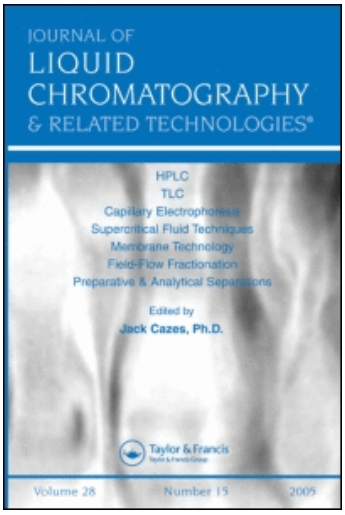

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Dual Parallel Liquid Chromatography/Dual Mass Spectrometry (LC2/MS2) of Bovine Brain Total Lipid Extract

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ABSTRACT

A commercially available bovine brain total lipid extract containing polar and non-polar lipid fractions has been separated in one analytical run using two high performance liquid chromatography (HPLC) systems in parallel, coupled to dual mass spectrometers, in parallel. The whole extract of bovine brain was separated on normal phase (NP, LC1) and reversed-phase (Rp, LC2) columns simultaneously, with detection using an ion trap mass spectrometer (MS1) and a tandem mass spectrometer (MS2) coupled to the two HPLC systems, respectively. A single injection was loaded onto an amine column to perform separation of phospholipid components. Non-polar components, such as triacylglycerols, were unretained on this polar column. Using the diverter valve on the front of the ion trap mass spectrometry (MS1), the bolus of neutral lipids was

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redirected to a RP liquid chromatography system (LC2) on which the neutral lipids were separated using a second chromatographic system at the same time that the polar lipids were separated on LC1. Electrospray ionization (ESI)-mass spectrometry (MS) on an ion trap mass spectrometer (MS1), with ammonium formate added as a sheath liquid, was used for analysis of the phospholipids. Electrospray ionization-mass spectrometry, with ammonium formate incorporated into the solvent system, was used on a tandem mass spectrometer (MS2) for identification of triacylglycerols (TAGs) and other neutral lipids. This dual liquid chromatography/dual mass spectrometry (LC2/MS2) system represents a unified approach for a total lipid analysis in one experiment using a single sample injection.

Key Words: Phospholipids; Triacylglycerols; Tandem mass spectrometry; Electrospray ionization; Atmospheric pressure chemical ionization.

INTRODUCTION

Total lipid extracts, such as those obtained using the methods of Folch et al.[1] or Bligh and Dyer,[2] produce a mixture of polar and non-polar lipids. Polar lipids are comprised mostly of phospholipids, while non-polar lipids consist of mostly triacylglycerols (TAGs), diacylglycerols (DAGs), cholesterol, and cholesterol esters. Often, the subject of analysis is either the class of polar lipids or the class of non-polar lipids, to the exclusion of the other. Typically, normal-phase (NP) chromatography with a silica or amine column is used for separation of the phospholipids and other polar components. In such cases, the neutral lipids elute as a bolus near the solvent front. Conversely, reversed-phase (RP) chromatography with an octadecylsilane (C_{18}) column is typically used for separation of the non-polar components. The polar components elute as a bolus near the solvent front under RP conditions. We have previously published methods for the analysis of phospholipids, with emphasis on sphingomyelin (SPM) and dihydrosphingomyelin (DHS), using a polar amine column.[3–5] In these separations, neutral lipids were eluted near the solvent front. The various classes of phospholipids were separated into distinct peaks, and the individual molecular species within each phospholipid class were identified by online mass spectrometry (MS). We were also able to use MS to identify the major species of the non-polar components in the bolus that eluted near the solvent front.[5] However, identification of the neutral lipids such as TAGs without prior separation was not ideal. The bolus contained numerous isobaric TAG that were not distinguished, and also the DAG fragment ions could not be attributed to the individual parent TAG molecular species. Separation of the TAG prior to mass spectrometric analysis is preferred. Thus, one column type and solvent system

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is not ideal for simultaneous separation of both polar lipids and neutral lipids on one column.

We have previously published numerous reports on the analysis of separated TAGs using liquid chromatography with online MS detection.[6,7] Separation of TAG on a non-aqueous RP system allows a large number of individual molecular species to be identified, and the DAG fragment ions that result from each species provides structural information to differentiate between isobaric species. The use of atmospheric pressure chemical ionization (APCI)-MS has become popular, because APCI-MS is effective at ionizing large neutral molecules. Atmospheric pressure chemical ionization is a soft ionization technique, which produces some protonated molecule ions, along with gentle fragmentation that yields large fragment ions that facilitate structural characterization. Applications of APCI-MS to lipid analysis have been recently reviewed.[8]

Methods have also been published that allow electrospray ionization (ESI)-MS to be used for analysis of neutral lipids, some of which have been previously reviewed.[9] It may seem counterintuitive that large neutral molecules, such as TAG, with no easily charged functional groups may be ionized using ESI-MS. Nevertheless, approaches have been found to produce ionic adducts of different types that allow ESI-MS to be used for TAG analysis. We have recently demonstrated the use of ESI-MS for analysis of normal TAG and of TAG oxidation products.[10] In this report, we showed the use of ammonium formate as a sheath liquid to promote ammonium adduct formation. Though not an extensive review, recent applications of ESI-MS to neutral lipids have been discussed in our recent report.[10] We showed that, with the use of ammonium formate as a sheath liquid, ESI-MS was more sensitive than APCI-MS for analysis of TAG and TAG oxidation products, especially high molecular weight TAG oxidation products.

Given the high degree of information obtainable by MS, in combination with the quality of separations offered by high performance liquid chromatography (HPLC), a single method that combines the separation of all polar and non-polar lipid classes with detection by MS has long been sought. Byrdwell and coworkers[5,10] have twice demonstrated "dual parallel mass spectrometer" methods that have utilized two mass spectrometers connected in parallel to one LC system, to obtain both ESI-MS and APCI-MS data simultaneously from either polar[5] or neutral lipids.[10] We have shown that these two ionization techniques produced complementary data, which together provided a wealth of information to allow molecular species to be identified. We refer to the instrument configuration used in our previous reports, that have employed a single liquid chromatographic system coupled to two mass spectrometers in parallel, as an LC1/MS2 arrangement. After our initial report of dual parallel mass spectrometers for polar lipid analysis,[5] it became

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obvious that separation of the bolus of neutral lipids, with mass spectrometric detection, would also be desirable.

We demonstrate, here, the first example of a method for simultaneous separation and analysis of polar and non-polar lipids from a commercially available total brain extract, using dual parallel liquid chromatographic systems attached to dual parallel mass spectrometers. Polar lipids were separated on a NP chromatographic system (LC1), with detection by positive and negative ion ESI ion trap mass spectrometry (MS1), MS/MS, and MS^3, at the same time that neutral lipids were separated on a RP system (LC2), coupled to a second mass spectrometer (MS2) using positive ion ESI-MS with MS/MS. We refer to this arrangement as an LC2/MS2 configuration.

EXPERIMENTAL

Bovine brain whole lipid extract was purchased from Avanti Polar Lipids (Alabaster, AL). The whole extract, ~25 mg/mL in chloroform as received, was transferred to a tared amber vial and taken to dryness under Argon. The sample was then reconstituted to a known concentration of 25.0 mg/mL in chloroform. All solvents, except water, were purchased from Aldrich Chemical Co. (St. Louis, MO). The HPLC grade solvents were purchased and were used without further purification. Water was obtained from a Millipore (Waters, Inc., Milford MA) Milli-Q Academic deionized water filtration system and was used without further purification.

Liquid Chromatography Instrumentation

A NP-LC system was attached to the ion trap mass spectrometer, while a RP-LC system was attached to the tandem mass spectrometer. The NP-HPLC system was composed of a P4000 quaternary pump with membrane degasser, an AS300 autosampler, and a UV 6000 photodiode array detector (Thermo Separation Products, Thermo Finnigan, San Jose, CA). The AS 3000 autosampler was used to make an injection of 20 μL onto the NP columns. The NP-HPLC system employed two amine columns in series, Adsorbosphere NH_2, 25 cm × 4.6 mm, 5 μm (Alltech Associates, Deerfield, IL). The gradient program used three solvents: solvent A, 40% H_2O, 60% isopropyl alcohol (IPA); solvent B, 40% methanol, 60% IPA; solvent C, 40% hexane, 60% IPA. Each solvent contained 0.1% NH_4OH. The gradient program used was as follows: from 0 to 10 min, 15% A, 15% B, 70% C; from 10 to 20 min, a linear change to 25% A, 25% B, 50% C, held until 30 min.; from 30 to 40 min, a linear change to 30% A, 45% B, 25% C; from 40 to 50 min, a linear change to

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30% A, 70% B, held until 75 min; recycled to initial conditions from 75 to 85 min. The flow rate was 0.80 mL/min throughout. The outlet from the amine columns was plumbed to the diverter valve on the front of the LCQ Deca mass spectrometer, described below. After the diverter valve, the flow went through the UV 6000 photodiode array detector (PDA). The flow was then split via a tee, with the straight-through exit connected to the ESI source on the LCQ Deca, and the 90° exit directed to waste. Splitting the flow kept the flow rate into the ESI source at a lower level, and also allows for the future use of a fraction collector to collect phospholipid fractions, instead of sending them to waste.

The RP-HPLC system used an LDC 4100 quaternary pump with membrane degasser (Thermo Separation Products, Thermo Finnigan, San Jose, CA). The RP-HPLC system employed two Inertsil ODS-3 columns in series, 25 cm × 4.6 mm, 5 μm (MetaChem Technologies). A binary gradient was developed which used: solvent A, methanol; solvent B, 40% hexane, 60% IPA. Both solvents contained 1 mM ammonium formate made by replacement of 1% of the pure solvent with 100 mM ammonium formate in H_2O. This was added to provide electrolyte for use in ESI mode and to promote adduct formation (this was used instead of a sheath liquid on the TSQ). The gradient program was as follows: from 0 to 15 min, 90% A, 10% B; from 15 to 30 min, a linear change to 35% A, 65% B; from 30 to 70 min, a linear change to 30% A, 70% B; from 70 to 80 min, a linear change to 10% A, 90% B; recycled to initial conditions from 80 to 90 min. The flow rate was 0.8 mL/min throughout. The effluent from the columns on LC2 was split via a tee, with the 90° exit of the tee delivered to an evaporative light scattering detector (ELSD), and the straight-through exit of the tee delivered to the ionization source of the tandem mass spectrometer. The ELSD data was acquired by both the tandem mass spectrometer (having low digital resolution for the auxiliary detector) and a stand-alone 24-bit data acquisition system (EZ-Chrom Elite). However, the levels of neutral lipids were below the detection limit on the ELSD under these conditions, so no ELSD chromatograms are presented here.

The interconnections between the two liquid chromatographic systems, the two auxiliary detectors (PDA and ELSD), and the two mass spectrometers (the ion trap mass spectrometer and tandem mass spectrometer) are shown in Fig. 1. The outlet of the NP-HPLC columns (LC1) was connected to port 2 of the diverter valve on the LCQ Deca. Port 3 went to a tee that split flow between the PDA and the ESI source on the LCQ Deca. In the load position, LC1 simply flowed through the diverter valve, from port 2 to 3, and to the UV 6000 PDA and the ESI source on the LCQ. The pump of the RP-HPLC system (LC2) was attached to port 6 of the diverter valve. The line to the RP columns was attached to port 1. The RP-HPLC system was located in proximity to the diverter valve to minimize dead volume (distances in Fig. 1

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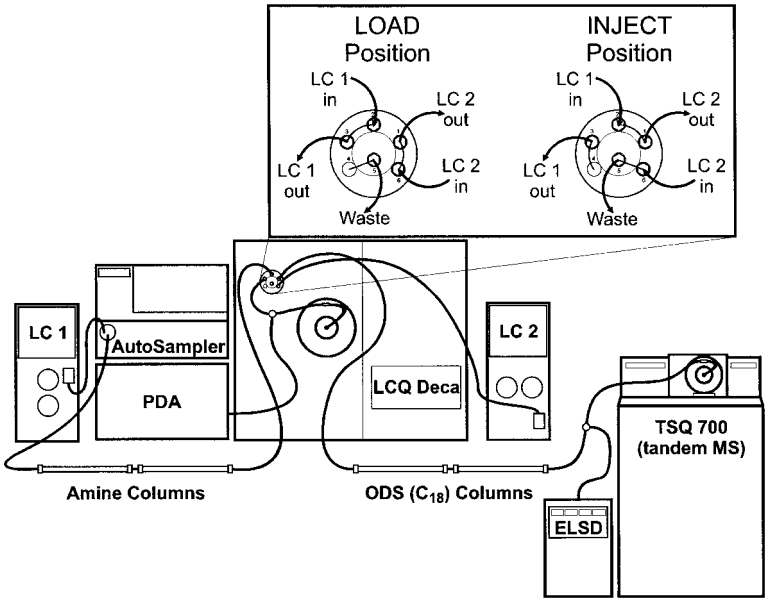

Figure 1. Arrangement of instruments for dual liquid chromatography/dual mass spectrometry (LC2/MS2) experiments.

are exaggerated for clarity). In the load position, LC2 simply flowed through the diverter valve, from port 6 to 1, and to the RP columns. In the inject position, flow from port 2 (LC1 in) was directed to port 1 (LC2 out to columns), while LC2 in was directed to waste (port 5). This arrangement allowed the neutral lipid bolus eluted from the columns on LC1 to be directed onto the columns attached to LC2, while the flow from LC2 was directed to waste. The diverter valve was open from 6.00 to 8.25 min.

Mass Spectrometry Instrumentation

LC1 was attached to an LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Scans were obtained in positive and negative ion mode from m/z 50 to 2000. The run was broken into three scan segments, with three different scan programs. In the first segment, from 0 to 25 min, the scan program was as follows: a positive MS scan, a positive MS/MS scan, a negative MS scan, and a negative MS/MS scan. In the second segment, from 25 to 55 min, the scan program was as follows: a negative MS scan, a positive MS scan, a positive MS/MS scan, and a positive MS^3 scan. The third segment, after

55 min, was the same as the first segment, having a positive MS scan, a positive MS/MS scan, a negative MS scan, and a negative MS/MS scan. These segments were selected because in the first part of the run, phospholipids which gave good positive and negative ion spectra eluted, so positive and negative MS/MS scans were sought. In the second segment, phospholipids containing a quaternary amine [phosphatidylcholine (PC), SPM, and DHS eluted. These provided excellent positive ion spectra, but poorer negative ion spectra, so emphasis was on positive ion MS, MS/MS, and MS^3 scans. After the sphingolipids eluted, the scan program returned to the original scan program. The heated capillary was operated at 250°C. For MS/MS and MS^3 scans, the activation energy was 58% (arbitrary units) with a "q factor" of 0.25. The activation time was 900 ms. Ammonium formate solution (20 mM) in H_2O/ACN (1 : 4) was added as a sheath liquid via syringe pump (AB 140B, Applied Biosystems, Foster City, CA), as previously described,[10] to facilitate the formation of negative ions from the quaternary amine phospholipids, as discussed below. Sheath liquid flow was delivered at 20 μL/min.

The tandem mass spectrometer attached to LC2 was a Finnigan MAT TSQ 700 (Thermo Finnigan, San Jose, CA). The TSQ 700 gave an optimal signal in Q3 low mass mode. For full scans using Q3, Q1 was operated in RF-only mode. Sheath and auxiliary gases were set to 35 psi and 5 mL/min, respectively. In ESI mode, the spray voltage was 5.5 kV. Electrospray ionization-mass spectrometry spectra were obtained from m/z 200 to 1200, with a scan time of 1.0 s. The heated capillary temperature was 265°C throughout. An instrument command Language (ICL) procedure was used that allowed automatic switching between MS and MS/MS modes when the signal passed a threshold. This procedure has been previously described.[5] The procedure allowed real-time modification of all parameters, including the cut-off threshold, parent ion mass selection range, and others. The parent quadrupole (Q1) offset was −5.0 V, the collision cell offset was −5.0 V in full scan mode and −25.0 V in MS/MS mode, and the product quadrupole (Q3) offset was −5.0 V. Argon was used as the collision-induced dissociation (CID) gas. Five scans were obtained in full scan mode, then the CID gas was turned on and four MS/MS scans of each of the two most abundant precursor ions were collected. Data obtained on the TSQ 700 were imported into the Xcalibur™ software on the LCQ, to allow the chromatograms and spectra from the two mass spectrometers to be more easily compared.

RESULTS AND DISCUSSION

The bovine brain total lipid extract sample was injected onto the NP columns containing an amine stationary phase, using the autosampler pictured in Fig. 1. The neutral lipids were unretained on this polar column, and so

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eluted as a bolus with short retention time. The TAGs and other non-polar molecules eluted between 6.00 and 8.25 min. Over this time window (labeled as "diverted" in Fig. 2), the electronically actuated valve on the front of the LCQ Deca was set to divert the effluent (valve in the inject position) from the first columns onto the head of the second set of columns, which contained (RP) packing. After diverting the neutral bolus, the valve closed (valve in the load position), and the original solvent system continued to drive the polar

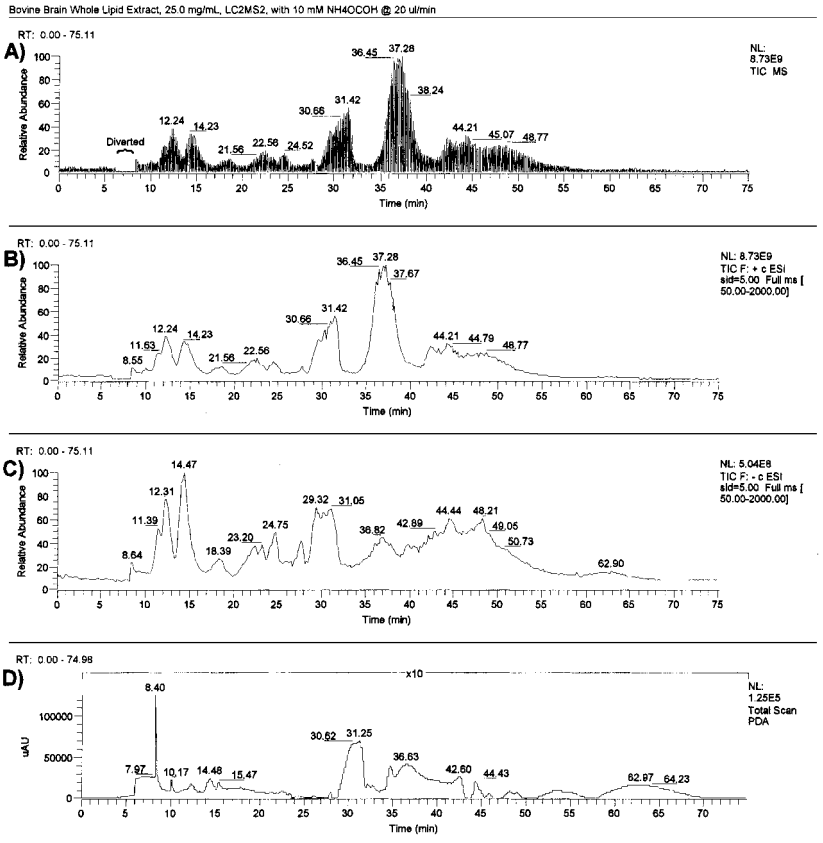

Figure 2. Electrospray ionization-mass spectrometry and UV chromatograms of polar components from commercially available bovine brain total lipid extract separated using NP-HPLC. (A) Electrospray ionization-mass spectrometry TIC showing all positive and negative MS, MS/MS, and MS^3 scans; (B) ESI-MS TIC showing only scans obtained in positive full scan (MS) mode; (C) ESI-MS TIC showing scans obtained in negative full scan mode; and (D) PDA detector UV chromatogram.

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lipids down the amine columns. At the same time, the second pump, also plumbed through the diverter valve, continued pumping a second solvent system down the RP system to perform the neutral lipid separation. Chromatograms showing elution of the polar components from the amine columns and the neutral components from the RP columns are shown in Figs. 2 and 3, respectively. Figure 2 shows the ESI-MS total ion chromatogram from the ion trap mass spectrometer, a chromatogram filtered to show only (+) ion scans, a chromatogram filtered to show only (−) ion scans, and a chromatogram from

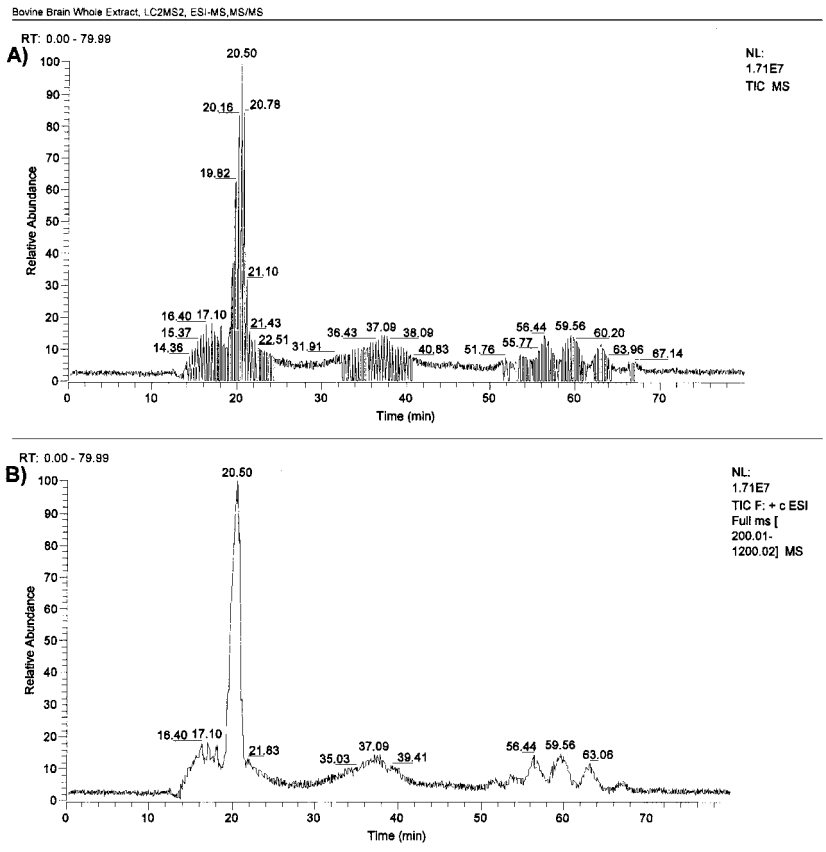

Figure 3. Electrospray ionization-mass spectrometry and UV chromatograms of polar components from commercially available bovine brain total lipid extract separated using RP-HPLC. (A) Electrospray ionization-mass spectrometry TIC showing all MS and MS/MS scans, and (B) ESI-MS TIC showing only scans obtained in positive mode.

the PDA. The data from the PDA are not discussed, because they provided no structural information. Figure 3 shows the ESI-MS TIC from the tandem mass spectrometer and the TIC filtered to show only (+) ion scans. The ELSD was not sufficiently sensitive to produce a useful chromatogram. Negative ion scans were not obtained on the tandem instrument. These two figures show that multiple classes of both polar and neutral lipids were separated using this combination of two LC systems with two mass spectrometers. Figures 4–6

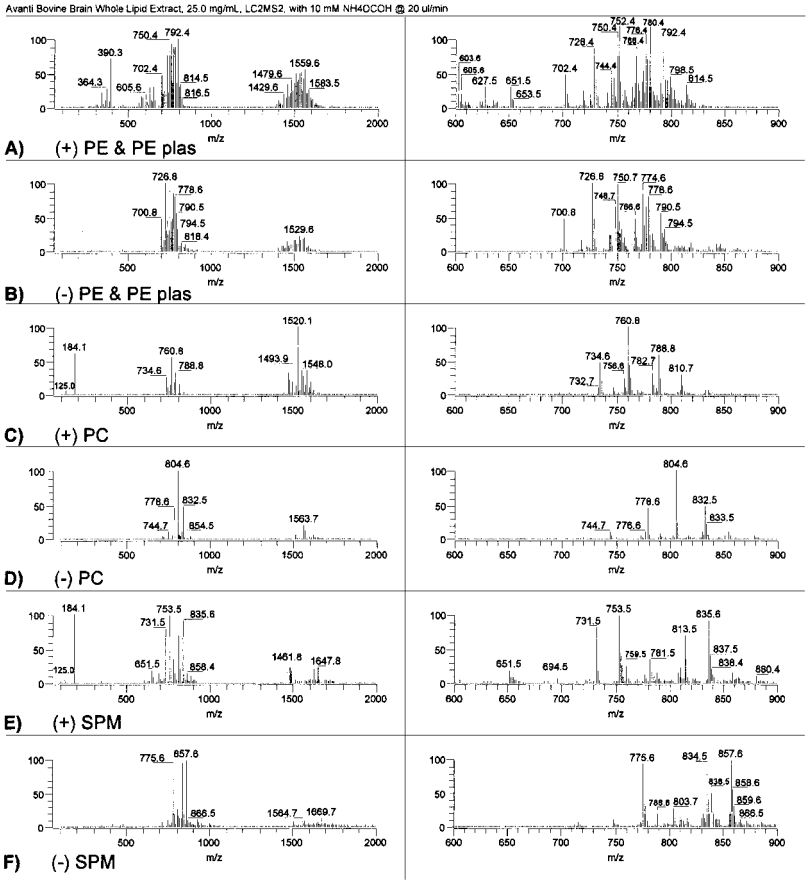

Figure 4. Positive and negative averaged ESI-MS mass spectra of bovine brain phospholipids. (A) positive ion mass spectrum of PE and PE plas; (B) negative ion spectrum of PE and PE plas; (C) positive spectrum of PC; (D) negative ion spectrum of PC; (E) positive ion spectrum of sphingolipids; (F) negative ion mass spectrum of sphingolipids. Left panel m/z 50–2000; right panel m/z 600–900.

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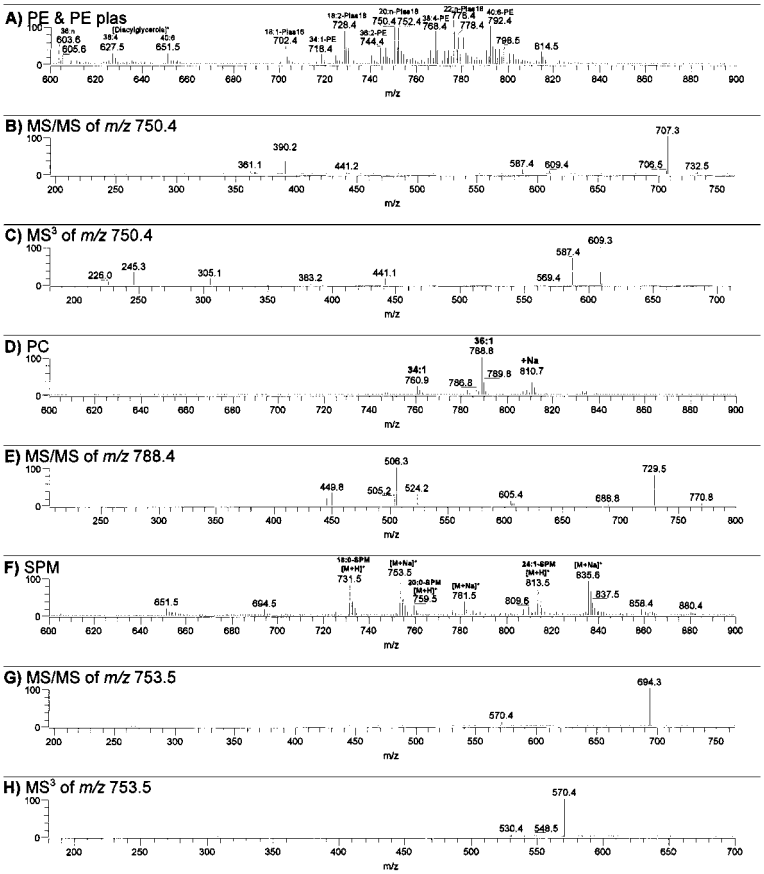

Figure 5. Electrospray ionization-mass spectrometry, MS/MS, and MS/MS/MS mass spectra of PE and PE plas, PC, and SPM. (A) Full-scan ESI-MS mass spectrum of PE and PE plas molecular species; (B) ESI-MS/MS mass spectrum of m/z 750.4 [20 : 5,18P PE plas + H]$^+$; (C) MS^3 mass spectrum of m/z 750.4; (D) full-scan MS spectrum of PC molecular species; (E) MS/MS of m/z 788.8 [18 : 0, 18 : 1—PC + H]$^+$; (F) full-scan MS of sphingolipid molecular species; (G) MS/MS of m/z 753.5 [18 : 0—SPM + Na]$^+$; (H) MS^3 of m/z 753.5. Identities of fragments suggested in Sch. 1.

show mass spectra of polar components separated on the amine column, while Figs. 7–9 show ion chromatograms and mass spectra of non-polar lipids.

We recently employed dual parallel mass spectrometers in an LC1/MS2 configuration with ESI-MS and APCI-MS to demonstrate that ESI-MS provided increased sensitivity for the analysis of TAGs compared to APCI-MS, when

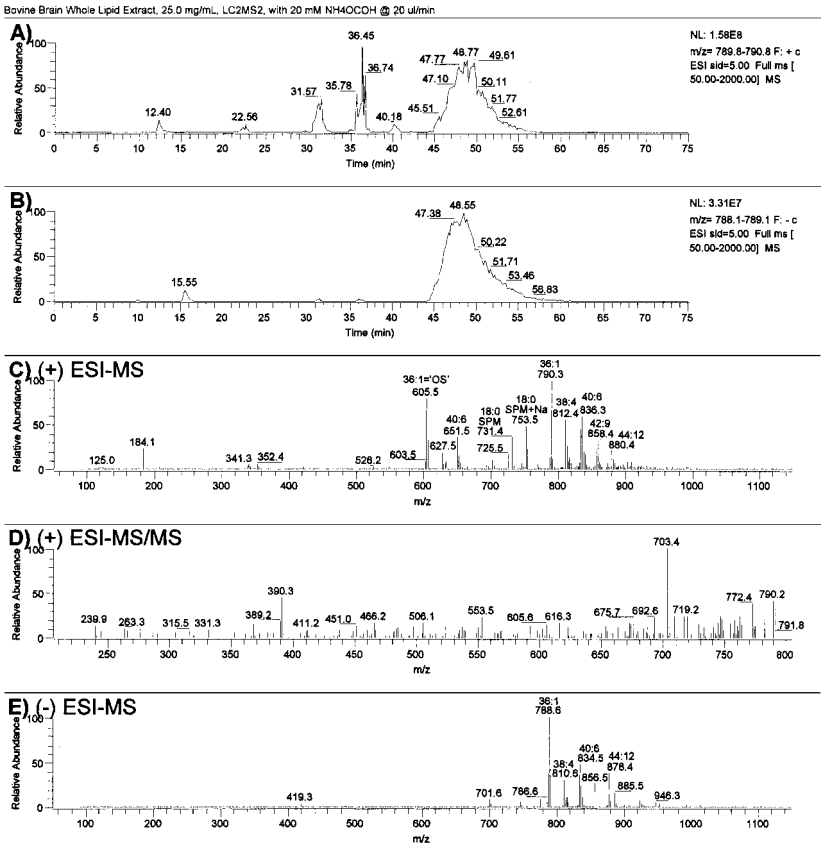

Figure 6. Extracted ion chromatograms and ESI-MS and MS/MS mass spectra of PS molecular species. (A) positive Ion EIC of m/z 790.3 representing oleoylsteroyl-PS (OSPS) $[M+H]^+$; (B) negative ion EIC of m/z 788.6, $[OSPS-H]^-$; (C) positive ion mass spectrum averaged across 45–48 min; (D) positive ion MS/MS mass spectrum averaged across all spectra having 790.3 as the base peak; (E) negative ion mass spectrum averaged from 45–48 min.

ammonium formate was added as a sheath liquid.[10] Commercially available bovine brain whole extract contained a small amount of neutral lipids, so maximum sensitivity was required. Therefore, we employed dual liquid chromatography with ESI mass spectrometry on both the ion trap mass spectrometer and the tandem mass spectrometer in this experiment. In the present example, ammonium formate was used as a sheath liquid on the LCQ mass spectrometer, whereas it was incorporated into the solvent system on the TSQ.

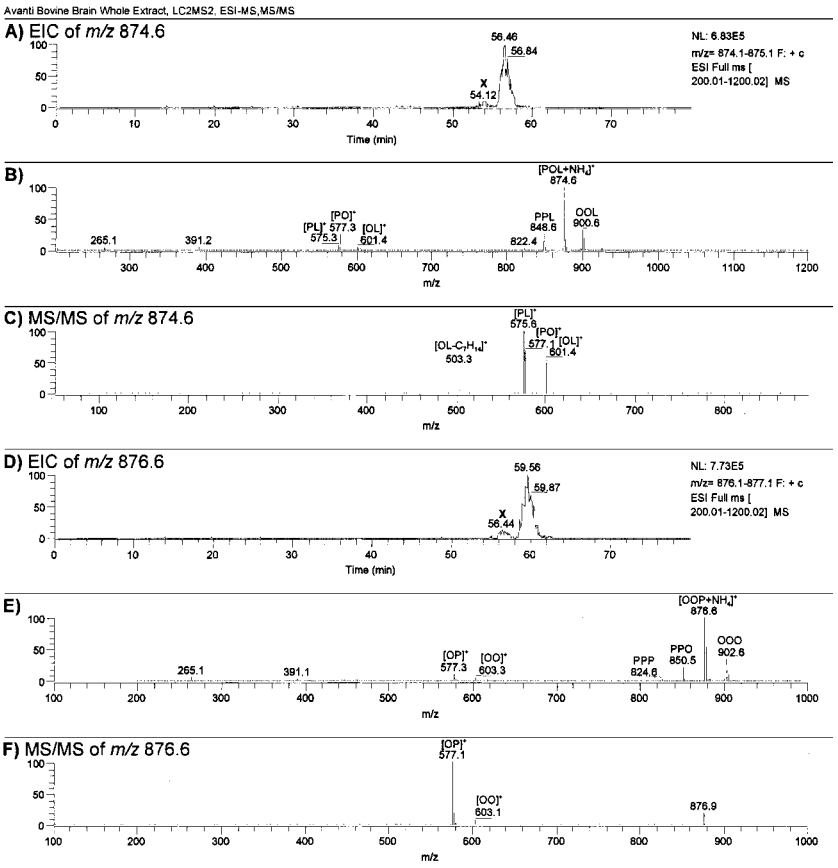

Figure 7. Electrospray ionization, ESI-MS, and MS/MS mass spectra of TAG ammonium adducts. (A) EIC of m/z 874.6 = $[POL + NH_4]^+$; (B) average mass spectrum across POL peak, showing m/z values of overlapped TAG; (C) MS/MS spectrum of m/z 874.6 parent; (D) EIC of m/z 876.6 = $[OOP + NH_4]^+$; (E) average mass spectrum across OOP peak, showing m/z values of overlapped TAG; (F) MS/MS spectrum of m/z 876.6 parent. x = peak arising from the $2x^{13}C$ isotope variant of M-2.

Figure 2 shows elution of phosphatidyl ethanolamine (PE) and its plasmalogen (PE plas), PC, and the sphingolipids, as well as other polar lipids. Phosphatidyl ethanolamine and PE plas occurred at 28.5 to 32 min, PC eluted between ~35 and 39 min, and sphingomyelin came off the column between 42 and 50 min. Within these time ranges, long-chain molecular species eluted with shorter retention times, and medium- and short-chain

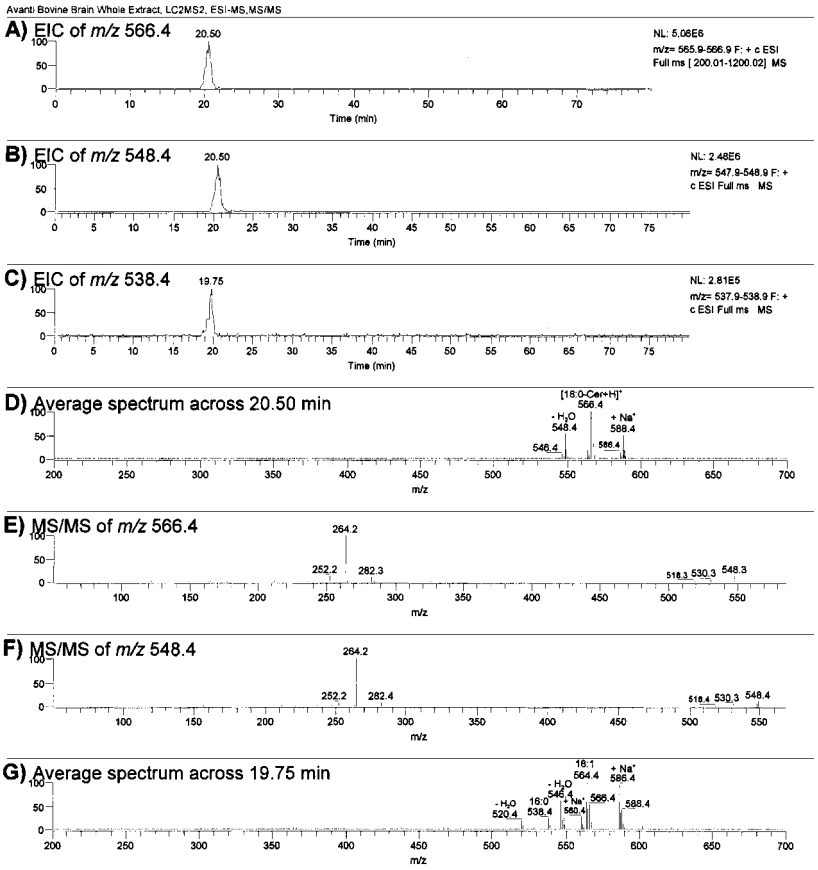

Figure 8. EICs and ESI-MS and MS/MS mass spectra of ceramides. (A) EIC of stearoylceramide, m/z 566.4; (B) EIC of m/z 548.4; (C) EIC of palmatoylceramide, m/z 538.4; (D) ESI-MS mass spectrum averaged across peak at 20.5 min; (E) ESI-MS/MS mass spectrum of m/z 566.4 parent; (F) ESI-MS/MS spectrum of m/z 548.4 parent; (G) ESI-MS mass spectrum averaged across peak at 19.75 min.

molecular species eluted later in the peaks. The shorter molecular species of one class of PL may overlap the long-chain species of the next eluted class of PL, but the two classes are easily distinguished by mass. Other classes of polar lipids, described below, eluted between 10 and 25 min, and phosphatidylserine (PS) eluted near 45 to 55 min, overlapped with SPMs. The medium- to short-chain SPM species overlapped the long- to medium-chain PS species. Figure 4 shows positive and negative full-scan MS mass spectra of PE, PC, and the

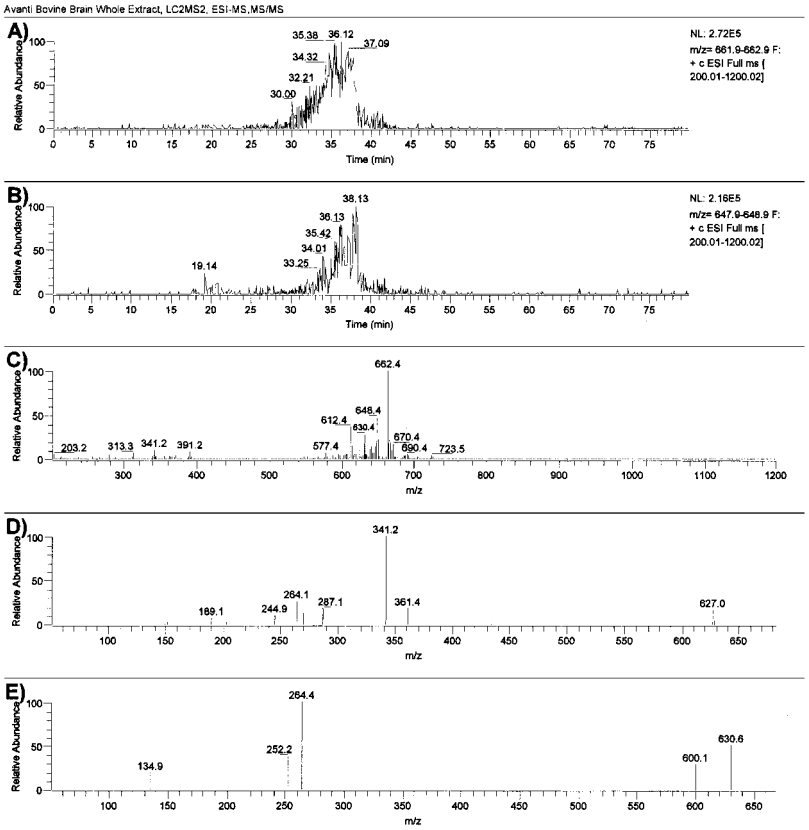

Figure 9. EIC and ESI-MS and MS/MS of other neutral lipids. (A) EIC of m/z 662.4 (18 : 0, 20 : 4 DAG); (B) EIC of m/z 648.4 (24 : 1 ceramide); (C) average ESI-MS mass spectrum from 34 to 38 min; (D) average ESI-MS/MS mass spectrum with m/z 662.4 parent; (E) average ESI-MS/MS mass spectrum with m/z 648.4 parent.

sphingolipids. The m/z range 50–2000 is shown in the left panel of Fig. 4, which includes the range of the dimers. Dimers were formed to a substantial extent in the ESI source. Dimer formation is concentration dependent, and can be greatly reduced by using a very dilute sample solution at a very low flow rate. However, at very low concentrations species present at low levels are not observed. Since neutral lipids were present at relatively low levels in this sample, a higher concentration was used to allow all possible molecular species of both neutral and polar lipids to be observed. For quantitative analysis, a dilute solution analyzed on a tandom mass spectrometer in negative

ion mode yields usable quantitative results, based on the demonstration by Han and Gross[11,12] that different molecular species of anionic phospholipids have nearly identical ionization efficiencies, after corrections for ^{13}C isotope abundances.

The right panel of Fig. 4 shows the protonated molecule m/z region for three classes of phospholipids. In addition to the protonated molecules and some fragments, the abundances of the m/z values of the dimers were substantial, but only in the case of the PC species were they larger than the protonated molecules. Phosphatidyl ethanolamine species produced protonated ionic molecules, $[M+H]^+$, in (+) ion mode, and deprotonated ionic molecules, $[M-H]^-$ in (−) ion mode, allowing facile identification of the molecular masses. The PC species and sphingolipid molecules both produced protonated, $[M+H]^+$, and sodiated molecules, $[M+Na]^+$, in (+) ion mode, while they gave almost exclusively formate adducts, $[M+45]^-$, in (−) ion mode. Masses of common molecular species of these classes are given in Table 1. In the mass spectra in Fig. 4, the molecular species were most easily identified by their formate adducts, $[M+45]^-$, and then masses confirmed by protonated and sodiated molecules.

Figure 4(C) shows that the most abundant PC species had m/z values of 734.7, 760.8, and 788.8. Table 1 shows that these m/z values corresponded to dipalmitoyl-PC (DPPC), palmitoyloleoyl-PC (POPC), and stearoyloleoyl-PC (SOPC), respectively. The negative ion mass spectrum in Fig. 4(D) showed m/z values that corresponded to the formate adducts, $[M+45]^-$, of each of these primary molecular species, also shown in Table 1. Phosphatidylcholine molecular species produced sodiated molecules having abundances that were 32% to 53% of the abundances of the protonated molecules. Sphingomyelin species, on the other hand, gave abundances of sodiated molecules that were larger than the abundances of the protonated molecules. For SPM molecular species, the protonated molecules had abundances that were 70% to 81% of the abundances of the sodiated molecules.

Figure 5 shows full-scan MS, MS/MS, and MS/MS/MS mass spectra of PE and PE plas, PC, and SPM. The suggested identities of the fragments observed from these three phospholipid classes are shown in Sch. 1. These identities are based on the recent work by Hsu and Turk[11] using deuterium-labeled PC to elucidate fragmentation mechanisms. Their recent work provided convincing evidence that the α-hydrogens on the fatty acyl chains were involved in loss of the phosphate head groups. This work replaced previous suggested pathways in which hydrogens on the glycerol backbone were thought to play a major role in the fragmentation/ionization process. Also, reflected in the fragment identities in Sch. 1, is the delocalization of the sodium ion on the sodiated SPM. For a number of years, several groups have represented alkali metals as being delocalized, to help account for the dramatic change in the fragment patterns observed when such a metal is attached.[13–16]

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Table 1. Calculated masses of phospholipid molecular species.

Abbreviations	$[M+H]^+$	$[M+NA]^+$	$[M+45]^-$	$[DAG]^+$
PE [Head group + 2H]$^+$ **142.0**				
PS-PE	720.6			
PO-PE	718.5			
PL-PE	716.5			
SS-PE	748.6			
SO-PE	746.6			
SL-PE	744.6			
OO-PE	744.6			
OL-PE	742.5			
OLn-PE	740.5			
LL-PE	740.5			
18 : 0,20 : 0-PE	776.6			
38 : 1	774.6			
38 : 2	772.6			
38 : 3	770.6			
38 : 4	768.6			
38 : 5	766.5			
38 : 6	764.5			
20 : 0,20 : 0-PE	804.6			
40 : 1	802.6			
40 : 2	800.6			
40 : 3	798.6			
40 : 4	796.6			
40 : 5	794.6			
40 : 6	792.6			
PE plas				
L-Plas(16)	700.5			
O-Plas(16)	702.5			
S-Plas(16)	704.6			
20 : 0-Plas(16)	732.6			
20 : 1-Plas(16)	730.6			
22 : 0-Plas(16)	760.6			
22 : 1-Plas(16)	758.6			
22 : 2-Plas(16)	756.5			
22 : 3-Plas(16)	754.4			
22 : 4-Plas(16)	752.3			
22 : 5-Plas(16)	750.2			
22 : 6-Plas(16)	748.1			
24 : 0-Plas(16)	788.7			
24 : 1-Plas(16)	786.6			

(continued)

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Table 1. Continued.

Abbreviations	$[M+H]^+$	$[M+NA]^+$	$[M+45]^-$	$[DAG]^+$
P-Plas(18)	704.6			
Ln-Plas(18)	726.5			
L-Plas(18)	728.6			
O-Plas(18)	730.6			
S-Plas(18)	732.6			
20 : 0-Plas(18)	760.6			
20 : 1-Plas(18)	758.6			
20 : 2-Plas(18)	756.6			
20 : 3-Plas(18)	754.6			
20 : 4-Plas(18)	752.6			
20 : 5-Plas(18)	750.6			
22 : 0-Plas(18)	788.7			
22 : 1-Plas(18)	786.6			
22 : 2-Plas(18)	784.6			
22 : 3-Plas(18)	782.6			
22 : 4-Plas(18)	780.6			
22 : 5-Plas(18)	778.6			
22 : 6-Plas(18)	776.6			
24 : 0-Plas(18)	816.7			
24 : 1-Plas(18)	814.7			
PC $[\text{Head group}+2H]^+$ **184.1**				
PP-PC	734.6	756.6	778.6	
PS-PC	762.6	784.6	806.6	
PO-PC	760.6	782.6	804.6	
PL-PC	758.6	780.6	802.6	
SS-PC	790.6	812.6	834.6	
SO-PC	788.6	810.6	832.6	
SL-PC	786.6	808.6	830.6	
OO-PC	786.6	808.6	830.6	
OL-PC	784.6	806.6	828.6	
OLn-PC	782.6	804.6	826.6	
LL-PC	782.6	804.6	826.6	
18 : 0,20 : 0-PC	818.7	840.6	862.7	
38 : 1-PC	816.6	838.6	860.6	
38 : 2-PC	814.6	836.6	858.6	
38 : 3-PC	812.6	834.6	856.6	
38 : 4-PC	810.6	832.6	854.6	
38 : 5-PC	808.6	830.6	852.6	
38 : 6-PC	806.6	828.6	850.6	
20 : 0,20 : 0-PC	846.7	868.7	890.7	

(continued)

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Table 1. Continued.

Abbreviations	$[M+H]^+$	$[M+NA]^+$	$[M+45]^-$	$[DAG]^+$
40 : 1-PC	844.7	866.7	888.7	
40 : 2-PC	842.7	864.6	886.7	
40 : 3-PC	840.6	862.6	884.6	
40 : 4-PC	838.6	860.6	882.6	
40 : 5-PC	836.6	858.6	880.6	
40 : 6-PC	834.6	856.6	878.6	
SPM [Head group + 2H]$^+$ **184.1**				
PSPM	703.6	725.6	747.6	
SSPM	731.6	753.6	775.6	
OSPM	729.6	751.6	773.6	
LSPM	727.6	749.6	771.6	
20 : 0-SPM	759.6	781.6	803.6	
20 : 1-SPM	757.6	779.6	801.6	
22 : 0-SPM	787.7	809.7	831.7	
22 : 1-SPM	785.7	807.6	829.6	
22 : 2-SPM	783.6	805.6	827.6	
24 : 0-SPM	815.7	837.7	859.7	
24 : 1-SPM	813.7	835.7	857.7	
24 : 1-SPM	811.7	833.7	855.7	
PS				
PP-PS	736.5			551.5
PS-PS	764.5			579.5
PO-PS	762.5			577.5
PL-PS	760.5			575.5
SS-PS	792.6			607.6
SO-PS	790.6			605.6
SL-PS	788.5			603.5
OO-PS	788.5			603.5
OL-PS	786.5			601.5
18 : 0,20 : 0-PS	820.6			635.6
38 : 1	818.6			633.6
38 : 2	816.6			631.6
38 : 3	814.6			629.6
38 : 4	812.5			627.5
38 : 5	810.5			625.5
38 : 6	808.5			623.5
20 : 0,20 : 0-PS	848.6			663.6
40 : 1	846.6			661.6
40 : 2	844.6			659.6

(continued)

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Table 1. Continued.

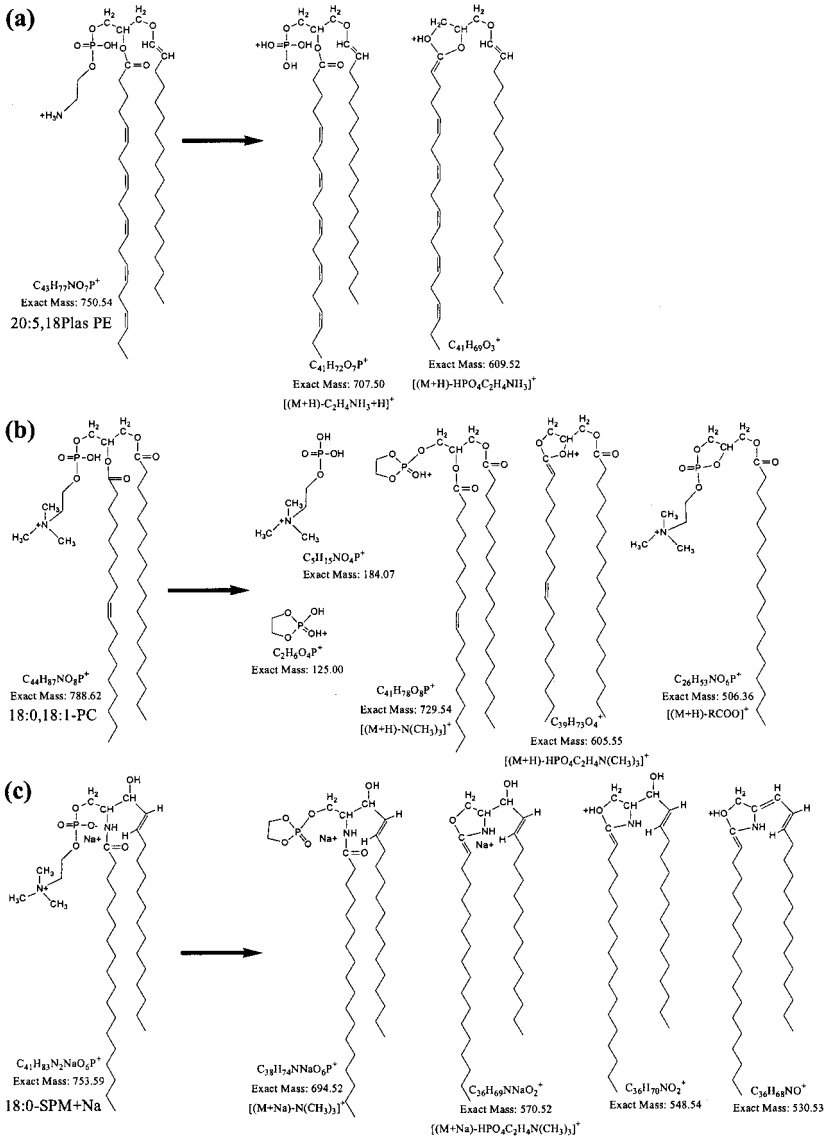
Abbreviations	$[M+H]^+$	$[M+NA]^+$	$[M+45]^-$	$[DAG]^+$
40 : 3	842.6			657.6
40 : 4	840.6			655.6
40 : 5	838.6			653.6
40 : 6	836.5			651.5
20 : 0-22 : 0-PS	876.7			691.7
22 : 0,22 : 0-PS	904.7			719.7

Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; SPM, sphingomyelin; P, palmitic acid, C16 : 0; S, stearic acid, C18 : 0; O, oleic acid, C18 : 1; L, linoleic acid, C18 : 2; Ln, linolenic acid, C18 : 3; $[DAG]^+$, diacylglycerol fragment. $[DAG]^+$ are the same for PE, PC, and PS.

Without an alkali metal, phosphocholine-containing phospholipids often yield only the m/z 184 ion corresponding to the phosphocholine head group [although, this is not the case in the the MS/MS spectrum of the PC in Fig. 5(E)]. In the mass spectra of PE plas molecular species, two sets of peaks appeared at m/z 362/364 and m/z 390/392. These were found to be consistent across PE plas molecular species, regardless of the identity of the fatty acyl chain attached to the plasmalogen, so it was determined that these peaks arose from the alkyl-ether fatty acyl chain attached to the PE head group. Full disclosure of the structure of this fragment will require more thorough study of the fragmentation mechanism.

Bovine Brain Phosphatidylserine Molecular Species

Examination of Fig. 2(B) and (C) shows that the chromatographic peaks arising from the sphingolipids did not appear as sharp as the peaks in the chromatogram of previous reports. Also, Fig. 4(F) shows that a peak at m/z 834.5 was present in this spectrum that did not have a mass corresponding to the sphingolipids. Further analysis revealed that another class of molecules eluted with similar retention times to the sphingolipids, under these chromatographic conditions. It was found that PS molecular species eluted just after, and partially overlapped with, the sphingolipids. Figure 6(A) and (B) shows positive and negative EIC of the molecular species having a base peak of m/z 790.3 in positive ion mode and m/z 788.6 in negative ion mode. These values corresponded to oleoylstearoyl PS (OSPS), as given in Table 1. If we assume that in its natural state, the PS is zwitterionic, having a negatively charged phosphate group, a neutral carboxylic acid, and a protonated primary amine, then a proton on the



Scheme 1. Suggested phospholipid fragment ion identities.

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phosphate group would form a + ion, $[M+H]^+$. Loss of the proton from the phosphate moiety would produce the negative ion, $[M-H]^-$.

Figure 6 shows positive and negative mass spectra averaged across the PS region. In Fig. 6(C), peaks arising from the most abundant molecular species can be observed, as well as DAG fragment ions that confirmed the DAG nature of these species and the identities of the fatty acyl chains. The peak at m/z 790.3 in Fig. 6(C) and m/z 788.6 in Fig. 6(E) arose from OSPS, as mentioned above. The peak at m/z 605.5 in Fig. 6(C) confirmed the "OS" DAG fragment ion. The peak at m/z 836.3 in Fig. 6(C) and m/z 834.5 in Fig. 6(E) identified the second most abundant PS molecular species as 40 : 6, which was confirmed by the peak at m/z 651.5 in Fig. 6(C). Numerous other molecular species were observable, such as 38 : 5, 38 : 4, 38 : 3, and 38 : 2 at m/z 810.4, 812.4, 814.4, and 816.3, respectively, and 40 : 8, 40 : 7, 40 : 6, 40 : 5, and 40 : 4, etc., at m/z 832.4, 834.4, 836.4, 838.3, 840.4, etc., respectively. The difference in m/z values between the fully protonated ionic molecule and the DAG fragment ion (e.g., 790 − 605) gave the mass of the protonated PS head group, 185 amu.

The MS/MS mass spectrum in Fig. 6(D) exhibited a primary fragment at m/z 703.4, corresponding to loss of the serine moiety to give the fully protonated oleoylstearoylphosphatidic acid, ("OS")$OPO_3H_3^+$. As Fig. 6(D) shows, the PS molecules underwent extensive charge-remote fragmentation. The MS/MS spectrum in Fig. 6(D) is an average mass spectrum across all MS/MS scans of m/z 790.3, occurring between 46 and 54 min. Also, the m/z 703.4 protonated phosphatidic acid (PA) fragment formed by loss of the serine moiety can be seen in the full MS scan in Fig. 6(C), and the deprotonated PA fragment, ("OS")OPO_3H^-, can be seen at m/z 701.6 in the negative ion spectrum in Fig. 6(E). Other authors[12] have reported negative ion MS/MS, in which the molecules underwent less fragmentation and produced more diagnostically useful fragment ions, as opposed to the positive ion spectrum in Fig. 6(D) (these authors employed a TSQ 700 tandem mass spectrometer). Our own experience has shown that for some classes of molecules, fragmentation in the ion trap mass spectrometer is more extensive than the fragmentation on our tandem mass spectrometer, though for other classes the fragmentation appears nearly identical. One other note regarding the PS molecular species: the chromatographic peaks for PS were more broad than those of other phospholipids. The presence of both carboxylic acid and amine functional groups on the PS gave rise to mixed modes of retention on the polar column and, therefore, broader peaks.

ESI-MS and MS/MS of Triacylglycerols

Triacylglycerols are large, neutral molecules that normally are not amenable to ESI-MS analysis. However, when ammonium formate was added to the

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solvent system, abundant ammonium adduct ions were formed. We recently demonstrated the use of ammonium adducts for analysis of TAG and their oxidation products.[10] We showed, that with ammonium formate as sheath liquid, ESI-MS was more sensitive than APCI-MS for TAG analysis. When APCI-MS was applied to analysis of the neutral lipids from bovine brain extract, insufficient signal was obtained to allow analysis of the neutral lipids. Therefore, ESI-MS was employed, with ammonium formate incorporated into the solvent system, instead of being added as a sheath liquid. Figure 7 shows EIC of the m/z values of the ammonium adducts of two of the most abundant TAG from the bovine brain extract, palmitoyloleoyllinoleoylglycerol (POL) and dioleoylpalmitoylglycerol (OOP). Figure 7 also shows the ESI-MS and MS/MS mass spectra of these two species. These mass spectra showed the same characteristics as those recently reported.[10] The spectra showed peaks representing the ammoniated molecules almost exclusively, with only very small abundances of the DAG fragment ions. The MS/MS mass spectra, on the other hand, exhibited primarily DAG fragment ions for every DAG contained in the TAG molecule. One difference between this and our previous report[10] was that previously we performed ESI-MS, MS/MS, and MS^3 of TAG on an the ion trap mass spectrometer, and APCI-MS and MS/MS on a tandem mass spectrometer. In this LC2/MS2 experiment, the LCQ was used for the phospholipid analysis, so the ESI-MS of TAG was carried out on the tandem mass spectrometer, also with ESI. The appearance of spectra such as shown in Fig. 7, obtained from tandem mass spectrometer, were essentially identical to the data obtained on the the ion trap mass spectrometer, even though completely different solvent systems were used, and the ammonium formate was delivered in a different way. However, the tandem mass spectrometer allowed only MS/MS, instead of MS^3.

On the RP-HPLC system the neutral lipids containing longer-chain fatty acyl chains eluted at longer retention time, while medium- and short-chain molecular species eluted earlier. This is in contrast to the elution orders during the polar lipid separation using the NP system, in which longer-chain species eluted with shorter retention times. Also, unsaturated species eluted earlier than their saturated homologs on the RP system.

ESI-MS and MS/MS of Ceramides and Diacylglycerols

Above, we have identified all of the classes of polar and non-polar lipids that we have most commonly encountered in the past. There are other classes, such as DAGs, that are known to accompany classes such as TAGs. There are other neutral lipids that we have not previously identified using either APCI-MS or ESI-MS, but which produced definitive mass spectra that allowed the molecules to be identified here.

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The largest peak in the ESI-MS chromatogram of neutral lipids in Fig. 3 occurred at 20.50 min. Figure 8 shows EIC and mass spectra of the species that eluted at this time. The identical elution time and peak shape of m/z 548.4 and 566.4 in Fig. 8(A) and (B) indicated that the m/z 548.4 peak probably was a fragment from the m/z 566.4 peak. Examination of the MS/MS spectrum of m/z 566.4 in Fig. 8(E) confirmed that the m/z 548.4 peak was a fragment produced from the m/z 566.4 ion. We have previously observed[17] (under APCI-MS conditions) that such a difference of 18 amu is often associated with a loss of a hydroxy group in the form of H_2O, by abstracting an additional hydrogen (leaving a double bond in place of the hydroxy). The MS/MS spectrum in Fig. 8(D) also showed further loss of 18 amu, the difference between m/z 548.3 and 530.3, indicating that the molecule likely possessed a second hydroxy group. The difference between the peaks m/z 282.3 and 264.2 in Fig. 8(D) further indicated the likely presence of a hydroxy group. In addition to loss of 18 amu from the m/z 548.3 ion, this ion also exhibited loss of 30 amu to give the ion at m/z 518.3. Similarly, the m/z 282.3 peak showed the same loss of 30 amu. The fact that the m/z values of the presumably protonated parent and fragment ions in both the ESI-MS and the MS/MS spectra were even, indicated the likelihood that the molecule contained one nitrogen atom. The presence of multiple hydroxy groups and a nitrogen, taken together, provided clues that these molecules were ceramides, which are known to be present in brain extracts. Calculated masses of prominent molecular species of this class of neutral lipids did give m/z values that were the same as those observed in mass spectra of the protonated molecule and fragment masses, which are depicted in Sch. 2. The fragmentation products in Sch. 2 reflect all of the fragmentation pathways shown by Couch et al.[18] under APCI-MS conditions, except in Sch. 2 we show protonated fragments, whereas Couch et al.[18] did not depict sites of protonation. We show the amine nitrogen protonated, as shown in some of the fragments represented by Domon and Costello,[19,20] obtained by FAB-MS/MS. These are similar to those of Fillet et al.,[21] obtained by ESI-MS/MS, but Fillet et al. also did not show ionic (protonated) fragments. Other authors have analyzed ceramides using negative ion tandem mass spectrometry.[22–25] We did not, however, perform negative ion tandem MS on the TSQ mass spectrometer for this experiment. This would require modification of the instrument program written in ICL for the TSQ, and TAGs are known to not give good response in negative ion mode. Negative ion mass spectrometry is much more readily accomplished using the LCQ Deca, ion trap mass spectrometer, which is why negative, as well as positive ion mass spectra of phospholipids are presented here, whereas only positive ion mass spectra are presented of TAGs, ceremides, and other neutral lipids. Nevertheless, the pathways shown by Hsu and Turk[23] in negative ion mode are very comparable to

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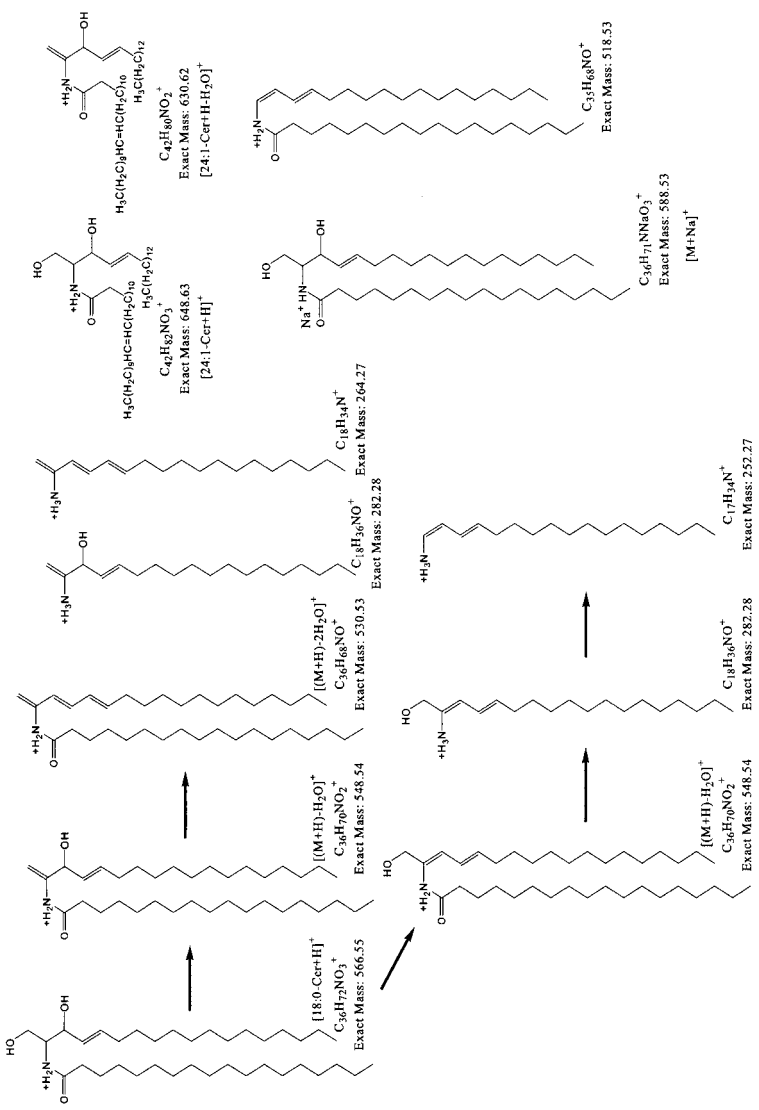

Scheme 2. Proposed protonated (and sodiated) molecules and fragment ions formed from ceramides under ESI-MS and MS/MS conditions.

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the positive fragments shown in Sch. 2. The pathways shown by of Raith and Neubert[22] in negative ion mode are also analogous to those structures shown in Sch. 2. Han,[24] Lee et al.[25] also showed negative ion fragmentation pathways of ceramides obtained by negative ion ESI-MS/MS. The protontated molecules in Sch. 2 have similarities to some of the lithiated adduct fragments proposed by Hsu and Turk.[26]

Protonated stearoylceramide ($[18:0\text{—Cer} + H]^+ = C_{36}H_{72}NO_3{}^+$) has a calculated protonated molecule mass of 566.6, and gave fragments of 548.5 and 530.5 in the MS/MS spectrum, from loss of one and two hydroxy groups, respectively, as shown in Sch. 2. It was interesting that the MS/MS spectrum of m/z 566.4 in Fig. 8(D) appeared virtually indistinguishable from the MS/MS spectrum of m/z 548.4 in Fig. 8(E), because the intact ceramide lost its first hydroxy group so readily. This molecule also produced fragments at m/z 282.3 and 264.3 that further specified the length of the sphingosine backbone. The mass spectrum in Fig. 8(D) shows peaks arising from the protonated stearoylceramide at m/z 566.4, the dehydroxylated stearoylceramide at m/z 548.4, and the sodiated stearoylceramide, $[Cer + Na]^+$, at m/z 588.4. The stearoylceramide was not the only molecular species present. Other amide-linked acyl chains formed molecular species which m/z values may be seen in Fig. 8(G). Protonated oleoylceramide ($[18:1\text{—Cer} + H]^+ = C_{36}H_{70}NO_3{}^+$) gave protonated, dehydroxylated and sodiated molecule m/z values at 564.4, 546.4, and 586.4, respectively. Similarly, protonated palmitoylceramide ($[16:0\text{—Cer} + H]^+ = C_{34}H_{68}NO_3{}^+$) gave protonated, dehydroxylated, and sodiated molecule m/z values at 538.4, 520.4, and 560.4, respectively. It was a characteristic of this class of molecules that they did not form ammoniated molecules, just as the sphingolipids, which contain the sphingosine backbone, also did not form ammoniated molecules. It was interesting that the ceramides also formed sodiated molecules, just as the sphingolipids formed sodiated molecules. As mentioned above, this sodium was tightly bound and arose from sodium in the cellular extract. Scheme 2 shows the sodium atom of the sodiated ceramide molecule as being delocalized, in accordance with the discussion of phosphocholine phospholipids, above. The combination of protonated and sodiated molecules, along with gentle fragmentation to give dehydroxylated molecules in the full scan spectra, along with the definitive structural fragments in the MS/MS spectra, allowed this class of molecules to be identified in a straightforward manner.

Another substantial peak, eluted between 32 and 38 min in Fig. 3 contained two classes of lipids. This peak contained intact DAGs, as shown below, but other components coeluted in this time region. Figure 9 shows EIC of the two most abundant ions that appeared in the average mass spectrum, Fig. 9(C), in this time range. The ion having the m/z 662.4 was the base peak. The ESI-MS/MS spectrum of this ion is given in Fig. 9(D). The even mass of

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the parent ion indicated the presence of a nitrogen. If the m/z 662.4 was the ammonium adduct ion, $[M + NH_4]^+$, this would indicate a molecular mass of 644.4, and corresponds to stearoylarachidonyl (18 : 0, 20 : 4) DAG. Given the ease with which DAG lose their —OH group to form the DAG fragment ions commonly observed from DAG and TAG, it was not surprising that the MS/MS spectrum, Fig. 9(D), did not exhibit a protonated molecular ion at m/z 645, but instead exhibited a peak at m/z 627.0, which was the DAG fragment ion of stearoylarachidonyl (18 : 0, 20 : 4) DAG. The peaks at m/z 341.2 and 361.4 in the MS/MS spectrum in Fig. 9(D) corresponded to $[RCO + 74]^+$ peaks for stearoyl and arachidonyl acyl chains, respectively. The arachidonyl acyl chain also produced an acylium ion, RCO^+, at m/z 287.1. Numerous other ammonium adducts of intact DAGs were identified in the time range between 32 and 40 min. Diacylglycerol that eluted later in the peak, near 40 min, were more saturated than the 18 : 0, 20 : 4 DAG.

Another class of molecules gave peaks in Fig. 9(C) that overlapped those of the DAGs. The MS/MS mass spectrum of m/z 648.4 is shown in Fig. 9(E). This MS/MS spectrum showed that the m/z 630.4 peak in the full-scan MS was a fragment from the m/z 648.4 peak. As seen above, the loss of 18 amu indicated the loss of a hydroxy group. The m/z 648.4 peak produced fragment ions at m/z 264 and 252 in Fig. 9(E), indicating the sphingosine backbone. Additionally, the peak at m/z 630.6 underwent loss of 30 amu, as did the m/z 548.4 peak discussed above. The m/z 648.4, 630.4, and 612.4 peaks in the full scan spectrum arose from the ceramide having a 24 : 1 fatty amide chain. The intact protonated molecule ion has a calculated mass of 648.6 (as shown in Sch. 2), the loss of one hydroxy group gave a calculated mass of 630.6 and loss of the second hydroxy group gave a mass of 612.6. These fragments are equivalent to those that were shown in Table 1 in the report by Gu et al.[27] The 24 : 1 ceramide also produced a peak at m/z 670.4 in Fig. 9(C) from the sodiated adduct. The 24 : 1-ceramide produced the analogous protonated and sodiated molecules and followed all of the same fragmentation pathways that were depicted in Sch. 2. The longer fatty chain was responsible for the longer elution time of this ceramide.

ESI-MS of Polar Sphingolipids

The ceramides discussed above were eluted unretained from the polar chromatographic system, and passed onto the columns in the RP system, where they exhibited the retention times mentioned above, and shown in Figs. 8 and 9. The useful near-molecular ions and rich fragmentation patterns in the MS/MS spectra allowed the ceramides to be identified and important fragments to be characterized. There were several peaks that eluted from the NP

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system that exhibited the same fragments, indicating that they contained homologous structural fragments, and more specifically contained sphingosine backbones. But these molecules had a polar head group, in addition to the sphingosine backbone, which caused them to be retained on the polar column instead of being diverted to the non-polar system. These sphingolipids eluted in several peaks early in the NP chromatographic run. Figure 2 showed that several peaks eluted before PE, which eluted around 30 min. Ion chromatograms of prominent peaks in the mass spectra, and the average mass spectra across these peaks, are shown in Fig. 10. Positive ion mass spectra across the four numbered peaks in Fig. 10(A)–(E) exhibited base peaks of either m/z 792.5 or m/z 810.5. Because of the similar appearances of the spectra in Fig. 10(A) and (D), and the similar appearances of Fig. 10(B) and (E), the positive ion spectra did not provide uniquely identifying peaks to allow the molecular species to be identified. However, the negative ion mass spectra did exhibit peaks arising from deprotonated molecules and from formate adducts, which allowed the molecular weights of the species to be determined. In combination with the negative ion mass spectra, the positive ion mass spectra were seen to exhibit fragments that provided important structural information. All of the polar sphingolipid peaks exhibited m/z values of 282.3, 264.3, and 252.3 that indicated the presence of the sphingosine backbone, as shown in Sch. 2. Furthermore, the species having the primary fragments at m/z 792.5 gave an abundant fragment of m/z 630.5 in the full mass spectra, Fig. 10(B) and (E), as well as in the MS/MS spectrum, Fig. 10(C). As indicated in Sch. 2, this fragment corresponded to a ceramide backbone containing a 24 : 1 fatty amide chain. The MS/MS mass spectrum of m/z 792.5 gave primary fragments at m/z 630.4, 612.4, and 600.3 analogous to the fragments having m/z 548.5, 530.5, and 518.5 depicted in Sch. 2. Furthermore, the fragments at m/z 630.4, 612.4, and 600.3 were the same as those shown in Fig. 9(C) for 24 : 1 ceramide.

The negative ion ESI-MS mass spectra were necessary for identifying the molecular weights of the glycosphingolipids. The molecules fragmented minimally in (−) ion mode, and so produced abundant $[M-H]^-$ peaks. Several $[M+H]^+$ and $[M-H]^-$ ions are presented in Sch. 3. The hydroxy-cerebrosides are depicted as having hydroxy-fatty amide chains, consistent with the Avanti Polar Lipids catalog that states that brain cerebrosides eluted as two TLC spots, one containing non-hydroxylated fatty acids and one containing hydroxylated fatty acids.[28] Hsu and Turk[26] also showed mass spectra of hydroxylated glycosphingolipids, as lithium adducts. Levery et al.[29] had also shown ESI-MS/MS of lithium adducts of hydroxy- and non-hydroxy containing cerebrosides, in agreement with the structures shown in Sch. 3.

For the 24 : 1 cerebroside, eluted at 12.4 min (see Fig. 10), and the hydroxy-cerebroside, eluted at 14.2 min, the difference between the

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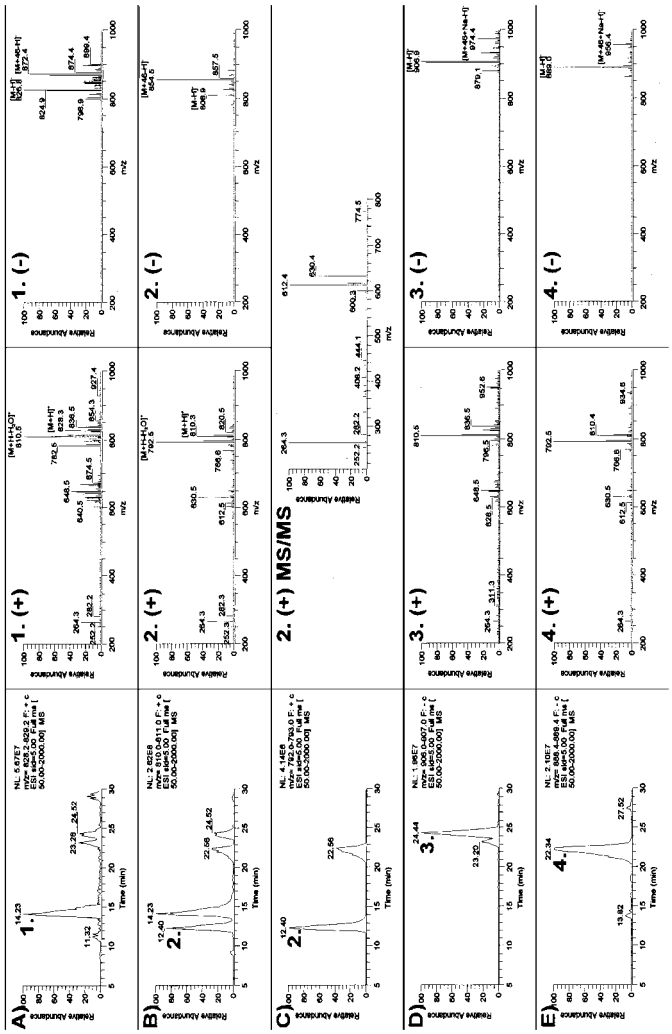

Figure 10. EIC and mass spectra of glycosphingolipids. (A) EIC of m/z 828.7 = [24 : 1—GalDH(OH)Cer + H]$^+$ and average (+) and (−) ESI-MS mass spectra of peak 1; (B) EIC of m/z 810.5 = [24 : 1—GalCerH]$^+$ and [24 : 1—GalDH(OH)Cer + H − H_2O]$^+$ and average (+) and (−) mass spectra of peak 2; (C) EIC of m/z 792.5 = [24 : 1—GalCer + H − H_2O and MS/MS mass spectrum of m/z 792.5 parent; (D) (−) ion EIC of m/z −06.8 = [24 : 1—SulGalDH(OH)Cer]$^+$ and average (+) and (−) mass spectra of peak 3; (E) (−) ion EIC of m/z −888.9 = [24 : 1 − SulGalCER9]$^+$ and average (+) and (−) mass spectra of peak 4.

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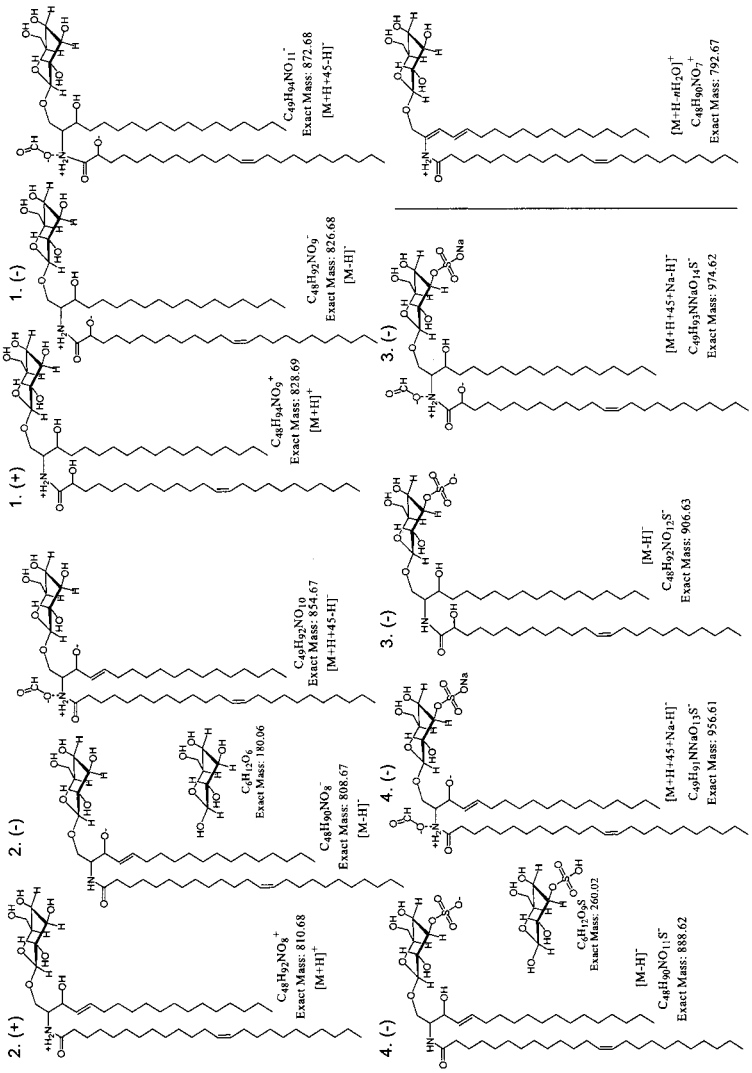

Scheme 3. Identities of protonated, deprotonated, and adduct ions form from glycosphingolipids and sulfatoglucosphingolipids. Numbers refer to peaks and mass spectra in Fig. 10.

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$[M+H]^+$ ion (m/z 810.3 and m/z 828.3, respectively) and the ceramide backbone fragment (m/z 630.5 and m/z 648.5, respectively) was $\Delta 180$, corresponding to a hexose head group. This could be either glucose or galactose, which could not be distinguished by mass. Scheme 3 depicts the galactose-containing cerebrosides, in agreement with Hsu and Turk, who reported lithiated adducts of bovine brain cerebrosides.[26] Scheme 3 reflects both the (+) ions shown by Domon et al.[20] for derivatized ceramides, and the (−) ions shown for glycosphingolipids.[19] An ion that has not been observed by others is the formate adduct formed due to the presence of ammonium formate. The exposed amide secondary nitrogen behaved similar to a choline quaternary amine. Thus, the fully protonated nitrogen formed an ammonium adduct similar to the adduct formed by choline phospholipids. Even more interesting were the sulphate-containing glycosphingolipids that formed not only $[M-H]^-$ ions, but also $[M+H+46+Na-H]^-$ ions that were adducts containing both formate and sodium ions. These ions were protonated at the amide nitrogen, where they formed an ammonium adduct, and they were deprotonated at a hydroxy oxygen (based on the references cited above). Again, the negative ion mass spectra were necessary for the identification of the molecular weights of the sulphatides, while the positive ion mass spectra provided confirmation of the presence of important structural features. In negative ion mode, the [M − H]-peak was the base peak in the mass spectra of sulphatides, with adducts formed with both sodium and ammonium formate also present. In positive ion mode, the m/z 810.5 and m/z 792.5 peaks indicated that these molecules contained α-hydroxy and non-hydroxy galactosylceramide fragments, upon loss of the sulphate group.

CONCLUSIONS

We have demonstrated, here, that an LC2/MS2 approach is feasible to perform a total lipid analysis, and that it allowed identification of numerous molecular species from a variety of polar and non-polar lipid classes. It is worth noting, that the process of eluting the neutral lipid bolus from the non-polar column and then switching it through the diverter valve to the non-polar columns did not result in a dramatic degradation of peak shape and loss of resolution. The peak shapes in EIC of neutral lipids in Figs. 7 and 8 clearly showed that sharp, narrow peaks could be obtained, even after elution down two pairs of columns having different polarities.

Although, we have successfully demonstrated the proof of concept for the LC2/MS2 analysis, we have performed only qualitative analysis here. Many details require further investigation to provide quantitative analysis of all of the identified components. For polar lipids, the main factors that require additional

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work are the elimination of the dimers and the sodium adducts, to produce only protonated molecules, $[M + H]^+$. Recent experiments have allowed us to identify sheath liquid additives that do successfully eliminate these artifacts.

Quantitative analysis of the non-polar lipids will require a different approach. As has been mentioned in several previous publications by us and others,[6–8] the fragmentation patterns of TAGs under APCI-MS conditions depend strongly on the degree of unsaturation within the TAG. Those TAG with more than four sites of unsaturation produced protonated molecules as base peaks, while those with less than three sites of unsaturation produced DAG fragment ions as base peaks. Triacylglycerols with three or four sites of unsaturation could have either protonated molecules or DAG fragment ions as base peaks, depending on the specific fatty acyl chains on the TAG. In the past, we were able to produce response factors for the fatty acyl chains that were then multiplied together to produce response factors for the TAGs. This was feasible because we analyzed only mixtures of TAGs. In a membrane total lipid extract, fatty acyl chains are present that arise from other classes of molecules. It is not possible to saponify the total lipid extract and produce fatty acid response factors that apply only to the TAG. Instead, a suitable approach is to collect a split portion of the HPLC effluent over the time range at which the TAG elute. This collected fraction would contain only TAG, so it may be saponified, and analysis of the fatty acid methyl esters performed by gas chromatography with flame ionization detection (GC-FID), to allow fatty acid response factors to be produced, and TAG response factors calculated in the usual way. Analysis of TAG under ESI-MS conditions has not yet been thoroughly addressed. The response factors required for ESI-MS of TAG are expected to be very different from those necessary for quantitative analysis by APCI-MS. All TAG, saturated and unsaturated, produced ammoniated molecules $[M + NH_4]^+$ as base peaks under ESI-MS conditions. We have recently shown[10] that polyunsaturated TAG give higher response than saturated TAG under ESI-MS conditions. This trend is the opposite of that observed using APCI-MS.

The separation of the TAG needs to be improved to increase the resolution to the point that it is similar to what we have previously reported for this class. We have successfully performed LC2/MS2 analyses, to be reported, that show that the acetonitrile/methylene chloride system, which produces a better separation of TAG, is sufficiently compatible with the NP system to allow use of the better solvent pair on the RP system. We also recognize the need to modify the NP system to allow the PS species to be fully resolved from the SPM species.

Although, there is still room for improvement in the separation and data acquisition programs, we have demonstrated, here, that it was possible to perform a single total lipid analysis in which both polar and non-polar lipids were separated on LC1 and RP (LC2) columns, simultaneously, followed by

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parallel detection using mass spectrometers with ESI (MS1 and MS2) ionization modes, acquiring MS, MS/MS, and MS^3 mass spectra. This analysis required no fabrication of special instrument components, but used common commercially available instruments with standard accessories. This "dual parallel liquid chromatography/parallel mass spectrometry," LC2/MS2, approach produced a prodigious amount of data that allowed the whole series of molecular species from a variety of lipid classes to be identified.

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