Dual parallel liquid chromatography with dual mass spectrometry (LC2/MS2) for a total lipid analysis

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1. ABSTRACT

Total lipid extracts containing both polar and non-polar lipids were separated using two liquid chromatographic systems joined together using a column-switching valve. Detection was accomplished using mass spectrometers attached to each of the two liquid chromatographic systems, for a dual liquid chromatography/dual mass spectrometry (LC2/MS2) arrangement. This experimental approach is demonstrated to be useful to identify many classes of polar and non-polar lipids, and numerous individual molecular species within each class, which is a primary goal of lipidomics. Data are presented from a bovine brain total lipid extract and a sand bream filet extract as examples. The use of both ESI-MS and APCI-MS are demonstrated for analysis of non-polar lipids, and advantages and shortcomings of each technique are discussed. The two parallel chromatographic separations are compared to lipidomics approaches that employ infusion without chromatography. The data show that the LC2/MS2 is one approach to a comprehensive total lipid analysis that can be performed using only commercially available instruments with no fabrication or modification required. The LC2/MS2 approach represents a uniquely valuable tool for monitoring the amounts of precursors and products of molecules involved in cell signaling processes. The dual liquid chromatography/dual mass spectrometry approach represents one of the most powerful tools for lipidomics analysis reported to date.

2. INTRODUCTION

The complete composition of all lipid classes and lipid molecular species within each class, in other words "the entire spectrum of lipids in a biological system" (1), is called the lipidome. The study of the complete set of all lipids in a system is therefore called 'lipidomics'. The complete sets of lipids within specific classes constitute subsets of the lipidome. For instance, the composition of all molecular species of triacylglycerols has been referred to as the 'triacylglycerol lipidome' (2), while the composition of all phospholipids classes and molecular species within each class can be called the 'phospholipidome'. But the synthesis and metabolism of lipids and the biological roles of the metabolites must also be considered, and so a complete understanding of the lipidome involves also characterizing the proteins that act on and are modulated by lipid components. A more comprehensive definition of lipidomics has been put forth as "the full characterization of lipid molecular species and of their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation" (1).

The study of all lipids includes polar lipids, such as phospholipids, and non-polar lipids, such as triacylglycerols (TAGs). This raises one of the fundamental problems of lipidomics: how to analyze molecules having radically different polarities using one unified approach. Typically, chloroform/methanol

extractions, such as those described by Bligh and Dyer (3) or Folch *et al.* (4), contain enough methanol that they draw out polar lipids into the chloroform-rich solvent, as well as non-polar lipids. A chloroform/methanol extraction is said to produce a 'total lipid extract'. In practice, however, substantial losses of phospholipids can occur with the aqueous wash step of biphasic extractions. Complete extraction of phospholipids is better achieved using a monophasic methanolic extraction (5). Nevertheless, biphasic chloroform/methanol/ water extractions are the most widely used extractions, and are considered the standard against which other extraction techniques are compared. Thus, it is these types of extractions that are most often used to obtain commercially available lipids offered by suppliers.

While the opposing polarity characteristics of disparate classes of lipids have an important impact on the solvents used to obtain a total lipid extract, they have an even greater impact on the methods used for analysis of the extracted lipids. Often, polar lipids are separated by class using normal-phase (NP) high-performance liquid chromatography (HPLC), while non-polar lipids are separated using reversed-phase (RP) HPLC. Usually, lipids of each type of polarity are separated to the exclusion of the other. When polar lipids are separated using a NP-HPLC column, such as a silica, diol or amine column, non-polar lipids elute essentially unretained as a bolus near the solvent front. On the other hand, when non-polar lipids are separated using RP-HPLC, polar lipids are essentially unretained and elute near the solvent front. The solvent composition in RP-HPLC can be modified to retain polar lipids based on their acyl chain lengths and degrees of unsaturation, but highly non-polar molecules, such as TAGs, do not elute under conditions used to separate molecules such as phospholipids by hydrocarbon chain length. Thus, the chromatographic conditions used for polar lipids are fundamentally incompatible with the conditions necessary to separate non-polar lipids. Chromatographic separation of all classes and molecular species of both polar lipids and non-polar lipids in a single analysis is one of the most elusive goals of lipidomics.

One approach to overcoming the problems imposed by contradictory chromatographic behavior of lipid classes of opposing polarities has been to forego the chromatographic separation altogether. This has been demonstrated with great success for some samples, and has given rise to the technique known as 'shotgun lipidomics' discussed at length previously (6,7). Using this approach, the difference in response of different classes of lipids to positive versus negative ionization allows some classes to be detected in positive-ion mode, while others are detected in negative-ion mode. Response of some classes is enhanced in negative-ion mode while others are suppressed. Conversely, response of some classes is enhanced in positive-ion mode while other classes are suppressed. Furthermore, each class of lipid produces a cluster of masses in a region of the mass spectrum particular to that class, so many classes and numerous species within each class can be identified. The identification can be greatly aided by the use of neutral loss scanning to identify characteristic moieties attributable to each class.

There are some drawbacks that keep the shotgun lipidomics approach from being the answer to lipidomics analysis of all samples. First, although the difference in response to positive versus negative ions suppresses some classes and enhances others, the signals from the classes that are suppressed are reduced but not entirely eliminated. Thus, species in the class of interest that are present at low levels may be masked by species present in larger amounts in the class that was suppressed but not eliminated. Species can be identified with an experimental error of approximately ± 5% in a straightforward manner (6,8). Species present in lower amounts may be masked by species in suppressed classes, but the situation can be greatly improved by derivatization and identification of individual classes (9). Second, some classes are so similar to each other in structure that many species of one class are isobaric with species of a similar class. This is especially true of sphingomyelin and dihydrosphingomyelin. Because of their similarities, dihydrosphingomyelin species containing monounsaturated fatty chains are isobaric with sphingomyelin species having saturated fatty chains. This makes these classes very difficult to distinguish by mass alone (10). For these and other reasons, it may be difficult to identify all molecular species of all classes of lipids using a 'shotgun lipidomics' approach. For some classes it is desirable, if not indispensable, to perform a chromatographic separation to allow all classes and species to be identified. However, the chromatographic separation of disparate classes suffers from the pitfalls mentioned above. One single chromatographic column is not ideally suited to separation of very polar, or even ionic and zwitterionic molecules, and simultaneously very non-polar molecules.

The need for different chromatographic separations that are each tailored to provide optimal separation of both polar (or ionic) and non-polar classes has given rise to an approach that employs 'dual parallel liquid chromatography' for separations of very different classes of lipids simultaneously from a single sample injection (11,12). This approach employs a column-switching technique in which two HPLC systems, a NP-HPLC system and an RP-HPLC system, are interconnected through an electronically actuated valve. Using this approach, a sample of a total lipid extract is injected onto a NP-HPLC system, in which the non-polar lipids, such as TAGs are essentially unretained, and pass immediately down the column to the switching valve built into the front of the ion trap mass spectrometer, where they are diverted to a RP-HPLC system. After the switching valve is closed, the polar and ionic lipids (i.e. phospholipids and others) are retained and move down the NP-HPLC column. Simultaneously, the non-polar lipids that were diverted move down the RP-HPLC system. Combinations of detectors including ultraviolet/visible (UV/Vis), an evaporative light scattering detector (ELSD) and tandem quadrupole or ion trap mass spectrometers are attached to the outlets of each of the two sets of HPLC columns.

LC2/MS2 total lipid analysis

This unified approach to lipid analysis has several advantages. First, lipid classes and molecular species are chromatographically separated, making their detection by mass spectrometry much easier. Second, the solvent composition and gradient program of each of the parallel chromatographic systems can be optimized for the classes and species of lipids to be separated, so no compromise in separation efficiency is required. Third, all species, even those present at low levels can be separated and identified since most classes do not overlap. Fourth, the type of ionization that is most suitable for each class of lipids can be utilized, since two different mass spectrometers are employed. We often use electrospray ionization (ESI) mass spectrometry (MS) for phospholipids analysis and atmospheric pressure chemical ionization (APCI) MS for triacylglycerols, although we demonstrate here the use of ESI-MS for both polar and non-polar lipid classes. Fifth, the duty cycle of the mass spectrometer can be most efficiently tailored to the needs of each class of lipid. ESI-MS/MS can be used for classes that fragment well, while parent ion scanning can be used for classes, such as glycerophosphocholines (GPCho), that produce a limited number of fragments from $[M+H]^+$ precursors (i.e. the phosphocholine head group at m/z 184.1). Positive and negative ion modes can be selected and changed during the chromatographic run to take advantage of preferences for individual classes to form positive or negative ions, but also to produce complementary data that aid in identification and structural elucidation of molecules. We summarize here the use of dual parallel liquid chromatography combined with dual mass spectrometry (LC2/MS2) for a unified approach to analysis of both polar and non-polar lipids in total lipid extracts. Special emphasis is placed on analysis of sphingomyelins and ceramides, since these two classes are the precursors and products, respectively, of the Sphingomyelin Cycle. Sphingomyelin species are polar and are separated by NP-HPLC, while ceramides are non-polar and are separated by RP-HPLC. This precursor/product pair serves as a particularly apropos example of the strengths of an LC2/MS2 approach to lipidomics.

3. MATERIALS AND METHODS

All solvents, except water, were purchased from either Aldrich Chemical Co. (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). 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. The bovine brain total lipid extract was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and was used without further purification.

The bream filet extraction was performed using methanol/chloroform/water (2:1:0.8) based on a modification of the method of Bligh and Dyer (3). A sand bream was purchased from a local fish market, and filleted. 2.2913 g of the filet were taken for the extraction. Initial volumes of extraction solvents were 6.67 mL H_2O and 25.0 mL MeOH/CHCl$_3$ (2:1) (=16.67 mL MeOH + 8.33 mL CHCl$_3$), for a composition of 0.8 H_2O : 1 CHCl$_3$: 2 MeOH.

The sample was assumed to have 80% moisture content, so 0.80 times the sample weight was subtracted from the 6.67 mL H_2O to give a total water volume of 6.67 mL including the moisture from the sample, to maintain the 0.8:1:2 proportions. 20 μL of tridecadeuterated (d_{13}) dipalmitoyl phosphatidylcholine (DPPC), 10.0 mg/mL, was added as an internal standard, and the sample was homogenized with the initial extraction solvent mixture, using a blade homogenizer (PowerGen 1000, Fisher Scientific, Hampton, NH, USA). Then, 8.33 mL of CHCl$_3$ was added and the sample was further homogenized. Next, 8.33 mL of H_2O was added and the sample was further homogenized. The final composition of the solvent mixture was 0.92 H_2O : 1 CHCl$_3$: 1 MeOH. The homogenate was filtered through a 1 in. Buchner filter funnel into a 50 mL centrifuge tube. The sample was centrifuged at 5000 rpm for 10 min and two clear layers without interfacial flocculence were obtained. The top layer was aspirated off. The remaining chloroform-rich layer was evaporated to ~ 1 mL and transferred to a tared 1.8 mL amber vial and then evaporated to dryness. 17.3 mg of extract was obtained. The extract was reconstituted in 500.0 μL of chloroform, to a final concentration of 34.6 mg/mL.

3.1. High performance liquid chromatography

For LC2/MS2 experiments, a normal-phase liquid chromatographic system was attached to the ion trap mass spectrometer, while a reversed-phase 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, now Thermo Fisher Scientific, San Jose, CA, USA). The AS 3000 autosampler was used to make an injection of 20 μL onto the normal-phase columns. The NP-HPLC system employed two amine columns in series, Adsorbosphere NH$_2$, 25 cm x 4.6 mm, 5 μm (Alltech Associates, Deerfield, IL, USA). The gradient program used three solvents: solvent A, 40% H_2O, 60% isopropyl alcohol (IPA); solvent B, 40% methanol (MeOH), 60% IPA; solvent C, 40% hexane (Hex), 60% IPA. Each solvent contained 0.1 % NH$_4$OH. The gradient program used was as follows: from 0 min to 10 min, 15% A, 15% B, 70% C; from 10 min to 20 min, a linear change to 25% A, 25% B, 50% C, held until 30 min.; from 30 min to 40 min, a linear change to 30% A, 45% B, 25% C; from 40 min to 50 min, a linear change to 30% A, 70% B, held until 75 min; recycled to initial conditions from 75 min 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 ion trap mass spectrometry (ITMS) instrument, described below and shown in Figure 1. 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 allowed use of a fraction collector, if desired, to collect phospholipid fractions instead of sending them to waste.
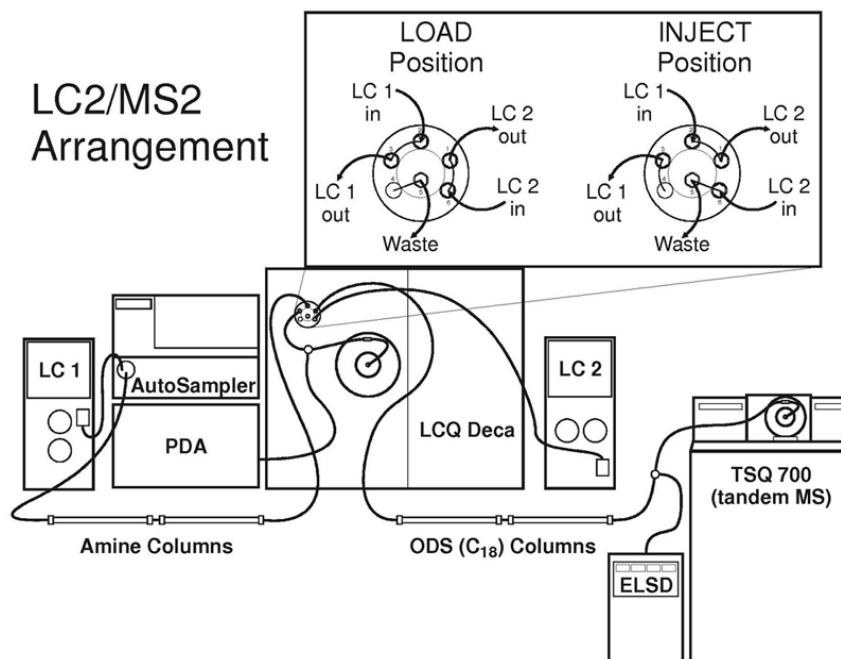
Figure 1. Arrangement of interconnections between two liquid chromatographs and two mass spectrometers for dual parallel liquid chromatography/ dual mass spectrometry experiments using the electronically actuated valve built into the LCQ Deca ion trap mass spectrometer.

The reversed-phase HPLC system used a Constametric 4100 quaternary pump with membrane degasser (Thermo Separation Products, now Thermo Fisher Scientific, San Jose, CA). The RP-HPLC system employed two Inertsil ODS-3 columns in series, 25 cm x 4.6 mm, 5 µm (MetaChem Technologies). A binary gradient was used: solvent A, MeOH; solvent B, 40% Hex, 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. 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 a Varex MKIII evaporative light scattering detector (ELSD) (Alltech Associates, Deerfield, IL, USA), and the straight-through exit of the tee delivered to the ionization source of the tandem sector quadrupole (TSQ) mass spectrometer. The levels of non-polar lipids in bovine brain were below the detection limit of the ELSD under these conditions, so no ELSD chromatograms for that sample 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 the tandem mass spectrometer) are shown in Figure 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, 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 reversed-phase columns was attached to port 1. The RP-HPLC system was located in proximity to the diverter valve to minimize dead volume (distances in Figure 1 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 non-polar 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 min to 8.20 min for the bovine brain extract, and from 5.75 to 7.75 for the sand bream total lipid extract.

3.2. LC2/MS2 mass spectrometry instrumentation

An LCQ Deca (Thermo Quest Corp., now Thermo Fisher Scientific, San Jose, CA) ion trap mass spectrometer was used for acquisition of ESI-MS data from the NP-HPLC system. Scans were obtained from *m/z* 50 to 2000, in centroided positive-ion mode for all runs. The method used for analysis of the fish filet used up-front collision energy of 20.0 V to try to reduce phospholipid dimer formation. The ion trap was set to automatically select the most abundant precursor ion and perform MS/MS, and in scan segment 2, to select the most abundant product ion and perform MS/MS/MS. The activation

energy was 58 % (arbitrary units) with a 'q factor' of 0.25. The activation time was 900 ms. 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 MS3 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, sphingomyelin, SPM, and dihydrosphingomyelin, DHS) eluted. These provided excellent positive-ion spectra, but poorer negative-ion MS/MS spectra, so emphasis was on positive-ion MS, MS/MS and MS3 scans. After the sphingolipids eluted, the scan program returned to the original scan program. The heated capillary was operated at 250 ^{o}C. 20 mM ammonium formate solution in H_2O/ACN (1:4) was added as a sheath liquid via syringe pump (AB 140B, Applied Biosystems, Foster City, CA), which improved sensitivity and facilitated the formation of negative adduct ions from the quaternary amine phospholipids, as discussed below. Sheath liquid flow was delivered at 20 µL/min. Manninen and Laakso (13) compared several chemical ionization reactant molecules by sparging the sheath gas through a bottle containing one of several solvents. They compared the signal given when methanol, isopropanol, water, or 0.5% NaOH in MeOH was used as the sparging solvent. The best signal by APCI-MS was produced when the sheath gas was bubbled through methanol. Also, Duffin *et al.* (14) had shown that ammonium adducts yielded better MS/MS spectra than sodium adducts, although the sodium adducts gave better sensitivity. Since we performed MS/MS analysis, we used an ammonium salt.

The tandem mass spectrometer attached to LC2 was a Finnigan MAT TSQ 700 (Thermo Finnigan Corp., now Thermo Fisher Scientific, San Jose, CA). The TSQ 700 gave 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. ESI MS spectra were obtained from *m/z* 200 to 1200, with a scan time of 1.0 s. The heated capillary temperature was 265 ^{o}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. 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 (~2 mTorr) and four MS/MS scans of each of the two most abundant precursor ions were collected. The time on the ICIS data

system on the TSQ 700 was not synchronized to the Xcalibur™ data system on the LCQ Deca. 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.

4. RESULTS

4.1. LC2/MS2 of polar lipids in bovine brain total lipid extract

Figures 2 and 3 show two parallel chromatographic runs obtained simultaneously from a single injection of bovine brain total lipid extract. Figure 2 shows the NP-HPLC separation detected using both positive- and negative-ion modes on the ITMS instrument, and Figure 3 shows the RP-HPLC separation detected using positive-ion mode on the TSQ instrument. In Figure 2A, the non-polar lipids that eluted in the region between 6.0 and 8.2 min were diverted to the RP-HPLC system, as indicated by the label. The chromatogram in Figure 3 was produced from the RP-HPLC separation of the non-polar lipid bolus that was diverted from the NP-HPLC system.

The largest peaks in the chromatograms in Figure 2 are due to phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM) molecular species. Positive-ion spectra of these phospholipid classes are given in Figure 4. The spectrum over the first half of the time range over which the PE species eluted, Figure 4A, showed protonated molecules, [M+H]$^+$, of mostly PE plasmalogen species, which contained a vinyl-ether linkage at *sn*-1 of the backbone, instead of the normal ester linkage. The most abundant masses in Figure 4A were *m/z* 776.4 and *m/z* 780.4, which corresponded to *p*18:0/22:6 and *p*18:0/22:4, respectively, as seen at www.Plasmalogens.com. The vinyl ether bond does not count as conventional fatty acid unsaturation, so the plasmalogen having an 18-carbon chain with no other sites of unsaturation is designated as *p*18:0. The next most abundant peaks were at *m/z* 752.4 and *m/z* 750.4, which corresponded to *p*18:0/20:4 and *p*18:0/20:5, respectively. Figure 4B shows an average spectrum across the time region from 29.5 to 31.5 min, at which time the PE Plasmalogens were diminishing and normal diacyl PE species predominated. The major peak at *m/z* 792.4 arose from a PE having a 40 carbons in the acyl chains, and six sites of unsaturation, or 40:6 (see www.PhosphatidylEthanolamines.com), and gave a corresponding diacylglycerol-like fragment ion, [DAG]$^+$, at *m/z* 651.5. The peak at *m/z* 768.4 corresponded to 38:4 PE, such as 20:4/18:0 PE or 20:3/18:1 PE, etc., and gave a [DAG]$^+$ ion at *m/z* 627.5. The peaks at *m/z* 744.4 and *m/z* 746.4 (not labeled) corresponded to 36:2 PE and 36:1 PE, respectively, such as 18:2/18:0 PE or 18:1/18:1 PE and 18:0/18:1 PE, respectively. These gave corresponding [DAG]$^+$ fragments at *m/z* 603.5 and *m/z* 605.6, respectively. Similarly, the peak at *m/z* 718.4 (not labeled) arose from 16:0/18:1 PE, which also produced the [DAG]$^+$ fragment at *m/z* 577.5.

Figure 4B also shows PE plasmalogen species overlapped with the diacyl PE species. The peak at *m/z*
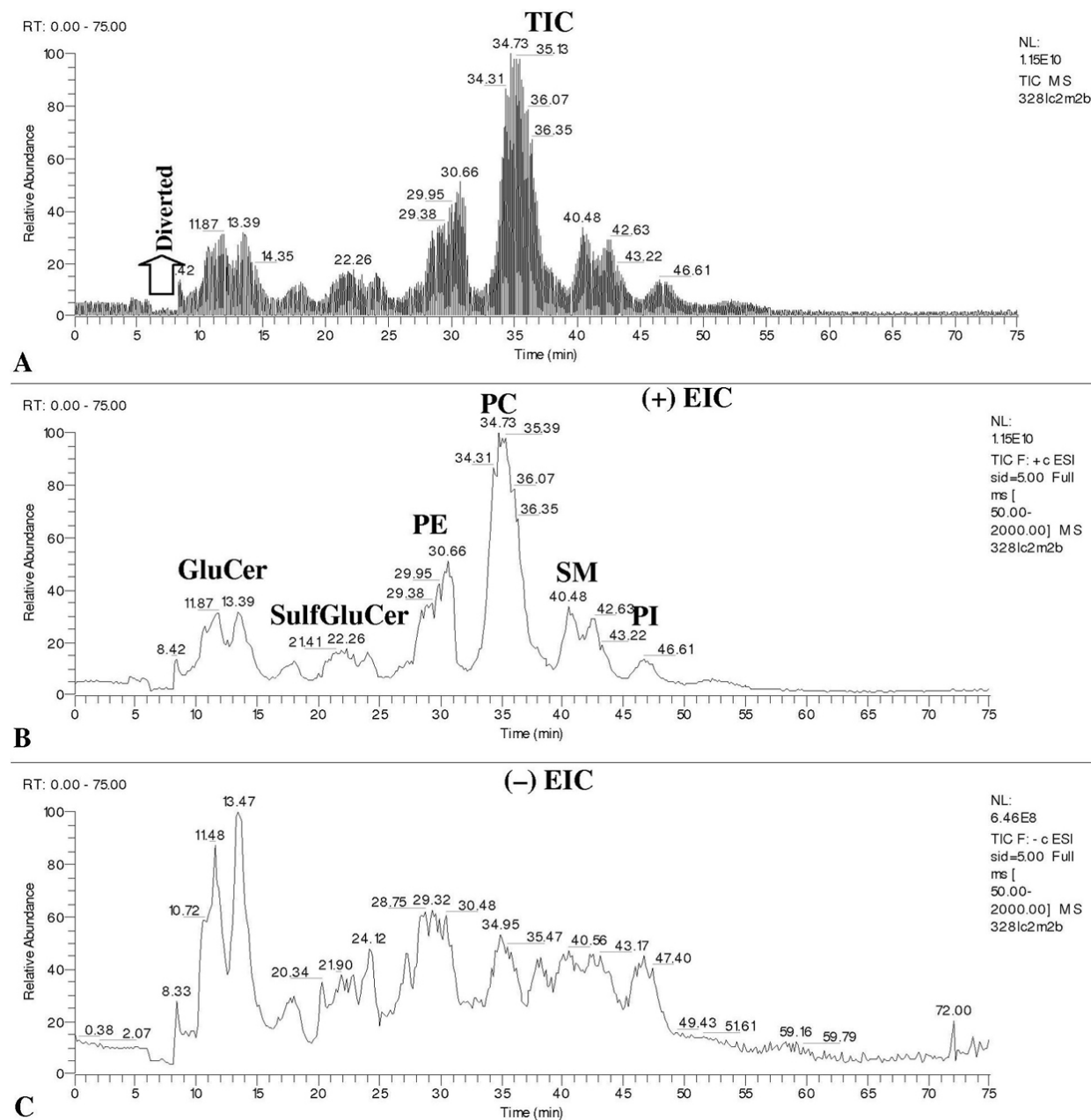
Figure 2. Normal-phase HPLC separation of polar lipids from bovine brain total lipid extract detected on the LCQ Deca ion trap mass spectrometer. (A) ESI-MS total ion chromatogram (TIC); (B) extracted ion chromatogram (EIC) of positive-ion scans extracted out of the TIC; (C) EIC of negative-ion scans extracted out of the TIC.

728.4 arose from p18:0/18:2 PE plasmalogen, while the peak at m/z 702.4 arose from p16:0/18:1 PE plasmalogen. Thus, both PE and PE plasmalogen species could be identified in the bovine brain total lipid extract. The spectra showed that the diacyl PE species produced larger abundances of the [DAG]⁺ fragments by loss of the PE head group than analogous fragments from plasmalogens produced by loss of the head group. Figures 4A and 4B showed that long-chain PE plasmalogen species eluted before long-chain diacyl PE species, which were overlapped with short-chain PE plasmalogen species.

Phosphatidylcholine species can be seen in Figure 4C. This class of phospholipid produced almost exclusively protonated molecule ions, [M+H]⁺, by ESI-MS, except the use of 5.0 V of in-source collision energy gave a large abundance of m/z 184.1 from the phosphocholine head group. The m/z 184.1 peak also arose from the later-eluting sphingolipid species, and clearly identified these classes as containing the phosphocholine head group. The PC protonated molecules identified the molecular weights of groups of isobaric PCs, and the MS/MS data (not shown) allowed the molecular species to be identified, as well as the locations of the fatty
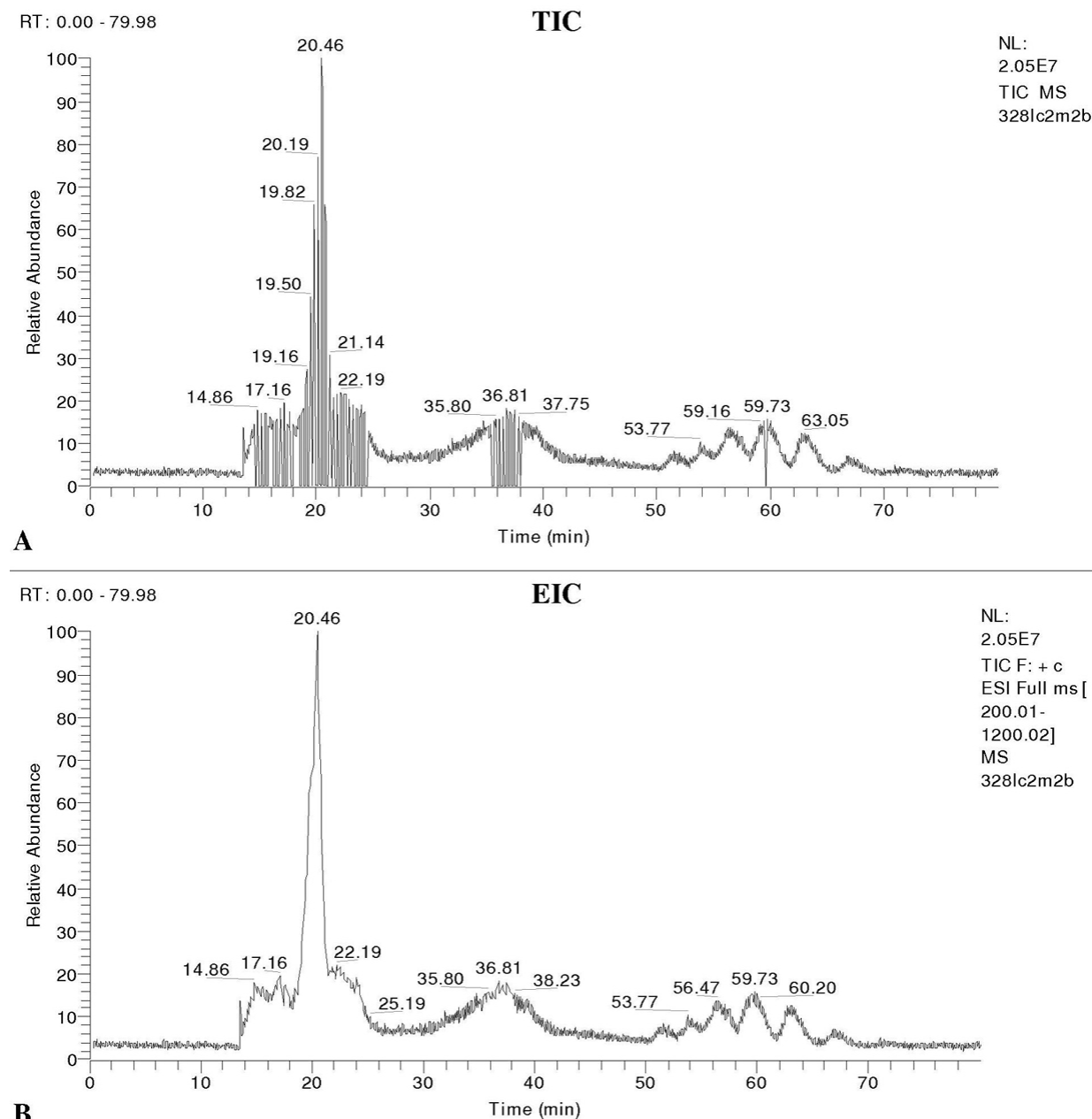
Figure 3. Reversed-phase HPLC separation of polar lipids from bovine brain total lipid extract detected on the TSQ 700 tandem sector quadrupole mass spectrometer. (A) ESI-MS total ion chromatogram (TIC) showing all MS and MS/MS scans; (B) extracted ion chromatogram (EIC) of MS scans extracted out of the TIC.

acyl chains on the glycerol backbone, based on the relative abundances of the fragments formed by loss of the fatty chains as ketenes, as suggested previously (15). The PC molecular species that correspond to the masses seen in Figure 4C are identified at www.PhosphatidylCholines.com.

Mass spectra of sphingolipid species are shown in Figure 4D and 4E. As mentioned above, the m/z 184.1 peak identified these molecules as possessing the phosphocholine moiety. Protonated molecules and sodium adducts of various molecular species can be seen in Figures 4D and 4E, and these

can be identified based on the mass lists given at www.Sphingomyelin.com. For instance, the peaks at m/z 815.5 and m/z 837.6 corresponded to the d18:0/24:1 dihydrosphingomyelin (DSM) protonated molecule, $[M+H]^+$, and sodiated molecule, $[M+Na]^+$, respectively. These masses were isobaric with the $[M+H]^+$ and $[M+Na]^+$ of d18:1/24:0 sphingomyelin (SM), but the use of chromatography separated the dihydrosphingomyelins from sphingomyelins, eliminating the potential ambiguity that would arise if these classes were analyzed without prior chromatographic separation. The identification of bovine brain sphingomyelin species has been discussed in great detail in our recent publication (10). In the
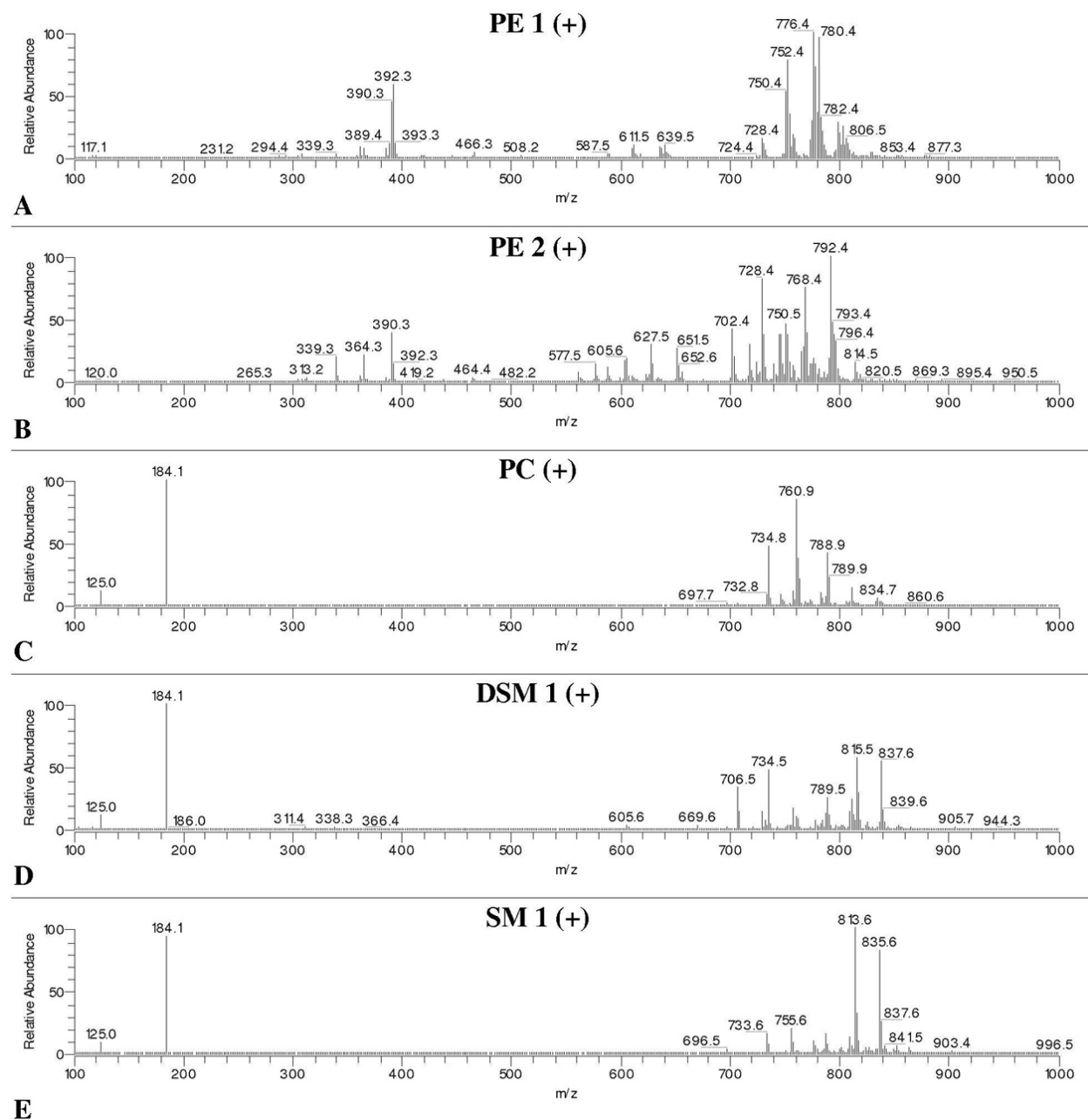
Figure 4. Positive-ion ESI-MS mass spectra averaged across chromatographic peaks of phospholipid classes in bovine brain total lipid extract. (A) (+) mass spectrum across phosphatidylethanolamine (PE) peak from 28.0 to 29.5 min; (B) (+) mass spectrum across PE peak from 29.5 to 31.5 min; (C) (+) mass spectrum across phosphatidylcholine (PC) peak from 33.5 to 37.0 min; (D) (+) mass spectrum across dihydrosphingomyelin (DSM) peak from 37.0 to 39.0 min; (E) (+) mass spectrum across sphingomyelin (SM) peak from 39.5 to 41.0 min. See (−) mass spectra across identical time ranges in Figure 5.

past, methods that employed infusion without chromatographic separation for sphingolipids have reported few dihydrosphingomyelin species, because many are isobaric with sphingomyelin species. Another pair of ions were observed at m/z 789.5 and m/z 811.6 that corresponded to the $[M+H]^+$ and $[M+Na]^+$ ions of d18:0 /22:0 DSM. The peaks at m/z 706.5 and 734.5 corresponded to short-chain PC species that overlapped with the long-chain DSM species.

Figure 4E shows an average mass spectrum from 39.5 to 41.0 min, which was in the second of three chromatographic peaks produced by the sphingolipids (SLs). In the second SL peak, long-chain SM species occurred overlapped with short-chain DSM species, as described in detail elsewhere (10). The ions at m/z 813.6 and m/z 835.6 corresponded to the $[M+H]^+$ and $[M+Na]^+$ of d18:1/24:1 SM, respectively, while the ions at m/z 733.6 and m/z 755.6
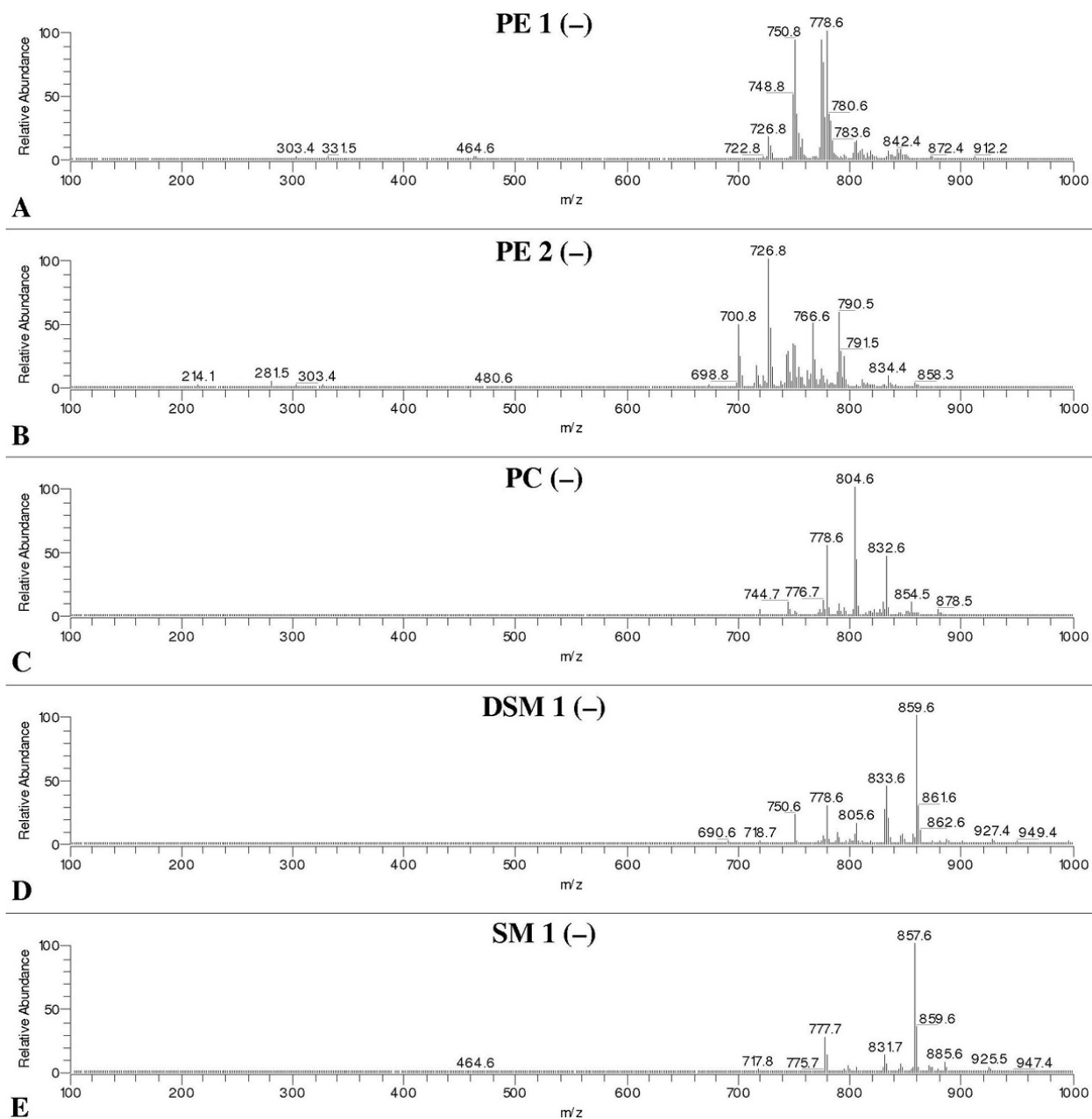
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Figure 5. Negative-ion ESI-MS mass spectra averaged across chromatographic peaks of phospholipid classes in bovine brain total lipid extract. (A) (−) mass spectrum across PE peak from 28.0 to 29.5 min; (B) (−) mass spectrum across PE peak from 29.5 to 31.5 min; (C) (−) mass spectrum across PC peak from 33.5 to 37.0 min; (D) (−) mass spectrum across DSM peak from 37.0 to 39.0 min; (E) (−) mass spectrum across SM peak from 39.5 to 41.0 min. See (+) mass spectra across identical time ranges in Figure 4.

corresponded to the $[M+H]^+$ and $[M+Na]^+$ of d18:0/18:0 DSM, respectively.

The spectra above demonstrated that PE, PC and DSM/SM phospholipids gave protonated molecules in positive-ion mode, and that phosphocholine phospholipids also gave abundant sodium adducts. In negative-ion mode the phospholipid classes behaved differently. Figures 5A and 5B demonstrate that PE species produced deprotonated molecules, $[M-H]^-$ in negative-ion mode, while the phosphocholine-containing phospholipid classes (PC, DSM, SM) exhibited almost exclusively formate adducts in negative-ion mode. Comparison of Figures 5A and 5B to Figures 4A and 4B showed that for every $[M+H]^+$ in Figures 4A and 4B, there was a corresponding $[M-H]^-$ ion at 2 m/z lower in the negative-ion mass spectra in Figures 5A and 5B. The $[M+H]^+$ and $[M-H]^-$ ions made it very easy for the 'M' to be determined for PE plasmalogen and
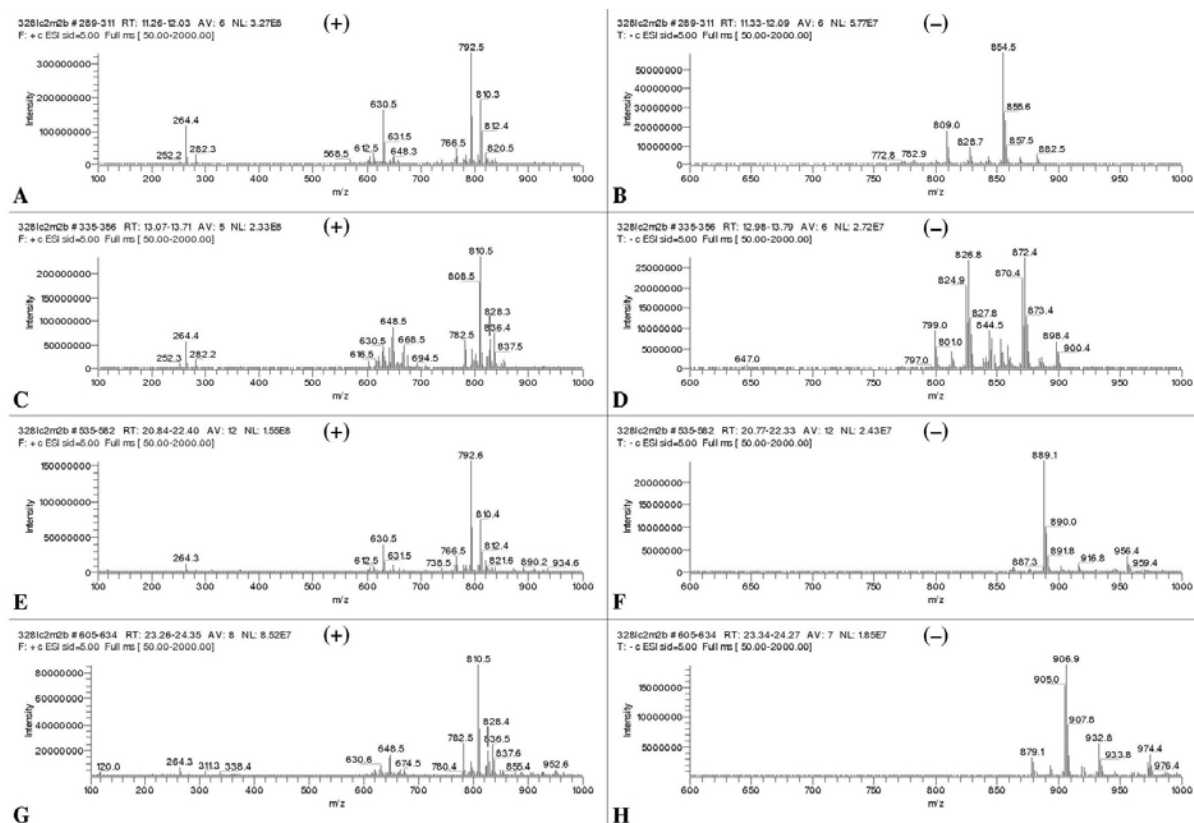
Figure 6. Mass spectra of early-eluting peaks from NP-HPLC detected by positive- and negative-ion ESI-MS. (A, B) (+,–) mass spectra of *d*18:1/24:1 GluCer, 11.3 to 12.0 min; (C,D) (+,–) mass spectra of *d*18:1/24:1-OH GluCer, 13.1 to 13.7 min; (E,F) (+,–) mass spectra of *d*18:1/24:1 SulfGluCer, 20.8 to 22.4 min; (G,H) (+,–) mass spectra of *d*18:1/24:1-OH SulfGluCer, 23.3 to 24.4 min.

PE molecular species. For PE species, the total ion abundance was ~9x10⁶ in positive-ion mode and ~1.5x10⁷ in negative-ion mode, demonstrating that negative-ion mode was more sensitive for PE species.

PC, DSM and SM did not produce [M-H]⁻ ions, but instead produced [M+45]⁻ ions. If the zwitterionic molecule was 'M', and a proton was added at the negative phosphate oxygen to give a positively charged [M+H]⁺ (due to the remaining quaternary amine), then removal of the phosphate proton to give 'M' along with addition of a formate moiety, HCOO⁻ to the quaternary amine gives a negatively charged formate adduct, [M+45]⁻. PC gave a maximum abundance of ~2.5x10⁸ in positive-ion mode and ~2.2x10⁸ as the formate adduct in negative-ion mode. DSM gave a maximum abundance of ~9.8x10⁷ in positive-ion mode and ~1.6x10⁷ in negative-ion mode. Thus, PC, DSM and SM gave better sensitivity in positive-ion mode, but nevertheless gave strong signal from the formate adduct in negative-ion mode. As long as the identity of the [M+45]⁻ is recognized in negative-ion mode, the adduct provides valuable data to identify the molecular weight from negative-ion mode to confirm the results obtained in positive-ion mode. The use of MS/MS for differentiation of DSM from SM in bovine brain has been discussed in great detail elsewhere (10) and so will not be mentioned in detail here. The positive- and negative-ion mass spectra in Figures 4 and 5, respectively, clearly allow the

phospholipid class and molecular species to be determined, which accomplishes a major goal of lipidomics.

There were some classes of glycosphingolipids that were identified in bovine brain that especially emphasized the importance of using a chromatographic separation in combination with mass spectrometry for lipidomic analysis. The mass spectra of glycosphingolipids are shown in Figure 6. Figure 6A shows a positive-ion ESI mass spectrum averaged across the time range from 11.3 to 12.0 min, and that mass spectrum was virtually indistinguishable from the mass spectrum in Figure 6E that was averaged from 20.8 to 22.4 min. Similarly, Figure 6C shows a positive-ion mass spectrum averaged from 13.1 to 13.7 min that was virtually indistinguishable from the mass spectrum in Figure 6G that was averaged from 23.3 to 24.4 min. If only positive-ion mass spectra were available, the species in Figure 6A versus 6C and in Figure 6E versus 6G could not be distinguished and identified. Furthermore, the ions that are common to all four mass spectra, such as *m/z* 810.5, would make it very difficult to distinguish the four species without prior chromatographic separation, using positive-ion mass spectra alone.

The negative-ion mass spectra, however, provided valuable information to allow four different classes to be distinguished. The negative-ion mass spectra
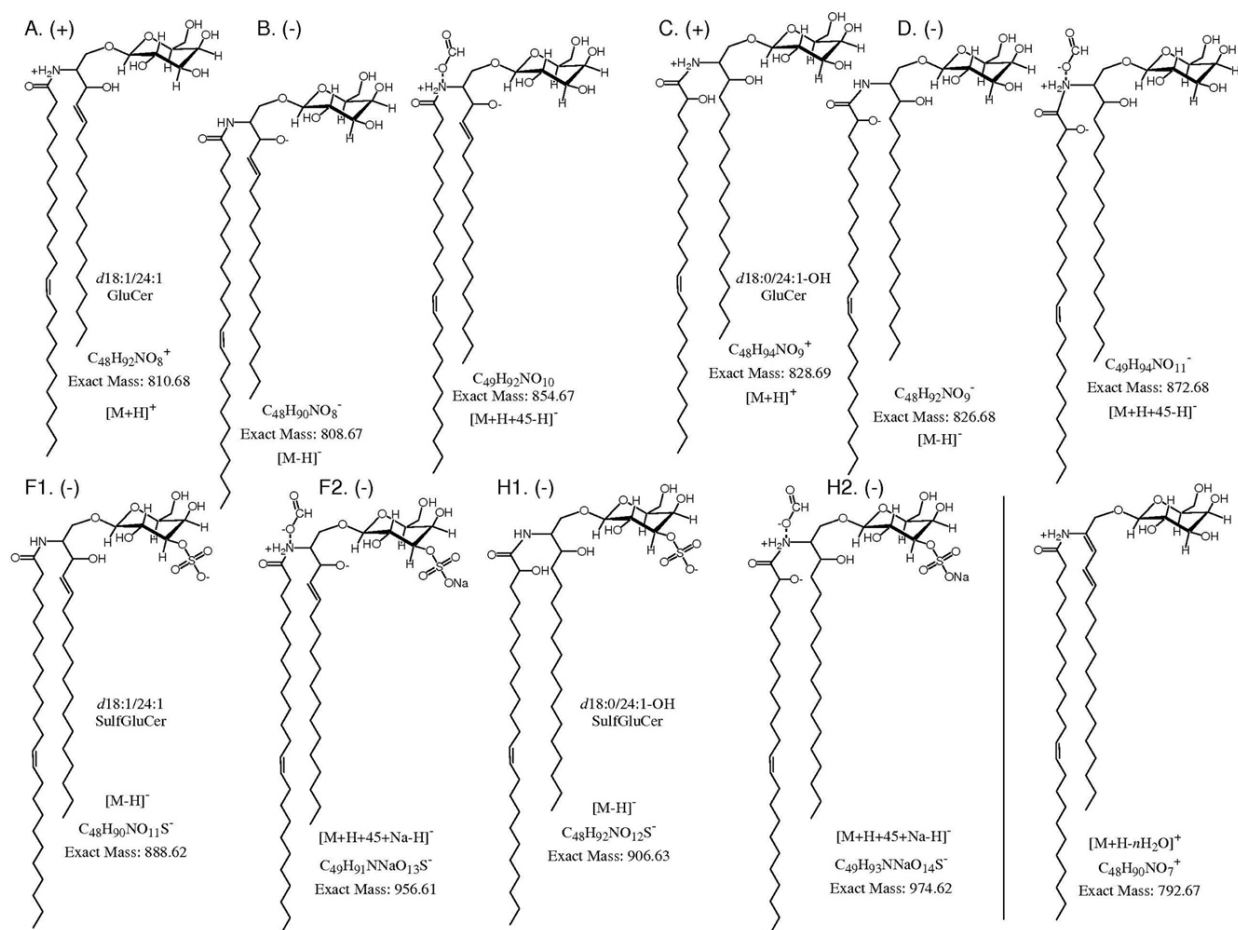
Figure 7. Proposed identities of glycosphingolipids in bovine brain total lipid extract eluted early in NP-HPLC chromatogram. Labels refer to panels in Figure 6.

exhibited two very useful ions that aided identification, which were the deprotonated molecule, [M-H]⁻, and a formate adduct, [M+H+45-H]⁻. The formate adduct was especially interesting, since the molecule was both protonated and deprotonated, at different sites. To make the anionic formate moiety 'stick' to the molecule, the ceramide amine group had to be protonated, similar to a normal ceramide, to give [M+H+45]. But the formate moiety neutralized the protonated amine, rendering the molecule neutral. To be detected by negative-ion mass spectrometry, the adduct had to be deprotonated at one of the hydroxyl groups, for [M+H+45-H]⁻, to give the molecule a net negative charge. For instance, the glucosylceramide *d*18:1/24:1 GluCer gave a deprotonated molecule at *m/z* 809.0 in Figure 6B (calculated monoisotopic mass = 808.7), and gave a [M+H+45-H]⁻ formate adduct ion at *m/z* 854.5. These two ions, taken with fragments in the positive-ion mass spectrum, allowed the structure of the molecule to be determined. The structures of the deprotonated molecules and the unusual formate adducts are pictured in Figure 7 for the four molecules for which the mass spectra are shown in Figure 6. Structures A and B in Figure 7 correspond to the mass

spectra in Figures 6A and 6B; structures C and D correspond to Figures 6C and 6D; structures F1 and F2 in Figure 7 both correspond to negative ions in Figure 6F and structures H1 and H2 in Figure 7 both correspond to negative ions in Figure 6H.

Figure 6D shows peaks at +16 and +18 *m/z* compared to Figure 6B. These masses and the fragments in the positive-ion spectra indicated that these were hydroxy-containing variants of the molecule shown in Figure 7B. Furthermore, there were two variants: those containing a dihydroceramide backbone (missing the 4,5 *trans* double bond) and those containing a normal ceramide backbone. The set of ions differing by two mass units were observed throughout fragments in both positive- and negative-ion mass spectra, Figures 6C and 6D, respectively. The later elution of the hydroxy-containing compounds compared to the non-hydroxy GluCer was expected based on principles of NP-HPLC, since the hydroxyl group rendered the variant more polar than the non-hydroxy analog.

There were two additional related molecules that eluted even later on the NP-HPLC system. These

molecules, for which negative-ion mass spectra are given in Figures 6F and 6H, each gave a [M-H]$^-$ ion that was 80 *m/z* larger than the molecules in Figures 6B and 6D. It was determined that this difference arose from having a sulfate group –OSO$_3^-$ on the glucosyl ring, to give sulfatoglucosylceramides, as shown in Figure 7. These molecules produced very interesting adducts containing both a formate moiety (like the molecules mentioned above) and a sodium atom, to give [M+H+45+Na-H]$^-$. This was very unusual and had not been reported before.

The peaks mentioned above highlight the importance of using a chromatographic separation to identify molecules that produce very similar positive-ion mass spectra but which have different functional groups and so different retention characteristics. The examples also highlight the importance of having both positive- and negative-ion mass spectra, as well as MS/MS in both ionization modes to allow identification of all molecular species.

4.2. LC2/MS2 of non-polar lipids in bovine brain total lipid extract

The RP-HPLC chromatogram of non-polar lipids shown in Figure 3 is relatively simple compared to the polar lipid separation discussed above. But several important lipid classes, and the molecular species within the classes can be separated and identified using the non-polar lipid separation. The largest peak in the chromatogram occurred at 20.46 min, and the average MS and MS/MS mass spectra across this peak are given in Figures 8A and 8B, respectively. The *m/z* 282.3 and *m/z* 264.2 peaks in the MS/MS spectrum in Figure 8B identified the molecule as having a *d*18:1 long-chain base, while the protonated molecule at *m/z* 566.4, the sodium adduct at *m/z* 588.4 and the dehydrated protonated molecule at *m/z* 548.4 in Figure 8A allowed the molecule to be identified at *d*18:1/18:0 ceramide. The identities of the fragments produced by *d*18:1/18:0 ceramide are given in Figure 9. The chromatography of this ceramide species revealed an important point: the ceramide-containing phospholipids and glycolipids eluted on the normal-phase system, while ceramide itself eluted on the reversed-phase system. The implications of this are discussed further below.

A sodium atom was tightly bound to a proportion of the ceramide molecules during the chromatographic run; the sodium adduct was not formed in the ESI source. This was evidenced by the fact that the peak in the extracted ion chromatogram (EIC) of the [M+Na]$^+$ adduct at *m/z* 588.4 was broader and eluted later than the peak in the EIC of the [M+H]$^+$ at *m/z* 566.4. The [M+H]$^+$ ion at *m/z* 566.4 was the base peak in mass spectra averaged from 19 to 21 min, while the [M+Na]$^+$ adduct at *m/z* 588.4 was the base peak in mass spectra averaged from 21 to 25 min.

On the RP-HPLC system, the molecular species of ceramides were well separated. The *d*18:1/18:0 ceramide eluted between 19 and 25 min, while the longer *d*18:1/24:1 ceramide was retained longer and eluted in the range from ~35 to 38.5 min. Furthermore, an abundant diacylglycerol,

stearoylarachidonylglycerol, or 18:0/20:4, eluted in the same time range. Nevertheless, the MS/MS mass spectra easily differentiated the ceramide from the diacylglycerol. Figure 8C shows the mass spectrum averaged from 35.0 to 38.5 min, which exhibited a base peak at *m/z* 662.4 due to the ammonium adduct of the diacylglycerol, and peaks at *m/z* 648.4, *m/z* 630.4 and *m/z* 612.4 that arose from the *d*18:1/24:1 ceramide. The MS/MS mass spectrum of *m/z* 648.4 in Figure 8D exhibited the characteristic long-chain base fragment, [LCB]$^+$, at *m/z* 282.3 and [LCB-H$_2$O]$^+$ at *m/z* 264.2 that identified the *d*18:1 long-chain base, as well as the [M+H-H$_2$O]$^+$ and [M+H-2H$_2$O]$^+$ peaks characteristic of ceramides. Fragments from *d*18:1/24:1 ceramide are also shown in Figure 9.

The MS/MS mass spectrum of stearoylarachidonyldiacylglycerol, Figure 8F, was entirely different from the spectrum of the ceramides, as expected. The DAG was observed as its ammonium adduct, [M+NH$_4$]$^+$ at *m/z* 662.4, and this produced a protonated molecule at *m/z* 645.2 in the MS/MS mass spectrum, along with a diacylglycerol-like fragment ion, [DAG]$^+$, at *m/z* 627.1, which is the same type of [DAG]$^+$ fragment formed from triacylglycerols. The [DAG]$^+$ fragment was equal to the normal intact DAG minus a mole of water. The MS/MS spectrum showed [R$_1$COO+58]$^+$ and [R$_2$COO+58]$^+$ fragments corresponding to the individual fatty acyl chains that allowed the fatty acids (FA) to be characterized according to carbon chain length and degree of unsaturation. The ion at *m/z* 341.3 was from an 18:0 FA, while the ion at *m/z* 361.1 was from a 20:4 FA. These FA identities were consistent with 18:0/20:4 DAG, which gave a protonated molecule at *m/z* 645.2. The above examples demonstrated that positive-ion ESI-MS and MS/MS mass spectra were useful to identify various molecular species of ceramides and diacylglycerols having different carbon chain lengths and degrees of unsaturation.

Triacylglycerols eluted in the time range from ~50 to 70 min in Figure 3. Figure 10 shows mass spectra of two typical TAGs. Since TAGs are non-polar and not readily ionized by ESI, ammonium formate was added to the solvent system to promote adduct formation. The mass spectra in Figures 10A and 10C show [M+NH$_4$]$^+$ adduct ions as the base peaks for OPO and OPS, respectively. The MS/MS spectra in Figures 10B and 10D showed a protonated molecule, [M+H]$^+$, formed from the ammonium adduct precursor ion. Figure 10B also showed diacylglycerol-like fragment ions, [DAG]$^+$, at *m/z* 577.3 and *m/z* 603.2 for [OP]$^+$ and [OO]$^+$, respectively. According to literature precedent (16,17,18), which indicates that loss of the FA from the *sn*-2 position is energetically disfavored, these fragments indicated that the 'P' was probably at the *sn*-2 position. This is because the abundance of the [OO]$^+$ fragment relative to the [OP]$^+$ fragment was less than the statistically expected ratio of 1:2, indicating that [OO]$^+$ was disfavored. Thus, ESI-MS/MS spectra may allow identification of regioisomers in a manner similar to APCI-MS. Figure 10D showed the MS/MS spectrum of *m/z* 878.6, which exhibited the protonated molecule for OPS and the [SP]$^+$ fragment at *m/z* 579.2. Additional fragments were not present in the
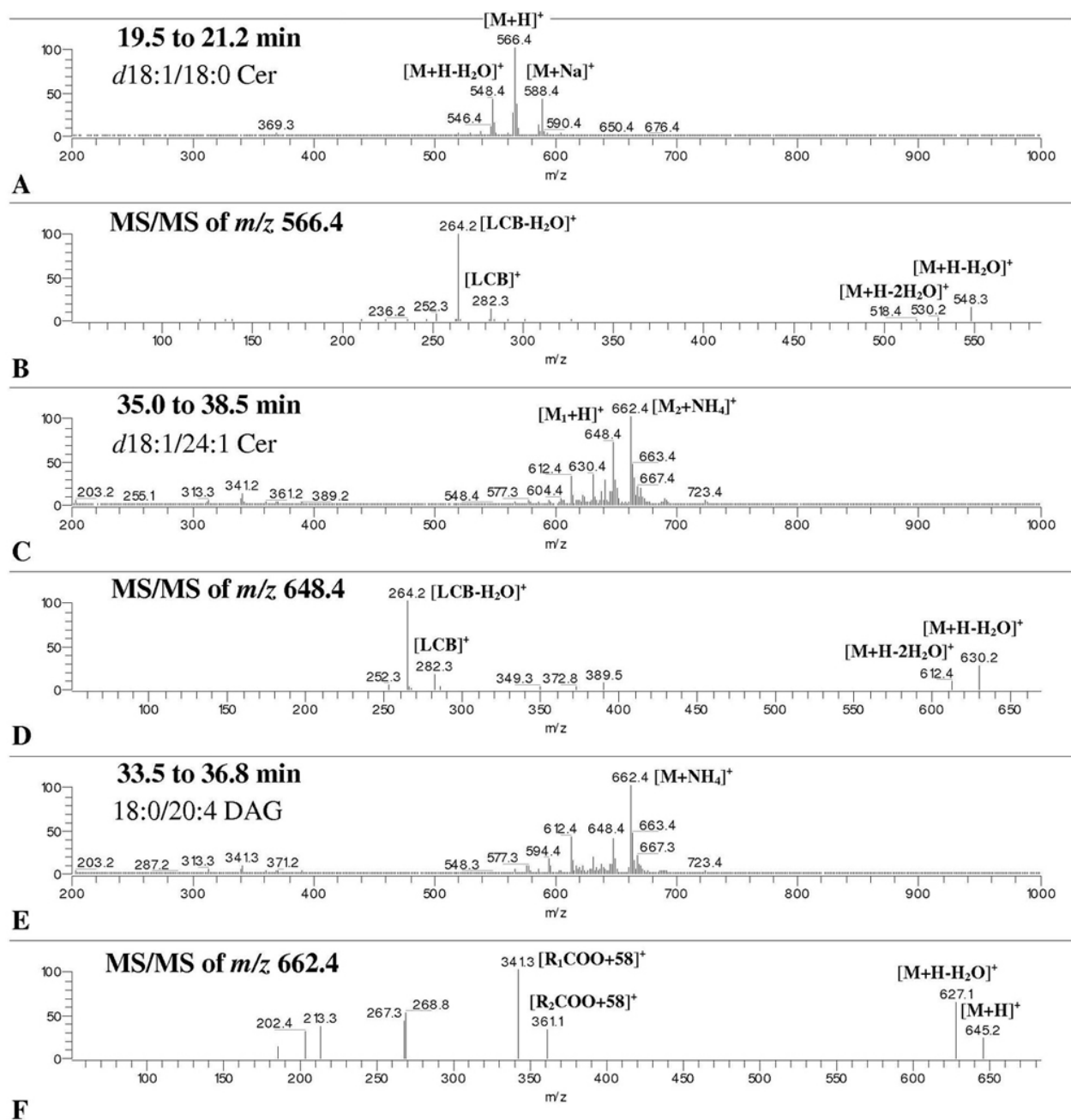
Figure 8. Average ESI-MS and MS/MS mass spectra from RP-HPLC separation of neutral lipids from bovine brain total lipid extract. (A) Average mass spectrum from 19.5 to 21.2 min; (B) MS/MS of m/z 566.4; (C) avg. mass spectrum from 35.0 to 38.5 min; (D) MS/MS of m/z 648.4; (E) avg. mass spectrum from 33.5 to 36.8 min; (F) MS/MS of m/z 662.4.

averaged spectrum in Figure 10D because only few MS/MS spectra were obtained and averaged across the TAGs in the run shown, due to the level of the cutoff threshold for MS/MS. Nevertheless, spectra such as that in Figure 10B clearly demonstrate the usefulness of ESI-MS/MS of ammonium adducts to identify i) the molecular species of TAGs, and often ii) the identity of the FA in the *sn*-2 position, or the specific regioisomer. These are two primary goals of lipidomics of triacylglycerols.

4.3. LC2/MS2 of polar lipids in sand bream filet total lipid extract

Another example of an LC2/MS2 approach for total lipid analysis is given that demonstrates the use of ESI-MS/MS for polar lipid analysis and APCI-MS for non-polar lipid analysis. Figures 11 and 12 show the dual chromatograms obtained from the NP-HPLC separation and the RP-HPLC separation, respectively. The time range from 5.75 to 7.75 min, where the non-polar bolus occurred
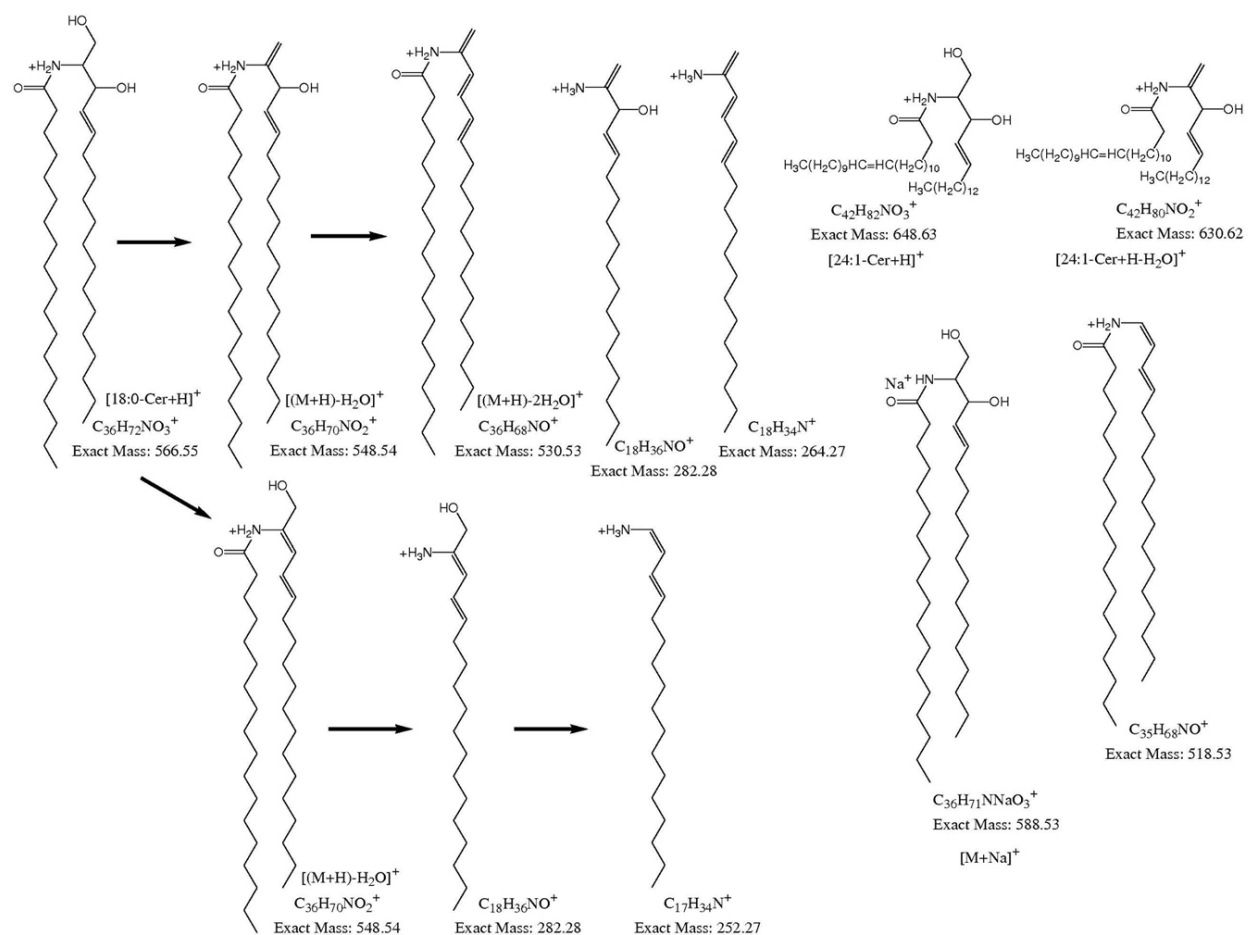
Figure 9. Proposed fragment identities from ceramides by ESI-MS/MS.

and was diverted to the NP-HPLC, is labeled as 'diverted' in Figure 11. Mass spectra of the phospholipids separated on the NP-HPLC system are given in Figure 13. An up-front collision energy of 20.0 V was employed to try to minimize dimer formation from the phospholipids, but it was not effective. We later found that dimer formation can be almost completely eliminated by using a solution of 50 mM glycine in 1M NH₄OCOH with a pH of 8.80, added post-column via a tee supplied by a syringe pump at 50 μL/min (10).

The up-front collision energy had a minor effect on the positive-ion mass spectra of phospholipids. The PC and SM species produced a slightly higher proportion of the phosphocholine head group at m/z 184.1 than in the mass spectra shown in Figure 4. The spectra of PE, PC and SM were very similar to those give above for bovine brain. Figure 13 shows positive- and negative-ion mass spectra of another phospholipid, phosphatidylinositol (PI). This was present in bovine brain but a spectrum was not given above due to space limitations. Figure 13D shows that PI gave sodium adducts and [DAG]⁺ fragment ions in positive-ion mode and deprotonated molecules, [M–H]⁻, in negative-ion mode.

The negative-ion mass spectrum of PE was very similar to that given above. The PE species produced almost exclusively deprotonated molecules, [M–H]⁻, in negative-ion mode, even with an up-front collision energy of 20.0 V. The mass spectra of PC and SM, on the other hand, showed distinct differences due to the increased collision energy. The phosphocholine-containing phospholipids produced two primary ions in negative-ion mode: i) formate adducts, [M+H+45–H]⁻, or [M+45]⁻, as described above, and ii) fragments formed by loss of a methyl group, –CH₃, from the quaternary amine of the zwitterionic molecule, M, to give a demethylated negative ion, [M–CH₃]⁻. The experiment showed that increasing the up-front collision energy did not reduce dimers, and so it should not be used, since it produced fragments that were no more useful for structural identification than the deprotonated molecules obtained when no up-front energy was supplied.

4.4. LC2/MS2 of non-polar lipids in sand bream filet total lipid extract

Figure 14 shows mass spectra averaged over five minute intervals across the range 40 to 70 min in Figure 12. One important difference between the TAG from bovine brain versus those from the sand bream filet total extract
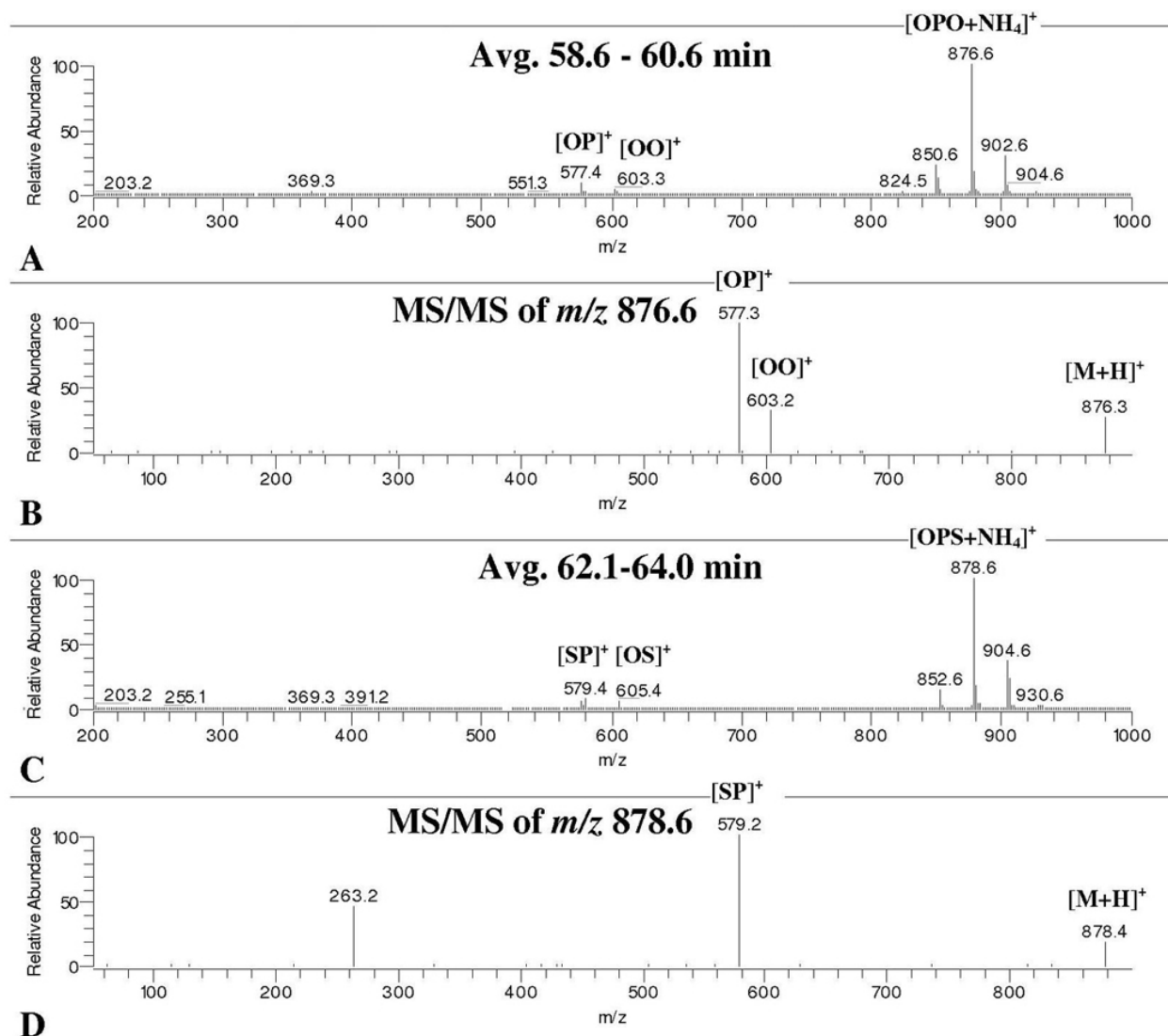
113

Figure 10. ESI-MS and MS/MS mass spectra of TAG molecular species. (A) Full-scan MS of OPO; (B) MS/MS of *m/z* 876.6; (C) MS of OPS; (D) MS/MS of *m/z* 878.6.

was that the sand bream TAG contained a wide variety of both odd- and even-chain TAG molecular species. These can be seen from the large number of peaks between the labeled even carbon chain species in Figure 14. TAG with odd carbon chains have similarly been reported in carp (19) and other fish species (20).

Another important difference was that the collision energy caused the early-eluted unsaturated TAG to exhibit protonated molecules, while saturated TAG eluted later and produced ammonium adducts, as described above. The collision energy also caused polyunsaturated TAG to produce [DAG]$^+$ fragments as the base peaks, whereas they would normally produce [M+H]$^+$ base peaks by APCI-MS.

Figure 14A shows the mass spectrum of the earliest-eluted TAG, which contained short-chain fatty

acids, such as lauric and myristic acids, and TAG containing polyunsaturated FA, such linolenic and arachidonic acids. Figures 14B through 14D were produced by TAG that contained monounsaturated and saturated short- to medium-chain fatty acids. Figures 14E and 14F were produced by TAG with FA having up to 20 and 22 carbons with one or no sites of unsaturation.

The dramatic difference between ESI-MS of TAG versus APCI-MS of TAG can be seen by comparing Figure 10 to Figure 14. ESI-MS produced practically no [DAG]$^+$ fragments in the full-scan mass spectra, whereas APCI-MS produced mostly [DAG]$^+$ fragments. These data were selected to highlight the differences between these ionization modes. These data indicate that ESI-MS with MS/MS provided both the molecular weight, based on the [M+NH$_4$]$^+$ adduct, and the [DAG]$^+$ fragments from MS/MS that allowed the individual fatty acid chains to be
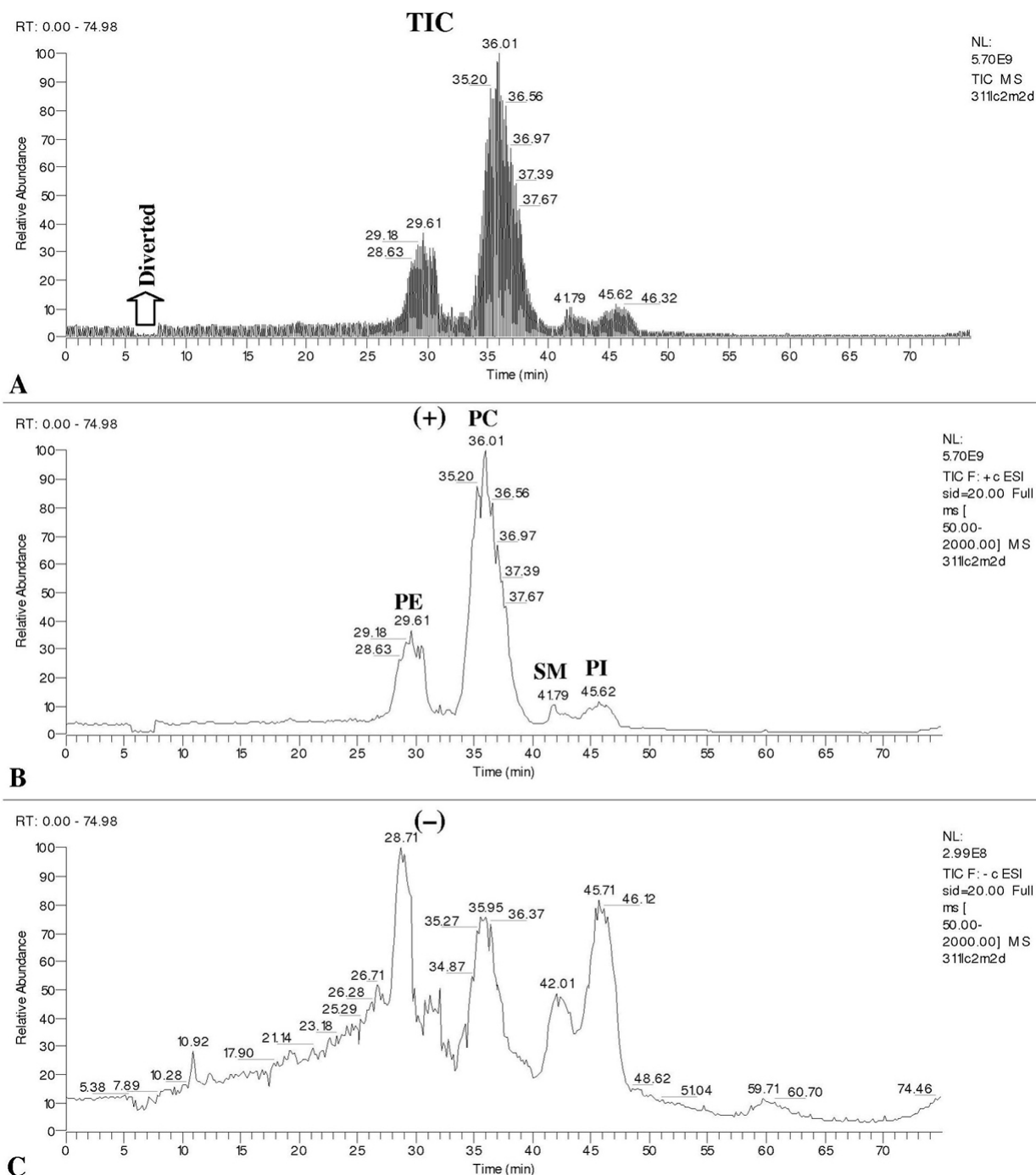
Figure 11. Positive- and negative-ion ESI-MS chromatograms of sand bream polar lipids. (A) TIC; (B) positive-ion full scan chromatogram; (C) negative-ion full scan chromatogram.

identified. But ESI-MS requires the use of an additive to provide the formate, since ESI-MS of TAG without additive produced very little signal. APCI-MS provided complementary information, by producing abundant [DAG]$^+$ fragments that allowed the molecular species to be identified, but up-front collision energy should be minimized, so that fragmentation of the protonated molecule is minimized. These two ionization sources provide an especially powerful combination of information when the two can be employed in parallel, in LC1/MS2 dual parallel mass spectrometer experiments.

The non-polar lipid bolus in the total lipid extract contained a class of molecules that was overlapped with the TAGs. These molecules produced a strong ion at *m/z* 369.4 that was characteristic of cholesterol-related molecules. An
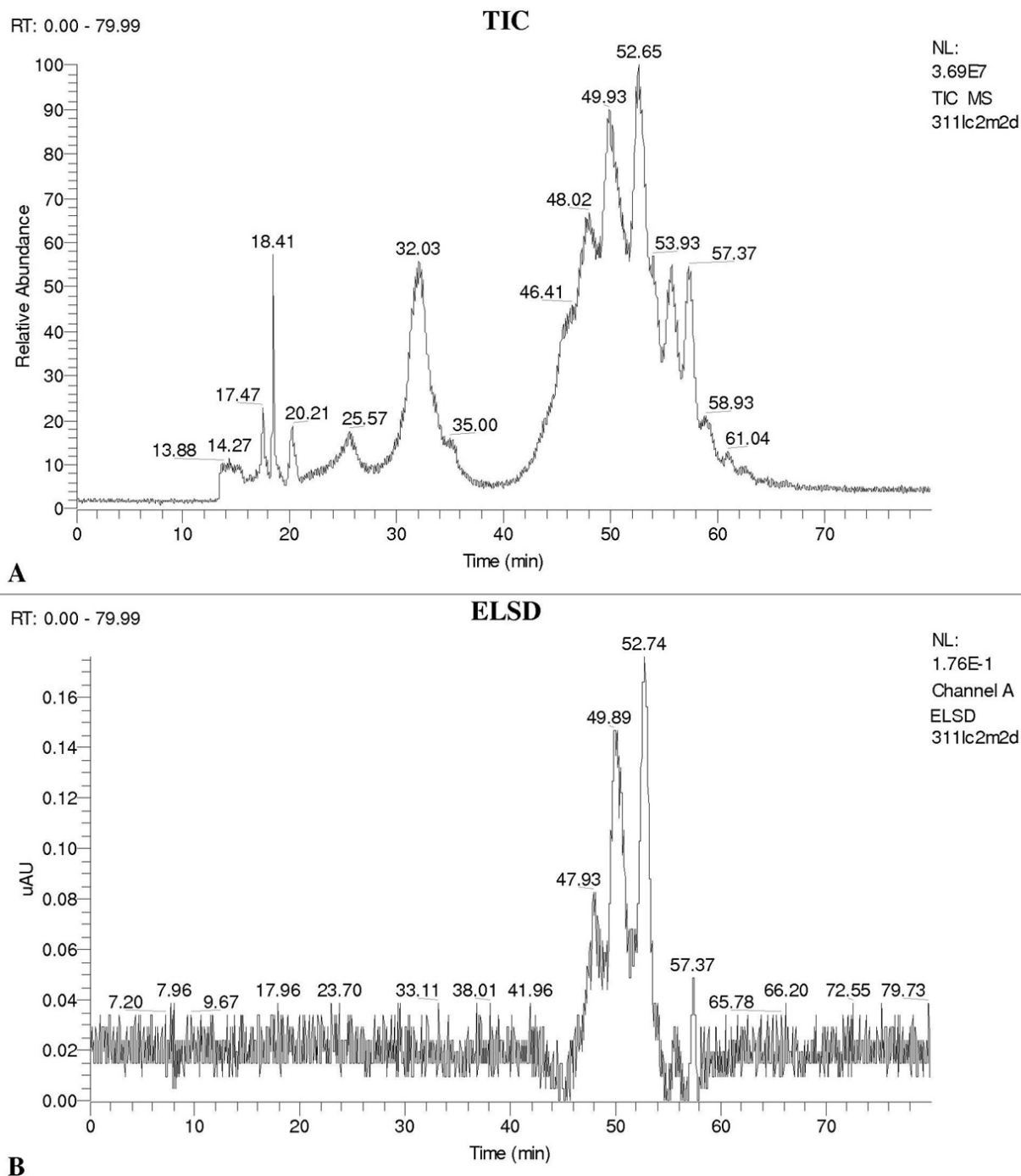
Figure 12. (A) Total ion chromatogram and (B) ELSD chromatogram of sand bream neutral lipids.

extracted ion chromatogram of *m/z* 369.3, Figure 15A, showed several peaks that produced this fragment. One type of molecule eluted in the range 30 to 35 min, and produced a simple mass spectrum that exhibited ions at *m/z* 383.3 and *m/z* 369.4. This molecule overlapped with diacylglycerols that eluted in the same time range. The molecule had structural similiarities to cholesterol, but it has not yet been fully characterized. In the time range 50 to 62 min, several molecules eluted that also produced a

primary ion at *m/z* 369.3. These later-eluting peaks are labeled in the EIC of *m/z* 369.3 in Figure 15A. EICs of the protonated molecules of three of the species are given in Figures 15B, 15C and 15D. The molecules represent a homologous series differing by *m/z* 14, or –CH₂. The protonated molecules at *m/z* 691.6, *m/z* 705.6 and *m/z* 719.6 have the same masses as 22:0, 23:0 and 24:0 didehydrocholesteryl ethers or 21:0, 22:0 and 23:0 didehydrocholesterol esters. But the mass spectra did not

Figure 13. Positive- and negative-ion ESI-MS mass spectra of sand bream filet phospholipids. (A) Phosphatidylethanolamines, 28.1 to 31.0 min; (B) phosphatidylcholines, 34.1 to 38.0 min; (C) long-chain sphingomyelins, 41.3 to 42.6 min; (D) phosphatidylinositols, 44.6 to 47.0 min.

correspond to mass spectra of authentic standards of cholesterol esters, so the molecules have not been further characterized. Nevertheless, the EICs and mass spectra were presented to demonstrate that the chromatographic separation allows numerous classes of non-polar lipid molecules to be separated into individual molecular species, even when one class of molecule co-eluted with another class.

5. DISCUSSION

The data above demonstrated that a column switching technique employing dual liquid chromatography systems can successfully separate polar lipids from non-polar lipids, and can separate individual molecular species within the lipid classes. The value of this approach for lipidomics is obvious. Each class of lipid and the molecular species within each class can be identified and compared.

Ever since the Sphingomyelin Cycle (21), which is the stimulus-regulated breakdown of sphingomyelin into ceramide, was discovered, there has been intense interest in the roles of ceramides in a myriad of cell signaling pathways. The enzyme sphingomyelinase acts on sphingomyelin molecules to produce the potent cell-signaling molecule ceramide. As can be seen from the bovine brain data above, sphingomyelin is a phospholipid that was separated using NP-HPLC, while ceramide eluted with the non-polar lipids on the RP-HPLC system. Using conventional chromatographic techniques, sphingomyelin and ceramide are not separated in the same run. However,

using the LC2/MS2 dual liquid chromatography/ dual mass spectrometry technique, sphingomyelin and ceramide could both be monitored and the levels and the identities of individual molecular species for both classes be determined from a single injection. Thus, the effects on cellular systems of different chain lengths and number of sites of unsaturation in sphingomyelin could easily be probed by monitoring both the precursor sphingomyelin molecules and the product ceramide molecules after treatment of a cellular system with specific SM species. It has already been reported that the carbon chain length and degree of unsaturation have a dramatic impact on the rate of sphingomyelinase turnover, and that the enzyme is equally, if not more effective on dihydrosphingomyelin as it is on sphingomyelin (22).

Sphingomyelin and dihydrosphingomyelin are especially difficult to differentiate, since they differ only by the presence or lack of a 4,5-*trans* double bond, respectively, in the long-chain base backbone of the molecule. DSM species having any sites of unsaturation are isobaric with SM species. In a recent report (10), we demonstrated that dihydrosphingomyelin has been overlooked or under-reported in many previous reports that used MS for sphingolipid analysis. A primary reason for this is that many researchers used infusion, without chromatographic separation, to identify the sphingolipids. By such an approach, only saturated DHS species have unique masses compared to SM species, and so in several cases, only saturated DSM species were reported.

Figure 14. Mass Spectra of triacylglycerols. (A) Average ESI-MS mass spectrum from 40 to 45 min; (B) avg. from 45 to 50 min; (C) avg. from 50 to 55 min; (D) avg. from 55 to 60 min; (E) avg. from 60 to 65 min; (D) avg. from 65 to 70 min.

The data above demonstrated that ESI-MS and APCI-MS are valuable tools that can allow the class of a lipid to be identified, and usually the lengths of the carbon chains and the number of sites of unsaturation can also be determined. The data demonstrate the complexity of mass spectral data for lipid analysis. If one imagines all of the spectra for PE, PE plasmalogen, PC, SM, DSM, GluCer, SulfGluCer, PI and other polar lipids plus TAG, ceramides, and cholesterol-related molecules all overlapped and giving peaks for every molecular species of all classes simultaneously, one begins to understand the difficulty of identifying all classes and molecular species by infusion, without the chromatographic separation. We have shown that the classification as polar or non-polar, along with the specific retention time on either the NP-HPLC system or the RP-HPLC system can provide valuable information that can allow all classes and molecular species within each class to be identified, even those species present at low levels. The retention times provide a valuable additional piece of information that is not available when infusion is used. Furthermore, the separation of lipids into classes by chromatography makes identification of the molecular species within each class simpler, and allows isobaric species to be differentiated chromatographically. An LC2/MS2 arrangement allows the goals of lipidomics, to identify the classes and molecular species of all lipid classes, to be accomplished from a single injection of a total lipid extract.

The data from the bovine brain total lipid extract demonstrated that ESI-MS is not only excellent for polar lipid analysis, but is also very useful for non-polar lipid analysis, as long as ammonium is supplied to facilitate adduct formation. ESI-MS/MS of the ammonium adducts of TAGs, for instance, can provide not only the identity of the TAG molecular species in terms of FA carbon chain length and degree of unsaturation, but also the regioisomeric position of the FA can be deduced from the $[DAG]^+$ fragment ratios. Alternatively, the sand bream filet total lipid extract demonstrated that APCI-MS full-scan mass spectra can be used to obtain the same kind of information, but up-front collision energy should be minimized, since sufficient fragmentation for structural elucidation already occurs in the APCI source.

The experiments above demonstrated the power of using dual liquid chromatographs with dual mass spectrometers for complete analysis of the lipidome of a total lipid extract. Although we did not identify every class of lipid present in these samples, we identified a variety of classes and numerous molecular species within each class to demonstrate the utility of the LC2/MS2 approach to lipidomics analysis. This approach clearly has benefits over techniques that do not utilize a chromatographic separation prior to mass spectral analysis. The chromatography not only provides retention data to aid in classification of lipids, but also produces spectra that are simpler and easier to interpret
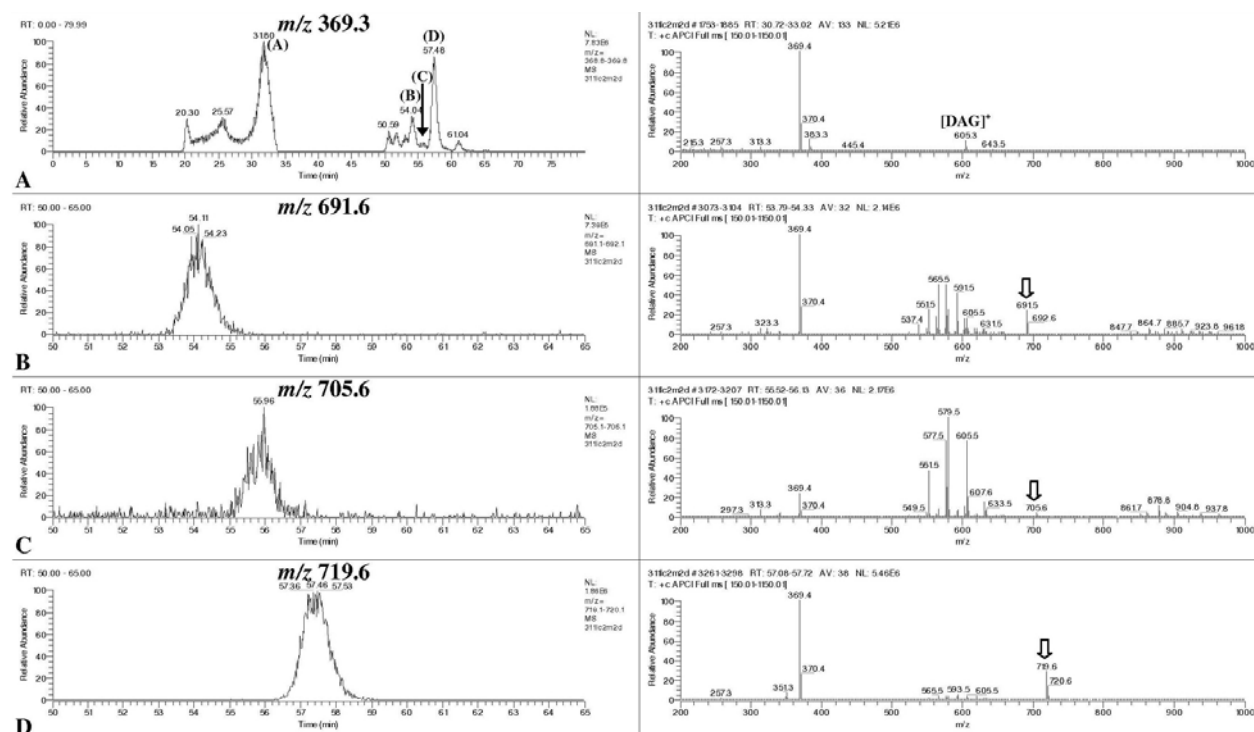
Figure 15. Cholesterol-related molecules that co-eluted with triacylglycerols. These molecules each produced an abundant fragment at *m/z* 369.3 and a protonated molecule (labeled with arrows). (A) EIC of *m/z* 369.3 and average mass spectrum across peak A, showing cholesterol-related molecule overlapped with OS diacylglycerol; (B) EIC of *m/z* 691.6 and average mass spectrum across peak B; (C) EIC of *m/z* 705.6 and average mass spectrum across peak C; (B) EIC of *m/z* 716.6 and average mass spectrum across peak D.

than data from infused samples, since the lipid classes are separated from each other.

The primary disadvantage to the LC2/MS2 approach is that two instruments are required. However, as mass spectrometry has become the detection method of choice in many laboratories, and instruments continue to evolve and to become more sensitive and less expensive, it is becoming more and more practical to use two instruments for one comprehensive analysis. A distinct advantage of the method reported above is that it requires no special or fabricated instrumentation. The LCQ series of mass spectrometers come factory-equipped with the programmable electronically actuated valve that allows the column switching to be accomplished using only standard HPLC instrumentation. We hope that in the coming years the LC2/MS2 approach will become more commonplace and that the value of this approach for lipidomics analysis will be realized on a wider scale.

6. ACKNOWLEDGEMENT

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