by scanning for molecules that underwent a neutral loss of 185 Da. The fatty acid distribution of individual molecular species was determined in the negative ion mode by product ion (daughter ion) analysis of $[M\text{-}CH_3]^-$ ions from PC or $[M\text{-}H]^-$ ions from PE and PS. Analysis of the negatively charged product ions was performed with collision energies ranging from 15 to 40 V. GPL samples were reconstituted to 2 nmol/ml in methylene chloride–methanol–water 45:45:10 (v/v/v) for analysis. Because the sensitivity of ion detection has been shown to vary with ion mass (8), a mass dependent correction was established by fitting a line to a plot of ion intensity at a constant molar GPL concentration versus *m/z*. The ion intensity was normalized to the first ion in the spectrum. The experimental ion intensities at each *m/z*

were corrected by dividing by the fitted equation. The relationship of ion intensity versus m/z for PC was roughly linear but gave a better fit to a polynomial equation: $10.494 + (-2.20 \times 10^{-2} * m/z) + (1.182 \times 10^{-5} * m/z^2)$. The relationship for PE was exponential: $0.372 + 8709.8 * \exp(-0.0131 * m/z)$.

RESULTS

Total phospholipid concentration affects quantitation by ESI-MS/MS

To establish the range of linear response, a series of 1,2-O-tetradecanoyl-*sn*-glycero-3-phosphocholine, di-14:0 PC, and 1,2-O-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, di-16:0 PE concentrations were analyzed. Phosphatidylcholine was detected by the formation of m/z 184 ions, while neutral loss of 141 Da from precursor ions was used to measure PE. At equal concentrations, employing collision energies of 25 and 20 eV to optimize signal intensity of PC and PE, respectively, PC was more easily detected. Ionization efficiency of PE was substantially increased by including 1% formic acid in the solvent. The PC standard had a linear response of ion count versus concentration from 0.004 to 2 nmol/ml **(Fig. 1A),** while the PE standard was linear from 0.1 to 1 nmol/ml (Fig. 1B). Beyond these concentrations, the response was nonlinear and began to plateau. Rat liver lipid extract, which includes neutral lipids and GPL, was also analyzed for PC (Fig. 1C) and PE (Fig. 1D). Increasing concentrations of total cellular phospholipid resulted in response curves of major PC and PE molecular species similar to the standards with optimum concentrations up to 1 and 2 nmol/ml, respectively.

Separation of GPLs by TLC and HPLC results in significant selective losses of molecular species

Lipid classes are routinely separated before quantifying phospholipid molecular species. However, the phospholipid classes are not quantitatively recovered after chromatography. In the past, we assumed that the individual molecular species were recovered in proportion to their concentration in the original sample. We tested this as-

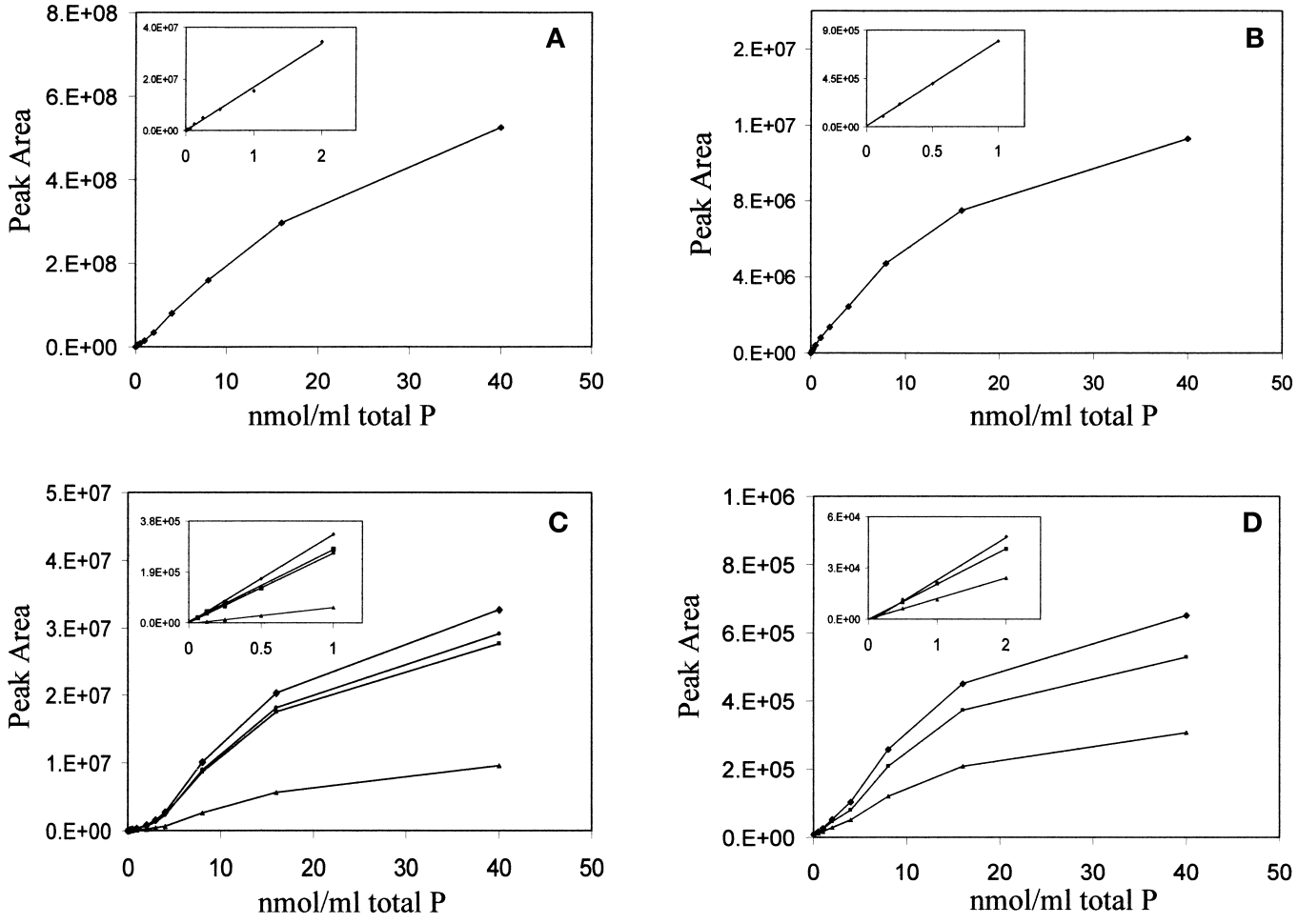

Fig. 1. Determination of working concentration range for quantitative phospholipid analysis. Increasing concentrations of di-14:0 PC (A) and di-16:0 PE (B) synthetic standards were analyzed by MS/MS. For analysis, each sample was reconstituted in a solution of methylene chloride–methanol–water 45:45:10 (v/v/v) containing 1% formic acid. Increasing concentrations of total rat liver lipid extract was also analyzed for PC (C) and PE (D). Four major PC molecular species and three major PE molecular species from the total PC and PE peak profiles of rat liver are plotted in C and D, respectively. The small graph insets are plots of the linear ranges derived from each larger plot. The correlation coefficients (R^2) of the linear plots are: A: 0.9968; B: 0.9997; C: average of 4 plots 0.9979; D: average of 3 plots 0.9979.

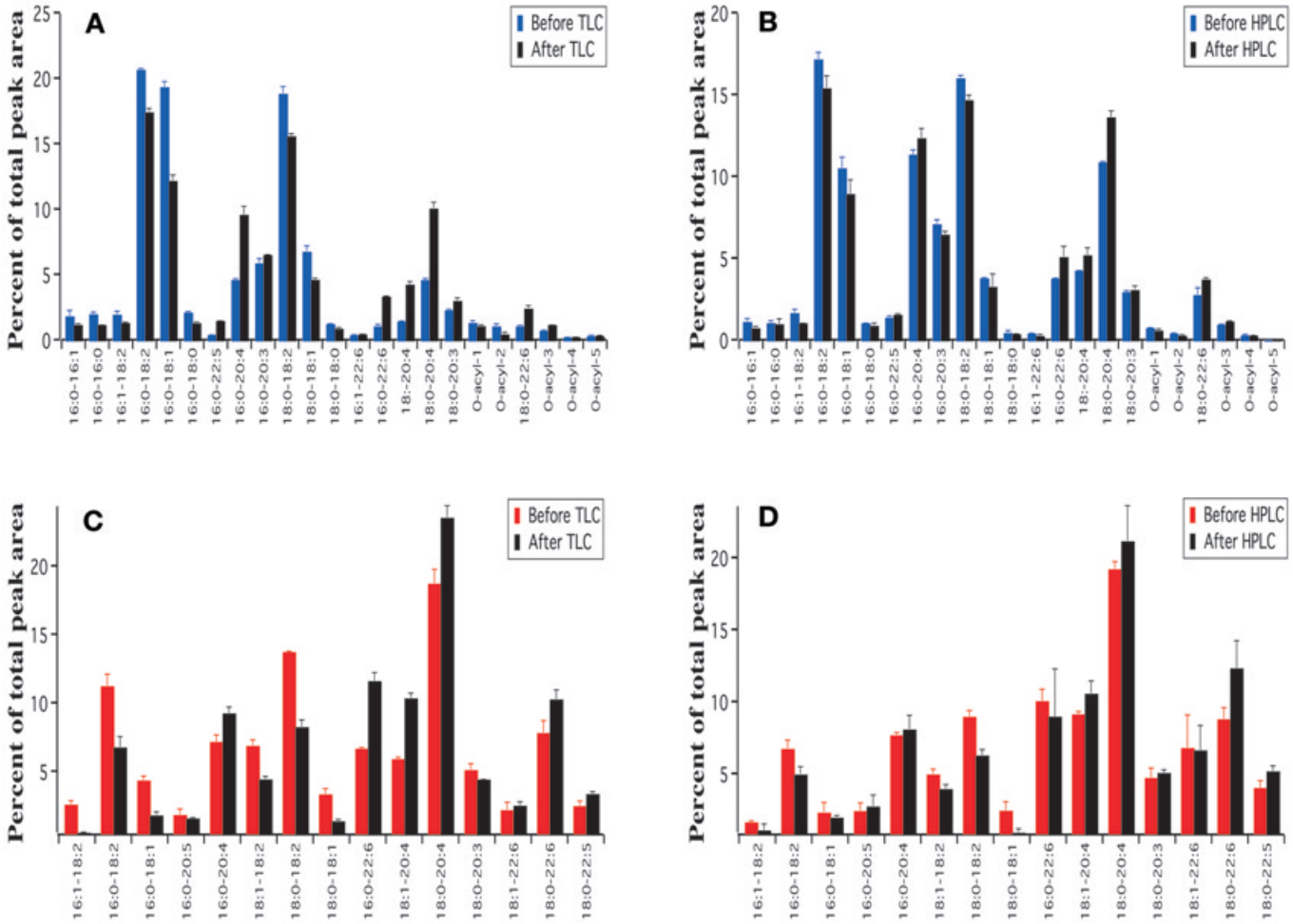

Fig. 2. Change in molecular species composition after phospholipid separation by TLC or HPLC. Aliquots of rat liver lipid extract were separated by TLC or HPLC, extracted, and fractions were pooled as described. The original extract and the TLC- and HPLC-separated phospholipids were analyzed by lipid phosphorus assay. Equimolar amounts were analyzed for PC and PE molecular species by precursor ion scanning of *m/z* 184 and neutral loss of 141 Da, respectively. Each sample was analyzed in triplicate. The major peaks were integrated and added together to get total peak area. The liver extract for analysis of before and after TLC separation, A: PC and C: PE, and HPLC separation, B: PC and D: PE, were from two different rats. Product ion analysis in the negative ion mode was performed to determine the fatty acid composition of each species.

sumption by analyzing PC and PE molecular species by ESI-MS/MS before and after chromatographic separation. Total rat liver lipid extract was separated by TLC or by normal-phase HPLC. Because it was not known whether one phospholipid class influences the ionization efficiency of other classes, all of the phospholipid fractions were pooled before MS analysis and the phospholipid distribution compared with the distribution in the untreated extract. All samples were diluted to 2 nmol/ml lipid phosphorus before analysis. The overall loss of phospholipid by chromatography was approximately 30% and 50% for HPLC and TLC, respectively. Adding acid to the extraction solvents would have undoubtedly improved the recovery from TLC plates, but increased the chance of catalyzing lipid hydrolysis or oxidation. **Figure 2** shows the percent contribution of each molecular species to the total PC (A and C) and PE (B and D) before and after TLC and HPLC. The percentages shown in Fig. 2 were corrected for reduced transmission efficiency with increase in phospholipid ion mass.

Figure 2 shows that there was a selective loss of certain phospholipid species after both TLC and HPLC separation. These losses were proportional to the total number of double bonds in the phospholipid. **Figure 3** is a plot of the number of total double bonds in the *sn*-1 and *sn*-2 fatty acids against the change in percent contribution to total PC or PE after TLC or HPLC. The percent change of molecular species in each double bond group, e.g., 0-, 1-, 2-, or 3-double bond(s), etc., were summed and the results are shown in Fig. 3. The net contribution of species having 0 to 3 double bonds was reduced after chromatography. The reduction for PC was 20% after TLC separation and 7.2% after HPLC, while PE lost 19.7% after TLC separation and 7.5% after HPLC. GPLs having a total of 4 to 7 double bonds had a proportionally greater contribution to the total after separation.

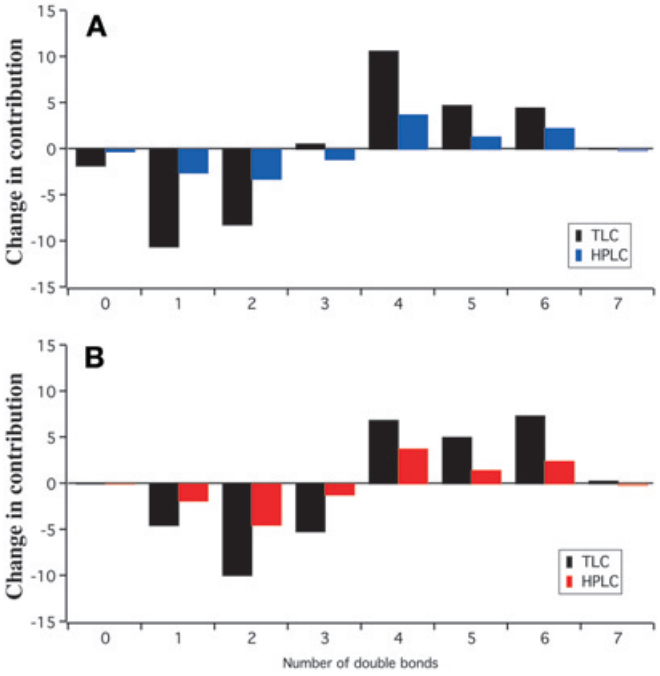

Fig. 3. Correlation between number of total double bonds in PC or PE molecular species and difference in percent contribution after TLC or HPLC separation. The rat liver PC (A) and PE (B) molecular species were grouped according to the total number of double bonds in the *sn*-1 and *sn*-2 fatty acids. The plot shows the change in the percent contribution of each molecular species after chromatography.

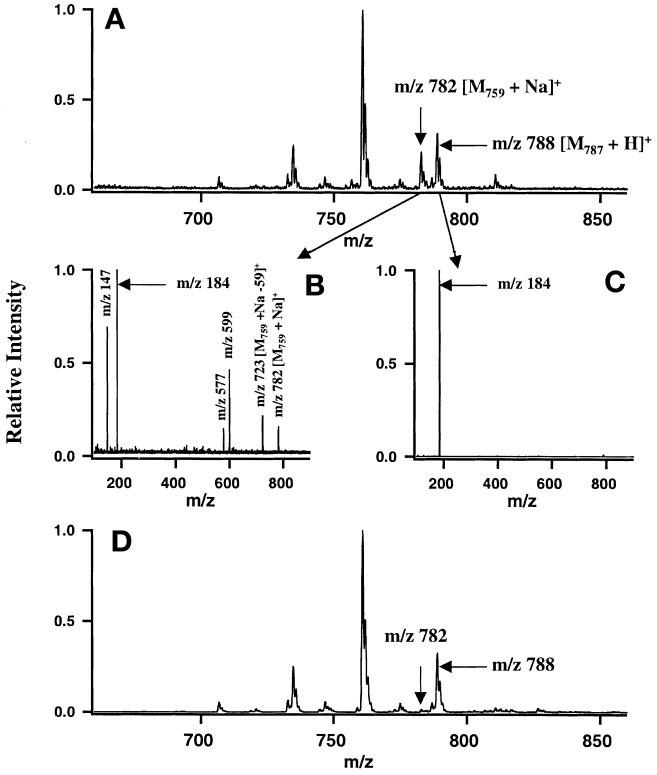

Fig. 4. Sodium adducts of PC. Bovine PC in methanol was allowed to stand with NaCl. The soluble material was diluted to 1:2 in methylene chloride–methanol. A: ES-MS1 spectrum of bovine PC treated with aqueous sodium chloride. B: Product ion spectrum of the *m/z* 782 ion. Na^+ has replaced H as the counter ion to the phosphate anion. C: Product ion spectrum of *m/z* 788 peak, which has H^+ as the counter ion to the phosphate anion. D: Precursor ion spectrum monitoring *m/z* 184. Note that the peak at *m/z* 782 is absent in this spectrum.

During the analyses it was apparent, particularly with PCs, that among samples the ion intensities would vary up to 20-fold. Reduction in the total precursor ion intensity correlated with a change in the apparent distribution of PC molecular species in the ES-MS1 spectrum as compared with the distribution in the precursor ion spectrum. The ES-MS1 spectrum contained ions that had masses 22 Da greater than in those in the precursor ion spectrum. A mass increase of 22 mass units suggested replacement of a proton by the sodium cation. We confirmed that sodium adducts are formed by adding sodium acetate to PC standards. Sodiated and unsodiated PC yield a different distribution of product ions as shown in **Fig. 4B** and **C**. Protonated PC showed only a strong *m/z* 184 ion, Fig. 4B. The intensity of the *m/z* 184 ion from the sodiated PC (Fig. 4C) was substantially reduced relative to other ions. The spectrum of sodiated PC was similar to that reported by Han and Gross (25). The neutral loss of 59 Da (M + Na − 59), *m/z* 723 in Fig. 4C, is characteristic of the sodiated PC. We now use the 59 Da neutral loss spectrum to ascertain the presence of sodium adducts in PC preparations (25). Sodium adducts can be prevented by treating PC samples dissolved in methylene chloride with 10 μl of formic acid, washing them with water, then diluting the recovered organic layer 1:2 with methanol and adding 5 μl formic acid. Analysis of samples treated in this fashion did not contain sodium adducts (data not shown).

Lipid oxidation

Preliminary studies of PC isolated from TLC plates occasionally showed the presence of lower molecular mass PCs that were not present in the unchromatographed extract. **Fig. 5A** and **B** show the *m/z* 184 precursor ion scans before and after TLC. The TLC plate was allowed to stand for an additional 30 min after drying to accentuate oxidation. To exaggerate the conversion and prepare sufficient product for chemical analysis, developed TLC plates were left in a fume hood overnight. Comparing **Fig. 6A** to that of lipid extract not subjected to TLC (Fig. 6B) suggests that most of the original PC was converted into lower molecular mass PCs. A multiple-step oxidation mechanism was suspected. This mechanism results in the loss of a significant mass of a fatty acid chain and the addition of an oxo-moiety. To establish the presence of an oxo-moiety, the lower molecular mass PCs were either reduced with $NaBH_4$ or converted into a 2,4-dinitrophenylhydrazone. Reduction with $NaBH_4$ gave new PCs having a mass 2 units higher than the starting PC, e.g., the mass of the predominant oxidized component was shifted to *m/z* 652 (Fig. 6C). Treatment with 2,4-dinitrophenylhydrazine gave PCs having masses 180 units greater than the underivatized

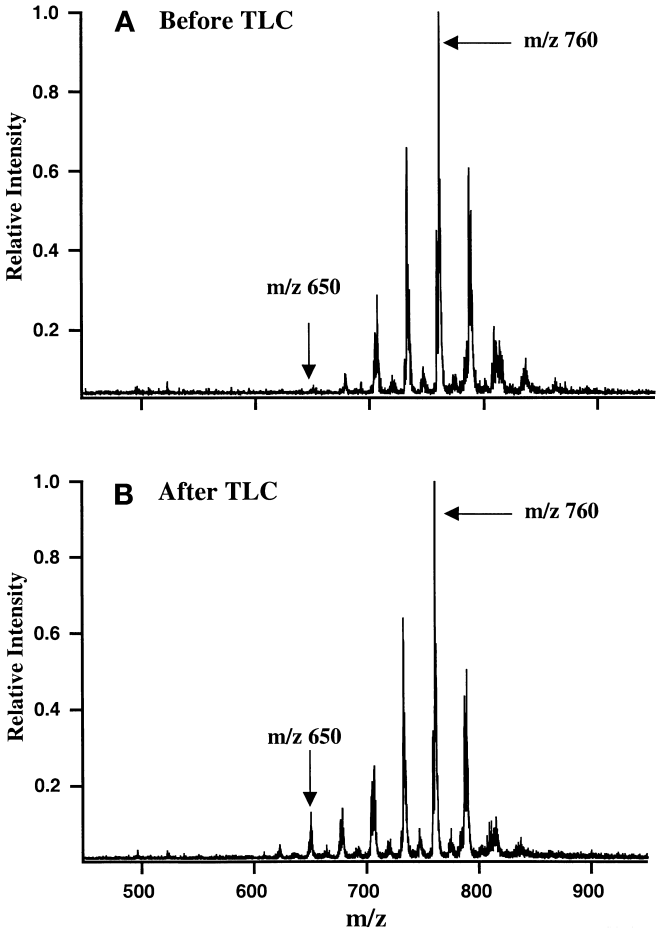

Fig. 5. Oxidation of PC during TLC separation. Whole cell lipid extract from MDA-231 cells that were separated by TLC. Panel A shows the PC *m/z* 184 ion precursor ion profile from whole cell lipid extract before separation by TLC. Panel B shows the *m/z* 184 ion PC precursor ion profile of extract that had been separated by TLC but left on the plate for 30 min before extraction. These profiles are representative of PC profiles from 3–6 independent experiments.

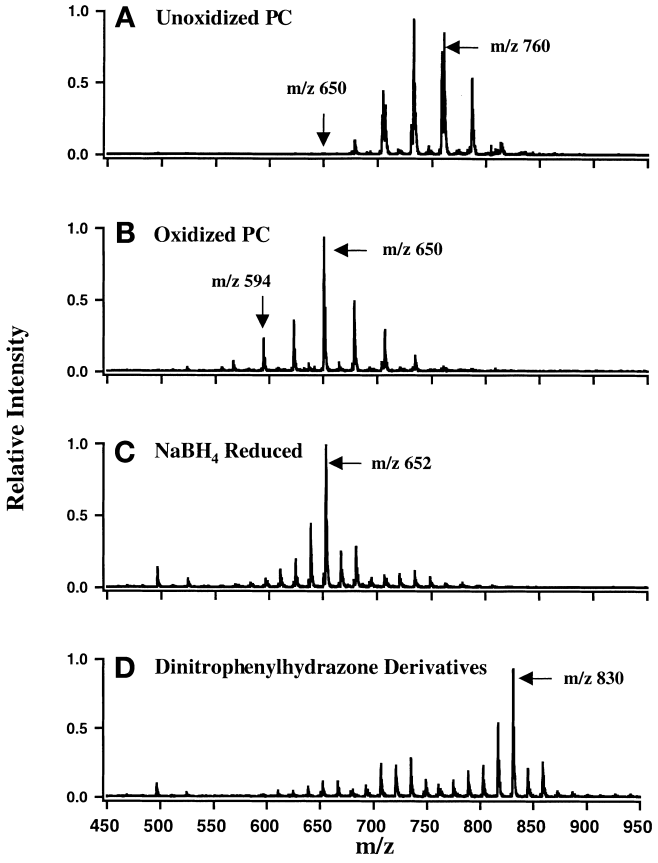

Fig. 6. Oxidation of unsaturated PC. Whole cell extract from MDA-231 cells was completely oxidized as described in Materials and Methods by exposing a resolved TLC plate to air overnight. The PC fraction was extracted from silica gel and analyzed by mass spectrometry. PC molecules were detected by precursor ion analysis for *m/z* 184. Panel A shows a typical, unoxidized PC profile obtained from PCs that were not separated by TLC. 1-O-hexadecanoyl-2-O-(9-octadecadecenoyl)-*sn*-glycero-3-phosphocholine, *m/z* 760, is the major molecular species in this sample. Panel B shows the PC precursor ion profile from PCs that were left on a TLC plate for 12 h after development. A glycerophosphocholine with *m/z* 650 is the predominant molecular species in this sample. Panel C shows the PC profile after reducing the material in Panel B with $NaBH_4$ to form alcohols. The masses of the resultant alcohols are 2 Da higher than their corresponding aldehydes, e.g., *m/z* 650 shifts to *m/z* 652. Panel D shows the PC profile generated by treating the material from Panel B with 2,4-dinitrophenylhydrazine. The formation of a 2,4-dintrophenylhydrazone derivative will increase the mass by 180 Da, e.g., *m/z* 650 shifts to *m/z* 830. The results are representative of two separate experiments.

compounds, e.g., the mass of the predominant oxidized product shifted to *m/z* 830 (Fig. 6D).

To further characterize the composition of oxidized PCs, we carried out product ion analysis of selected PCs. This procedure uses negative ion detection of the carboxylate anions generated from the demethylated anion $[M\text{-}15]^-$ (9, 11). The product ion analysis of the negative ion *m/z* 634, which corresponds to the protonated ion *m/z* 650, gave an anion *m/z* 171 at *sn*-2 and *m/z* 255 at *sn*-1. Other low molecular mass PCs had positive ion *m/z* values of 594, 622, 678, and 690. Ion *m/z* 594, which corresponds to the *m/z* 578 ion in the negative ion mode, yielded product anions of *m/z* 115 and *m/z* 255.

PC molecular species differentially migrate on TLC silica gel plates

To assess the location of sample GPL on a TLC plate after the plate has been developed, phospholipid standards were run in parallel on a separate TLC plate and then stained. It has been suggested that the migration of phospholipid subspecies within a TLC band may differ from the migration of a single, pure standard. We used ESI-MS/MS to determine whether sample bands of phospholipid classes were homogeneous or if separation of molecular species had taken place. Two different activated TLC silica gel H plates were spotted with egg PC and the plates developed. One of the plates was stained with iodine vapor to visualize PC and the corresponding region of the second TLC plate band was divided into six 1-cm zones. These sections were immediately scraped into separate tubes, extracted, and the GPL analyzed by ESI-MS/MS. **Figure 7**

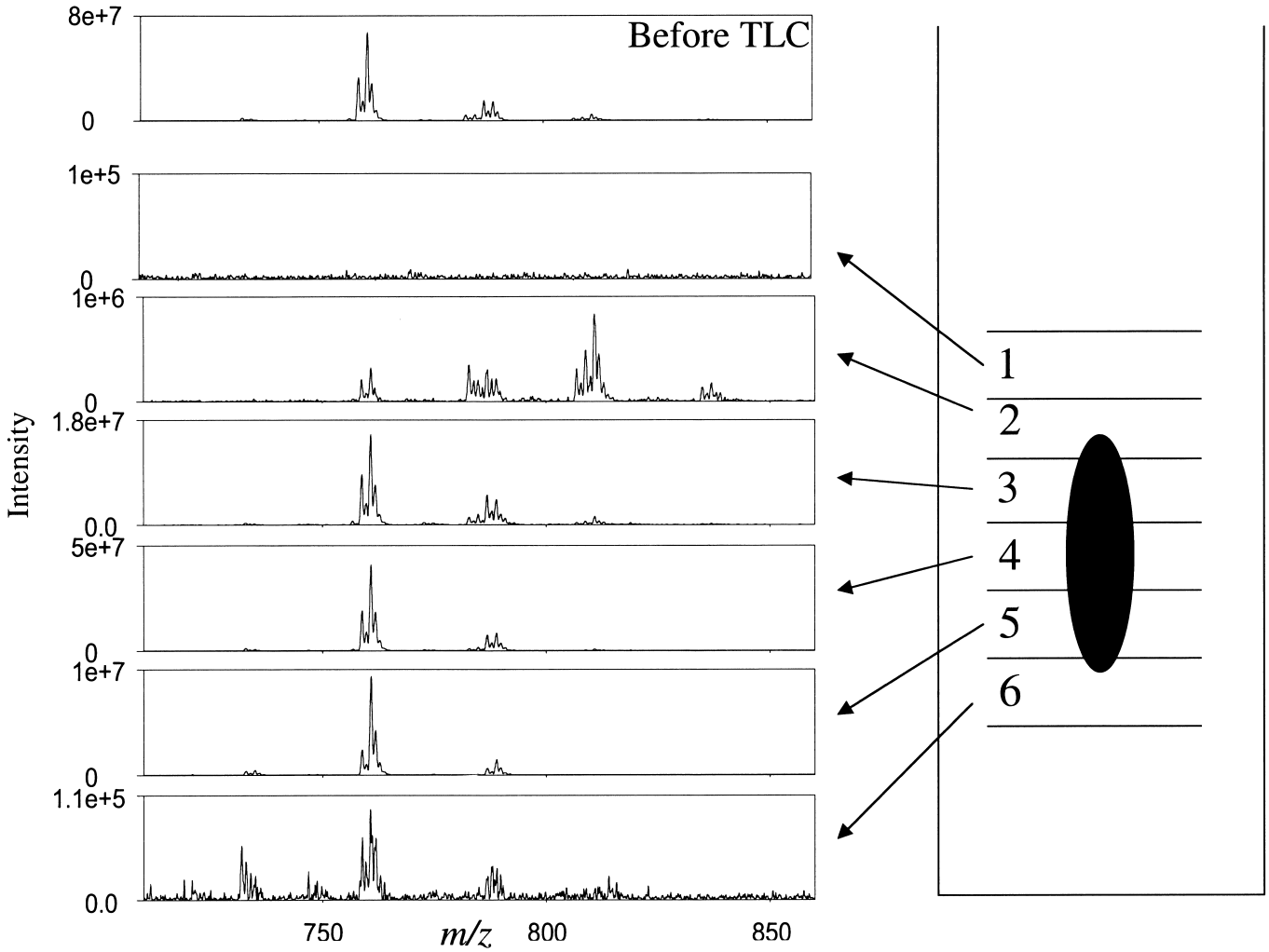

Fig. 7. Differential separation of PC molecular species on silica gel H plates. Egg PC (15 nmol) was spotted onto two silica gel H plates and run in a solvent system of chloroform–methanol–ammonium hydroxide 65:35:8 (v/v/v). The plates were air-dried for 5 min and one plate was exposed to iodine vapor for approximately 1 min. The PC band on the second, unexposed plate was divided into 6 1-cm fractions according to the location of the iodine-stained band. The fractions were scraped into separate glass tubes and the PC was extracted from the silica gel as described and analyzed by ESI-MS/MS.

shows the PC profiles of the six zones. PC subspecies containing 20- and 22-carbon fatty acids migrated at the front of the PC band in fraction 2, while bands 3 through 6 contained almost exclusively shorter chain PCs composed of palmitate and/or stearate.

Head group fragmentation efficiency

At a collision energy that gives the greatest precursor ion intensity, the sensitivity of PC detection decreases with increasing molecular mass (8, 26). However, by a judicious choice of collision energy, similar ion intensities can be obtained over small mass ranges. The effect of collision energy on fragmentation was demonstrated by analyzing at collision energies ranging from 15 to 40 volts equimolar mixtures of 14:0-14:0 PC and 1-O-hexadecanoyl-2-O-(9-octadecenoyl)-*sn*-glycero-3-phosphocholine (16:0-18:1 PC), 16:0-16:0 PE and 1-O-octadecanoyl-2-O-docosahexenoyl-*sn*-glycero-3-phosphoethanolamine (18:0-22:6 PE), and 1,2-O-tetradecanoyl-*sn*-glycero-3-phosphoserine (14:0-14:0 PS), and 1-O-hexadecanoyl-2-O-(9-octadecenoyl)-*sn*-glycero-3-phosphoserine (16:0-18:1 PS). Each pair of phospholipid standards differed in mass by approximately 100 Da. The results are plotted in Fig. 6 as the intensity of the high molecular mass component divided by the intensity of the low molecular mass component versus collision energy. For each phospholipid subclass, the ratio increased with collision energy as shown in **Fig. 8A–C**. Unfortunately, to achieve equal intensity of high and low mass ions required a considerable sacrifice of the total ion intensity (data not shown).

DISCUSSION

The results of this study demonstrated several important and previously undocumented findings about GPL chromatography. First, at concentrations of GPL less than 2 nmol/ml there was a linear relationship between GPL

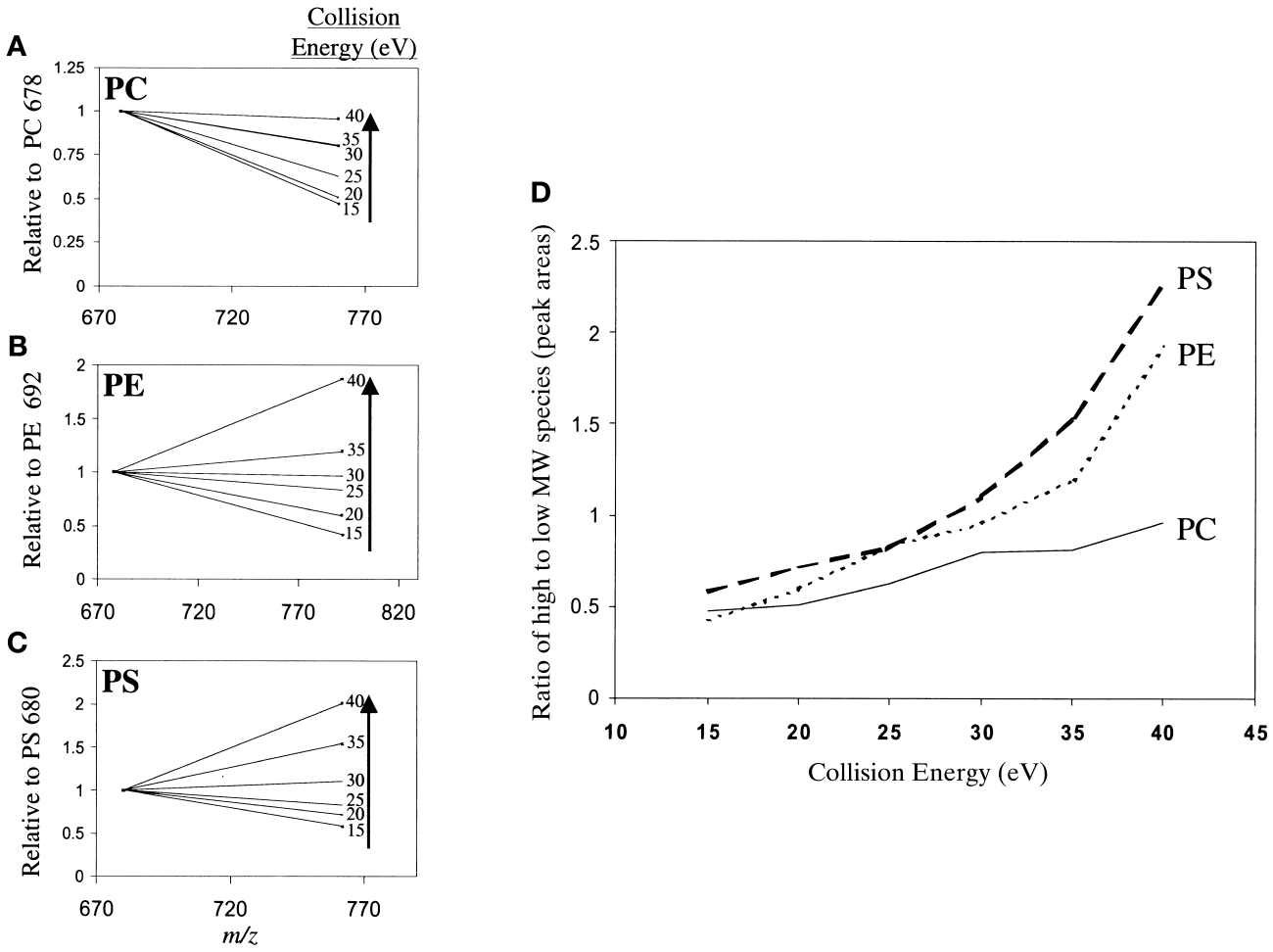

Fig. 8. Differential effect of collision energy on head group fragmentation efficiency. Equimolar amounts of 1,2-O-ditetradecanoyl-*sn*-glycero-3-phosphocholine (*m/z* 678) and 1-O-hexadecanoy-2-O-(9-octadecenoyl)-*sn*-glycero-3-phosphocholine (*m/z* 760) (A), 1,2-O-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (*m/z* 692) and 1-O-hexadecanoy-2-O-(docosahexenoyl)-*sn*-glycero-3-phosphoethanolamine (*m/z* 792) (B), and 1,2-O-ditetradecanoyl-*sn*-glycero-3-phosphoserine (*m/z* 680) and 1-O-hexadecanoy-2-O-(9-octadecenoyl)-*sn*-glycero-3-phosphoserine (*m/z* 762) (C) were analyzed using precursor ion scans at collision energies ranging from 15 to 40 eV. The profiles were acquired twice and the results were averaged. Plots A, B, and C show the increase in slope as collision energy increases. The slopes at different collision energies were derived by plotting each pair of phospholipids relative to the peak area of the low molecular mass species. The peak area ratios of the higher molecular mass species to the lower molecular mass species in each pair are plotted in D as a function of collision energy to determine the collision energy at a ratio of 1.

and ion intensity. Second, there was a selective loss of GPL molecular species with less than 3 double bonds from samples treated by either TLC or HPLC. The losses ranged from 7% to 20% and were smaller for HPLC and up to 3-fold greater for TLC. Third, oxidation of unsaturated GPL on TLC plates can occur within 30 min after exposure to ambient air. Fourth, molecular species within a single GPL class migrate differentially on TLC plates. We have also shown that although direct analysis by ESI-MS/MS bypasses these problems, sample concentration and collision chamber energy are critical factors in obtaining dependable quantitation.

Phospholipid standards of PC and PE gave a linear response from 0.004 to 2 nmol/ml (Fig. 1A) and 0.1 to 1 nmol/ml (Fig. 1 B), respectively. Beyond these concentrations, the response was nonlinear and began to plateau. Increasing concentrations of total phospholipid from rat liver extracts gave response curves of major PC and PE molecular species similar to the standards with optimum concentrations at 1 to 2 nmol/ml of total phospholipid. Koivusalo et al. (21) reported similar ion count versus concentration curves for phospholipid molecular species. They showed that the ion count versus concentration curves began to plateau above 5 nmol/ml total phospholipid. Because of the nonlinear response above 1 to 2 nmol/ml (1–2 pmol/μl) total GPL, we performed quantitative studies at or below 2 nmol/ml rather than employ individual internal standards for each phospholipid.

A unique advantage of ESI-MS/MS is the rapid analysis of total lipid extracts without preliminary purification or derivatization. The analysis yields the molecular species composition of different phospholipid subclasses with minimal distortion. Significant losses, up to 50%, were incurred when GPL were purified by chromatography. An

oft-made assumption is that these losses are uniformly distributed among the phospholipid molecular species. Using ESI-MS/MS, we have shown that the chromatographic procedures routinely used to separate phospholipid classes affect the distribution of molecular species. Figure 3 shows that the distribution of losses among phospholipid molecular species was greatest for the more saturated GPL, e.g., those having from 0 to 3 double bonds in the fatty acids. Selective losses to molecular species having 0 to 3 double bonds caused an apparent enrichment in molecular species having from 4 to 6 double bonds. Higher molecular mass, more unsaturated PC molecular species migrated faster, in a more concentrated band, than did other PCs. We propose that the PCs containing more unsaturated fatty acids have a weaker interaction with the silica gel and/or they are more soluble in the mobile phase. The larger area occupied by the smaller, less unsaturated PCs suggests that strong, nonspecific binding to silica gel contributed to the lower recovery. Plasmalogens are a subclass of the 1-O-alkyl GPL that are particularly sensitive to hydrolysis. In the presence of the formic acid and water used to introduce samples into the electrospray interface, plasmalogens are likely to be rapidly hydrolyzed. The hydrolysis products would not be detected in the mass range associated with the more common diacyl and 1-O-alkyl PCs and PEs.

Allowing the PCs to remain on the plates for an extended period of time caused the oxidation of unsaturated PCs and the formation of small molecular mass PCs. These oxidation products added 2 Da when reduced to alcohols by $NaBH_4$, suggesting that a single oxo-moiety was present in the fragment. Facile formation of 2,4-dinitrophenylhydrazones suggests that the oxo-moiety is an aldehyde rather than a ketone. Oxidative cleavage of the double bonds would be expected to give either aldehydes or acids, but not keto-moieties. Analysis of two of the oxidized PCs showed that they contained small, carboxylic acids in addition to a full-length fatty acid. Assuming that oxidation yields an aldehyde, the *m/z* 115 ion may be 5-oxopenanoic acid and the *m/z* 171 ion 9-oxononanoic acid. The presence of ion *m/z* 115 is consistent with oxidation of the double bond closest to the carboxyl moiety of eicosatetraenoic acid as reported by Khaselev and Murphy (27). Ion *m/z* 171 is likely formed by the oxidation of 9,12-octadecadienoic acid. Re-analysis of a large number of TLC-purified PC spectra showed that approximately half of the samples contained trace amounts of the smaller PCs. None of the small PCs were present in freshly extracted, unchromatographed samples. These observations suggest that the commonly used methods for TLC separation form only small amounts of these oxidation products. However, these small amounts may be important for studies that quantify in vivo lipid oxidation.

There are several possible consequences of the oxidation process. The first is that oxidation of PC on TLC plates will cause a small underestimation of the total mass of unsaturated fatty acids when MS is used. Analysis by measuring lipid phosphorus would probably not detect significant changes in mass. Because oxidation would create small, volatile hydrocarbon fragments, techniques that rely on counting radioactive lipids would be prone to undercounting. Again, in most instances oxidation would introduce a small error. However, when the GPL are left in contact with the TLC plate for extended periods, the errors could become more significant. A possibly more significant consequence of oxidation during chromatography would be the formation of minute amounts of oxidized PC like those that are reported to have biological activity (18, 28).

The loss of lipid and change in molecular species composition caused by sample manipulation and chromatographic separation were overcome by directly analyzing a total cellular lipid extract. However, we found that total phospholipid concentration, rather than the concentration of any single class, determined the linear range for analysis. For example, the optimum range of PC quantification in a total cellular extract, in which PC makes up 60% of phospholipid, was less than that of a PC standard alone. Working at or below the maximum concentration permits direct quantitative comparisons between samples.

The fatty acyl compositions for Fig. 2 were determined in the negative ion mode using collision-induced fragmentation to release carboxylate anions. The intensities of the product ions provide positional information, because the *sn*-2/*sn*-1 intensity ratio is usually greater than one (12, 29). However, exceptions to this general rule exist and the ratio depends on collision energy (30). For example, although 1-O-octadecanoyl-2-O-docosahexenoyl-*sn*-glycero-3-phosphoethanolamine was reported to have a *sn*-2/*sn*-1 ratio less than 1 at a collision energy of 30 eV (30), the phosphatidylcholine analog had an *sn*-2/*sn*-1 ratio greater than 1 (data not shown). Therefore, as suggested by Hvattum et al. (30), positional assignments for GPL should be verified with standards.

An additional complication of interpretation in the negative ion mode comes from the mass equivalence of certain PE and PC molecular species. In the negative ion mode, PC species lose a proton and a methyl group from the phosphohead group moiety. This loss of 16 *m/z* units can result in a higher molecular mass PC species giving a negative ion that is equal to the negative ion mass of a lower molecular mass PE species. Product ion analysis in this situation will always give three or four fatty acid anions. It is possible to predict the fatty acid composition of overlapping phospholipid species based on the *m/z* values obtained from precursor ion scans because the true masses of PC and PE will always differ by 28 mass units. Verification of molecular species composition can be obtained by separating GPL subclasses using online HPLC or by recording a spectrum of the lithium adducts (31).

These studies show that chromatographic separation of PC and PE before molecular species analysis will introduce artifacts due to non-random losses of certain molecular species. As suggested by Brügger et al. (8), direct analysis of lipid extracts gives an unbiased composition for PC and PE. However, for analysis discussed in this work, the total load of ionizable species introduced into the electrospray interface should not exceed 1–2 nmol/ml.

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