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Global Analysis of Retina Lipids by Complementary Precursor Ion and Neutral Loss Mode Tandem Mass Spectrometry

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Summary

Despite an increasing recognition of the causative and diagnostic role of lipids in the onset and progression of retinal disease, information on the global lipid profile of the normal retina is quite limited. Here, a “shotgun” tandem mass spectrometry approach involving the use of multiple lipid class-specific precursor ion and neutral loss scan mode experiments has been employed to analyze lipid extracts from normal rat retina, obtained with minimal sample handling prior to analysis. Redundant information for the identification and characterization of molecular species in each lipid class was obtained by complementary analysis of their protonated or deprotonated precursor ions, or by analysis of their various ionic adducts (e.g., Na^+ , NH_4^+ , Cl^- , $\text{CH}_3\text{OCO}_2^-$). Notably, “alternative” precursor ion or neutral loss scan mode MS/MS experiments are introduced that were used to identify rat retina lipid molecular species that were not detected using “conventional” scan types typically employed in large-scale lipid-profiling experiments. This chapter outlines the principles and advantages of utilizing complementary/redundant identification of lipid species as a strategy to overcome inherent challenges and limitations of shotgun lipid analysis, and provides examples of the application of this strategy in the analysis of the retina lipidome.

Keywords

Retina; Lipidomics; Lipid analysis; Electrospray ionization; Tandem mass spectrometry

1. Introduction

Retina has a unique fatty acid profile with the highest levels of long chain polyunsaturated fatty acids (LCPUFA), including docosahexaenoic acid ($\text{DHA}_{22:6n3}$), and arachidonic acid ($\text{ARA}_{20:4n6}$), observed in the body (1–5). Of these, $\text{DHA}_{22:6n3}$ is the most abundant fatty acid in both neural and vascular elements of the retina (2), retinal pigment epithelial cells (6,7) and retinal photoreceptor outer segment disc membranes (8,9). Extensive studies clearly demonstrate the important role of lipids in retinal health and disease (5). However, most studies to date have focused on the role of LCPUFA's, and DHA in particular, measuring total fatty acid levels without obtaining structural information. The reason for this is likely methodological – LCPUFA are very abundant in the retina and relatively easy to measure by well developed high-performance liquid chromatography (HPLC) or gas chromatography (GC) techniques. The limitations of the traditional techniques have precluded comprehensive complex lipid analysis from the limited amount of retinal material that can be obtained from animal models such as rats and mice. Thus, there is surprisingly little information available regarding the lipid composition of the normal retina, and only limited information describing changes in global lipid profiles between normal and diseased retinal tissue (See Note 1).

Recent advances in the application of electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) (10–20) techniques, coupled with the use of tandem mass spectrometry methods employing selective precursor ion and neutral loss scan mode analysis strategies, have enabled the development of “shotgun” lipidomics approaches for rapid and sensitive monitoring of the molecular compositions and abundances of individual lipid species in unfractionated lipid extracts (10–17). While shotgun approaches allow for high-throughput analysis of multiple lipid classes without prior chromatographic separation of lipid analytes, the large number of lipid molecular species present in crude extracts presents a significant challenge for the analyst. In addition to possible overlap of the molecular ions and the ^{13}C isotope peaks of numerous lipid species, even greater extract complexity can arise due to the presence of individual lipid species in multiple ionic forms, via adduction with a variety of cationic (e.g., $+\text{H}^+$, $+\text{Na}^+$, $+\text{NH}_4^+$) or anionic (e.g., $-\text{H}^-$, $+\text{Cl}^-$) species in positive or negative ion modes, respectively, that may be present in small amounts following extraction of lipids from tissues or cells. Additional complexity may also be observed for certain lipids due to the presence of adducts formed by reaction of the solvents and buffer additives that are commonly employed for sample analysis. For example, phosphocholine-containing lipids are readily adducted with methylcarbonate ($\text{CH}_3\text{OCO}_2^-$) anions (21), formed by the *in vitro* reaction of hydroxide or bicarbonate salts (22,23) with methanol, effectively increasing the number of lipid species observed in negative ion mode analysis.

In our studies, relatively limited tissue availability has prompted us to develop strategies for attaining a thorough accounting of the global lipid composition of retina without requirement for multiple sample fractionation or processing steps, such as chromatographic separation, or destruction of glycerophospholipids for enhanced sphingolipid analysis. Utilizing a triple quadrupole mass spectrometer to perform multiple precursor ion and neutral loss scan mode MS/MS experiments, we have found that complementary/redundant detection of a given lipid class based on the unique fragmentation behaviors of various ionic forms (e.g., $[\text{M} + \text{H}]^+$, $[\text{M} - \text{H}]^-$, $[\text{M} + \text{Na}]^+$, $[\text{M} + \text{NH}_4]^+$, $[\text{M} + \text{Cl}]^-$, $[\text{M} + \text{CH}_3\text{OCO}_2]^-$) of various lipid classes facilitates (a) the ability to identify molecular lipid species that may not be detected when only one scan mode is used, (b) a more thorough accounting of the various lipid species that may be present in multiple ionic forms when “absolute quantification” is desired, and (c) simplification of relative quantification by selection of an MS/MS scan mode in which lipid species are present in only one ionic form. Furthermore, the ability to identify lipids in more than one ionic form greatly increases the confidence for peak identification, even for ions observed at low (<5%) relative abundance.

Here, we outline the general approach and the results obtained by performing complementary precursor ion and neutral loss scan mode tandem mass spectrometry analyses. Although presented within the context of defining the normal rat retina lipidome, the analytical principles could benefit the analysis of virtually any tissue or cell lipid extract. In this chapter, our discussion will be limited to analysis of rat retina sphingomyelin (SM), glycerophosphatidylcholine (GPCCho), glycerophosphatidylethanolamine (GPEtn), glycerophosphatidylinositol (GPIIns), glycerophosphatidylserine (GPSer), and diacylglycerol (DG) and triacylglycerol (TG) molecular lipid species. Complementary sets of precursor ion and neutral loss scan mode MS/MS experiments will be described for the analysis of these lipid classes. Analogous sets of complementary precursor ion and neutral loss experiments will also be suggested for additional lipid classes including ceramides, cholesteryl esters, glycerophosphatidylglycerol (GPGro), glycerophosphatidic acid (GPA), and

¹Although disruption of lipid metabolism is known to play a key role in the onset and progression of a variety of retinal diseases, the use of retinal lipid profiles has not been sufficiently explored. This lack of knowledge is largely due to the diversity of lipids and to limits in the sensitivity of previously available analytical methods. The methodology described in this chapter for lipid profile analysis of normal retina will be further applied to various diseased retinal states to aid in developing effective therapeutic strategies for retinal disorders.

monoacylglycerols. The results of the analyses for these additional lipid classes in rat retina lipid extracts may be obtained upon request from the authors.

2. Materials

2.1. Equipment

1. Teflon/glass tissue homogenizer (Glas-Col®, Sigma-Aldrich St. Louis, MO).
2. Vortex.
3. Centrifuge with swinging bucket rotor (Sorvall RC 5B Plus, Thermo Fisher Scientific Inc., Waltham, MA).
4. Evaporating Unit (Reacti-Vap, Pierce, Thermo Fisher Scientific Inc., Waltham, MA).
5. SpeedVac SC110 (Savant, Thermo Fisher Scientific Inc., Waltham, MA).
6. Chip-based nano-electrospray ionization (nESI) source (Advion NanoMate, Ithaca, NY).
7. Thermo Scientific model TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA).
8. Anesthesia System Isoflurane Vapor 19.1 (G.A.S. Inc., Bridgeville).

2.2. Reagents and Chemicals

1. Methanol (MeOH), HPLC grade, J.T. Baker (Phillipsburg, NJ).
2. Deionized water, HPLC grade, J.T. Baker (Phillipsburg, NJ).
3. Sodium chloride (NaCl), HPLC grade, J.T. Baker (Phillipsburg, NJ).
4. Ammonium hydroxide (NH₄OH), EMD Chemicals (Gibbstown, NJ).
5. Chloroform (CHCl₃), EMD Chemicals (Gibbstown, NJ).
6. Isopropanol, Fisher Scientific (Pittsburgh, PA).
7. Lipid standards from Avanti Polar Lipids (Alabaster, AL).

2.3. Animals

Male *Wistar* rats were maintained on Harlan-Teklad laboratory chow (#8640) and water *ad libitum*. At 12 weeks of age, rats were sacrificed under anesthesia (isoflurane/Vapomatic), and eyeballs were removed and used for retinal preparations. All procedures for the use and care of animals for laboratory research were approved by the All University Committee for Animal Use and Care at Michigan State University.

3. Methods

3.1. Lipid Extraction from Rat Retinal Tissue

Lipids were extracted using a modified method of Folch (24). Whole retinas (~12 mg tissue) from 12-week old male *Wistar* rats were placed in an ice-chilled Teflon/glass tissue grinder and homogenized on ice in 50 µL/mg tissue of 40% MeOH in H₂O. Retina homogenates were extracted with 200 µL/mg tissue of CHCl₃:MeOH (2:1 v/v) by vortexing for 1.0 min. After centrifugation at 3,000 × *g* for 10 min, the lower organic phase was recovered and transferred to a new glass tube. The aqueous upper phase was re-extracted with 200 µL/mg tissue of CHCl₃, then vortexed and centrifuged as before. The lower organic phase was collected and combined with the lower phase from the first extraction. The pooled organic phases were

evaporated under nitrogen and further dried in a speedvac overnight. Dried lipid extracts were resuspended in 50 μ L/mg tissue of isopropanol: methanol:chloroform (4:2:1 v/v/v) and stored under nitrogen in glass vials in the dark at -80°C until further use.

3.2. nESI-MS and MS/MS Conditions

Immediately prior to analysis, aliquots of retina lipid extracts were diluted 1:20 in a solution of isopropanol: methanol: chloroform (4: 2: 1 v/v/v) containing 20 mM NH_4OH . Synthetic lipid standards were diluted in the same solution to a final concentration of 2 μM . For some experiments, samples were analyzed in the presence of 0.5 mM NaCl. All samples were centrifuged, loaded into Whatman Multichem 96-well plates (Fisher Scientific, Pittsburgh, PA) and sealed with Teflon Ultra-Thin Sealing Tape (Analytical Sales and Services, Pompton Plains, NJ). Lipids were then introduced to a Thermo Scientific model TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA) via a chip-based nano-electrospray ionization (nESI) source (Advion NanoMate, Ithaca, NY) operating in infusion mode, using an ESI HD_A chip, a spray voltage of 1.4 kV, a gas pressure of 0.3 psi, and an air gap of 2 μL . The ion transfer tube of the mass spectrometer was maintained at 150°C . All MS and MS/MS spectra were acquired using methods controlled by the Xcalibur software (Thermo, San Jose, CA). In MS mode, the Q1 peak width was maintained at 0.5 u. For neutral loss and precursor ion MS/MS scans, the peak widths of both Q1 and Q3 were maintained at 0.5 u. For product ion scan mode MS/MS experiments, Q1 and Q3 were operated with peak widths of 1.0 u. The Q2 collision gas pressure was set at 0.5 mTorr. MS and MS/MS scans were typically acquired using scan rates of 500 m/z /s. Collision energies were individually optimized for each product ion, neutral loss, or precursor ion scan mode MS/MS experiment using commercially available lipid standards. The MS/MS spectra shown were typically averaged over 100–300 scans.

3.3. Identification of Lipid Molecular Species

Peak finding and correction for ^{13}C isotope effects was performed on complementary sets of precursor ion and/or neutral loss scan mode MS/MS data using the Lipid Mass Spectrum Analysis (LIMS) v.1.0 software (25) peak model fit algorithm in conjunction with an expanded user-defined database of hypothetical lipid compounds. Assignment of glycerophospholipid acyl chain constituents was achieved by precursor ion scans to monitor for the formation of specific m/z product ions corresponding to deprotonated fatty acid anions in negative ion mode; these scans identified glycerophospholipids as $[\text{M} - \text{H}]^{-}$, $[\text{M} + \text{Cl}]^{-}$, and $[\text{M} + \text{CH}_3\text{OCO}_2]^{-}$ ions. Assignment of diacylglycerol and triacylglycerol acyl chain constituents was achieved by neutral loss scans to monitor for the loss of m/z values corresponding to the loss of a fatty acid + NH_3 from DG and TG $[\text{M} + \text{NH}_4]^{+}$ ions in positive ion mode.

3.4. Application of Complementary Precursor Ion and Neutral Loss Mode Tandem Mass Spectrometry to Analysis of Rat Retina Lipids

3.4.1. Mass Spectrum of a Crude Rat Retina Lipid Extract—The use of an animal model, such as the rat, for studying changes in global retina lipid profiles as a function of the onset and progression of disease is attractive for several reasons. These include the ability to control or systematically evaluate the effect of a number of variables, such as genetic background, environment, age, and diet that are known to alter the presence and abundance of particular lipid species at any given time. In order to obtain an initial “snapshot” of the global lipid profile of the normal rat retina, a crude lipid extract isolated from a whole retina was subjected to analysis by using nanoelectrospray ionization coupled to a triple quadrupole mass spectrometer in both positive and negative ionization modes. The mass spectra obtained from these analyses are shown in Fig. 1a, b respectively. Analysis of lipid extracts from three separate

rat retinas resulted in essentially identical spectra to those shown in Fig. 1a, b, indicating that minimal biological variability was observed between these animals (data not shown). This was expected given that these samples were obtained from animals with identical genetic backgrounds that were maintained under essentially identical conditions.

The resolution and mass accuracy associated with the mass spectra shown in Fig. 1 precluded the identification of individual lipid species on the basis of their masses alone. Indeed, in many cases, the product ion scan mode MS/MS experiments and the precursor ion and neutral loss scan mode MS/MS experiments discussed below indicated that multiple lipid species having the same nominal mass values, as well as multiple lipids with isobaric masses, were present at a given m/z value (see Notes 2 and ³). Furthermore, the presence and the identities of low abundance lipid species, such as mono-, di-, and tri-acylglycerols, ceramides, and cholesteryl esters that were present at or below the level of chemical noise in these spectra could not be determined.

3.4.2. Complementary Sets of Precursor Ion and Neutral Loss Scans for Global Lipid Analysis—In order to obtain a more comprehensive profile of the lipid composition of the crude rat retina lipid extract, a series of 27 precursor ions and neutral loss scan mode MS/MS experiments were performed in both positive and negative ionization modes (11,13, 14,21,26–30). A complete description of these scans, including the specific lipid class to be identified, the precursor ion type selected for each scan (e.g., $[M + H]^+$, $[M + Na]^+$, $[M + NH_4]^+$, $[M - H]^-$, $[M + Cl]^-$ and $[M + CH_3OCO_2]^-$), the mass of the specific product ion or the neutral loss that was detected, and their identities, is given in Table 1 (see Notes 4, ⁵, and

²From the data described above, it is clear that crude lipid extracts from whole rat retinas contain an extremely complex mixture of molecular species, representing a wide array of lipid classes at varying abundances. The observation of multiple ionic adducts of a given lipid class in both positive (e.g., $[M + H]^+$, $[M + Na]^+$, and $[M + NH_4]^+$) and negative (e.g., $[M - H]^-$, $[M + Cl]^-$ and $[M + CH_3OCO_2]^-$) ionization modes adds further complexity, effectively increasing the number of “spectral features” to the resultant mass spectra obtained by analysis of these extracts. However, the ability to identify specific lipid species in both positive and negative ionization modes, as well as in their multiple adducted forms, enables unambiguous lipid identification, particularly important when lipids were present at low abundance.

³In many cases, multiple lipid species were found to be present at a single nominal m/z value. For example, m/z 834 in the positive ion mode MS spectrum shown in Fig. 1a, was found to contain diacyl GPCCho (18:0/22:6) $[M + H]^+$, GPSer (18:1/22:6) $[M + H]^+$, and GPSer (18:0/20:4) $[M + Na]^+$ ions. Likewise, m/z 834 in the negative ionization mode spectrum shown in Fig. 1b was found to consist of $[M - H]^-$ ions of GPSer (18:0/22:6) and GPEtn (22:6/22:6), as well as the methyl carbonate adduct ($[M + CH_3OCO_2]^-$) of GPCCho (16:0/18:1). Detection of lipid classes in multiple ionization forms was necessary to fully elucidate the identities of ions present in both positive and negative ion mass spectra at numerous m/z . This point becomes especially important when comparisons must be made across numerous samples, such as comparisons between normal and disease states, in which case any observed change in ion abundance between two or more groups of samples must be accurately characterized.

⁴To assist in the implementation of complementary analysis of major lipid classes within the lipidome, we have assembled multiple pairings of possible precursor ion and neutral loss scan mode MS/MS experiments to enable redundant detection of each lipid class. The advantages and disadvantages of many of these scan types have been discussed, and in several cases, the use of redundant detection of a lipid class minimized the shortcomings of one or more MS/MS experiments. For example, positive ion analysis of GPEtn $[M + H]^+$ ions by neutral loss scan mode MS/MS was found to be more sensitive than negative ion mode precursor ion analysis of GPEtn $[M - H]^-$ ions in terms of signal/noise and absolute ion abundance. The more sensitive positive ion neutral loss scan is therefore optimal for analysis of low abundance GPEtn ions. However, the additional presence of $[M + Na]^+$ ions, even at very low abundance, complicates analysis of the resultant positive ion MS/MS spectra and necessitates complementary analysis of GPEtn $[M - H]^-$ ions for clarity. Negative ion MS/MS analysis of GPEtn enables the identification of alkenyl species that are not observed in positive ion GPEtn MS/MS analysis; yet the significantly lower sensitivity of the negative ion mode GPEtn precursor ion scan precludes analysis of lipid species that may be present at relatively low abundance.

⁵Most of the individual MS/MS experiments we have paired for use in complementary analysis of rat retina lipid classes were obtained from the available literature. In several cases we have employed novel precursor ion and neutral loss scans derived from previously published gas-phase fragmentation pathways to detect lipid classes in various ionic forms or modification states. To our knowledge, this analysis of the rat retina lipidome was the first analysis to include the negative ion neutral loss of 135 Da for detection of SM and GPCCho species as methylcarbonate adducts; likewise, it was the first analysis to explore differential detection of SM and GPCCho as methylcarbonate adducts by use of the negative ion neutral loss of 161 Da for preferential detection of SM species; and it was the first analysis to include detection of sodiated GPCCho and GPSer species by positive ion mode precursor ion scanning of m/z 147 and m/z 208, respectively. Additionally, we introduced the positive ion mode neutral loss of 35 Da for simultaneous detection of diglyceride species as ammonium adducts. In this chapter we have provided a preliminary outline of the possible benefits and shortcomings of each of these novel MS/MS experiments, although a more rigorous examination of each proposed scan would be beneficial for assessment of the viability of these scans for use in large-scale lipid-profiling experiments.

⁶). Specific examples of these experiments, for determining the presence of SM, GPCho, GPEtn, GPIs, GPSe, DG, and TG lipids, are discussed below.

In each case, multiple precursor ion and neutral loss scan mode experiments were used to identify and characterize the individual components of a particular lipid class. The use of multiple scans enabled the compositions of the overlapping ions present at a particular m/z value in either the positive or negative ionization mode mass spectra to be more fully elucidated, and allowed the list of spectral features (i.e., molecular ions and their various adducts) that were observed in both the positive and negative ionization mode mass spectra to be significantly expanded. Particularly noteworthy was an observation that the use of complementary precursor ion and neutral loss scans enabled the identification and characterization of molecular species that were not detected using the 'typical' scan types that have been most commonly employed previously in the literature for lipid-profiling experiments.

3.4.3. Complementary Precursor Ion and Neutral Loss Scan Mode Analysis of Rat Retina SM and GPCho Lipids

3.4.3.1. Negative Ion Analysis of SM and GPCho: The identification of abundant SM and GPCho species in negative ion mode is useful when it is necessary to (a) identify spectral features of a negative ion mass spectrum; (b) perform fatty acid analysis of lipid species by negative ion product ion tandem mass spectrometry or precursor ion scanning of m/z corresponding to deprotonated fatty acyls; and (c) identify m/z at which one or more lipid species may be present in a negative ion mass spectrum or tandem mass spectrum. Analysis of these two lipid classes is often complicated by overlap of a GPCho first ^{13}C isotope peak with a putative SM molecular ion. This can be overcome by destruction of GPCho (and all glycerophospholipids) by alkaline hydrolysis (29). This approach is not useful, however, when analysis of both SM and GPCho species are needed from limited amounts of tissue. While algorithms to correct for ^{13}C isotope abundances have been used to distinguish putative SM molecular ions from GPCho first ^{13}C isotope peaks (25,31), the extremely low abundance of retinal SM molecular ions detected by typical positive ion analysis (*see Subheading 3.3.2*) has precluded the successful reliance upon deisotoping algorithms alone for thorough analysis of rat retina SM species.

The ability of SM and GPCho species to form adducts with chloride (Cl^-) and methyl carbonate ($\text{CH}_3\text{OCO}_2^-$) anions enabled a convenient method for negative ion mode detection of SM and GPCho molecular species. Chloride ions are often present in small amounts in lipid extracts. While Cl^- ions may be minimized by multiple sample washing and desalting steps, we have found the presence of Cl^- to be beneficial for negative ion analysis of SM and GPCho. Figure 2a demonstrated negative ion mode collisionally induced dissociation (CID) of the $[\text{M} + \text{Cl}]^-$ ion of a synthetic GPCho(14:0/14:0) species in the presence of 0.5 mM NaCl. Collisionally induced dissociation of chloride adducts of GPCho species led to an abundant neutral loss of 50 Da, corresponding to loss of methyl chloride. Dissociation of this species also resulted in ions corresponding to the loss of the GPCho 14:0 fatty acid moieties as neutral ketene species, observed at m/z 452, as well as abundant ions at m/z 227 corresponding to the deprotonated fatty acids. By contrast, CID of chloride-adducted synthetic SM(d18:1/12:0) revealed the

⁶We have provided, as a reference, pairings of MS/MS experiments in Table 1 that can be used in the complementary analysis of lipid classes that were not discussed in this chapter, including cholesteryl esters, glycerophosphatidic acid (GPA), glycerophosphatidylglycerol (GPGro), and ceramides. As these lipid classes are of low abundance in many tissues, and especially in rat retina, their analysis certainly benefits from complementary analysis of multiple ion forms where possible, or by redundant detection of the same ion form by multiple scans. The pairings suggested for complementary analysis of lipid classes are certainly not exhaustive, and other sets of complementary pairs could be substituted for analysis of various lipid classes. Furthermore, as the understanding of gas-phase fragmentation pathways of lipid classes and their ionic adducts progresses, it will expand the ability to perform complementary analysis to lipid classes that have not been addressed in this chapter.

abundant neutral loss of 50 Da was the primary product ion observed over a wide range of collision energies (Fig. 2b). Dissociation of GPCho(14:0/14:0) $[M + CH_3OCO_2]^-$ ions resulted in the spectra shown in Fig. 2c. Abundant neutral losses of 76 Da (loss of $CH_3OCO_2 + H$), 135 Da (loss of $CH_3OCO_2 + (CH_3)_3N$), and 161 Da (loss of $CH_3OCO_2 + (CH_3)_3NCHCH_2$) were observed, as well as ions representing the loss of the neutral 14:0 fatty acid (m/z 391) and the fatty acid anion (m/z 227). Fragmentation of the methyl carbonate adduct of synthetic SM (d18:1/12:0) by CID Fig. (2d) revealed phosphocholine-derived ions similar to those observed for GPCho, however at significantly different abundances. Abundant neutral losses of 76, 135, and 161 Da were all observed, yet here the loss of 161 Da dominated, as opposed to the loss of 135 Da that was seen for GPCho.

The negative ion mode gas-phase fragmentation behavior of SM and GPCho species, as demonstrated in Fig. 2, enable the use of neutral loss scan mode MS/MS for class-specific detection of SM and GPCho. Performing the conventionally used neutral loss scan (NLS) of 50 Da (scan number 1 in Table 1) in rat retina lipid extracts in the presence of 0.5 mM chloride resulted in detection of both SM and GPCho species (Fig. 3a). From the NLS 50 scan a total of eight SM peaks, highlighted with bold text in Fig. 3a, were readily discernable from GPCho species. As the neutral loss of 135 Da was the most abundant loss observed for GPCho $[M + CH_3OCO_2]^-$ ions across a wide range of collision energies, it enabled negative ion detection of rat retina GPCho species as methyl carbonate adducts by performing NLS 135 (Fig. 3b, scan number 2 in Table 1) when ammonium hydroxide was added to the rat retina lipid extract at a concentration of 20 mM. Five low abundance SM peaks, highlighted with bold text in Fig. 3b, were also detected with this scan. We next employed the neutral loss of 161 Da (scan number 3 in Table 1) to detect SM and GPCho $[M + CH_3OCO_2]^-$ ions in a rat retina lipid extract, which resulted in the spectrum shown in Fig. 3c. Here, seven additional SM peaks could be detected that were not observed in the NLS 135 scan, as well as four SM peaks not detected by NLS 50. Additionally, the relative abundances of SM species at m/z 777 and 805 (corresponding to SM(d18:1/16:0) and SM(d18:1/18:0) $[M + CH_3OCO_2]^-$ ions) were increased approximately tenfold and 20-fold, respectively, compared to their relative abundances in the NLS 135 scan. The differential fragmentation behavior of methylcarbonate-adducted anions of SM and GPCho was thus exploited to preferentially detect either a predominance of GPCho ions via the neutral loss of 135 Da, or a predominance of SM ions via the neutral loss of 161 Da.

Both chloride and methylcarbonate adducts of GPCho species were suitable for performing negative ion mode product ion scans to confirm GPCho fatty acid assignments. Likewise, GPCho species were detected as both chloride and methylcarbonate-adducted anions when precursor ion scanning of m/z corresponding to deprotonated fatty acyls was performed in rat retina lipid extracts (scan number 18 in Table 1, data not shown.) Therefore, it was advantageous to assess rat retinal GPCho content by utilizing both NLS 135 and NLS 50 for detection of GPCho species as $[M + CH_3OCO_2]^-$ and $[M + Cl]^-$ ions, respectively. Performing the additional NLS 161 for detection of SM $[M + CH_3OCO_2]^-$ ions allowed for the most thorough identification of rat retinal SM species. Under the conditions employed here, negligible formate or acetate adducts of PC and SM lipids were observed (11,32).

3.4.3.2. Positive Ion Analysis of SM and GPCho: Collisionally induced dissociation of GPCho and SM $[M + H]^+$ ions yields an abundant characteristic product ion at m/z 184 corresponding to the protonated phosphocholine headgroup. Product ion spectra of synthetic GPCho(14:0/14:0) and SM(d18:1/12:0) are shown in Fig. 4a, b, respectively. As observed for chloride and methylcarbonate adducts of GPCho and SM, product ion spectra of synthetic GPCho(14:0/14:0) and SM(d18:1/12:0) $[M + Na]^+$ ions yielded similar product ions, though at differing relative abundances (Fig. 4b, c). Abundant ions corresponding to loss of 59 Da ($N(CH_3)_3$), 183 Da (neutral phosphocholine), and an ion corresponding to sodiated cyclophosphane at m/z 147 were observed in the product ion spectra of both GPCho and SM.

In the case of GPCho, the ion at m/z 147 was the most abundant ion observed in the product ion spectrum. In the case of SM, however, the neutral losses of 183 and 59 Da were more abundant than the ion at m/z 147. Additionally, the loss of 205 Da (corresponding to the neutral loss of sodium choline phosphate) was abundant from dissociation of GPCho $[M + Na]^+$ ions, yet was present at less than 1% relative abundance upon dissociation of SM $[M + Na]^+$ ions.

The spectra obtained from various positive ionization mode precursor ion and neutral loss scan mode MS/MS experiments for the identification of SM and GPCho lipid ions from crude whole rat retina lipid extract are shown in Fig. 5. The spectrum obtained from the conventional precursor ion scan (PIS) mode MS/MS experiment (scan number 4 in Table 1), to monitor for the formation of the characteristic phosphocholine product ion at m/z 184 from dissociation of SM and GPCho $[M + H]^+$ ions, is shown in Fig. 5a. After isotopic correction, only four SM peaks were identified (m/z 703, 731, 759, and 815, corresponding to d18:1/16:0, d18:1/18:0, d18:1/20:0, and d18:1/24:0 species of SM, respectively) at low (<5%) relative abundance. However, PIS 184 provided excellent sensitivity for GPCho detection, as it provided the highest signal/noise observed for all methods of GPCho detection.

As sodium (Na^+) ions are often present in lipid extracts due to carryover from the aqueous phase during lipid extraction, sample washing steps, and the mass spectrometer matrix, additional precursor ion and neutral loss scan mode MS/MS analyses of SM and GPCho $[M + Na]^+$ ions were also performed (see Fig. 5b–d; scan numbers 5–7 in Table 1.) Notably, each scan type varied greatly in terms of utility for SM analysis, consistent with the analysis of product ion spectra from GPCho and SM $[M + Na]^+$ ions. We found that PIS 147 (Fig. 5b, scan number 5 in Table 1), resulted in relative abundances of SM and GPCho species that were overall very similar to those obtained by the PIS 184 scan; however, only two SM species could be identified with this MS/MS scan. Figure 5c demonstrates the virtually exclusive detection of GPCho species as $[M + Na]^+$ ions with no detection of SM $[M + Na]^+$ ions. This was achieved by utilizing the constant neutral loss of 205 Da (scan number 6 in Table 1). Exclusive detection of GPCho species by NLS 205 was consistent with the expected result based on the product ion spectra of GPCho and SM $[M + Na]^+$ ions. Conversely, the product ion spectra of GPCho and SM $[M + Na]^+$ ions suggested that the neutral loss of 183 Da could allow enhanced detection of SM ions relative to GPCho ions. The use of NLS 183 in rat retina lipid extracts resulted in the spectrum shown in Fig. 5d. Here, four SM species could be detected, and the relative abundances of SM and GPCho were similar to those observed for PIS 184. The same results were obtained when NLS 183 was performed across a wide range of collision energies, which implies that this MS/MS scan mode does not afford a significant improvement over PIS 184 in terms of detection of SM species. Additionally, the ion at m/z 856 (corresponding to GPCho(18:0/22:6)) was observed at greater relative abundance in NLS 183 relative to the other MS/MS scans utilized, which may implicate an acyl chain contribution to the fragmentation behavior of these ions. All of the scans used to detect SM and GPCho as $[M + Na]^+$ ions were equally useful in providing redundant identification of GPCho molecular species, yet none of these scans matched the PIS 184 MS/MS scan in terms of overall sensitivity.

As observed for negative ion analysis, complementary precursor ion and neutral loss scan mode MS/MS analysis of SM and GPCho in the positive ion mode enabled redundant detection of SM and GPCho species, and the use of NLS 205 enabled specific detection of only GPCho without lipid extract fractionation prior to analysis. The ability to redundantly detect multiple molecular species, especially in the case of GPCho, by using multiple lipid class-specific product ions increased confidence in peak identification for low abundance ions. It should be noted, however, that negative ion analysis of SM species as either $[M + CH_3OCO_2]^-$ or $[M + Cl]^-$ ions proved more useful than positive ion analysis in terms of readily identifying the greatest number of distinct SM molecular species. In contrast, positive ion analysis was more effective for identification of GPCho species in terms of overall sensitivity (signal/noise);

however, negative ion analysis of GPCho was useful for determining fatty acid substituents by product ion scanning and/or precursor ion scanning of deprotonated fatty acyl species. If “absolute quantitation” of retinal SM and GPCho were to be attempted here, it would be necessary to analyze all ionic forms of SM and GPCho relative to internal standards and sum the contributions of each ionic form of SM or GPCho molecular species. Relative comparisons of lipid species across numerous samples could be achieved by selecting optimal scan modes for the desired lipid classes (i.e., NLS 161 for analysis of SM and PIS 184 for analysis of GPCho).

The MS/MS spectra collected for analysis of SM and GPCho species described here were analyzed between m/z 700–1,200 for positive ion mode, and m/z 700–1,300 for negative ion mode. To allow these spectra to be viewed in greater detail, smaller mass ranges are shown in Figs. 3 and 5. However, it should be noted that complementary analysis of rat retina SM and GPCho species verified the presence of GPCho species containing 32:6 and 34:6 very long chain polyunsaturated fatty acids; in each case, a 22:6 fatty acid was identified at the remaining glycerol position of the GPCho species. These species were observed as abundant ions in the positive ion MS spectrum Fig. (1a) at m/z 1,018 and 1,046, respectively. These species were identified by all positive ion MS/MS scan modes employed here for the analysis of SM and GPCho. These high molecular weight GPCho species were also identified by NLS 50 as $[M + Cl]^-$ ions, which subsequently were used for product ion mode MS/MS analysis to verify the identities of the fatty acid moieties. However, these GPCho species were not detected as methylcarbonate adducts by either NLS 135 or NLS 161. By combining both positive and negative ion mode shotgun MS/MS analyses of all phosphocholine-containing lipids, identification of 13 SM and 29 GPCho lipids was achieved in rat retina crude lipid extracts with a significant degree of redundancy (*see* Note 7). A summary of the SM and GPCho molecular species detected by all MS/MS scan modes described above is provided in Table 2.

3.4.4. Complementary Precursor Ion and Neutral Loss Scan Mode Analysis of Rat Retina GPEtn and GPIIns Lipids—Pairs of complementary neutral loss and precursor ion scans were also employed for the identification and characterization of GPEtn and GPIIns lipid species. The detection of GPEtn lipids has typically been achieved by monitoring for the neutral loss of 141 Da (i.e., phosphoethanolamine) from their $[M + H]^+$ precursor ions in positive ion mode MS/MS analysis (scan number 8 in Table 1). The spectrum obtained from analysis of the crude rat retina lipid extracts using this scan is shown in Fig. 6a. After correction for ^{13}C isotope contributions, a total of 20 GPEtn lipid species were identified in this experiment. The use of a complementary precursor ion scan to monitor for the presence of a characteristic m/z 196 product ion, corresponding to a glycerol phosphoethanolamine derivative Fig. (6b) from the $[M - H]^-$ precursor ions of GPEtn (scan number 9 in Table 1), enabled the detection of three additional GPEtn lipids. The observed discrepancy in identified GPEtn lipids between the two scan types was due, in part, to the fragmentation behavior of GPEtn alkenyl species. While collisionally induced dissociation of GPEtn alkenyl $[M - H]^-$ ions gives rise to the GPEtn-specific fragment at m/z 196, the neutral loss of 141 Da is virtually absent from CID of GPEtn alkenyl $[M + H]^+$ ions (38). GPEtn alkenyl species detected by the negative ion PIS 196 included those at m/z 748, 750, and 774 in Fig. 6b (*see* Table 3). Although scanning for the constant neutral loss of 141 Da affords the most sensitivity for detection of GPEtn species in terms of signal/noise ratio and absolute ion abundance (data not shown), the

⁷Clearly, the use of redundant identification of rat retina lipid classes from multiple precursor ion types enabled the identification of molecular species that would not have been observed if only one MS/MS scan mode had been employed in the analysis of each lipid class. This was especially true in the analysis of SM and GPCho species, in which only four rat retina SM species could be readily detected by the conventional positive ion precursor ion scan of m/z 184; four SM species were detected by neutral loss of 183 Da from sodiated SM ions; two SM species were detected by PIS 147 from sodiated SM ions; and 12 SM species were detected by the negative ion neutral loss of 161 Da from SM methylcarbonate adducts.

utility of the positive ion NLS 141 is effectively limited to detection of diacyl GPEtn species. Furthermore, GPEtn $[M + Na]^+$ ions, if present, are also detected by NLS 141, further complicating spectrum interpretation and quantification against an internal standard. m/z 814 and 858 in Fig. 6a represent abundant ions corresponding to sodium adducts of GPEtn (18:0/22:6) and GPEtn(22:6/22:6), respectively. It is possible to decrease the abundance of $[M + Na]^+$ ions in this scan mode by using low collision energy CID (<30 V under the conditions employed); however, the use of lower collision energies in the analysis of rat retina GPEtn lipids resulted in underrepresentation of GPEtn species containing 22:6 fatty acid constituents due to the lower fragmentation efficiency of these species. While the negative ion precursor of m/z 196 scan affords detection of both diacyl and alkyl/acyl GPEtn species, this scan mode may also detect GPEtn $[M + Cl]^-$ ions formed when chloride is present in a lipid extract (m/z 826 and 870 in Fig. 6b). It should be noted that the signal-to-noise associated with the spectrum obtained by negative ion mode PIS 196 was approximately two orders of magnitude lower than that of the positive ion mode neutral loss scan, thereby placing a limitation on the utility of this scan mode for the detection of additional low abundance GPEtn lipid ions. Due to the potential complications arising from the presence of various ionic adducts, as well as the differential fragmentation behavior of alkenyl GPEtn species in positive vs. negative ionization mode, the use of both of these MS/MS scan modes to redundantly detect GPEtn lipids allowed for more thorough evaluation of GPEtn lipids and greater confidence in peak identification (see Note 2). The GPEtn lipids identified in rat retina are summarized in Table 3. Fatty acid constituents were analyzed in negative ion mode as described in **Subheading 3.3**. In this analysis, a total of 24 GPEtn species were identified in rat retina after accounting for isobaric species, with redundant identification achieved for all diacyl species.

The detection of GPIs lipids is typically achieved using a negative ion precursor ion scan mode MS/MS experiment, by monitoring for the formation of a characteristic product ion at m/z 241 (corresponding to a dehydrated phosphoinositol) from their $[M - H]^-$ ions (scan number 10 in Table 1). The spectrum resulting from this scan, shown in Fig. 6c, enabled the identification of 14 GPIs lipids. The use of a complementary scan (number 11 in Table 1) to monitor for the characteristic neutral loss of 277 Da (corresponding to the combined losses of phosphoinositol + NH_3) from the $[M + NH_4]^+$ adducts of GPIs Fig. (6d) enabled nine of the lipids observed in the negative ion mode precursor ion scan experiment to be confirmed. This was especially useful in confirming ions of relatively low abundance. For example, the ions at m/z 909 and 929 in the spectrum shown in Fig. 6c represent putative GPIs $[M - H]^-$ (Total carbon: total double bond) 40:6 and 42:10 species, respectively. These ions were confirmed as likely GPIs species by the presence of their corresponding $[M + NH_4]^+$ ions after conducting NLS 277 (m/z 928 and 948 in Fig. 6d). Further complementary negative ion mode fatty acid analysis by scanning for precursor ions corresponding to deprotonated fatty acyl groups (scan number 18 in Table 1) confirmed the fatty acid constituents of these GPIs species as diacyl 18:0/22:6 and 20:4/22:6, respectively (data not shown.) Taken together, these complementary sets of data enabled confident identification of the ions in question despite their low relative abundance. A compilation of all rat retina GPIs species identified by positive and negative ion mode analysis is provided in Table 4.

3.4.5. Complementary Precursor Ion and Neutral Loss Scan Mode Analysis of Rat Retina GPSer Lipids—Detection of glycerophosphatidylserine (GPSer) lipid species is typically achieved in either positive or negative ion mode by the use of neutral loss scan mode MS/MS experiments. Figure 7a demonstrates the collisionally induced dissociation of the $[M + H]^+$ ion of synthetic GPSer(14:0/14:0) at m/z 680. The fragmentation of this ion is dominated by the neutral loss of 185 Da corresponding to loss of the phosphoserine headgroup. Figure 7b shows the spectrum obtained from collisionally induced dissociation of the $[M + Na]^+$ ion of the same GPSer(14:0/14:0) species. While a neutral loss of 185 Da was observed, the fragmentation of the GPSer(14:0/14:0) $[M + Na]^+$ ion was dominated by the formation of

the ion at m/z 208, corresponding to a sodium phosphoserine cation. Additionally, loss of neutral sodium phosphoserine (207 Da) was observed at m/z 495. Figure 7c demonstrates CID of the GPSer(14:0/14:0) $[M - H]^-$ ion observed at m/z 678. The most abundant ion observed in this spectrum results from the loss of 87 Da, corresponding to a serine derivative. Additional abundant ions were observed at m/z 381 and 363, corresponding to losses of the 14:0 fatty acids as ketene and neutral species, respectively; m/z 227, corresponding to the deprotonated fatty acid ions; and m/z 153, corresponding to a cyclic glycerolphosphate derivative.

As the neutral loss of 185 Da was observed from dissociation of both GPSer $[M + H]^+$ and $[M + Na]^+$ ions, performing the typical positive ion mode analysis of rat retina GPSer by utilizing NLS 185 (scan number 12 in Table 1), resulted in detection of both GPSer $[M + H]^+$ and $[M + Na]^+$ ions (see Fig. 8a). As observed in the positive ion analysis of GPEtn lipids, the presence of GPSer species in two different ionic forms within the same spectrum complicated interpretation of the spectrum, as ions that differ by 22 mass units could represent a difference of an adducted sodium ion, or the mass difference represented by the addition of two fatty acid methylene groups and three double bonds. The spectrum in Fig. 8a contains several sets of ions that differ by 22 mass units, such as m/z 790 and m/z 812; m/z 812 and m/z 834; m/z 836 and m/z 858; m/z 858 and m/z 880; and m/z 880 and m/z 902. In each case, the ion observed at an increase of 22 mass units could represent either a sodium-adducted species of the ion at the lower nominal mass, or a unique GPSer molecular species with one or more fatty acids that contain additional carbons and double bonds relative to the GPSer species at the lower nominal mass.

Additionally, the spectrum obtained by NLS 185 contained several ions of very low relative abundance (denoted by bold type), including m/z 762, m/z 790, and m/z 936. In order to provide confirmation of low abundance rat retina GPSer species and clarify the identities and ionic forms of ions observed in the NLS 185 spectrum, we employed two additional complementary GPSer-specific MS/MS scans. Figure 8b demonstrates use of a precursor ion scan for m/z 208 to detect formation of the sodium phosphoserine ion from GPSer $[M + Na]^+$ ions (scan number 13 in Table 1.) The spectrum obtained by performing this scan contained ions of only a single ionic form and elucidated m/z at which sodium-adducted GPSer species contributed to total ion current of the NLS 185 spectrum. The use of this complementary precursor ion scan would be useful in any positive ion mode analysis of GPSer in order to confirm the presence or absence of GPSer $[M + Na]^+$ ions, or to simplify relative quantitation of GPSer species against an internal standard. Furthermore, the use of PIS 208 enabled redundant detection of the low abundance ions observed in the spectrum obtained by scanning for the neutral loss of 185 Da. The ions in bold in Fig. 8b at m/z 784, m/z 812, and m/z 958 represent sodiated adducts of the protonated GPSer ions observed at m/z 762, m/z 790, and m/z 936, respectively, detected by the neutral loss of 185 Da.

Negative ion mode analysis of rat retina GPSer was achieved by the commonly used neutral loss of 87 Da (scan number 14 in Table 1). The spectrum obtained from this neutral loss mode MS/MS experiment is shown in Fig. 8c. Performing NLS 87 results in detection of GPSer molecular species in only one ionic form (i.e., $[M - H]^-$), which offers the same advantage as the positive ion mode PIS 208 in terms of spectrum simplification and ease of relative quantification against an internal standard. The detection of GPSer $[M - H]^-$ ions by NLS 87 offers an additional advantage in that ions identified by this MS/MS scan may in turn be used for direct analysis of esterified fatty acid constituents by product ion mode MS/MS experiments, or by precursor ion scanning of individual fatty acid anions. The use of NLS 87 to detect rat retina GPSer also provided another means of confirming the presence of low abundance ions detected by positive ion GPSer analysis. The ions in bold in Fig. 8c at m/z 760, m/z 788, and m/z 934 represent deprotonated equivalents of the low-abundance ions detected by NLS 185 and PIS 208. Subsequent analysis of the fatty acid constituents of these GPSer

species from their deprotonated ions revealed that the identities of these low abundance ions were diacyl GPSer(16:0/18:1), GPSer(18:0/18:1), and GPSer(24:6/24:6).

Notably, an abundant ion at m/z 810 was observed in the spectrum obtained from the negative ion NLS 87 MS/MS experiment. This ion represents the deprotonated form of the protonated ion observed at m/z 812 in the NLS 185 MS/MS experiment. However, an ion at m/z 812 was also observed in the PIS 208 MS/MS experiment, indicating that a small contribution of the ion current for m/z 812 in the NLS 185 spectrum was made by a GPSer sodium adduct of the ion at m/z 790. The quantification of m/z 812 in the NLS 185 spectrum against an internal standard would necessitate accommodation for the presence of underlying the GPSer $[M + Na]^+$ ion; however, the presence of the underlying GPSer $[M + Na]^+$ ion would not have been detected without performing the PIS 208 MS/MS scan. Furthermore, complementary positive and negative ion mode analysis of GPSer utilizing only NLS 185 and NLS 87, respectively, would have falsely confirmed that the ion detected at m/z 812 in the NLS 185 MS/MS scan consisted entirely of GPSer $[M + H]^+$ species. This analysis underscores the advantages of employing lipid class-specific MS/MS detection methods in which lipid species are detected as only one ionic form.

The ability to redundantly detect low abundance rat retina GPSer species in multiple ionic forms enabled increased confidence in peak calling and subsequent inclusion of these ions in analysis of esterified fatty acid groups. The ability to identify both protonated and sodium-adducted GPSer molecular ions in positive ion mode MS/MS analysis expanded the number of spectral features that could be assigned in the spectrum obtained from the initial MS analysis. Importantly, the use of a precursor ion scan of m/z 208 to identify GPSer $[M + Na]^+$ ions simplified positive ion GPSer analysis by clarifying the identities of lipid species at m/z where potential overlap existed between protonated and sodiated GPSer ions of differing fatty acid composition. Use of the negative ion NLS 87 scan for detection of GPSer enabled the identification of spectral features of the negative ion mode MS spectra, and provided additional confirmation of GPSer species observed at very low abundance. Relative quantification of GPSer would be most straightforward when using either the negative ion NLS 87 MS/MS experiment, or the positive ion PIS 208 MS/MS experiment when known amounts of exogenous salts have been added to a lipid extract; this scan is also useful for determining the presence of endogenous salts that may be present following lipid extraction. After taking into account the redundant lipid species identified in both the negative ion scan mode neutral loss experiment and the positive ion scan mode neutral loss and precursor ion experiments, a total of 29 GPSer molecular species could be identified in rat retina, with 22 redundant identifications. See Table 5 for a detailed catalog of all identified rat retina GPSer species. This example clearly highlights the utility of alternative or complementary MS/MS scans that are not commonly employed in lipidome-profiling studies to provide more detailed insights into the composition of complex lipid mixtures.

3.4.6. Complementary Neutral Loss Scan Mode Analysis of Rat Retina DG and TG Lipids—Nonpolar lipids, such as diacylglycerols (DG) and triacylglycerols (TG), have typically required derivatization and/or chromatographic separation prior to ESI-MS analysis (33–36). This limits the ability to analyze both polar and nonpolar lipid classes from limited sample amounts. We have applied the principle of complementary neutral loss scan mode MS/MS to the analysis of DG and TG species in the same unfractionated rat retina extract that was used for analysis of the polar lipid classes described above. The addition of ammonium hydroxide (other other ammonium salts) to lipid extracts prior to mass spectrometry analysis provides abundant ammonium cations that readily adduct neutral lipids such as DG's and TG's. Redundant detection of DG and TG $[M + NH_4]^+$ 4 ions was achieved by neutral loss scanning for multiple m/z corresponding to the loss of a neutral fatty acid + NH_3 (scan number 15 in Table 1.) By comparing spectra obtained from neutral loss scans corresponding to multiple

naturally occurring fatty acids, the fatty acid moieties esterified to the glycerol backbone of species at each m/z were elucidated. Additionally, we have found that mono- and diacylglycerol $[M + NH_4]^+$ ions are readily identified by neutral loss scanning of 35 Da, corresponding to an abundant loss of $NH_3 + H_2O$ (scan number 17 in Table 1.) This neutral loss scan was advantageous when simultaneous comparison of all MG/DG species to an internal standard was desired. Use of the NLS 35 MS/MS experiment was also useful for identifying DG species present in the ammonium-adducted fatty acid neutral loss spectra, as scanning for loss of a fatty acid + NH_3 may also detect cholesteryl esters present at very low abundance in rat retina. Thus, NLS 35 enabled differentiation between putative DG and cholesteryl ester ions detected by positive ion mode fatty acid analysis of neutral lipid species. This neutral loss mode MS/MS scan also provided another means of verifying low abundance putative DG peaks identified in one or more fatty acid neutral loss scan. The limitation of NLS 35 was that DG species were only identified in terms of total carbons: total double bonds, which could be deduced from the nominal mass of a given species.

Figure 9a shows the spectrum obtained from simultaneous analysis of all abundant rat retina DG species by scanning for a neutral loss of 35 Da between m/z 580 and 700. m/z 614 was detected as a putative diglyceride in this scan, consistent with the mass of a 34:0 (TC:TDB) diglyceride as an $[M + NH_4]^+$ ion. The fatty acid compositions of DG and TG species were then determined by scanning for the neutral loss of a 16:0 fatty acid + NH_3 (Fig. 9b), loss of an 18:0 fatty acid + NH_3 (Fig. 9c), and the loss of a 22:6 fatty acid + NH_3 (Fig. 9d), over a wider mass range (m/z 580–980). m/z 614 was redundantly detected in scans for the neutral loss of 16:0 and 18:0 fatty acids, confirming the identification of this m/z as DG(16:0/18:0). Analysis of the high mass range of the spectra shown in Fig. 9b–d also revealed the presence of m/z 924 in all three fatty acid neutral loss scans. This led to the identification of the TG species as TG(16:0/18:0/22:6). However, an ion at low relative abundance was observed at m/z 670 in the spectrum obtained from scanning for a neutral loss of an 18:0 fatty acid (Fig. 9c) that was not observed in the NLS 35 MS/MS experiment used to identify DG species. While m/z 670 is within the expected mass range for putative DG molecular species, the lack of an ion at m/z 670 in the NLS 35 spectrum cast doubt that this particular ion represented an 18:0 fatty acid-containing diglyceride. Direct product ion MS/MS analysis of m/z 670 was precluded by the exceedingly low abundance of this ion relative to polar lipid ion abundances in the unfractionated rat retina lipid extract. As the expected mass range for cholesteryl esters is similar to the expected mass range of diglycerides, we conducted an additional precursor ion scan of m/z 369 (scan number 16 in Table 1) to detect cholesteryl esters as $[M + NH_4]^+$ ions. A peak at m/z 670 was observed in the PIS 369 spectrum, corresponding to an 18:0 cholesteryl ester (data not shown). This was in agreement with the original observation of m/z 670 in a neutral loss scan of 18:0 fatty acid neutral species. Hence, the use of complementary precursor ion and neutral loss scan mode MS/MS experiments enabled detailed analysis and redundant confirmation of low abundance ions corresponding to rat retina neutral lipids without chromatographic separation of polar and nonpolar analytes. Note that the fatty acids selected for analysis by neutral loss scanning presented here were for demonstrative purposes only; a thorough analysis was conducted by scanning for neutral losses of all naturally occurring fatty acids. From this set of complementary analyses, we delineated a list of 29 diacylglycerol and 111 triacylglycerol distinct molecular species (see Table 6).

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Fig. 1. Mass spectra obtained from unfractionated rat retina lipid extract by scanning from m/z 400 to m/z 1,200 in (a) positive ion or (b) negative ion mode.

Fig. 2. Negative ion mode product ion MS/MS spectra of $[M + Cl]^-$ ions of synthetic GPCho (14:0/14:0) (a), SM(d18:1/12:0) (b), and $[M + CH_3OCO_2]^-$ ions of synthetic GPCho (14:0/14:0) (c), and SM(d18:1/12:0) (d).

Fig. 3.

Complementary negative ion mode MS/MS analysis of rat retina SM and GPCho species.

Bold text indicates *m/z* of identified SM species. (a) Detection of SM and GPCho as $[M + Cl]^-$ ions by neutral loss scanning of 50 Da in the presence of 0.5 mM NaCl. (b) Detection of SM and GPCho as $[M + CH_3OCO]^-$ ions by neutral loss scanning of 135 Da. (c) Detection of SM and GPCho as $[M + CH_3OCO]^-$ ions by neutral loss scanning of 161 Da.

Fig. 4. Positive ion mode product ion MS/MS spectra of $[M + H]^+$ ions of synthetic GPCho(14:0/14:0) (**a**) and SM(d18:1/12:0) (**b**) and $[M + Na]^+$ ions of synthetic GPCho(14:0/14:0) (**c**) and SM (d18:1/12:0) (**d**).

Fig. 5. Complementary positive ion mode MS/MS analysis of rat retina SM and GPCho species. **Bold** text indicates m/z of identified SM species. **(a)** Detection of SM and GPCho as $[M + H]^+$ ions by precursor ion scanning of m/z 184. **(b)** Detection of SM and GPCho as $[M + Na]^+$ ions by precursor ion scanning of m/z 147. **(c)** Detection of GPCho as $[M + Na]^+$ ions by neutral loss scanning of 205 Da. **(d)** Detection of SM and GPCho as $[M + Na]^+$ ions by neutral loss scanning of 183 Da.

Fig. 6.

Complementary positive and negative ion mode MS/MS analysis of rat retina GPEtn and GPIns species. **(a)** Detection of GPEtn as $[M + H]^+$ and $[M + Na]^+$ ions by positive ion mode neutral loss scanning of 141 Da. **(b)** Detection of GPEtn as $[M - H]^-$ ions by precursor ion scanning of m/z 196. **(c)** Detection of GPIns as $[M - H]^-$ ions by negative ion mode precursor ion scanning of m/z 241. **(d)** Detection of GPIns as $[M + NH_4]^+$ ions by positive ion mode neutral loss scanning of 277 Da.

Fig. 7.

Product ion mode MS/MS spectrum of synthetic GPSer(14:0/14:0) $[M + H]^+$ ions (a), $[M + Na]^+$ ions (b), and $[M - H]^-$ ions (c).

Fig. 8. Complementary positive and negative ion mode MS/MS analysis of rat retina GPSer species. **Bold** text indicates m/z of species at very low relative abundance that were redundantly detected in multiple ion forms. **(a)** Detection of GPSer as $[M + H]^+$ and $[M + Na]^+$ ions by positive ion mode neutral loss scanning of 185 Da. **(b)** Detection of GPSer as $[M + Na]^+$ ions by positive ion mode precursor ion scanning of m/z 208. **(c)** Detection of GPSer as $[M - H]^-$ ions by negative ion mode neutral loss scanning of 87 Da.

Fig. 9.

Complementary positive ion mode MS/MS analysis of rat retina DG and TG species. **(a)** Simultaneous detection of all abundant DG species as $[M + NH_4]^+$ ions by neutral loss scanning of 35 Da. **(b)** Detection of all 16:0-containing DG and TG as $[M + NH_4]^+$ ions by neutral loss scanning of 273 Da. **(c)** Detection of all 18:0-containing DG and TG as $[M + NH_4]^+$ ions by neutral loss scanning of 301 Da. **(d)** Detection of all 22:6-containing DG and TG as $[M + NH_4]^+$ ions by neutral loss scanning of 345 Da.

Description of the precursor ion and neutral loss scan mode MS/MS experiments employed for the class-specific identification of lipid molecular species

Table 1

#	Lipid class	Precursor ion	MS/MS scan type	Product ion / Neutral loss	Ref
1	SM, GPCho	[M + Cl] ⁻	NLS 50	CH ₃ Cl	(21)
2	SM, GPCho	[M + CH ₃ OCO ₂] ⁻	NLS 135	CH ₃ OCO ₂ H+(CH ₃) ₃ N	(21)
3	SM, GPCho	[M + CH ₃ OCO ₂] ⁻	NLS 161	CH ₃ OCO ₂ +(CH ₃) ₃ NCHCH ₂	(21)
4	SM, GPCho	[M + H] ⁺	PIS 184	Phosphocholine	(13)
5	SM, GPCho	[M + Na] ⁺	PIS 147	Sodium cyclophosphane	(37)
6	GPCho	[M + Na] ⁺	NLS 205	Sodium cholinephosphate	(13)
7	SM, GPCho	[M + Na] ⁺	NLS 183	Neutral phosphocholine	(37)
8	GPEtn	[M + H] ⁺ , [M + Na] ⁺	NLS 141	Phosphoethanolamine	(14)
9	GPEtn	[M - H] ⁻	PIS 196	Glycerol phosphoethanolamine derivative	(13)
10	GPIns	[M - H] ⁻	PIS 241	Dehydrated phosphoinositol	(13)
11	GPIns	[M + NH ₄] ⁺	NLS 277	Phosphoinositol+NH ₃	(38)
12	GPSer	[M + H] ⁺ , [M + Na] ⁺	NLS 185	Phosphoserine	(11)
13	GPSer	[M + Na] ⁺	PIS 208	Sodium phosphoserine	-
14	GPSer	[M - H] ⁻	NLS 87	Serine	(13)
15	Di-/Triacylglycerols, Cholesteryl esters	[M + NH ₄] ⁺	NLS (<i>m/z</i> varies)	Fatty acid+NH ₃	(28)
16	Cholesteryl esters	[M + NH ₄] ⁺	PIS 369	Cholestane cation	(13)
17	Mono-/Diacylglycerols	[M + NH ₄] ⁺	NLS 35	H ₂ O+NH ₃	-
18	Glycerophospholipids	[M - H] ⁻ , [M + Cl] ⁻ , [M + CH ₃ OCO ₂] ⁻	PIS (<i>m/z</i> varies)	Fatty acyl anion	(13)
19	GPGro	[M + NH ₄] ⁺	NLS 189	Phosphoglycerol+NH ₃	(38)
20	GPA	[M + NH ₄] ⁺	NLS 115	NH ₃ +H ₃ PO ₄	-
21	GPGro, GPA	[M - H] ⁻	PIS 153	Cyclic glycerophosphate derivative	(13)
22	18:0-based ceramides	[M + H] ⁺	PIS 266	Sphinganine derivative	(29)
23	18:1-based ceramides	[M + H] ⁺	PIS 264	Sphingosine derivative	(29)
24	18:2-based ceramides	[M + H] ⁺	PIS 262	Sphingadine derivative	(27)
25	18:0-based ceramides	[M - H] ⁻	NLS 258	16:0 aldehyde	(30)
26	18:1-based ceramides	[M - H] ⁻	NLS 256	16:1 aldehyde	(30)

#	Lipid class	Precursor ion	MS/MS scan type	Product ion / Neutral loss	Ref
27	18:2-based ceramides	[M - H] ⁻	NLS 254	16:2 aldehyde	(30)

P/S parent ion scan, *NLS* neutral loss scan

Table 2

Rat retina SM and GPCCho molecular species

Lipid species ^a	Scan #1 NLS 50 (m/z)	Scan #2 NLS 135 (m/z)	Scan #3 NLS 161 (m/z)	Scan #4 PIS 184 (m/z)	Scan #5 PIS 147 (m/z)	Scan #6 NLS 205 (m/z)	Scan #7 NLS 183 (m/z)
SM(d18:1/16:1)	-	-	775	-	-	-	-
SM(d18:1/16:0)	737	777	777	703	725	-	725
GPCCho(14:1/16:0)	738	-	778	704	-	-	726
GPCCho(14:0/16:0)	-	-	780	706	728	-	-
SM(d18:1/18:1)	763	-	803	-	-	-	751
SM(d18:1/18:0)	765	805	805	731	753	-	753
GPCCho(16:0/16:1)	766	806	806	732	754	-	754
GPCCho(16:0/16:0)	768	808	808	734	756	756	756
SM(d18:1/19:0)	-	-	819	-	-	-	-
GPCCho(O-18:0/16:0)	-	-	820	746	-	-	-
SM(d18:1/20:1)	-	-	831	-	-	-	-
GPCCho(16:1/18:1)	-	832	832	758	780	780	780
GPCCho(16:0/18:2)	-	832	832	758	780	-	780
SM(d18:1/20:0)	793	833	833	759	-	-	781
GPCCho(16:0/18:1)	794	834	834	760	782	782	782
GPCCho(16:0/18:0)	796	836	836	762	784	784	784
GPCCho(O-18:0/18:0)	-	-	-	774	-	-	-
GPCCho(16:0/20:4)	816	856	856	782	804	804	804
SM(d18:1/22:2)	-	857	857	-	-	-	-
SM(d18:1/22:1)	-	-	859	-	-	-	-
GPCCho(18:1/18:1)	820	860	860	786	808	808	808
GPCCho(18:0/18:2)	820	860	860	786	808	808	808
GPCCho(18:0/18:1)	822	862	862	788	810	810	810
GPCCho(18:0/18:0)	824	864	864	790	812	812	812
GPCCho(16:0/22:6)	840	880	880	806	828	828	828
GPCCho(16:1/22:5)	840	880	880	806	828	828	828
SM(d18:1/24:4)	841	881	881	-	-	-	-
GPCCho(38:5)	-	-	-	808	-	830	830

Lipid species ^a	Scan #1 NLS 50 (m/z)	Scan #2 NLS 135 (m/z)	Scan #3 NLS 161 (m/z)	Scan #4 PIS 184 (m/z)	Scan #5 PIS 147 (m/z)	Scan #6 NLS 205 (m/z)	Scan #7 NLS 183 (m/z)
GPCho(18:0/20:4)	844	884	884	810	832	832	832
SM(d18:1/24:2)	845	-	-	-	-	-	-
SM(d18:1/24:1)	847	-	887	-	-	-	-
SM(d18:1/24:0)	849	-	889	815	-	-	-
GPCho(18:1/20:0)	-	-	890	816	-	-	-
GPCho(18:1/22:6)	-	906	-	832	854	854	854
GPCho(18:0/22:6)	868	908	908	834	856	856	856
GPCho(18:1/22:5)	868	908	908	834	856	856	856
GPCho(40:4)	-	-	-	838	860	860	860
GPCho(42:10)	-	-	-	854	876	876	876
GPCho(42:4)	-	-	-	866	-	-	-
GPCho(22:6/22:6)	912	952	952	878	900	900	900
GPCho(22:6/32:6)	1,052	-	-	1,018	1,040	1,040	1,040
GPCho(22:6/34:6)	1,080	-	-	1,046	1,068	1,068	1,068

Table 3

Rat retina GPEtn species detected by complementary positive and negative ion mode MS/MS analysis

Lipid species ^a	Scan #8 NLS 141 (m/z)	Scan #9 PIS 196 (m/z)
GPEtn(16:0/18:1)	718	716
GPEtn(16:0/18:0)	720	718
GPEtn(O-16:1/20:4)	-	722
GPEtn(16:0/20:4)	740	738
GPEtn(18:1/18:1)	744	-
GPEtn(18:0/18:1)	746	744
GPEtn(O-16:1/22:6)	-	746
GPEtn(O-16:0/22:6)	750	748
GPEtn(O-18:0/20:4)	752	750
GPEtn(16:0/22:6)	764	762
GPEtn(18:1/20:4)	766	764
GPEtn(18:0/20:4)	768	766
GPEtn(O-18:2/22:6)	774	772
GPEtn(O-18:1/22:6)	776	774
GPEtn(O-18:1/22:5) GPEtn(O-18:0/22:6)	778	776
GPEtn(O-20:1/20:4)	-	778
GPEtn(18:2/22:6)	788	786
GPEtn(18:1/22:6)	790	788
GPEtn(18:0/22:6)	792	790
GPEtn(20:0/20:4)	796	794
GPEtn(20:4/22:6)	812	810
GPEtn(22:6/22:6)	836	834
GPEtn(22:4/22:6)	840	838

^a GPEtn fatty acid substituents were determined by negative ion mode MS/MS experiments as described in Subheading 3. Alkenyl species are denoted by O- preceding the acyl chain. A *dash* indicates a species that was not detected.

Table 4

Rat retina GPIs species detected by complementary positive and negative ion mode MS/MS analysis

Lipid species ^a	Scan #10 PIS 241 (<i>m/z</i>)	Scan #11 NLS 277 (<i>m/z</i>)
GPIs(16:0/18:1)	835	-
GPIs(16:1/20:4)	855	874
GPIs(16:0/20:4)	857	876
GPIs(18:2/20:4)	881	900
GPIs(16:0/22:6)	881	900
GPIs(18:1/20:4)	883	902
GPIs(18:0/20:4)	885	904
GPIs(18:2/22:6)	905	-
GPIs(18:1/22:6)	907	-
GPIs(18:0/22:6)	909	928
GPIs(20:0/20:4)	913	932
GPIs(20:4/22:6)	929	948
GPIs(20:4/22:6)	931	-
GPIs(22:6/22:6)	953	-

^aGPIs fatty acid substituents were determined by negative ion mode MS/MS experiments as described in Subheading 3. A *dash* indicates a species that was not detected.

Table 5

Rat retina GPSer molecular species detected by complementary positive and negative ion mode MS/MS analysis

Lipid species ^a	Scan #12 NLS 185 (m/z)	Scan #13 PIS 208 (m/z)>	Scan #14 NLS 87 (m/z)
GPSer(16:0/18:1)	762	784	760
GPSer(16:1/18:0)	762	784	760
GPSer(16:0/18:0)	-	-	762
GPSer(18:0/18:1)	790	812	788
GPSer(16:1/20:4)	-	-	808
GPSer(16:0/20:4)	812	834	810
GPSer(16:0/22:0)	-	842	818
GPSer(18:0/20:0)	-	842	818
GPSer(O-18:0/22:6)	-	842	818
GPSer(18:1/22:6)	834	856	832
GPSer(18:0/22:6)	836	858	834
GPSer(18:0/22:5)	838	860	836
GPSer(18:0/22:4)	840	862	838
GPSer(20:0/20:4)	840	862	838
GPSer(20:4/22:6)	856	878	854
GPSer(20:4/22:5)	858	-	856
GPSer(20:4/22:4)	-	-	858
GPSer(20:0/22:6)	-	886	862
GPSer(20:0/22:5)	866	-	864
GPSer(22:6/22:6)	880	902	878
GPSer(22:5/22:6)	882	904	880
GPSer(22:4/22:6)	884	906	882
GPSer(22:3/22:6)	-	908	884
GPSer(22:6/24:6)	908	930	906
GPSer(22:5/24:6)	910	932	908
GPSer(22:4/24:6)	912	934	910
GPSer(22:3/24:6)	-	-	912
GPSer(24:6/24:6)	936	958	934
GPSer(24:5/24:6)	938	-	936

^aGPSer fatty acid substituents were determined by negative ion mode MS/MS experiments as described in the Subheading 3. Alkenyl species are denoted by O-preceding the acyl chain. A *dash* indicates a species that was not detected.

Table 6

Rat retina DG and TG molecular species detected as $[M + NH_4]^+$ ions by complementary positive ion mode neutral loss scanning of m/z corresponding to the mass of naturally occurring fatty acids+ NH_3 (scan #15)

m/z	Lipid species
584	DG(16:0/16:1)
586	DG(16:0/16:0)
612	DG(16:0/18:1)
612	DG(16:1/18:0)
614	DG(16:0/18:0)
634	DG(16:0/20:4)
638	DG(18:1/18:1)
640	DG(16:1/20:0)
640	DG(18:0/18:1)
642	DG(18:0/18:0)
642	DG(16:0/20:0)
658	DG(16:0/22:6)
660	DG(18:1/20:4)
660	DG(16:0/22:5)
662	DG(18:0/20:4)
664	DG(18:0/20:3)
666	DG(18:2/20:0)
668	DG(18:1/20:0)
670	DG(18:0/20:0)
682	DG(20:4/20:4)
684	DG(18:1/22:6)
686	DG(18:0/22:6)
688	DG(18:0/22:5)
690	DG(20:0/20:4)
698	DG(20:0/20:0)
706	DG(20:4/22:6)
714	DG(20:0/22:6)
720	DG(20:0/22:3)
730	DG(22:6/22:6)
768	TG(14:0/14:0/16:0)
794	TG(14:0/14:0/18:1)
794	TG(14:0/14:1/18:0)
794	TG(14:0/16:1/16:0)
818	TG(14:0/16:1/18:2)
818	TG(16:1/14:1/18:1)
818	TG(16:1/16:1/16:1)
820	TG(14:0/16:0/18:2)
820	TG(14:0/16:1/18:1)

m/z	Lipid species
820	TG(14:0/16:0/18:2)
820	TG(14:1/16:1/18:0)
820	TG(16:0/16:1/16:1)
822	TG(14:0/16:0/18:1)
822	TG(16:0/16:0/16:1)
824	TG(14:0/16:0/18:0)
824	TG(16:0/16:0/16:0)
844	TG(16:0/16:1/18:3)
844	TG(16:1/16:1/18:2)
844	TG(18:1/18:2/14:1)
844	TG(18:2/14:0/18:2)
846	TG(16:0/18:2/16:1)
846	TG(16:1/16:1/18:1)
846	TG(18:1/14:0/18:2)
846	TG(18:1/14:1/18:1)
848	TG(14:0/18:1/18:1)
848	TG(16:0/16:0/18:2)
848	TG(16:0/16:1/18:1)
848	TG(18:1/14:0/18:1)
850	TG(16:0/16:0/18:1)
850	TG(16:0/18:0/16:1)
850	TG(18:0/14:0/18:1)
852	TG(16:0/16:0/18:0)
870	TG(16:0/20:4/16:1)
870	TG(16:1/16:1/20:3)
870	TG(16:1/18:2/18:2)
870	TG(16:1/18:3/18:1)
870	TG(18:2/18:3/16:0)
872	TG(16:0/16:0/20:4)
872	TG(16:0/18:1/18:3)
872	TG(16:0/18:2/18:2)
872	TG(16:0/20:3/16:1)
872	TG(16:1/18:0/18:3)
872	TG(16:1/18:1/18:2)
874	TG(16:0/18:1/18:2)
874	TG(16:1/18:0/18:2)
874	TG(16:1/18:1/18:1)
876	TG(16:0/18:0/18:2)
876	TG(16:0/18:1/18:1)
876	TG(16:0/18:0/18:1)
876	TG(16:1/16:1/20:0)
878	TG(16:1/18:0/18:1)

m/z	Lipid species
878	TG(16:1/16:0/20:0)
880	TG(16:0/18:0/18:0)
894	TG(16:0/22:6/16:1)
894	TG(16:1/18:3/20:3)
894	TG(18:0/14:1/22:6)
894	TG(18:1/14:1/22:5)
896	TG(16:0/16:0/22:6)
896	TG(16:0/18:2/20:4)
896	TG(18:2/18:2/18:2)
898	TG(16:0/16:0/22:5)
898	TG(16:0/18:1/20:4)
898	TG(16:0/18:2/20:3)
898	TG(16:0/20:3/18:2)
898	TG(16:1/20:1/18:3)
898	TG(16:1/20:3/18:1)
898	TG(18:2/18:2/18:1)
900	TG(16:0/18:0/20:4)
900	TG(16:0/18:1/20:3)
900	TG(18:0/18:1/18:3)
900	TG(18:0/18:2/18:2)
900	TG(18:1/18:1/18:2)
902	TG(18:1/18:1/18:1)
902	TG(18:1/18:2/18:0)
902	TG(18:2/20:1/16:0)
904	TG(16:0/20:1/18:1)
904	TG(18:0/18:0/18:2)
904	TG(18:0/18:1/18:1)
906	TG(16:0/18:1/20:0)
906	TG(16:0/18:0/20:1)
906	TG(18:0/18:0/18:1)
922	TG(16:0/18:1/22:6)
922	TG(16:0/20:3/20:4)
922	TG(16:0/16:1/24:6)
922	TG(16:1/18:0/22:6)
922	TG(16:1/20:2/20:4)
922	TG(16:1/18:1/22:5)
922	TG(18:1/20:4/18:2)
924	TG(16:0/16:0/24:6)
924	TG(16:0/18:0/22:6)
924	TG(16:1/18:0/22:5)
926	TG(16:0/18:0/22:5)
926	TG(16:0/20:1/20:4)

m/z	Lipid species
926	TG(18:0/18:1/20:4)
928	TG(18:0/18:0/20:4)
944	TG(18:1/22:6/18:3)
946	TG(18:1/20:4/20:4)
948	TG(18:0/18:2/22:6)
948	TG(18:1/18:1/22:6)
950	TG(18:0/18:1/22:6)
950	TG(18:0/18:2/22:5)
952	TG(18:0/18:0/22:6)
956	TG(18:0/20:0/20:4)
966	TG(20:4/20:4/20:5)
968	TG(18:2/22:6/20:4)
968	TG(18:3/20:3/22:6)
968	TG(22:6/22:6/16:0)
970	TG(16:1/22:5/22:5)
972	TG(18:0/20:4/22:6)
974	TG(18:0/20:4/22:5)
978	TG(18:2/22:5/20:0)

Stereochemistry of fatty acids has not been verified