RAPID COMMUNICATIONS IN MASS SPECTROMETRY, VOL. **12**, 256–272 (1998)

Dual Parallel Mass Spectrometers for Analysis of Sphingolipid, Glycerophospholipid and Plasmalogen Molecular Species

Wm. Craig Byrdwell*
FQS, NCAUR, ARS, USDA 1815 N. University Street, Peoria, Illinois 61604, USA

Analysis of phospholipids was performed using a liquid chromatographic separation with two mass spectrometers in parallel providing electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) data simultaneously from a triple quadrupole instrument and a single quadrupole instrument, respectively. The output from UV-Vis and evaporative light scattering detectors were also acquired by the two mass spectrometers, respectively, for four detectors overall. This arrangement was used to identify and calculate area percents for molecular species of dihydrosphingomyelin (DHS) and sphingomyelin (SPM) in commercially available bovine brain SPM, in human plasma extract and in porcine lens extract. Molecular species of phosphatidylethanolamine and its plasmalogen, and phosphatidylcholine and its plasmalogen were identified and semi-quantitative analysis performed. Commercially available bovine brain SPM was found to contain 11.5% DHS and 88.5% SPM. The only DHS molecular species identified in human plasma was 16:0-DHS, at or below 1% of the sphingolipid content. Porcine lens membranes were found to contain 14.4% DHS and 85.6% SPM. Other findings reported here include: (1) phospholipids were found to undergo dimerization in the electrospray source, giving masses representing combinations of species present. (2) Triacylglycerols gave usable mass spectra under electrospray ionization conditions, as well as under APCI-MS conditions. (3) Triacylglycerols gave ammonium adducts as base peaks in their APCI mass spectra, which reduced fragmentation and increased the proportions of molecular ions. (4) Mass spectra were obtained for phospholipids which underwent both protonation and sodium adduct formation in different chromatographic runs. © **1998 John Wiley & Sons, Ltd. This paper was prepared under the auspices of the US Government and it is therefore not subject to copyright in the US.**

Received 12 December 1997; Revised 7 January 1998; Accepted 10 January 1998
Rapid Commun. Mass Spectrom. **12**, 256–272 (1998)

Dihydrosphingomyelins (DHSs), which differ from sphingomyelins (SPMs) only by the absence of 4,5-unsaturation in the sphingoid backbone, are difficult to analyze because they usually occur chromatographically overlapped and indistinguishable from sphingomyelins. In mixtures, identification of some DHS species has been previously reported using mass spectrometry (MS),[1] but only saturated molecular species of DHS could be identified. Without prior chromatographic separation, only DHS species with no unsaturation in the acyl chains have unique masses, which are two mass units higher than sphingomyelins. Reversed-phase (RP) high performance liquid chromatography (HPLC) has been used successfully for separation of molecular species of SPMs (and coeluting DHSs).[2] However, differentiation of DHSs from SPMs required that the fractions be collected, and then different analyses were performed after derivatization to identify (i) the fatty acyl chain, and (ii) the sphingosine base. This method was very effective at elucidating the identities of the species, but was labor-intensive and time consuming. A similar RP-HPLC separation, but which utilized chemical ionization (CI) MS instead of chemical analysis of the sphingolipids, identified many SPM and DHS species, but the data still resulted in some ambiguities between SPM and DHS species.[3] And again, the method utilized fraction collection followed by analysis.

*Correspondence to: W. C. Byrdwell, FQS, NCAUR, ARS, USDA, 1815 N. University Street, Peoria, IL 61604, USA.

As technology has advanced, online liquid chromatography/mass spectrometric analysis has been employed. Thermospray mass spectrometry following the RP-HPLC separation of sphingomyelin did accomplish the identification of three minor DHS components in bovine brain SPM.[4] This represented a significant advance in the analysis of sphingolipids, but, since this was a RP-HPLC separation, prior phospholipid class separation was still required before molecular species could be separated and identified. A more recent study[5] also demonstrated the application of RP-HPLC/ thermospray MS to sphingomyelins, but in this report no data were given for dihydrosphingomyelins. Fast atom bombardment (mostly of unseparated mixtures), plasmaspray and other ionization methods have also been applied to phospholipids. Many of these applications have been recently reviewed,[6,7] and are not discussed here since they shed no light on the differentiation of DHS from SPM.

One of the most common interface/ionization sources for LC/MS analysis of phospholipids, including sphingomyelins, has become electrospray ionization (ESI).[1,8–10] Because most common phospholipids are naturally charged, they are particularly amenable to ESI-MS. However, most studies employing ESI-MS have either focused on unseparated mixtures, or have not included sphingomyelin in the samples. Thus, overall in the literature, there is a paucity of data which demonstrate the separation and identification of molecular species of DHSs differentiated from SPMs. We recently reported[11] the application of an HPLC separation using an amine column, adapted from a previous method,[12]

CCC 0951–4198/98/050256–17 $17.50

which allowed the partial separation of DHS species from SPM. We then demonstrated[13] that this partial resolution of the mixture of DHS species from SPM species was entirely sufficient to allow differentiation of all DHS and SPM species by mass using either ESI-MS or atmospheric pressure chemical lonization (APCI)-MS. A recent study preceded our report of the use of APCI-MS for phospholipid analysis by presenting data for dioleoyl phosphatidylcholine standard,[8] but SPM was not resolved from other phospholipids, and DHS was not identified, if present. In our investigation, we found that the reason for the partial separation of DHS from SPM was that the long chain (20:0–26:0) DHS species, which eluted first, were separated from the short chain (14:0–18:0) DHS species, and the DHS species eluted before SPM species (which were also separated into long and short chain species). The short chain DHS species remained chromatographically overlapped with the long chain SPM species, but these were easily differentiated by mass. Using this separation, the column allowed separation by class, so that several types of phospholipids were represented, and within each class, long chain species eluted before short chain species. The ESI-MS and APCI-MS data were seen to be complementary to each other, with ESI-MS providing only protonated molecules, while APCI-MS produced diagnostic fragments as well as small amounts of $[M+H]^+$ ions. In order to accomplish both types of ionization, separate runs were performed[13] and the ionization sources were changed between runs. All data were obtained using a single quadrupole instrument.

The extension and improvement of the previously demonstrated methodology[13] are reported here. In the current study, two mass spectrometers utilizing the same eluent stream in parallel are employed. APCI-MS data were obtained using a single quadrupole instrument, while ESI-MS data were obtained using a triple-quadrupole instrument. The triple quadrupole instrument was operated in both full scan and MS/MS modes. Collision-induced dissociation of parent ions was performed on the ESI-MS instrument using an automated procedure which selected the most abundant parent ions in the preceding scans for use as precursors for daughter ion formation. Additional data were obtained from UV-vis and evaporative light scattering detectors. Three types of tissue sphingolipids were examined: human plasma, bovine brain and porcine eye lens. Changes in the chromatographic performance led to some unexpected results.

EXPERIMENTAL

Materials

All solvents except iso-propanol (IPA) and water were of HPLC quality and were purchased from Sigma-Aldrich Chemical (Milwaukee, WI, USA), or EM Science (Cherry Hill, NJ, USA) and were used without further purification. Water was obtained from purification of house de-ionized water using a Millipore purification system. A.C.S. reagent grade isopropanol (IPA) (Fisher Chemical, Fairlawn, NJ, USA) was used until anomalies began, at which time HPLC grade IPA was used. Phospholipid standards were from Avanti Polar Lipids (Alabaster, AL, USA) and were used without further purification. Two sets of standard solutions were made: one based on approximate plasma phospholipid proportions, referred to as the Plasma Model solution, and one based on approximate human lens phospholipid proportions, referred to as the Lens Model solution. The Plasma Model solution contained 19.9% bovine brain SPM, 5.0% egg yolk phosphatidylethanolamine (PE), and 75.1% bovine brain phosphatidylcholine (PC) in methanol. The Lens Model solution contained 69.4% bovine brain SPM, 5.3% bovine brain PC and 25.3% PE, made up from the combination of bovine brain and egg PE, in methanol. Initial solutions were diluted 1:3 in methanol, so the injected concentrations were 8.44 mg/mL and 8.36 mg/mL of the Lens Model and Plasma Model, respectively.

Human plasma was extracted using the procedure of Folch *et al.*[14] Whole plasma lipid extract was used without prior separation of lipid classes. Porcine globes were obtained from a local slaughterhouse and the lenses were excised within 24 hours post-mortem and kept under liquid N_2 until extraction. Five lenses were homogenized using a blade homogenizer and were extracted according to the method of Folch *et al.*[14]

Liquid chromatography

HPLC was performed using an LDC-4100-MS quaternary pump with membrane degasser (Thermo Separation Products, Schaumburg, IL, USA) and an HP 1050 autosampler (Hewlett-Packard, Wilmington, DE, USA). The LC was operated under the control of the single quadrupole instrument. The triple quadrupole instrument was started via a 24 V relay attached to the autosampler to produce a contact closure. The autosampler start signal started data acquisition on both mass spectrometers. The column was an Adsorbosphere NH_2, 25 cm × 4.6 mm, 5 mm particle size (Alltech Associates, Deerfield, IL, USA). A gradient program was used which was composed of: (a) 33% ammonium hydroxide solution/isopropanol (40:60), (b) hexane/isopropanol (40:60), (c) methanol/isopropanol (40:60), and (d) water/isopropanol (40:60). The gradient was as follows: 5% **A** throughout; initial 68% B, 12% C and 15% D held for 10 minutes; ramp to 52% B, 16%C, 27%D at 15 minutes, held until 25 minutes; ramp to 95%C at 45 minutes, held until 60 minutes; recycled to initial conditions at 75 minutes. This resulted in a composition of 0.66% NH_4OH throughout. The effluent flow rate was 0.85 mL/min throughout. 10 μL of each sample was injected.

The column eluent split was accomplished by use of different lengths and diameters of polyether ether ketone (PEEK) and capillary tubing and tee-junctions as follows: a Valco Tee (Valco Instruments, Houston, TX, USA) was attached to the outlet of the column. To the tee side of the junction was attached a length (1.5 ft.) of 0.005 in. i.d. PEEK tubing which went directly to the UV-vis detector, the outlet from the UV-vis detector was connected via 0.005 in. i.d. PEEK tubing to the inlet of the evaporative light scattering detector (ELSD). The straight-through side of the first Valco Tee was attached to a second Valco Tee via a short piece of stainless steel 0.005 in. i.d. tubing, from each of the two other outlets of the second tee, an equal length (3 ft.) of 0.1 mm (=0.0039 in.) i.d. deactivated fused silica capillary tubing was attached via an adapting ferrule; one capillary was attached to the APCI-MS inlet, the other was attached to the ESI-MS inlet.

The UV-vis detector was a Spectroflow 757 (Applied Biosystems, Foster City, CA, USA) operated at a wavelength of 206 nm. The ELSD was a model ELSD MKIII (Varex, Burtonsville, MD, USA). The drift tube was set to 140°C, and the nebulizer gas (nitrogen) was set to 3.0

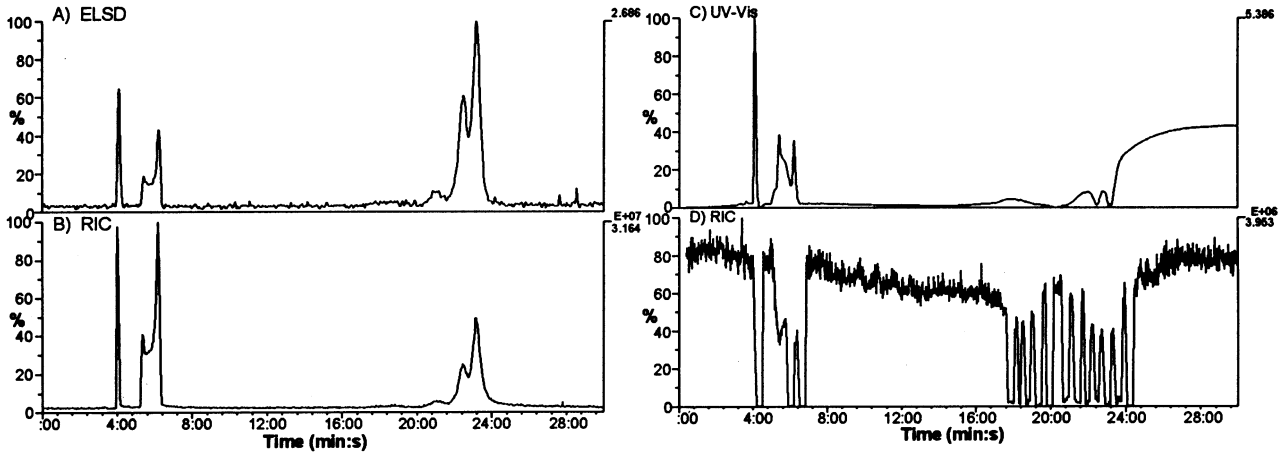

Figure 1. Chromatograms from four detectors of phospholipid standards in approximate proportions to human lens membrane. (a) Evaporative light scattering detector (ELSD) chromatogram, (b) reconstructed, or total, ion chromatogram (RIC) obtained by APCI-MS, (c) UV-Vis detector chromatogram (set to 206 nm) and (d) ESI-MS/MS reconstructed ion chromatogram. ESI-MS/MS obtained in automatic parent/daughter cycling mode. Phospholipid mixture of 25.3% egg/bovine brain phosphatidylethanolamine 5.3% bovine brain phosphatidylcholine and 69.4% bovine brain sphingomyelin. All Chromatograms obtained simultaneously using amine column with gradient as given in experimental.

standard liters per minute and a pressure of 26.6 psi. The UV-vis spectrophotometer was interfaced to the triple quadrupole instrument for data acquisition, while the ELSD was interfaced to the single quadrupole instrument.

Mass Spectrometry

A Finnigan MAT SSQ-710C (Finnigan MAT, San Jose, CA, USA) was used for acquisition of APCI-MS data. A Finnigan MAT TSQ 700 was used for acquisition of ESI-MS data. All conditions used for both APCI and ESI ionization sources were the same as recently described,[13] except the ESI spray voltage was set to 5.5 kV. For acquisition of daughter ion data using ESI-MS, an automatic acquisition program was written using the Instrument Control Language (ICL) which is part of the Finnigan MAT software. A brief description of the acquisition program is given. During automatic acquisition, the collision-induced dissociation (CID) gas was off during full scan mode, and turned on during daughter analysis. With the CID gas off in full scan mode, signal level was increased by a factor of ~100. The CID gas pressure valve was set to 2.0 mtorr, and required several seconds to reach this value, during which MS/MS acquisition began. The full scan parameters were as follows: scan range *m/z* 200 to 950 in 1.0 s; number of parent scans before threshold test = 9; parent offset = −5.5; collision offset = −10.0; daughter offset = −10.0; parent msms correction factor = 70. At the start of each run, the MS/MS threshold value was automatically set to two threshold increments (1 200 000 counts) above the initial baseline. If the total signal detected for masses above *m/z* 600 (signal cutoff value) passed the threshold level, then MS/MS acquisition began. The CID gas valve opened, and the program determined the masses of the three most abundant parents from the preceding scans. The parent masses were sorted high to low (since longer chains eluted first) and the program obtained daughter ion spectra for these masses in sequence. The parameters used for daughter ion analysis were as follows: scan range *m/z* 50 to 'parent mass + 25' in 1.0 s; number of daughter scans for each mass = 5; parent offset = −5.5; collision offset = −35.0; daughter offset = −42.3; parent msms correction factor = 5. All parameters were fully adjustable during a run, most by using soft buttons.

RESULTS AND DISCUSSION

Standards

An ELSD chromatogram and APCI-MS reconstructed ion chromatogram for the Lens Model solution are shown in Fig. 1(a) and 1(b). AUV-Vis chromatogram and ESI-MS/MS reconstructed ion chromatogram for the Lens Model Solution are shown in Fig. 1(c) and 1(d). Figure 1(d) demonstrates the cycling between full scan and daughter ion modes performed by the automatic acquisition program. Similarly, Fig. 2(a) and 2(b) represent ELSD and APCI-MS chromatograms obtained for the phospholipid mixture made in the approximate proportions of human plasma, while Fig. 2(c) and 2(d) represent the corresponding UV-Vis and ESI-MS chromatograms for the Plasma Model solution. Figure 2(d) again demonstrates the automatic cycling between full scan and daughter ion modes performed by the triple quadrupole instrument. The four detectors showed different sensitivities for the various classes of phospholipids and exhibited different susceptibilities to background changes caused by the gradient run, discussed below. Although Fig 1(d) and 2(d) exhibit a high background level in the reconstructed ion chromatograms (RIC) obtained by ESI-MS, this arose primarily from column bleed at *m/z* 425, so the upper mass region showed a much better signal to noise ratio, demonstrated in Fig. 3. Although the thrust of the present report is the demonstration of the utility of the dual mass spectrometer system for phospholipid molecular species identification, especially of dihydrosphingomyelins, extensive detail is given for all phospholipids studied.

APCI mass spectra of the three commercially available phospholipids in the model mixtures are shown in Fig. 4. The first phospholipid which eluted from the column was PE (PE plus PE plasmalogen). Figure 1 shows that these species eluted in two unresolved peaks. Figure 4(A) and 4(B) represent average mass spectra across the first and second unresolved peaks, respectively. Both spectra show that these species produced mostly protonated molecules,

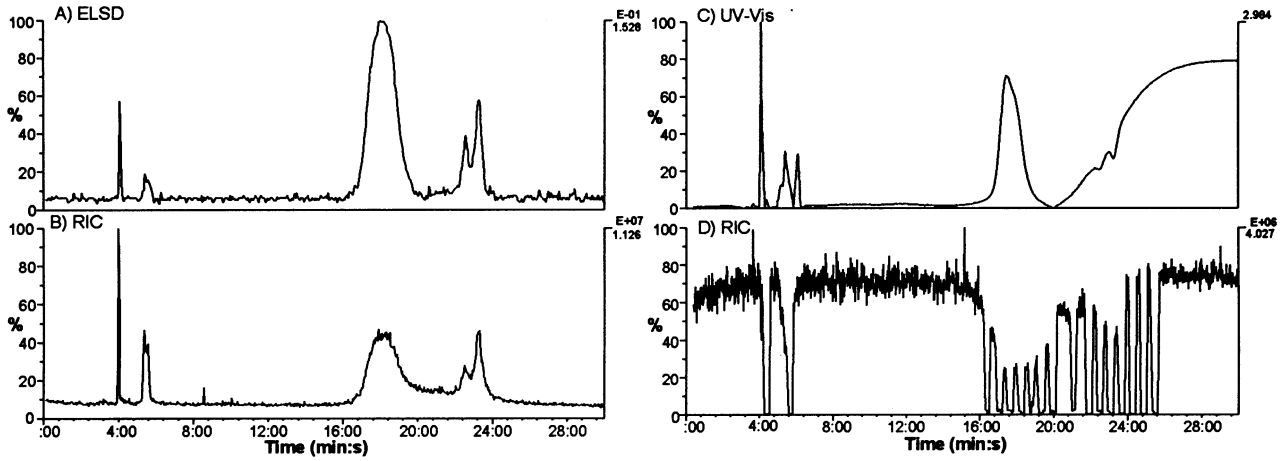

Figure 2. Chromatograms from four detectors of phospholipid standards in approximate proportions to human plasma: (a) ELSD chromatogram, (b) RIC obtained by APCI-MS, (c) UV-Vis detector chromatogram and (d) ESI-MS/MS reconstructed ion chromatogram. ESI-MS/MS obtained in automatic parent/daughter cycling mode. Phospholipid mixture of 5.0% egg PE, 75.1% bovine brain PC and 19.9% bovine brain SPM.

with little fragmentation. The protonated molecules allowed the identification of the species in the first peak as PE plasmalogens, while the second peak contained primarily PE, with some PE plasmalogen. The PE plasmalogen species exhibited less fragmentation than the PE species. The lack of fragmentation of both of these phosphoethanolamines is in contrast to the APCI-MS spectra previously shown[13] and to spectra shown below for PC. The only major difference in the starting conditions between this study and that previously reported[13] was that the NH_4OH was not premixed with the solvents in this study, but was kept as a separate solvent channel and incorporated into the effluent in the mixing valve. Whether such a small difference can account for the unusual phenomenon observed during this study will require further investigation. Despite the differences in the abundances, the fragments which the PE did produce were primarily diacylglycerol fragments identical to those reported earlier for PC and shown below, and are the same as those produced by triacylglycerols.[15] The diacylglycerol fragments confirmed the identification of the molecular species, while the difference of *m/z* 141 between the fragments and the corresponding protonated molecules confirmed the presence of the phosphoethanolamine head group. The masses given for the loss of head group fragments represent the fully protonated head groups. For PE molecules, this means one proton at the phosphate oxygen, and one proton on the nitrogen to form the ionized amine. For PC molecules, the masses include one proton on the phosphate oxygen. When head group fragments were observed in daughter ion spectra, they included an additional hydrogen on the phosphate oxygen which cleaved from the glycerol backbone. Extracted ion chromatograms (EICs) taken from the RIC in Fig. 1(b) are shown in Fig. 5. Figure 5(a) revealed that, using this column, 24:*n* and 22:*n* PE plasmalogen species eluted in the first peak between 5 and 6 1/2 minutes, 20:*n* species eluted between the two peaks, and 18:*n* and 16:*n* species eluted in the second peak. All PE species eluted in the second peak in this sample. The area percentage of the molecular species in egg PE (from the Human Plasma Model solution), calculated from the areas under peaks in EICs of the masses of protonated molecules obtained by APCI-MS, is given in Table 1. The EICs in Fig. 5(a) depict the seven most abundant molecular species of PE plasmalogen and PE in the Lens Model solution. The elution of these species was affected by the relative proportions of the two classes, so the appearance of the overall peak in PE from egg was narrower, and there was less difference in the elution of PE plasmalogen versus PE. All PE plasmalogen molecular masses were calculated based on a 16:1 ether-linked fatty chain, the most common fatty chain for plasmalogens. Daughter ion spectra obtained from ESI-MS/MS did indicate the possible presence of an 18:1 plasmalogen backbone, but further discussion is beyond the scope of this initial report.

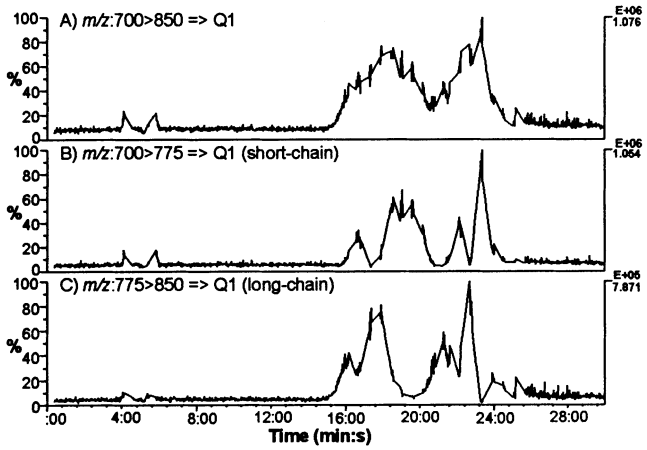

Figure 3. Filtered ESI-MS/MS extracted ion chromatograms (EICs). All chromatograms filtered to display only scans obtained in full scan mode. (a) Mass region from 700 to 850 Da which contained most phospholipid protonated molecular ion masses, (b) lower mass region from 700 to 775 Da which included masses of short-chain (14:0–18:0) protonated molecular ions. (c) Upper mass region from 775 to 850 Da which included long-chain (20:0–26:0) protonated molecular ions.

The PE plasmalogen/PE peak is seen to exhibit very different responses from the four detectors used. Figure 1(b) illustrates that this peak was larger than expected in the APCI-MS chromatogram based on its known concentration in the Lens Model solution, and the quantitative analysis from APCI-MS data revealed that the sum of the areas of the molecular species accounted for 58.0% of the total area for all phospholipids, though it was known to be present at 25.3% by weight. In the Plasma Model solution, which

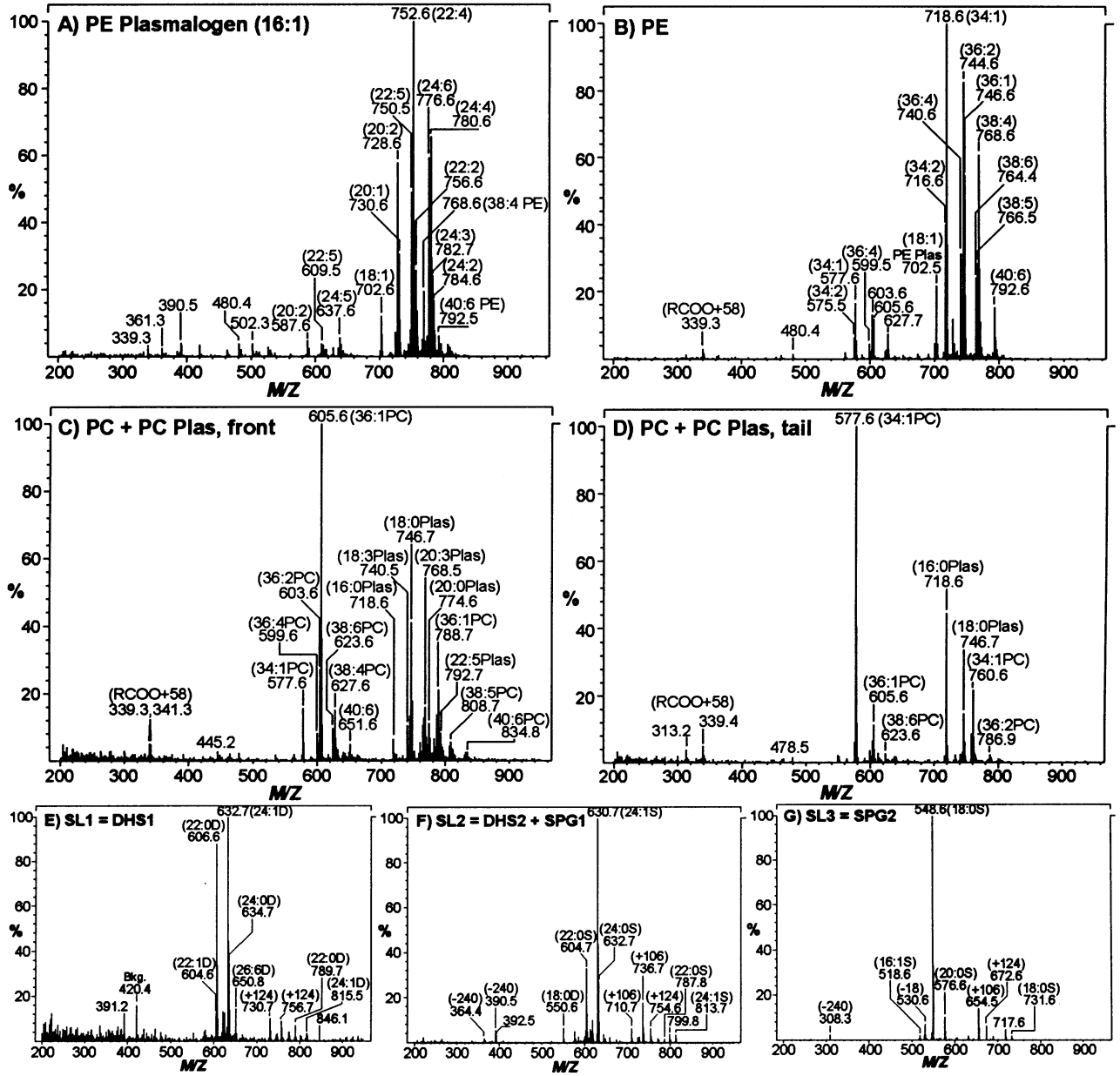

Figure 4. Mass spectra obtained using APCI-MS of phospholipid standards. Bovine brain/egg PE mixture shown in Fig. 1: (a) front peak eluted between 5.25 to 5.5 min, (b) second PE peak eluted at 6 to 6.5 min (see also Fig. 5(a)). Bovine brain PC and PC plas shown in Fig. 2: (c) front of PC peak eluted from 17 to 17.5 min, (d) section of PC peak eluted from 18.5 to 19 min (see also Fig. 5(b)). Bovine brain SPM shown in Fig. 1: (e) first sphingolipid peak (SL1) eluted near 21 min. Peak composed of long chain dihydrosphingomyelin (DHS, or D). (f) Second sphingolipid peak (SL2) eluted from 22 to 22.75 min. Peak composed of short-chain DHS and long-chain SPM. (g) Third sphingolipid peak (SL3) eluted from 22.75 to 23.75 min. Peak composed of short-chain SPM (see also Fig. 5(c)).

contained 5.0% PE by weight, the area percentage analysis by APCI-MS gave a composition of 11.7%. Thus, in both samples, the PE gave a percent composition which was near 2.3 times higher than the known composition. Within this class, however, the quantitation appeared to be more reliable. The Lens Model solution, which contained mostly bovine brain PE, showed 48.7% plasmalogen and 51.3% PE, which is in agreement with the nearly 50% plasmalogen indicated by the supplier. The egg PE in the Plasma Model solution gave area percent compositions of 12.7% plasmalogen and 87.3% PE. The Avanti Polar Lipids catalog reported that, using their HPLC method, they found the PE to be $>$ 99% pure PE. This demonstrates the difficulty in separation of molecular species of plasmalogens apart from the parent class. The UV-Vis detector can be seen in Fig. 1(c) to give more response from polyunsaturated plasmalogens at the front of the peak than normal PE at the back (see the elution demonstrated for this sample in Fig. 5(a). The great disparity between the diacyl phospholipids versus the sphingolipids, as well as the background drift, made quantitative analysis using the UV-Vis detector fruitless. The ELSD detector proved to be better at yielding percentage composition data. The chromatogram in Fig. 1(a) showed 15.8% PE, and that in Fig. 2(a) showed 4.8% PE. However, use of the mass spectrometer's data system for integration of ELSD chromatograms, although excellent for mass chromatogram integration, produced inadequate resolution in the area units of ELSD chromatograms.

Based on the spectra obtained for the PE plasmalogen and PE species, we can now conclusively identify the uni-

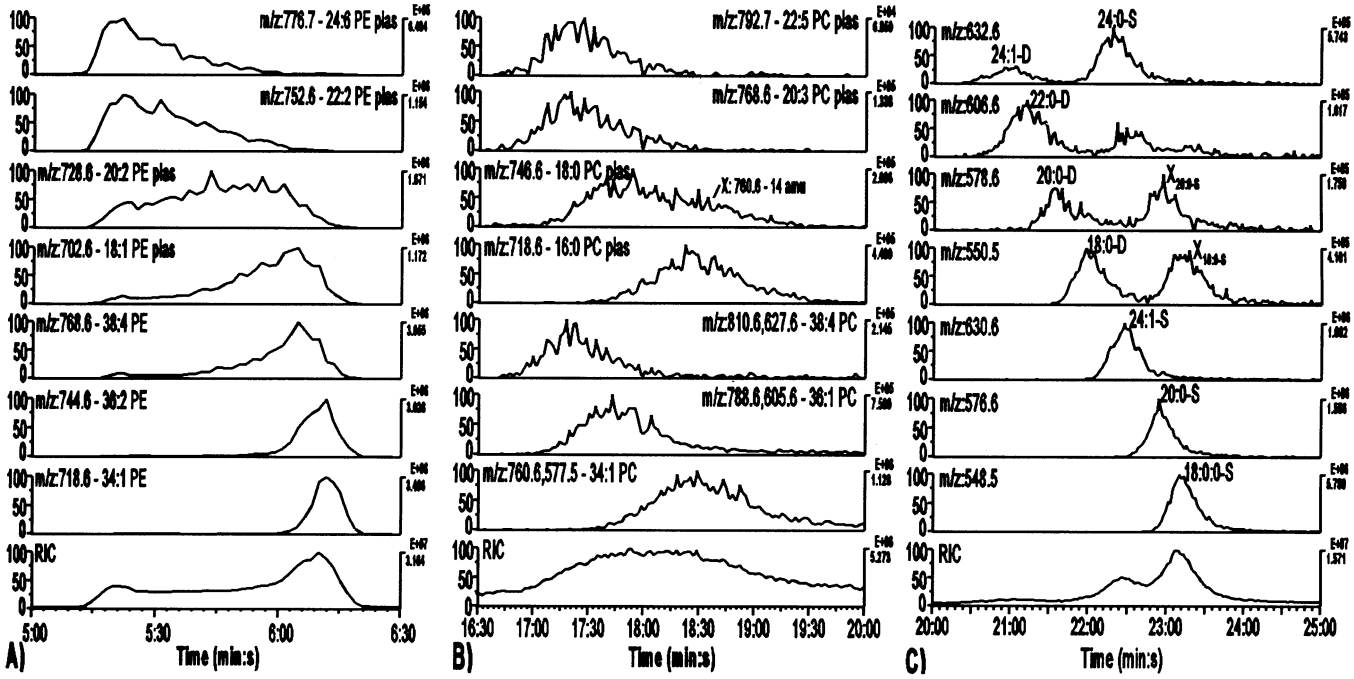

Figure 5. Extracted ion chromatograms obtained under APCI-MS conditions. (a) Phosphatidyl ethanolamine peak seen in Fig. 1(b). Long-chain PE plasmalogen (plas) eluted first, followed by shorter-chain PE plas and all PE species. (b) Bovine brain PC and PC plas peak seen in Fig. 2(b). Long chain species eluted before short chain species. (c) Bovine brain SPM peak seen in Fig. 1(b). Long-chain DHS eluted as the first peak, short-chain DHS and long-chain SPM eluted as the second peak, and short-chain SPM eluted as the third peak.

dentified phospholipid which was reported earlier[13] to have structural features similar to phosphatidyl ethanolamine plasmalogen as definitely being composed of PE plasmalogen, as well as some PE. The mass spectrum shown earlier may now be interpreted to show that the peak arose mostly from PE plasmalogen having 20:1 (*m/z* 589.6, 704.6) and 18:0 (*m/z* 563, 730.6) acyl chains. These identifications were not given conclusively in the previous report because earlier results[16] from a commercially available PE standard had exhibited contradictory chromatographic behavior. However, in the first report, the eluent did not contain any NH_4OH which was added later as electrolyte for the ESI-MS. Such a difference can change the ionization state of the phospholipid, and cause a change in the chromatographic behavior on the amine column. This possibility is supported by the similarity between the peak broadening for PE plasmalogen seen in the first report[16] to that shown in Fig. 5(a).

Bovine brain phosphatidyl choline eluted as a broad peak, with long-chain species eluted before short-chain species, as shown in Fig. 3 and 5(b). Most PC plasmalogens eluted only slightly before the similar normal PC, but the short-chain species eluted before the long-chain species, and these ran together to form one broad peak, which appeared homogeneous. The ESI-MS data clearly showed the presence of PC plasmalogen along with PC. While easily identified by ESI-MS, quantitation was performed using APCI-MS fragments. Since ESI-MS is more sensitive than APCI-MS, more species could be qualitatively identified using ESI than were quantified using APCI, such as 24:*n* plasmalogen species and 40:*n* PC species which were observed in EICs obtained by ESI-MS, but not quantified. Using APCI-MS data to give area percent results, it was found that bovine brain PC in the Plasma Model solution gave a total area percent of 29.0% PC plasmalogen and 71.0% for diacyl PC. Figure 4(c) and 4(d) show that abundant protonated molecules were apparent in the mass spectra of PC plasmalogen and PC. As with the PE plasmalogen, the PC plasmalogen did not exhibit significant fragmentation, but rather formed protonated molecules. The diacyl PC, conversely, formed abundant diacylglycerol fragment ions. As with PE, these fragments were used to confirm the identities of the acyl chains and the difference of *m/z* 183 between these fragments and the corresponding protonated molecules confirmed the presence of the phosphocholine head group (as did the daughter ion spectra obtained by ESI-MS/MS). The percent composition of the molecular species of bovine brain PC, determined by APCI-MS, is given in Table 1. Area percent calculations were performed using the protonated molecules for PC plasmalogen, while the areas under the peaks for the diacylglycerol fragments and the protonated molecules were summed to determine the total areas for PC, similar to the method used for triacylglycerol quantification. The phosphocholines, as a class, showed less response than expected based on the percent by weight. The Plasma Model was calculated from APCI-MS data to contain 1.3%, while the Lens Model was calculated to contain 62.5%. These solutions were known to contain 5.3% and 75.1% by weight, respectively. The compositions calculated from the ELSD chromatograms were 5.3% and 76.2%, respectively, in good agreement with expected results. It was fortuitous that, in the cases of both PE and PC classes, the plasmalogen molecular masses fell in between the parent class molecular masses. This allowed facile differentiation of the species. Only the most highly unsaturated plasmalogen species (6 or more sites of unsaturation) had masses the same as those of saturated parent species. The highly unsaturated PE plasmalogen species, if they occurred at all, were chromatographically separated from their isobaric parents. However, in the case

Table 1. Phospholipid molecular species area percent for commercial phospholipid standards

PE from Egg (Head Group $+2H)^+$ = 142 amu						PC from Bovine Brain (Head Group $+H)^+$ = 184 amu						SPM from Bovine Brain (Head Group $+H)^+$ = 184 amu					
PE plas	$[M+2H]^+$	%	PE	$[M+2H]^+$	%	PC plas	$[M+H]^+$	%	PC	$[M+H]^+$	%	DHS	$[M+H]^+$	%	SPM	$[M+H]^+$	%
24:0	788.7	0.4	40:0	804.7	0.2	24:0	830.7	0.5	40:0	846.7	0.4	26:0	845.8	0.0	26:0	843.8	0.2
24:1	786.7	1.9	40:1	802.7	0.1	24:1	828.7	0.3	40:1	844.7	0.2	26:1	843.8	1.1	26:1	841.7	0.9
24:2	784.7	2.3	40:2	800.6	0.0	24:2	826.7	0.3	40:2	842.7	0.2	26:2	841.7	0.0	26:2	839.7	0.1
24:3	782.7	3.7	40:3	796.6	0.1	24:3	824.7	0.4	40:3	840.7	0.3	26:3	839.7	0.0	26:3	837.7	0.0
24:4	780.7	5.0	40:4	796.6	0.7	24:4	822.7	0.4	40:4	838.7	0.6	26:5	835.7	1.0	26:4	835.7	0.0
24:5	778.7	6.0	40:5	794.6	2.5	24:5	820.7	0.6	40:5	836.6	0.7	24:0	817.7	10.5	26:5	833.7	0.1
22:0	760.6	0.6	40:6	792.6	1.4	22:0	802.7	0.3	40:6	834.6	1.9	24:1	815.7	16.6	26:6	831.7	0.1
22:1	758.6	2.0	40:7	790.6	0.3	22:1	800.7	0.4	40:7	832.6	1.0	24:2	813.7	1.0	24:0	815.7	5.7
22:2	756.6	1.8	38:0	776.6	0.5	22:2	798.7	0.5	38:0	818.7	0.4	24:3	811.7	0.3	24:1	813.7	14.5
22:3	754.6	3.7	38:1	774.6	0.2	22:3	796.7	2.0	38:1	816.7	0.6	24:4	809.7	0.2	24:2	811.7	0.8
22:4	752.6	6.6	38:2	772.6	0.3	22:4	794.6	1.8	38:2	814.7	0.9	24:5	807.7	1.6	24:3	809.7	0.1
22:5	750.6	7.5	38:3	770.6	3.7	22:5	792.6	3.7	38:3	812.6	1.0	22:0	789.7	14.7	24:4	807.7	0.1
20:0	732.6	7.5	38:4	768.6	19.9	20:0	774.7	4.1	38:4	810.6	3.9	22:1	787.7	3.6	24:5	805.7	0.1
20:1	730.6	15.5	38:5	766.6	6.1	20:1	772.6	2.2	38:5	808.6	2.4	22:2	785.7	0.0	22:0	787.7	5.4
20:2	728.6	11.8	38:6	764.5	3.0	20:2	770.6	2.0	38:6	806.6	2.9	22:5	779.7	1.2	22:1	785.7	1.9
20:3	726.6	2.4	38:7	762.5	0.2	20:3	768.6	7.5	38:7	804.6	0.6	20:0	761.7	9.9	22:2	783.7	0.0
20:4	724.6	1.3	36:0	748.6	1.5	20:4	766.6	3.8	36:0	790.7	2.4	20:1	759.7	0.6	22:3	781.7	0.0
18:0	704.6	4.8	36:1	746.6	11.8	20:5	764.6	5.1	36:1	788.6	16.3	20:5	751.6	3.5	22:4	779.7	0.0
18:1	702.6	10.8	36:2	744.6	14.3	18:0	746.6	19.7	36:2	786.6	6.9	18:0	733.6	30.9	20:0	759.7	11.2
18:2	700.6	3.9	36:3	742.6	3.0	18:1	744.6	6.5	36:3	784.6	1.7	18:1	731.6	1.4	20:1	757.6	0.6
18:3	698.5	0.0	36:4	740.5	6.2	18:2	742.6	1.6	36:4	782.6	3.6	18:2	729.6	0.4	20:2	755.6	0.2
16:0	676.6	0.4	36:5	738.5	0.2	18:3	740.6	5.0	36:5	780.6	0.6	18:3	727.6	0.4	20:3	753.6	0.0
16:1	674.5	0.0	36:6	736.5	0.2	16:0	718.6	27.2	36:6	778.6	0.3	16:0	705.6	1.3	20:4	751.6	0.0
			36:7	734.5	0.0	16:1	716.6	1.8	36:7	776.6	0.6	16:1	703.6	0.0	20:5	749.6	0.5
Sum		100.0	34:0	720.6	1.3	14:0	690.6	2.2	34:0	762.6	5.1				18:0	731.6	52.0
			34:1	718.6	13.9				34:1	760.6	31.8	Sum		100.	18:1	729.6	2.4
			34:2	716.5	7.4	Sum		99.9	34:2	758.6	4.3				18:2	727.6	0.8
			34:3	714.5	0.3				34:3	756.6	0.3				18:3	725.6	0.3
			32:0	692.5	0.0				32:0	734.6	3.7				16:0	703.6	2.0
			32:1	690.5	0.2				32:1	732.6	3.4				16:1	701.6	0.0
			32:2	688.5	0.2				32:2	730.6	0.4				14:0	675.6	0.1
			32:3	686.5	0.0				30:0	706.6	0.2						
															Sum		100.1
			Sum		99.7				Sum		99.6						
Percent of phosphoethanolamines						Percent of phosphocholines						Percent of sphingolipids					
PE plas = 12.7 PE = 87.3						PC plas = 29.0 PC = 71.0						DHS = 11.5 SPM = 88.5					

of PC plasmalogen, only partial chromatographic separation was achieved and overlap occurred between these highly unsaturated plasmalogens with their parent compounds, if they were both present. In the cases of overlap (e.g. 22:6 PC plas = 36:0 PC), the ESI-MS chromatogram was used to make the assignment (area assigned to 36:0 PC). For most species this was not a concern and analysis was straightforward. Another potentially ambiguous overlap of masses which required careful interpretation was that, for PC species, there was a small percentage of fragment formation which occurred from the replacement of a methyl group from the choline head group with a hydrogen atom, for a mass difference of 14 Da. This presented the possibility that a saturated PC plasmalogen could be confused with a PC parent with one more site of unsaturation. In these cases, although the species were isobaric, the chromatography was sufficient to partially differentiate the contribution to a plasmalogen peak from the parent, as shown in Fig. 5(b). However, because resolution of PC plasmalogen species from PC species was not complete, it is possible that some area attributed to PC plasmalogen species arose from demethylated PC species. Similarly, a protonated PC molecule in which all three choline methyl groups have been replaced by hydrogen atoms in the APCI source would have the same mass as a plasmalogen having two fewer carbons (e.g. (38:1 PC - 42) = 20:0 PC plas, *m/z* 774.7). The possibility exists that the area attributed to 20:0 PC plasmalogen could arise from the tri-demethylated parent PC. However, if this were the case all PC protonated molecules would give this fragment without regard to the identities of the fatty acyl chains, so the PC plasmalogen species would be in a constant proportion to the PC parent ions. This was not observed in the data. For instance, the ratio of the 20:0 PC plasmalogen percentage to the percentage of 38:1 PC is 7.2, while the ratio of 20:4 PC plas to 38:5 PC is 1.6. Most importantly, the ESI-MS/MS daughter ion spectra of 20:0 PC plasmalogen (*m/z* 774.7) and 18:0 PC plasmalogen (*m/z* 746.6) exhibited base peaks at *m/z* 184, clearly indicating the intact (not demethylated) choline head group.

In the chromatograms in Figs 1 and 2, the region over which SPM eluted (from 20 –25 minutes) showed three peaks, a small leading peak and two larger peaks. As was the case with human lens membranes, the first peak in this region represented the presence of long-chain dihydrosphingomyelins. The second peak represented long-chain sphingomyelins and short-chain dihydrosphingomyelins, and the third peak represented short-chain sphingomyelins.

The presence of these species is demonstrated in Fig. 3 which represents the filtering of the ESI-MS chromatogram in Fig. 2(d) to show only scans obtained in full scan mode. The mass region from *m/z* 700 to 850 (Fig. 3(a)), which contained all protonated molecules for PC, DHS and SPM species, was subdivided into regions from *m/z* 700 to 775 (Fig. 3(b)), which contained masses of short-chain species, and *m/z* 775 to 850 (Fig. 3(c)), which contained masses of long-chain species. Between 20 and 25 minutes in the chromatograms in Fig. 3 two peaks in the short-chain mass region (Fig. 3(b)) and two peaks in the long-chain mass region (Fig. 3(c)) may clearly be seen. The first peak in this region in each chromatogram represents DHS, while the second peak represents SPM. Figure 3 also demonstrates the elution of long-chain and short chain PC and PC plasmalogen between 15 and 20 minutes. Figure 5(c) shows EICs for the most abundant DHS and SPM species. Since the sample contained about 8 times as much SPM as DHS, the contributions from isotope peaks of SPM may be seen in chromatograms of completely saturated DHS species. Also, unsaturated DHS species were isobaric with SPM species having one less site of unsaturation in the amide-linked acyl chain (e.g., 24:1-DHS = 24:0-SPM). However, the DHS species were completely chromatographically separated from their isobaric SPM counterparts so no ambiguity arose. The integrated areas under extracted ion chromatograms indicated that bovine brain sphingomyelin was composed of 11.5% DHS and 88.5% SPM. The molecular species compositions of these sphingolipids is given in Table 1. The major species in DHS and SPM were similar, with 18:0, 24:1, 22:0 and 20:0 species predominant. However, there were substantial differences in the relative proportions of species such as 22:0 and 18:0. Because DHS has previously been difficult to identify, the importance of such compositional differences between these sphingolipids has not been addressed at all. It has been found that DHS leads to a more ordered membrane structure,[17] but the importance of this finding, and the impact that different molecular species have on membrane order, is still to be determined. It is interesting to note that egg yolk SPM was found to contain 8.2% DHS, but this was composed only of short-chain species, having none of the longer-chain species shown to be present in bovine brain SPM.

Mass spectra of the first, second and third sphingolipid peaks are shown in Fig. 4(e)–(g) respectively. The mass spectra presented here are similar to the spectra for these compounds which were previously reported,[13] but there are differences. First is the presence in these spectra of adducts formed from the addition of 106 Da to the diacylglycerol fragments, with another adduct at 18 Da more, or 124 Da. As mentioned above, the primary difference in the LC/MS method applied previously versus that used for this study was that the NH_4OH was not pre-mixed with the solvents in this chromatographic system. Whether this difference produced the undesirable side effect of adduct formation will have to be further explored using APCI-MS/MS. Another difference in the spectra was the loss of 14 Da from the protonated molecules, assumed to be replacement of a methyl moiety from the choline head group by a hydrogen atom, as mentioned above for PC. This mechanism appeared to occur to a larger extent with sphingolipids than with PC. The last difference observed in these spectra was a fragment arising from the loss of 18 Da from the sphingoid fragment. This occurred from loss of the hydroxy group in the form of dehydration. Loss of the hydroxy group by dehydration has been reported as the primary fragmentation pathway for hydroxy-containing triacylglycerols during APCI-MS.[18] However, the primary fragments in these mass spectra still definitively identified the DHS and SPM molecular species, and were used for semi-quantitative analysis.

The chromatographic separation using the amine column is the key difference which allowed us to report the presence of numerous intact DHS species in bovine brain and in egg yolk. Since all species eluted over a short time period, it was easier to identify species present at low levels. For example, 26:5-DHS, present as 1.0% of DHS, represented 67.3 ng, or 80.6 pmol, based on the 10 μL of sample injected. We were unable, however, to confirm the presence of a sphingolipid containing a 20:1 sphingoid backbone which has been reported in bovine brain. Jungalwala *et al.* reported[2] the identification of a 20:1 sphingolipid as the biphenylcarbonyl derivative of one fraction from a reversed-phase separation. The 20:1 sphingolipid was reported to occur only with an 18:0 fatty acid. This would have a mass isobaric with 20:0-SPM, so the sphingoid fragment would appear at *m/z* 576.6. Since 18:0 and 18:1 sphingolipids are separated so effectively using the amine column, if the 20:1 were present, it would be expected to be differentiable. The only peak apparent in the mass chromatogram for *m/z* 576.6 in Fig. 5(C) is that for 18:0-SPM. On the other hand, we were able to identify more polyunsaturated DHS and SPM species than have been reported, especially species containing 5 sites of unsaturation. These give peaks in their EICs which have longer retention times than their saturated homologs. The chromatographic data provide valuable confirmation of the mass data to support the presence of these species.

Most daughter ion spectra of phosphocholine-containing phospholipids (PC, PC plas, DHS and SPM) obtained by positive ion ESI-MS/MS showed only a base peak at *m/z* 184 arising from the intact fully protonated choline head group, and some fragments of the head group, similar to the head group region in the daughter spectrum shown for Lyso-PC, below. In some spectra, small amounts of useful fragmentation were observed, but most often the daughter spectra were simple and showed only phosphocholine fragments. Plasmalogens produced more useful spectra than the diacylglycerol compounds. Plasmalogens more often gave fragments useful for identification of the acyl chains along with the head group fragments. Useful fragmentation was only achieved as the CID gas approached 2.0 mtorr. Before the collision cell pressure rose to this level, only head group spectra were observed. Sphingolipids produced virtually only head group fragments in daughter ion spectra. Given the large number of experiments possible using dual parallel mass spectrometers, use of negative ion MS/MS mode for identification of $RCOO^-$ fragments is an obvious extension of the demonstrated methodology.

Human plasma extract

Figure 6 shows ELSD and APCI-MS chromatograms of human plasma whole extract. Because of the magnitude of the peak eluted between 3 and 4 min, chromatograms with expanded vertical scale are also given. Figure 6(e) and 6(f) show the UV-Vis and ESI-MS chromatograms obtained during the same run. Figure 7(e) shows the ESI-MS chromatogram for the mass range between *m/z* 700 and 850, the range containing the protonated molecules of PC, PC plas, DHS and SPM species. The chromatograms in Fig.

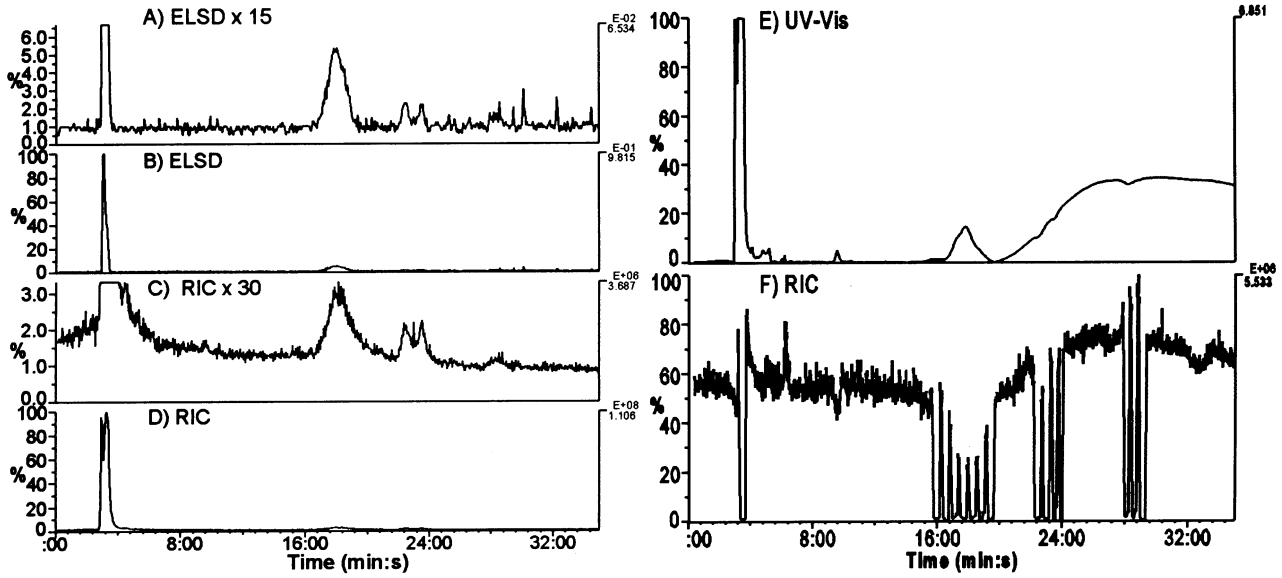

Figure 6. Chromatograms of human plasma whole extract by four detectors. (a) Expanded ELSD chromatogram, (b) raw ELSD chromatogram, (c) expanded RIC by APCI-MS, (d) raw RIC by APCI-MS, (e) UV-Vis chromatogram, (f) RIC by ESI-MS/MS, ESI-MS/MS obtained in automatic parent/daughter cycling mode.

7(a)–(d) represents chromatograms which have been filtered to show only data obtained in full scan mode. For the purposes of this study, the most important observation arising from these data is that in the time between 20 and 25 minutes, when DHS and SPM eluted, only one peak is seen in the low mass range (Fig. 7(b)), associated with short-chain DHS and SPM species, and only one peak is seen in the higher mass range (Fig. 7(c)), associated with long-chain DHS and SPM species. This indicates that human plasma contains virtually no dihydrosphingomyelin. Examination

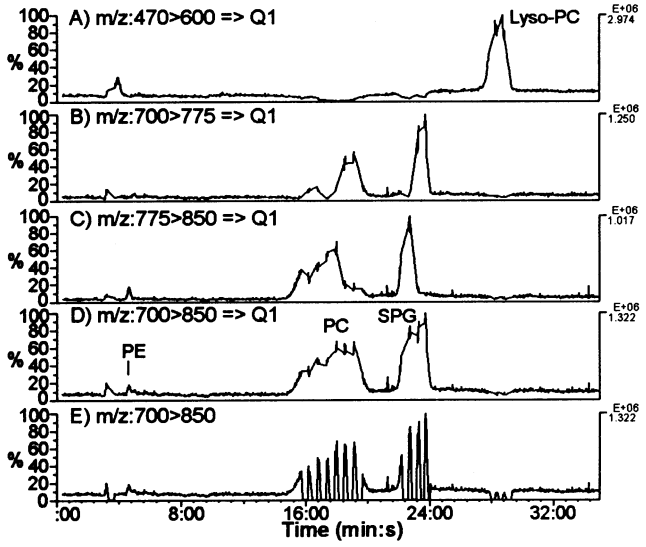

Figure 7. Extracted ion chromatograms obtained by ESI-MS/MS and filtered to display only scans obtained in full scan (parent) mode. (a) Mass region from 470 to 600 Da which contained the protonated molecular ions of lyso-phospholipids, as well as fragments of diacyl and acyl-alkenyl phospholipids. (b) Mass region from 700 to 775 Da which contains the protonated molecular ion short acyl chain species. (c) Mass region from 775 to 850 Da which contains the long acyl chain molecular species. (d) Mass region from 700 to 850 Da which contains the protonated molecular ion masses for short and long-chain diacyl, acyl-alkenyl, and sphingolipid species. (e) Unfiltered, raw ESI-MS/MS chromatogram showing the mass region from 700 to 850 Da.

of EICs for DHS species indicated that a small amount of 16:0-DHS could be identified, and this was at a very low level. Semi-quantitative analysis, presented in Table 2, shows the area percentages of the molecular species of SPM and indicates that 16:0-DHS was present at a level of about 1% of total sphingolipid. Small amounts of other DHS species may be present which are below the detection limit in the whole extract sample. Nevertheless, because of the clear separation of the isobaric DHS and SPM species, this column allowed unambiguous confirmation of the general lack of DHS in plasma, just as it indicated its presence in other samples. Previously, failure to identify DHS did not mean it was not present, as has been shown using the amine column for analysis of numerous species of DHS which were not previously identified. Using this method, chromatographic and mass data both support the lack of DHS in plasma.

As in brain PC, Fig. 7(b) and (c) indicate the presence of long and short chain PC plasmalogen and PC species. The area percent composition of these species is given in Table 2. In Fig. 7(a), another peak may be seen to elute near 29 minutes, having lower masses than the other phospholipids discussed thus far. Daughter ion spectra obtained across this peak allowed its identification. ESI mass spectra of the parent compound and an averaged daughter ion spectrum are shown in Fig. 8. These data clearly identify this phospholipid as lyso-phosphatidylcholine. The full scan spectrum provided protonated molecules from $m/z \sim 500$ to 600, while the daughter spectra showed a peak at m/z 184 (Fig. 8(b)) which identified it as a phosphocholine, and a peak at m/z 339 which arose from loss of the phosphocholine head group, and indicated the acyl-linked fatty chain. Because of the structure of lyso-compounds, this fragment had the same mass as $[RCOO+58]^+$ ions observed for diacylphospholipids.

The most abundant lipids in human plasma were cholesterol, cholesterol-related compounds (cholesterol esters, etc.) and triacylglycerols (TAGs). These coeluted in the large peak at short retention times in Fig. 6. Cholesterol eluted first, followed by cholesterol esters, with

Table 2. Phospholipid molecular species area percent of two human plasma phospholipids

		PC (Head Group $+H)^+$ = 184 amu						SPM (Head Group $+H)^+$ = 184 amu			
PC plas	$[M+H]^+$	%	PC	$[M+H]^+$	%	DHS	$[M+H]^+$	%	SPM	$[M+H]^+$	%
22:0	802.7	0.0	38:0	818.7	0.0	16:0	705.6	100.0	24:0	815.7	6.3
22:1	800.7	0.0	38:1	816.7	0.1				24:1	813.7	16.0
22:2	798.7	0.8	38:2	814.7	0.8				24:2	811.7	8.3
22:3	796.7	1.5	38:3	812.6	3.5				24:3	809.7	0.5
22:4	794.6	1.0	38:4	810.6	5.3				24:4	807.7	0.0
22:5	792.6	1.3	38:5	808.6	3.1				24:5	805.7	0.3
22:6	790.6	0.7	38:6	806.6	2.4				24:6	803.7	0.4
20:0	774.7	0.9	38:7	804.6	0.5				22:0	787.7	7.8
20:1	772.6	4.3	36:0	790.7	0.0				22:1	785.7	6.3
20:2	770.6	5.3	36:1	788.6	3.8				22:2	783.7	1.4
20:3	768.6	6.9	36:2	786.6	14.9				22:3	781.7	0.6
20:4	766.6	3.2	36:3	784.6	11.1				20:0	759.7	4.1
20:5	764.6	3.3	36:4	782.6	8.0				20:1	757.6	2.0
18:0	746.6	6.1	36:5	780.6	1.1				20:2	755.6	0.3
18:1	744.6	16.9	36:6	778.6	0.2				20:5	749.6	0.2
18:2	742.6	11.0	36:7	776.6	0.0				20:6	747.6	1.0
18:3	740.6	8.8	34:0	762.6	1.2				18:0	731.6	4.8
16:0	718.6	11.1	34:1	760.6	16.2				18:1	729.6	3.1
16:1	716.6	16.7	34:2	758.6	22.9				18:2	727.6	0.5
14:0	690.6	0.3	34:3	756.6	2.5				16:0	703.6	28.8
			32:0	734.6	0.0				16:1	701.6	4.4
Sum		100.1	32:1	732.6	1.5				16:2	699.6	0.5
			32:2	730.6	0.9				14:0	675.6	2.2
			30:0	706.6	0.0						
									Sum		99.8
			Sum		100.0						
		Percent of phosphocholines						Percent of sphingolipids			
		PC plas = 32.4 PC = 67.6						DHS = 1.1 SPM = 98.9			

TAGs coeluted over the first two-thirds of the large peak. APCI mass spectra for these compounds are shown in Fig. 9. Figure 9(b) showed unexpected results obtained for TAGs. Instead of the protonated molecules and diacylglycerol fragments normally observed for TAGs using APCI–MS,[15,19,20] ammonium adducts were observed, instead. The ammonium adducts gave much more molecular ion intensity than is normally obtained for TAGs with few sites of unsaturation. The formation of ammonium adducts of TAGs is not without precedent. Recently, Laakso and Manninen reported[21] that $[M+18]^+$ adducts could be formed when ammonium hydroxide in methanol was used as a reagent by running the sheath gas through this solution. For the work presented here, as for the previous work,[13] ammonium hydroxide was chosen simply as an electrolyte to produce a stable current under ESI-MS conditions, and because it allowed the use of a UV-Vis detector, in contrast with acetic acid. As the spectrum in Fig. 9(b) indicates, the system produced ammonium adducts exclusively, with no protonated molecule formation, and greatly reduced amounts of diacylglycerol fragments. The ammonium adducts acted to produce much larger molecular ion adduct peaks than have previously been reported using APCI-MS. Usually, under APCI-MS conditions, triacylglycerols gave

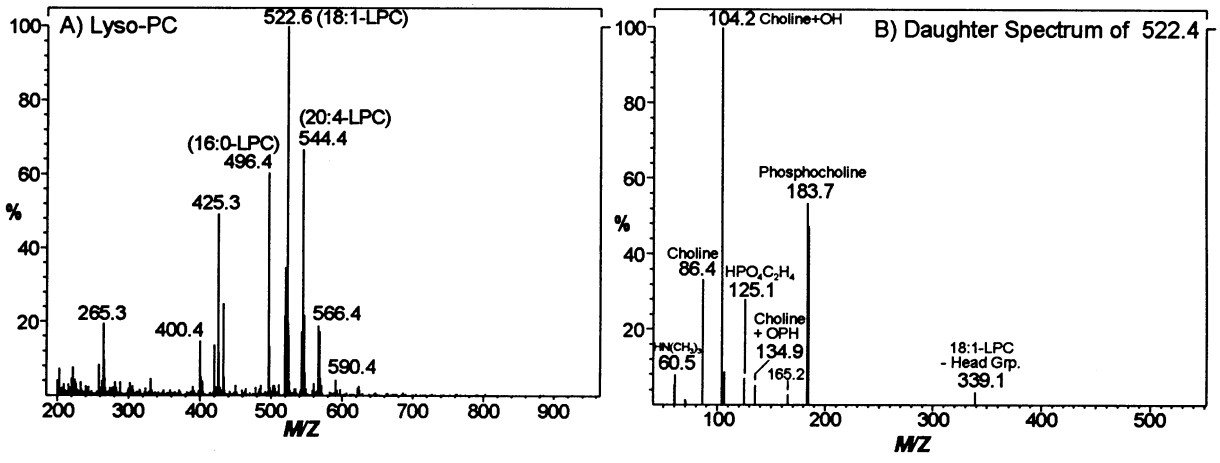

Figure 8. Mass spectra of lyso phosphatidyl choline obtained by ESI-MS and ESI-MS/MS. (a) Average of ESI-MS mass spectra across peak at 26 to 26.5 min in Fig. 7 obtained in full scan (parent) mode. (b) Daughter ion mass spectrum obtained by ESI-MS/MS of ion at 522.4 Da. Collision induced dissociation (CID) using argon collision gas used for ESI-MS/MS.

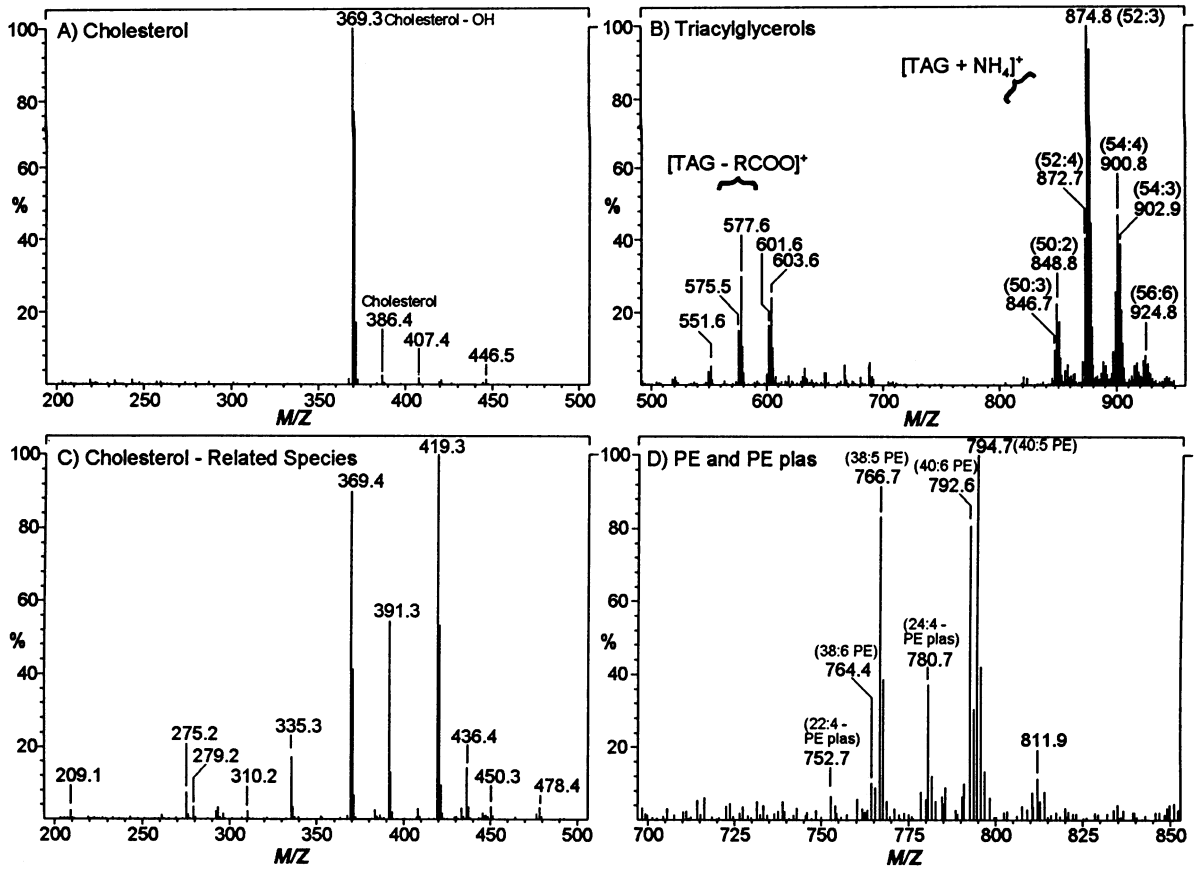

Figure 9. APCI-MS mass spectra of lipid constituents of human plasma whole extract. (a) Mass spectrum of first part of peak eluted at 2.5 to 4 min in Fig. 6; peak contains mostly cholesterol. (b) Mass spectrum showing ammonium adducts of triacylglycerols (TAGs) and fragments formed from loss of fatty acyl chains $[M - RCOO]^+$ from TAGs. (c) Mass spectrum of the second half of the peak eluted between 2.5 and 4 min; contains cholesterol-related species. (d) Mass spectrum showing molecular ions of phosphatidyl ethanolamine species.

very low abundances of protonated molecules for species with little unsaturation, while highly unsaturated species gave mostly protonated molecules. This behavior has been reported extensively.[15,19–21] It was an unexpected ancillary benefit of this study to observe the enhancement of molecular ion adduct abundances brought about by the ammonium hydroxide incorporated into the LC system. Although the TAGs are not resolved into molecular species, they can be identified by overall acyl chain length and degree of unsaturation. Quantitative analysis of the TAG molecular species in the sample used for this study was previously performed (Ref. 22, and manuscript in preparation) using the RP-HPLC/APCI-MS methodology previously established.[20,21] The complete TAG composition thus obtained was converted to the format of (carbon number:sites of unsaturation) for comparison with quantitative analysis performed using the ammonium adducts shown in Fig. 9(b). The results using both methods are shown in Table 3. While the specificity allowed by a full RP-HPLC/APCI-MS run is invaluable for identification of individual molecular species of TAGs, the ability to characterize all TAGs by acyl carbon number in the same run as phospholipid molecular species could be useful as a total lipid screening procedure.

In addition to the lipids discussed above, phosphatidyl ethanolamines were also observed in the human plasma sample. Though present at comparatively low levels, the major individual molecular species could be identified. As seen in Fig. 9(d), these species were similar to those observed in egg PE shown in Fig. 4(a) and 4(b) (as well as in bovine brain, not shown).

Table 3. Human plasma triacylglycerol composition comparison

TAG	APCI-MS $[M + NH_4]^+$	from APCI-MS complete TAG composition
54:0	0.0	0.3
54:1	0.1	0.5
54:2	1.8	2.6
54:3	8.7	8.6
54:4	9.7	10.3
54:5	3.7	5.7
54:6	0.7	2.1
52:0	0.2	0.4
52:1	1.6	3.6
52:2	22.4	20.5
52:3	23.7	22.0
52:4	7.4	9.0
52:5	0.5	1.4
50:0	0.2	0.7
50:1	5.7	3.9
50:2	8.1	4.9
50:3	3.1	2.2
50:4	0.4	0.4
48:0	0.4	0.2
48:1	1.1	0.5
48:2	0.8	0.4
Sum	100.3	100.2

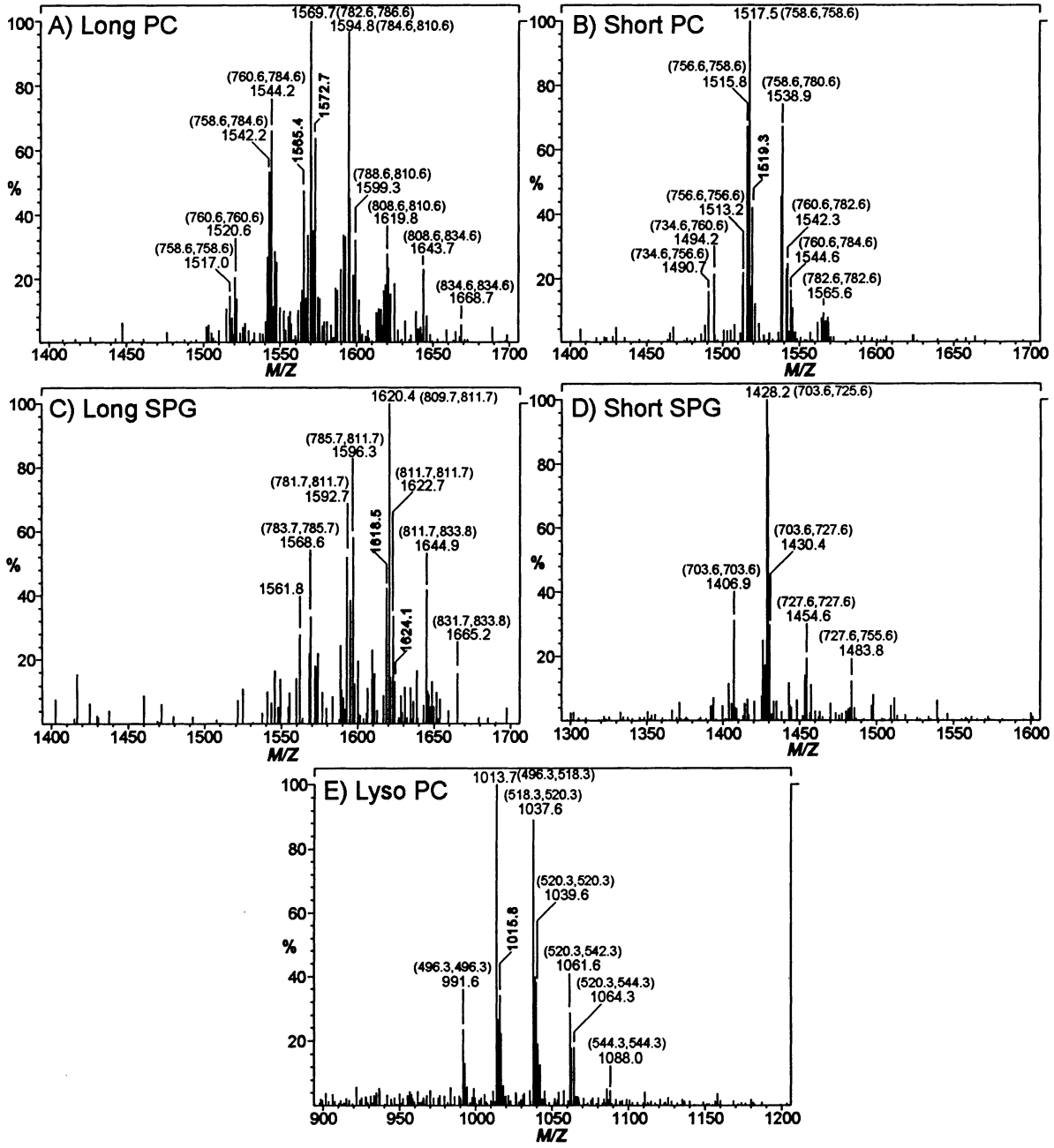

Figure 10. ESI-MS mass spectra showing formation of dimers of phospholipid molecular species of human plasma in ESI source. (a) Mass spectrum of range from 1400 to 1700 Da, showing peaks arising from the combination of PC phospholipids eluted between 15 and 17 minutes in Fig. 7. (b) Mass spectrum showing peaks arising from the combination of PC phospholipids eluted between 18 and 20 minutes in Fig. 7. (c) Mass spectrum of second SPM peak showing combination of sphingolipids. (d) Mass spectrum of third SPM peak showing combination of shorter-chain sphingomyelins. (e) Mass spectrum of range from 900 to 1200 Da showing combination of lyso-PC species eluted from 28 to 29.5 min in Fig. 7. For masses of PC and SPM species, see Tables. For lyso-PC use these masses: 16:0-LPC: 496.3 Da; 18:0-LPC: 524.4 Da; 18:1-LPC: 522.4 Da; 18:2-LPC: 520.3 Da; 18:3-LPC: 518.3 Da; 20:0-LPC: 552.4 Da; 20:4-LPC: 544.3 Da.

Other observations

Two other observations arose from this work. First, it was found that when the scan mass range was extended sufficiently, peaks arising from dimerization of phospholipids, presumably in the ESI source, were observed. Figure 10 shows dimers formed from long and short chain PC species, long and short chain SPM species and LPC species from the human plasma extract. In these spectra, almost all combinations of the species present are observed. The dimer masses represented either the sum of a protonated and non-protonated phospholipid or both protonated phospholipids. The labels on the figures give the masses for both protonated phospholipids. For this experiment, the ESI-MS instrument was run in full scan mode only. In some cases these dimers were present at levels as high as 5 or 6 percent of the base peak abundance. While the analytical utility of these dimers is marginal, their occurrence under ESI conditions provides an indication of a low energy of formation, which might have biological implications. Formation of similar ion

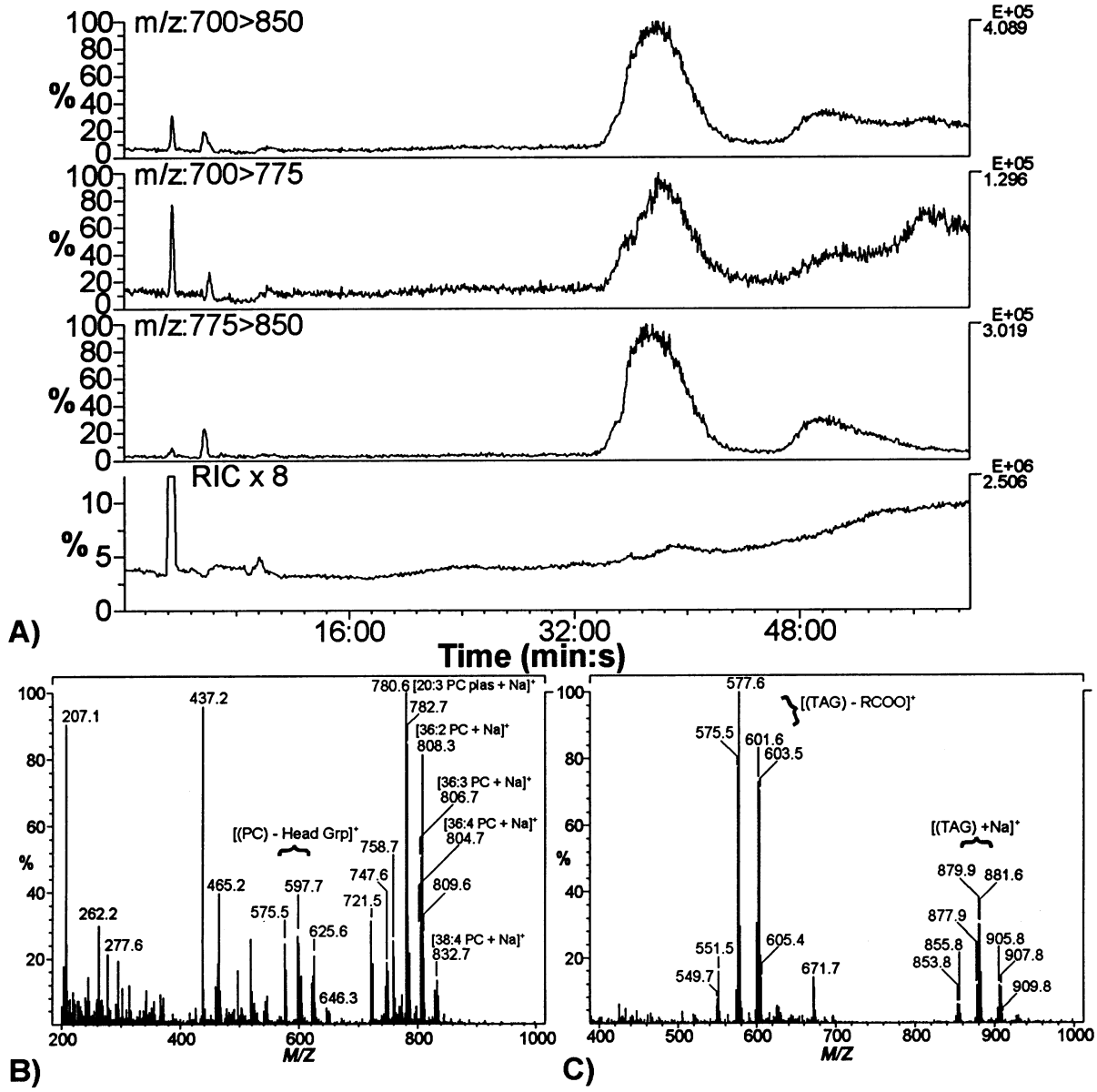

Figure 11. ESI-MS chromatograms and mass spectra of human plasma extract sodiated adducts. (a) ESI-MS chromatogram showing the RIC and short and long chain mass regions. (b) Mass spectrum showing sodiated molecular ions for PC species eluted between 36 and 40 minutes. (c) Mass spectrum showing sodiated triacylglycerol molecular ions and normal diacylglycerol fragments.

clusters from several phospholipid standards was recently reported using liquid secondary ion mass spectrometry (LSIMS).[23]

Another observation which arose was less benign. After allowing the column to sit unused for a week, the characteristics of the chromatographic system changed dramatically. When further analyses were performed, the chromatographic retention times were dramatically extended. Examination of the resultant spectra indicated that sodium adducts were being eluted instead of the protonated molecules to which we had become accustomed. A chromatogram of the human plasma sample is shown in Fig. 11 along with an ESI mass spectrum of PC species obtained across the top of the peak which now eluted between 34 and 42 min (compared to 16 to 20 min in Fig. 6). The elution of SPM in this run was not complete after 60 min. Figure 11(b) shows exclusively masses associated with sodium adducts, $[M+23]^+$, of the PC species instead of protonated molecules, $[M+1]^+$. APCI mass spectra similarly showed sodiated molecules for phospholipids, but still provided the normal fragments resulting from loss of the head group. While trying to resolve the problematic chromatographic behavior, a new column was installed and all solvents were changed. Attempts to regenerate the old column using acetic acid, phosphoric acid or ammonium hydroxide solutions had little effect or resulted in an admixture of sodiated and protonated molecules, with continued poor chromatography. This result was rather unexpected since this chromatographic system had previously been applied to different studies over a period of years. On the other hand, many other authors have reported sodium adducts of phospholipids exclusively when using other chromatographic systems. After several attempts to solve the problem, it was found that the proportioning valve on the pump had malfunctioned, and that Channel D on the pump, which had been used to deliver the H_2O/IPA portion

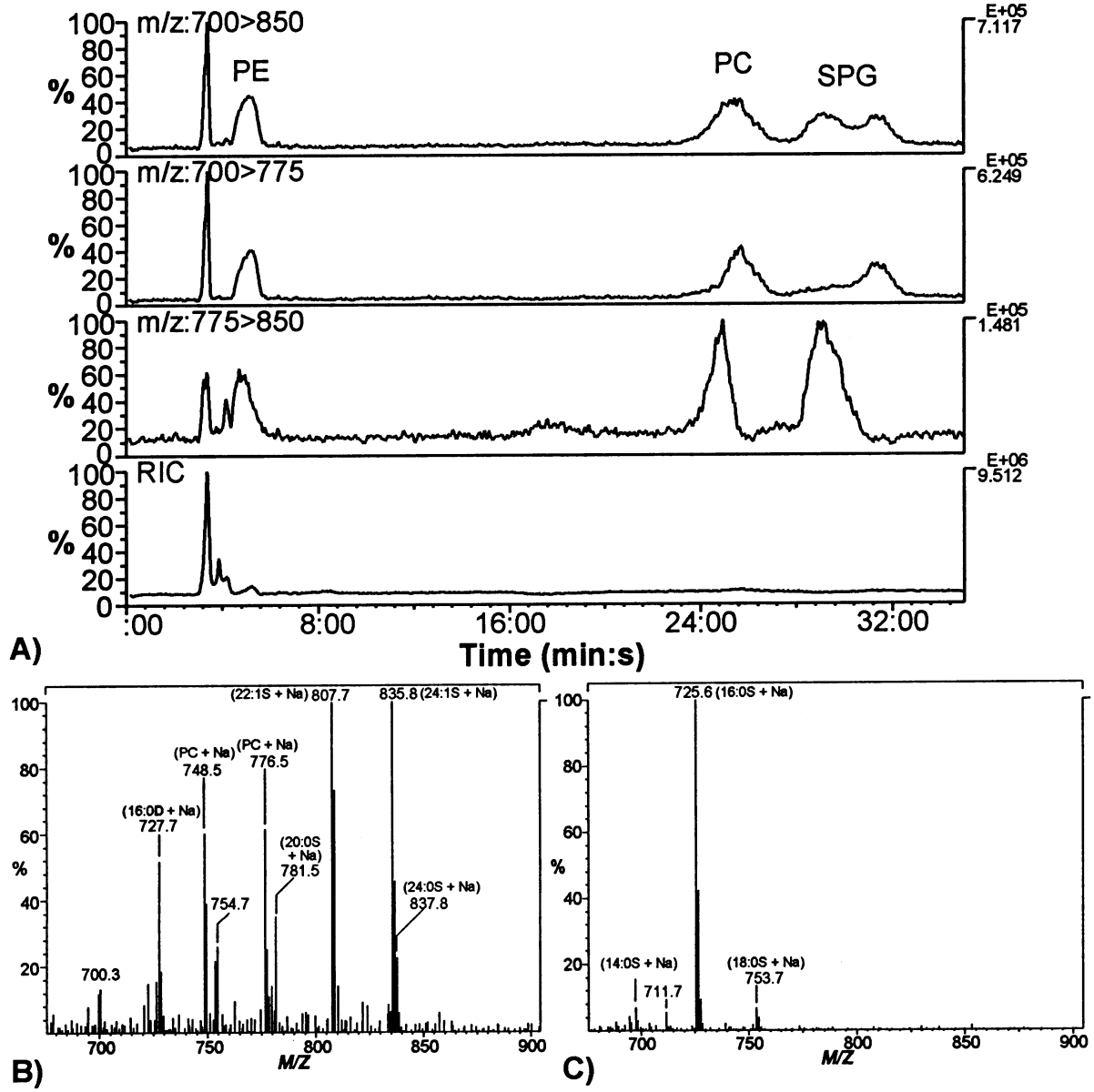

Figure 12. ESI-MS chromatograms and mass spectra of porcine lens extract sodiated adducts. (a) ESI-MS chromatogram showing the RIC and short and long chain phospholipid mass regions. (b) Mass spectrum of the second of three sphingolipid peaks. Peak contained short chain DHS and long chain SPM species, as well as residual PC, (c) Mass spectrum of the third of three sphingolipid peaks. Peak contained short chain SPM species.

of the eluent, was not properly delivering flow. Thus, the absence of a sufficient amount of aqueous component led to the chromatographic change. From this change in behavior, it appeared that the amine column has previously acted as an ion exchange column to remove sodium from phospholipid preparations, thus yielding only protonated molecules. The malfunction of the HPLC instrument thus provided an unexpected and useful insight into the retention mechanism of the column.

Concomitant with the change in the appearance of phospholipid spectra was a change in the appearance of triacylglycerol spectra obtained by the ESI-MS instrument. One would not expect large neutral TAGs to give good response under ESI-MS conditions, although ESI/MS has previously been used for analysis of TAGs.[24–26] However, when the chromatographic system changed its behavior, we obtained spectra for TAGs which showed both sodiated molecules and diacylglycerol fragments. Figure 11(c) shows a mass spectrum for the TAG species in human plasma obtained using ESI. This spectrum may be compared to that in Fig. 9(b), which showed the ammonium adducts of TAG obtained using APCI. The difference of 5 Da between the quasi-molecular ions in Fig. 11(c) versus 9(b) represents the difference between sodium adducts (+23) versus ammonium adducts (+18). During this same run, while sodium adducts appeared in ESI-MS spectra, the APCI mass spectra of TAGs continued to show ammonium adducts, and appeared the same as the spectrum in Fig. 9(b).

Porcine lens extract

Although the sodium adduct formation was not eliminated until the proportioning valve was replaced, washing the column with ammonium hydroxide solution did at least give

Table 4. Porcine lens sphingolipid molecular species area

		[Head Group $+H]^+$ = 184 amu			
DHS	$[M+H]^+$	%	SPM	$[M+H]^+$	%
24:0	817.7	1.0	26:0	843.8	0.0
24:1	815.7	6.8	26:1	841.7	0.6
24:2	813.7	2.6	26:2	839.7	0.5
24:3	811.7	0.0	26:3	837.7	0.2
22:0	789.7	2.3	26:4	835.7	0.1
22:1	787.7	7.8	24:0	815.7	3.4
22:2	785.7	1.1	24:1	813.7	15.1
20:0	761.7	4.2	24:2	811.7	3.5
20:1	759.7	4.2	24:3	809.7	0.2
20:2	751.6	0.0	22:0	787.7	4.3
18:0	733.6	12.3	22:1	785.7	13.4
18:1	731.6	2.0	22:2	783.7	0.7
18:2	729.6	1.2	20:0	759.7	3.7
16:0	705.6	41.6	20:1	757.6	3.3
16:1	703.6	2.9	20:2	755.6	0.3
14:0	677.6	9.0	18:0	731.6	10.0
14:1	675.6	0.9	18:1	729.6	1.9
			16:0	703.6	30.6
Sum		99.9	16:1	701.6	3.4
			14:0	701.6	4.9
			Sum		100.1
		Percent of sphingolipids			
	DHS = 14.4			SPM = 85.6	

better peak shapes and sphingomyelin could be eluted. An HPLC/ESI-MS chromatogram of porcine lens extract is shown in Fig. 12. The chromatogram and resultant mass spectra allowed the identification of both DHS and SPM species. Example spectra of the sodiated sphingolipids are shown in Fig. 12(b) and (c). Though sodium adducts of the molecules were observed (both by ESI-MS and APCI-MS, though only small abundances in APCI spectra), the sodium ion was associated with the head group, so the sphingoid backbone fragments formed during APCI were the exact same as those previously observed. Therefore, quantification was performed using the same masses as before the change in the chromatographic behavior. The molecular species are given in Table 4. Extracted ion chromatograms of the sphingoid backbone fragments obtained under APCI-MS conditions are shown in Fig. 13(a), while EICs for the sodiated molecules simultaneously obtained under ESI conditions, in parallel, are shown in Fig. 13(b). All molecular species identified were confirmed by the presence of simultaneous peaks in the APCI-MS and ESI-MS extracted ion chromatograms. Porcine Lens sphingolipids were calculated to be composed of 14.4% DHS and 85.6% SPM. The most abundant molecular species were 16:0, 18:0, 22:1 and 24:1, although the relative percentages of these differed between the DHS and SPM.

Conclusions

The dual mass spectrometer system allowed data to be obtained under different ionization conditions using one chromatographic run. This provided the benefit of virtually identical retention times between the different systems. Compared to the method previously used[13] for obtaining this same type of data, the amount of time, as well as the amount of solvent and sample used, were cut by half. More importantly, the amount of information obtained per run was increased dramatically. For components for which no standards had been run (e.g. lyso-PC in human plasma) the acquisition of daughter ion spectra obtained by ESI-MS/MS was decisive in identification of the compounds. The two ionization methods demonstrated here produced complementary data for several types of molecules. PC, DHS and SPM produced only intact ionized molecules under ESI-MS conditions, while they produced diagnostically useful fragments under APCI-MS conditions. Having both types of data was especially useful for the sphingolipids which produced very low abundances of protonated molecules in APCI-MS spectra. The use of EICs of sodiated molecules from ESI data, along with EICs of fragments in APCI data, prevented misinterpretation of potentially ambiguous masses in porcine lens extract.

We have chosen only the most simple conditions to demonstrate the application of the dual mass spectrometer system. The number of permutations is large when one considers that both positive and negative ion analysis may be performed on either instrument, APCI and ESI may be performed on either machine (the sources are interchangeable), and the triple quadrupole machine may be run in full

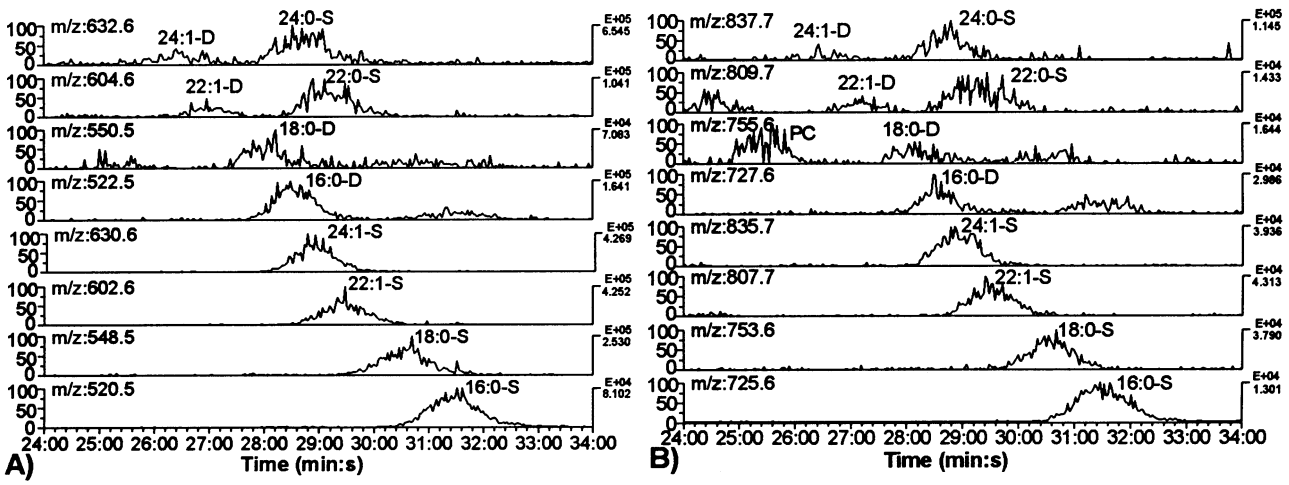

Figure 13. Extracted ion chromatograms of porcine lens sphingolipids by APCI-MS and ESI-MS corresponding to the most abundant species given in Table 4. (a) APCI-MS EICs of sphingolipid backbone ($[M\text{-head group}]^+$) fragments for sphingolipids eluted between 26 and 33 minutes. (b) ESI-MS EICs of the masses of sodiated molecular ions of the most abundant species given in Table 4.

scan mode exclusively or MS/MS may be performed. Given the large number of possible experiments, the acquisition of data from two machines simultaneously is an important time-saving arrangement. Of course, dedicating two complete mass spectrometer systems to analysis is not feasible in most laboratories. The cost of such instrumentation is often prohibitive. On the other hand, the advent of benchtop APCI and ESI ion trap technology makes use of dual instruments more feasible than ever before. Given the extensive amount of data provided, future studies of phospholipids in our laboratory will certainly employ the dual mass spectrometric system as a matter of course.

The use of two other detectors had significant advantages, as well. The quantitation of the relative proportions of the different classes of molecules was best performed using the ELSD, because we observed substantial differences in the responses of different phospholipid classes under MS conditions. However, within each phospholipid class, identification and quantification were best done using mass chromatograms. Since we used ESI-MS for MS/MS analysis in this study, APCI-MS alone was used for quantitative analysis. Using ESI-MS in full scan mode does, however, allow quantitative analysis to be performed in addition to APCI, as previously shown.[13] The UV-Vis detector showed severe limitations in its selective response to different phospholipid classes, as well as its susceptibility to impurities in the solvent system. If available, a second ELSD detector or a flame ionization detector for LC should be substituted for the UV-Vis detector. This would also allow more flexibility in the choice of electrolyte for ESI.

The use of both mass spectrometers allowed the identification of numerous dihydrosphingomyelin molecular species in bovine brain, and to determine that human plasma contains virtually no DHS. Use of both systems was especially valuable for the porcine lens extract, with both machines operating in full scan mode. As the DHS molecular species are identified in an increasing number of tissue types, distinct tendencies for several common species start to become evident. However, there are distinct compositional differences between tissue types. The relative proportion of DHS to SPM from porcine eye lens membranes was much more similar to bovine brain than to human eye lens membranes. But the identity of the molecular species was somewhere in between the compositions of bovine brain and human lens (high amounts of 16:0 like the human lens, but the levels of 18:0 were more like brain extract). The roles of the individual molecular species need to be elucidated and, given the effect of DHS on membrane fluidity, its biological significance needs to be further assessed. The nature of sphingomyelins from other sources should be re-examined in light of the new analytical capabilities which have evolved.

Acknowledgment

The work of Lynne Copes on the extraction of the human plasma sample is gratefully acknowledged.

REFERENCES

1. J. L. Kerwin, A. R. Tuininga and L. H. Ericsson, 'Identification of Molecular Species of Glycerophospholipids and Sphingomyelin Using Electrospray Mass Spectrometry', *J. Lipid Res.* **35**, 1102–1114 (1994).
2. F. B. Jungalwala, V. H. Hayssen, J. M. Pasquini and R. H. McCluer, 'Separation of Molecular Species of Sphingomyelin by Reversed-Phase High Performance Liquid Chromatography', *J. Lipid Res.* **20**, 579–587 (1979).
3. J. I. Teng and L. L. Smith, 'Improved High-Performance Liquid Chromatography of Sphingomyelin', *J. Chromatogr.* **322**, 240–245 (1985).
4. H.-Y. Kim and N. Salem, 'Application of Thermospray High-Performance Liquid Chromatography/Mass Spectrometry for the Determination of Phospholipids and Related Compounds', *Anal. Chem.* **59**, 722–726 (1987).
5. A. Valeur, N. U. Olsson, P. Kaufmann, S. Wada, C.-G. Kroon, G. Westerdahl and G. Odham, 'Quantification and Comparison of Some Natural Sphingomyelins by On-line High-Performance Liquid Chromatography/Discharge-Assisted Thermospray Mass Spectrometry', *Biol. Mass Spectrom.* **23**, 313–319 (1994).
6. A. Kuksis and J. J. Myher, 'Application of Tandem Mass Spectrometry for the Analysis of Long-Chain Carboxylic Acids', *J. Chromatogr. B* **671**, 35–70 (1995).
7. N. U. Olsson and N. Salem, 'Molecular Species Analysis of Phospholipids', *J. Chromatogr. B* **692**, 245–256 (1997).
8. A. A. Karlsson, P. Michelson, A. Larsen and G. Odham, 'Normal-Phase Liquid Chromatography Class Separation and Species Determination of Phospholipids Utilizing Electrospray Mass Spectrometry/Tandem Mass Spectrometry', *Rapid Commun. Mass. Spectrom.* **10**, 775–780 (1996).
9. X. Han and R. W. Gross, 'Structural Determination of Picomole Amounts of Phospholipids Via Electrospray Ionization Tandem Mass Spectrometry', *J. Am. Soc. Mass Spectrom.* **6**, 1202–1210 (1995).
10. H.-Y. Kim, T.-C. L. Wang and Y.-C. Ma, 'Liquid Chromatography/Mass Spectrometry of Phospholipids Using Electrospray Ionization', *Anal. Chem.* **66**, 3977–3982 (1994).
11. W. C. Byrdwell, D. Borchman, R. A. Porter, K. G. Taylor and M. C. Yappert, 'Separation and Characterization of the Unknown Phospholipid in Human Lens Membranes', *Invest. Ophthalmol. Vis. Sci.* **35**, 4333–4343 (1994).
12. V. L. Hanson, J. Y. Park, T. W. Osborn and R. M. Kiral, 'High-Performance Liquid Chromatographic Analysis of Egg Yolk Phospholipids', *J. Chromatogr.* **205**, 393–400 (1981).
13. W. C. Byrdwell and D. Borchman, 'Liquid Chromatography/Mass Spectrometric Characterization of Sphingomyelin and Dihydrosphingomyelin of Human Lens Membranes', *Ophthalmic Res.* **29**, 191–206 (1997).
14. J. Folch, M. Lees and G. H. Sloane Stanley, 'A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissue', *J. Biol. Chem.* **226**, 497–509 (1957).
15. W. E. Neff and W. C. Byrdwell, 'Soybean Oil Triacylglycerol Analysis by Reversed-Phases High Performance Liquid Chromatography Coupled with Atmospheric Pressure Chemical Ionization Mass Spectrometry', *J. Am. Oil Chem. Soc.* **72**, 1185–1191 (1995).
16. W. C. Byrdwell, D. Borchman, R. A. Porter, K. G. Taylor and M. C. Yappert, 'Separation and Characterization of the Unknown Phospholipid in Human Lens Membranes', *Invest. Ophthalmol. Vis. Sci.* **35**, 4333–4343 (1994).
17. D. Borchman, W. C. Byrdwell and M. C. Yappert, 'Thermodynamic Phase Transition Parameters of Human Lens Dihydrosphingomyelin', *Ophthalmic Res.* **28**(suppl 1), 81–85 (1996).
18. W. C. Byrdwell and W. E. Neff, 'Analysis of Hydroxy-Containing Seed Oils Using Atmospheric Pressure Chemical Ionization Mass Spectrometry', *J. Liq. Chromatogr. Rel. Technol.*, in press.
19. W. C. Byrdwell, E. A. Emken, W. E. Neff and R. O. Adlof, 'Quantitative Analysis of Triglycerides Using Atmospheric Pressure Chemical Ionization Mass Spectrometry', *Lipids* **31**, 919–935 (1996).
20. W. C. Byrdwell and W. E. Neff, 'Qualitative and Quantitative Analysis of Triacylglycerols Using Atmospheric Pressure Chemical Ionization Mass Spectrometry', in *New Techniques and Applications in Lipid Analysis*, R. E. McDonald and M. M. Mossoba (Eds), pp. 45–80, AOCS Press, Champaign, IL (1997).
21. P. Laakso and P. Manninen, 'Identification of Milk Fat Triacylglycerols by Capillary Supercritical Fluid Chromatography-Atmospheric Pressure Chemical Ionization Mass Spectrometry', *Lipids* **32**, 1285–1295 (1997).
22. W. C. Byrdwell and E. A. Emken, 'Liquid Chromatography/Atmospheric Pressure Chemical Ionization Mass Spectrometric Characterization of Human Chylomicron Triacylglycerols', 88th AOCS Annual Meeting Abstracts, p. 58, AOCS Press, Champaign, IL (1997).
23. Y. Wu, J. Wang and S. Sui, 'Characterization of Phospholipids by

Electron Impact, Field Desorption and Liquid Secondary Ion Mass Spectrometry', *J. Mass Spectrom.* **32**, 616–625 (1997).
24. K. L. Duffin, J. D. Henion and J. J. Shieh, 'Electrospray and Tandem Mass Spectrometric Characterization of Acylglycerol Mixtures That Are Dissolved in Nonpolar Solvents', *Anal. Chem.* **63**, 1781–1788 (1991).
25. J. J. Myher, A. Kuksis and P. W. Park, 'Stereospecific Analysis of Docosahexaenoic Acid-Rich Triacylglycerols by Chiral-Phase HPLC With Online Electrospray Mass Spectrometry', in *New Techniques and Applications in Lipid Analysis*, R. E. McDonald and M. M. Mossoba (Eds), pp. 100–120, AOCS Press, Champaign, IL (1997)
26. A. Kuksis and J. J. Myher, 'Application of Tandem Mass Spectrometry for the Analysis of Long-Chain Carboxylic Acids', *J. Chromatogr.* **671**, 35–70 (1995).

