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Enhancing detection and characterization of lipids using charge manipulation in electrospray ionization-tandem mass spectrometry

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Abstract

Heightened awareness regarding the implication of disturbances in lipid metabolism with respect to prevalent human-related pathologies demands analytical techniques that provide unambiguous structural characterization and accurate quantitation of lipids in complex biological samples. The diversity in molecular structures of lipids along with their wide range of concentrations in biological matrices present formidable analytical challenges. Modern mass spectrometry (MS) offers an unprecedented level of analytical power in lipid analysis, as many advancements in the field of lipidomics have been facilitated through novel applications of and developments in electrospray ionization tandem mass spectrometry (ESI-MS/MS). ESI allows for the formation of intact lipid ions with little to no fragmentation and has become widely used in contemporary lipidomics experiments due to its sensitivity, reproducibility, and compatibility with condensed-phase modes of separation, such as liquid chromatography (LC). Owing to variations in lipid functional groups, ESI enables partial chemical separation of the lipidome, yet the preferred ion-type is not always formed, impacting lipid detection, characterization, and quantitation. Moreover, conventional ESI-MS/MS approaches often fail to expose diverse subtle structural features like the sites of unsaturation in fatty acyl constituents or acyl chain regiochemistry along the glycerol backbone, representing a significant challenge for ESI-MS/MS. To overcome these shortcomings, various charge manipulation strategies, including charge-switching, have been developed to transform ion-type and charge state, with aims of increasing sensitivity and selectivity of ESI-MS/MS approaches. Importantly, charge manipulation approaches afford enhanced ionization efficiency, improved mixture analysis performance, and access to informative fragmentation channels. Herein, we present a critical review of the current suite of solution-based and gas-phase strategies for the manipulation of lipid ion charge and type relevant to ESI-MS/MS.

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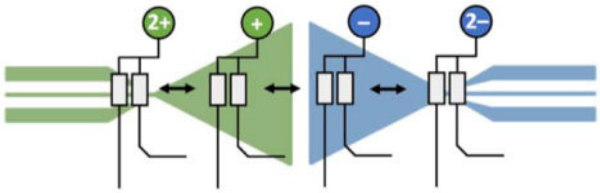
Graphical Abstract



Keywords

lipids; lipidomics; electrospray ionization; liquid chromatography; collision-induced dissociation; charge inversion; charge manipulation

Introduction

Lipids are universal cellular components, playing central roles in all organisms ranging from the smallest microorganisms such as bacteria and algae to complex animals like humans.[1] Perhaps best known for their fundamental roles as architectural components of cellular membranes, lipids also perform vital functions as signaling and energy storage molecules.[1–3] Based on the classification scheme devised by the LIPID MAPS[4, 5] consortium, lipids are divided into eight distinct categories: fatty acids (FAs), glycerolipids, glycerophospholipids (GPLs), sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (Figure 1). Noting that each individual lipid category also contains unique classes and subclasses of molecules, lipids exhibit extensive structural diversity. For example, just considering FAs, variations in aliphatic chain length, degree of unsaturation, site(s) of unsaturation, and modification such as hydroxylation[6], cyclopropanation[7, 8], nitrosylation[9, 10], and methyl chain branching[11] are observed. This structural complexity is further compounded as FAs serve as building blocks for complex lipids like GPLs. As illustrated with Figure 2, the general GPL structure includes a central glycerol backbone, a functionalized phosphate ester group, and fatty acyl (or alkyl ether) chains. Esterified at the *sn*-3 position of the glycerol backbone is a phosphate moiety. Also coupled to this phosphate group is a polar functional group (*e.g.*, choline, ethanolamine, serine, inositol, or glycerol), that is often referred to as the headgroup. The most common GPL structure observed in eukaryotes is the diacyl (phosphatidyl) subclass wherein fatty acids (FAs) are esterified at the *sn*-1 and *sn*-2 positions of the glycerol backbone, but additional GPL subclasses incorporate alkyl ether chains at the *sn*-1 position while a fatty acyl chain remains esterified at the sn-2 position (Figure 2).[2, 12] Explicitly, the plasmanyl and plasmenyl (*i.e.*, plasmalogen) GPL subclasses contain a 1-*O*-alkyl or a 1-*O*-alk-1′-enyl group, respectively, at the *sn*-1 position. In turn, a broad range of discrete GPL molecular structures arise from not only variations in fatty acyl constituents but also alterations both in the headgroup and *sn*-1 bond type.[13] While extensive lipid molecular structural diversity within a class itself presents a considerable challenge, lipidomics analysis of biological mixtures is further complicated by the presence of multiple classes of lipids (*i.e.*, mixture complexity) over a wide range of concentrations. Figure 3 emphasizes the analytical requirements for lipidome analysis. In particular, noting that biological systems can contain many hundreds and

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perhaps even thousands of structurally diverse lipids at vastly different concentrations, mixture complexity and associated issues of dynamic range pose formidable challenges for modern lipidomics.[14, 15]

Recently, modifications to lipid composition and production, namely FAs and GPLs, have been associated with the onset and progression of numerous human-related genetic[16, 17], neurodevelopmental[18, 19], and metabolic disorders[20–22] in addition to various other chronic pathologies[23–25], including several types of cancer.[26–35] Therefore, many lipidomics studies aim to characterize and quantify lipids, including lipid profiling for biomarker discovery. For instance, Chen *et al.* profiled serum samples from patients with early-stage breast cancer using LC-ESI-MS/MS, unveiling significant fold changes in a combined total of 15 phosphatidylcholines and cholesterol ester species in comparison with benign breast disease samples that could serve as potential biomarkers for diagnosis of early stage breast cancer.[33]

In a more recent example, the Xia group, while examining human plasma samples from breast cancer patients, discovered that the ratio of double bond positional lipid isomers may serve as reliable biomarkers for breast cancer.[36] Therefore, increasing evidence for the critical biochemical and physiological roles of lipids, specifically regarding their function in health and disease, has highlighted the demand for analytical techniques that facilitate the rapid, unambiguous identification and quantitation of lipids in complex biological samples.

Even though thin-layer chromatography (TLC)[37] and nuclear magnetic resonance (NMR)[38] have played significant roles in the advancement of lipid science, mass spectrometry has emerged as the premier tool for contemporary lipid analytics.[15, 39] Frequently coupled to some form of chromatography, mass spectrometry (MS) has largely facilitated the rapid expansion of the lipidomics field. Owing to unparalleled versatility, MS-based approaches have been widely adapted for lipid identification and quantitation, as mass spectrometry experiments allow for the individual selection and interrogation, by means of fragmentation, of target analytes in complex mixtures. Ideally, product ions generated via the dissociation of a precursor lipid ion enables the reconstruction of original lipid structure, though notable limitations prohibit complete structural elucidation. Since the 1950s[40], the method of choice for FA analysis has been gas chromatography (GC). However, GC-based strategies are restricted to the analysis of volatile, low molecular weight compounds. In turn, a rich history of derivatization chemistries have been developed to enhance FA volatility, improve chromatographic separation, and ameliorate chemo-selectivity of fragmentation.[41–43] Most commonly, GC is coupled to mass spectrometers equipped with electron ionization (EI) sources, as the combination with MS reduces ambiguity for FA identification when compared to GC alone.[39, 44] While powerful, growing interest in high molecular weight and structurally complex lipids demanded the development of alternate MS-based approaches, noting that GC approaches are not amenable to intact complex lipids analysis, as FAs must be hydrolyzed from complex lipid precursors prior to derivatization, resulting in the loss of valuable information concerning the molecular origin of fatty acyl constituents.

In the pursuit of enhanced lipid structural elucidation, Gross and co-workers laid the foundation for current understanding of lipid structural elucidation and fragmentation utilizing fast atom bombardment (FAB) in conjunction with tandem-MS (MS/MS).[45–55]

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Performed on sector instruments, initial studies showed that the collisional activation of lipid ions via high-energy CID (> 1keV) afforded the identification of both GPL and FA structures. In particular, FA carboxylate anions derived from the ionization of non-esterified FAs or liberated from a GPL precursor anion dissociate, revealing the presence and location(s) of a variety of functional groups such as carbon-carbon double bonds, acyl chain branching, cyclopropyl rings, epoxides, and hydroxyl groups. Explicitly, deprotonated FA anions undergo unimolecular 1,4-hydrogen eliminations to produce terminally unsaturated and structurally diagnostic carboxylate product ions in a process referred to as charge remote fragmentation (CRF).[46, 56–58] However, fatty acyl identification utilizing this approach is limited to FA structures with three of fewer sites of unsaturation, as underivatized PUFAs containing four or more double bonds decompose primarily by uninformative neutral losses such as decarboxylation (− 44 Da).[47]

To overcome these limitations, metal-cationized FAs were generated in the FAB source by doping the sample and matrix with selected salts. Once formed, these metal adduct ions were interrogated via high-energy CID. As the primary requirements for CRF were found to be a tightly localized charge site and access to keV collision energies, FA cationization with both alkali[52] and alkaline earth[54] metals yielded metal-adducted FA ions which upon interrogation via high-energy CID, generate product ion spectra that permit the localization of carbon-carbon double bonds and unambiguous isomeric discrimination. While high-energy CID of alkaline earth metal cationized FA ions affords identification of double bond position, the CID spectra of $[FA - H + 2Li]^+$ are arguably more straightforward. In turn, the utilization of lithium as a cationization reagent are favored over alternate alkali and alkaline earth metal counterparts. To localize carbon-carbon double bond positions repeatable spectral patterns obtained via interrogation of the $[FA - H + 2Li]^+$ cation were exploited, as carbon-carbon single bond cleavage proximal (*i.e.*, on the carboxyl end of the aliphatic chain) and vinylic to the last double bond in the aliphatic chain produced the heaviest fragment ion (highest *m/z*).[52, 55] The advantages of FAB-MS/MS of charge-switched FAs are highlighted with Figure 4.

Despite the clear successes demonstrated by high-energy CID, both instrumentation and ionization methods traditionally utilized to access CRF have become less common. In addition, both the ionization and fragmentation efficiency were low (*cf.* the magnification of the diagnostic product ions to the precursor ion abundance in Figure 4). Specifically, modern lipidomics workflows have largely replaced multisector instruments with triple quadrupole (QqQ), quadrupole time-of-flight (Q-TOF), and linear ion trap (LIT) mass spectrometers that operate under low-energy CID conditions (*i.e.*, <100 eV) while FAB has been primarily replaced with ionization methods like electrospray ionization (ESI) that offer increased sensitivity. Tandem-TOF (TOF/TOF) instruments are a notable exception that can achieve collision energies comparable to those accessed by multisector mass spectrometric platforms.[59, 60] In their report, Trimpin *et al.* explore the dissociation of charge-switched FA ions on a TOF/TOF platform, reporting that CID of $[FA - H + 2Li]^+$, produced by matrix-assisted laser desorption/ionization, results in CRF product ions that permit the assignment of subtle structural features such as carbon-carbon double bond position and acyl chain branching.[59] While this approach facilitates the characterization of a series of isomeric FA, not all carbon-carbon double bond positions in PUFAs, particularly those near the methyl

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end of the aliphatic chain, can be qualitatively assigned. Moreover, wide isolation windows on TOF/TOF instruments hinder complex mixture analysis and the fragmentation efficiency remains low.

As FAB-MS/MS provided limited sensitivity for lipidome analysis, recent advances and novel applications of ESI-MS and MS/MS technologies have greatly facilitated the expansion of lipidomics, improving upon many of the shortcomings associated with the aforementioned platforms.[2] Lipid extracts are admitted to a (tandem) mass spectrometer using liquid chromatography (LC) or directly infused via ESI employing an approach commonly referred to as shotgun lipidomics. Though not explicitly discussed herein, we note that ion mobility spectrometry coupled with MS has emerged as a powerful, alternative technique to LC-MS for the separation and identification of lipid isomers, representing a rapidly growing lipidomics approach. Importantly, ESI achieves partial fractionation of the lipidome, a concept coined “intrasource separation” by Han and Gross, as differences in lipid functional groups influence ionization efficiency, observed ion-type, and charge-state.[61–63] In the absence of a fixed charge site, the preference for a lipid to form a protonated or deprotonated precursor ion is largely dictated by acid-base chemistry.[64] For example, acidic lipids like FAs, glycerophosphoethanolamines (PEs), glycerophosphoglycerols (PGs), glycerophosphoserines (PSs), glycerophosphoinositols (PIs), and glycerophosphatidic acids (PAs) are detected in the negative ion mode as singly deprotonated $[M - H]^-$ species.[64] In contrast, choline-containing lipids like glycerophosphocholines (PCs) and sphingomyelin (SM) incorporate a fixed positive charge and are readily detected as gas-phase cations. Independent of ionization mode, lipid precursor ions can be detected and identified at a sum compositional level via accurate mass measurements (*i.e.*, observed mass-to-charge (*m/z*) ratios). Moreover, utilizing collision-induced dissociation (CID), class-specific fragmentation, pertaining to headgroup composition, allows for the detection of individual GPL classes.[65]

Further lipid structural elucidation is enabled through conventional ESI-MS/MS or MS^n experiments.[66–77] For example, in negative ion mode, low-energy CID of deprotonated GPL anions results in the cleavage of ester bonds at the *sn*-1 and *sn*-2 positions of anionic GPL, liberating fatty acyl chains and yielding abundant carboxylate anions that permit the assignment of fatty acyl composition and, in some cases, GPL subclass. While informative, such experiments fail to fully elucidate GPL structure. Explicitly, conventional ESI-MS/MS experiments do not generate unique product ions to assign the relative position of acyl chains on the glycerol backbone (*i.e.*, *sn*-position) and subtle structural features within the acyl chain constituents, like the positions and geometries of carbon-carbon double bonds among others, are not revealed. Furthermore, it is widely recognized that the native ion types formed upon ESI are not always the most advantageous for lipid detection and identificaiton.[52, 78] For example, the favorable ionization mode for FAs is the negative ion mode, as the carboxylic acid moiety can be readily deprotonated. However, there are two major obstacles for ESI-MS/MS analysis of $[FA - H]^-$ anions. First, when considering LC-ESI-MS/MS approaches, the best chromatographic resolution for FAs is achieved under acidic mobile phase conditions, which unfortunately, suppresses FA ionization efficiency.[79–81] Second, singly deprotonated FA anions undergo undesirable fragmentation when collisionally activated via low-energy CID. Specifically, $[FA - H]^-$ product ion spectra are dominated by

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water and carbon dioxide neutral losses and are largely devoid of structurally informative product ions.[82] As a consequence, various charge manipulation techniques have been developed to enhance lipid analysis. Reminiscent of early studies utilizing high-energy CID, charge manipulation of lipids in ESI, including charge-switching strategies, can provide (i) increased ionization efficiency; (ii) improved chemo-selectivity and dynamic range; and (iii) access to preferred fragmentation that afford enhanced structural elucidation. To access these benefits, several solution-based derivatizations have now become widely adopted for ESI-MS workflows in lipidomics. In these approaches the lipid analyte is either noncovalently or covalently modified prior to, or during, the introduction into the ESI source. In an alternative approach, lipids are first ionized via ESI and then derivatized post-ionization within the mass-spectrometer via gas-phase chemistries.

In this review, we examine the application and benefits of charge manipulation strategies, including charge-switching, for the mass spectrometric analysis of lipids. The approaches summarized herein are focused on those implemented with ESI as this ionization method is the most widely adopted for modern lipidomics experiments. We note, however, that many of the same chemistries can, and are, being translated to other ionization modalities, e.g., MALDI. Here we provide, an overview of both solution-based and gas-phase derivatization, with a focus on the transformation of lipid precursor ions, namely those derived from polar lipids including FAs and phospholipids. Collectively, the manipulation of lipid precursor ions via the modification of charge state or ion type provides the primary advantages of enhanced sensitivity, isomeric resolution, mixture analysis performance, and structural characterization.

2. Charge Switching

Most charge switching strategies for lipid analysis employ solution-based derivatization, as this form of derivatization is more widely accessible compared to their gas-phase counterparts that require instrument modifications. In particular, two main techniques have been developed in order to generate charge-switched lipid ions. While both strategies target lipid modification with a fixed-charge or easily ionizable functional group, the manner in which charge-switched derivatives are generated differs between the two described approaches. In the first approach, noncovalent lipid adducts are generated in solution prior to ionization, typically via lipid complexation with a metal cation. This is typically achieved by adding a dopant to the ESI spray solution and is thus an online modification. The second tactic involves covalent derivatization of the lipid at a marked functional group via wet-chemical modification. This approach predominantly requires additional preparative and even clean-up steps prior to sample injection into the mass spectrometer. Ultimately, in combination with conventional mass spectrometers, both covalent and non-covalent charge-switched lipids provide increased ion currents and expanded structural information that in favorable cases, enables isomer discrimination. More recently charge-switch derivatization utilizing gas phase ion/ion reactions has been explored for lipid detection, characterization, and quantitation. As described in more detail below, gas-phase charge inversion chemistry offers a number of advantages over parallel solution-based approaches, including enhanced derivatization efficiency, specificity, and sensitivity.[83]

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2.1. Negative to Positive Charge Inversion

2.1.1. Solution-based Metal Cationization—As stated above, workflows to access CRF are uncommon with modern instrumentation that operate on relatively low kinetic energy ions. However, charge-switching strategies employing solution additives have been extensively explored with the aims of improving lipid detection, identification, and quantitation using contemporary mass spectrometers. Acidic lipids such as FA, PS, PI, PA, PG, PE, typically form abundant $[M - H]^-$ anions with negative mode ESI but can be charge-switched to the positive ion polarity employing numerous solution phase additives. Explicitly, alkali, alkaline earth, and transition metal cations have been demonstrated to complex with a variety of polar lipid structures, facilitating acidic lipid ionization in the positive ion mode, resulting in increased ionization efficiency and sensitivity. Furthermore, as the low-energy CID spectra of lipid adduct cations contain structurally informative product ions, negative to positive mode charge-reversal has proven highly advantageous for lipid structural elucidation.

Paralleling previous approaches that employ high-energy CID, formal lithium fixation to lipid ions provides the notable advantages of enhanced structural characterization of unsaturated GPL and FA structures under low-energy CID conditions. First described by Hsu and Turk[84], abundant charge-switched FA cations were generated upon direct positive ESI of a solution containing a FA standard and lithium acetate. Low-energy CID of $[FA - H + 2Li]^+$ ions gives rise to product ion spectra that facilitate the identification of carbon-carbon double bond position(s) and, in turn, unambiguous isomeric discrimination via direct interpretation of a reproducible and relatively straightforward spectral pattern (Figure 5). Explicitly, the CID spectra of dilithiated adduct ions of monounsaturated FAs contain a series of closed-shell product ions, spaced 14 Da apart, signifying sequential carbon-carbon single bond cleavage along the hydrocarbon chain. As fragmentation approaches the unsaturation site, this pattern is terminated, as carbon-carbon bond cleavages distal to the double bond (*i.e.*, on the methyl end) are rarely observed. Moreover, an abundant product ion reflecting cleavage of the carbon-carbon single bond vinylic to the carbon-carbon double bond is observed in the resulting $[FA - H + 2Li]^+$ CID spectrum. When multiple double bonds are present and separated by a single methylene group, cleavages between double bonds yield abundant product ions reminiscent of those observed under high-energy CID conditions. However, congested spectra of $[FA - H + 2Li]^+$ cations resulting from non-selective fragmentation of the aliphatic chain can complicate carbon-carbon double bond localization, especially when examining complex mixtures where multiple isomers are often simultaneously present. In a recent example, the Reid group deployed 193 nm ultraviolet photodissociation tandem MS (UVPD-MS/MS), a selective fragmentation technique, to reduce the spectral complexity of dilithiated-FA cations.[85] Here, the UVPD MS/MS spectra of $[FA - H + 2Li]^+$ cations contain marked pairs of diagnostic product ions that readily facilitate the localization of double bond position(s), as shown in Figure 6. Notably, application of this strategy to colorectal cancer cell lines revealed relative changes in the ratio of FA double bond positional isomers without prior fractionation of the isomers.

Extending the lithium cationization strategy to intact GPL analysis, Hsu and Turk described the generation of mono-, di-, and tri-lithiated adduct ions by direct ESI and subsequent

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interrogation of the charge-switched lipid cations using a multiple stage LIT mass spectrometric approach.[73, 86] Ultimately, several factors influence the formation of lithium-adducted lipid cations, including GPL class, fatty acyl composition, and lithium cation concentration. In total, this strategy enabled the detailed structural elucidation of GPL, including the plasmalogen and lyso GPL subclasses. In particular, the MS^n (n = 2,3) spectra of lithium-adducted GPL cations permit the assignment of lipid class (*i.e.*, headgroup composition) and the identification of fatty acyl composition and the more abundant *sn*-regioisomer. To localize carbon-carbon double bond positions in unsaturated fatty acyl substituents, MS^n (n = 3,4) experiments performed on product ions that carry the unsaturated fatty acyl moiety are exploited. While effective, the propensity to form various lithiated adduct ion types and extensive fragmentation can complicate spectral interpretation particularly where multiple isomers are present. Moreover, as multiple stages of CID are required to assign unsaturated GPL structures, longer integration times are required, making this approach more compatible with direct infusion (shotgun) rather than LC-MS workflows.

Barium has also been used as a cationization agent for FA analysis. In the first example, Zehethofer *et al.* developed a UPLC-MS/MS method in which aqueous barium acetate was added post-column via an HPLC system to facilitate the solution-based derivatization of FAs prior to ionization and introduction to a hybrid triple quadrupole/linear ion trap (QqLIT) via ESI.[87] The fragmentation behavior of $[FA - H + Ba]^+$ adduct ions are comparable to those observed upon interrogation of lithium adducted FA complex cations, as detailed above. Briefly, for monounsaturated FAs, the CRF product ion series terminates at carbon-carbon single bond cleavage proximal (*i.e.*, on the carboxyl side) and vinylic to the double bond, while additional carbon-carbon single bond cleavages between double bonds are observed for PUFAs. Importantly, $[FA - H + Ba]^+$ adduct ions fragment upon collisional activation with low-energy CID to provide isomer-specific product ions, and in turn, sensitive multiple reaction monitoring (MRM) transitions could be established to easily identify FAs in which LC separation could not be achieved, as highlighted with Figure 7. In an additional study, Krogh and coworkers report the utilization of barium ion chemistry to selectively identify carboxylic acids, including FA structures, directly in waste and surface water and without chromatographic fractionation.[88]

In an additional approach, ESI-MS/MS of copper (II) adducted FAs performed on an ion-trap mass spectrometer facilitated structural characterization and notably, the differentiation of stereochemical isomers (*i.e.*, *cis* and *trans*). The CID spectra of $[FA - H + Cu(II)]^+$ ions contain product ions similar to those provided by CRF with high-energy CID. Specifically, the location of the unsaturation position is indicated by a pair diagnostic ions generated via carbon-carbon bond cleavage vinylic to the double bond with a characteristic mass differential of 26 Da. Interestingly, this approach also facilitates the discrimination amongst stereochemical isomers. Briefly, Afonso *et al.* report that Cu(II) coordination is seemingly dependent on double bond geometry. The copper dication is suggested to be coordinated by the carboxylate and double bond site(s) in unsaturated FA with *trans* double bond geometry, and as shown with Figure 8, this Cu(II)-coordination hinders the neutral loss of CO_2 (− 44 Da) from the $[FA - H + Cu(II)]^+$ ion with *trans* geometry.[89] In contrast, dissociation of the copper-containing unsaturated FA with *cis* geometry yields a dominant decarboxylated product ion, suggesting the presence of a free carboxylic group. While this stereospecific

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approach is effective, this strategy has not been extended to PUFAs containing three or more double bonds. Moreover, the significant natural abundance of the ^{64}Cu isomer and extensive fragmentation of the $[FA - H + Cu(II)]^+$ adduct ion results in congested CID spectra that require deconvolution and, thus, complicates FA identification.

Other solution-based charge switching strategies have also been developed for complex lipid detection and identification. For example, Deng and coworkers described the metal cationization of PS, PE, and PG lipids with a series of mono- and di-valent metals, including Li^+, Na^+, K^+, Sr^{2+}, Ba^{2+}, and the first transition series (*i.e.*, Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+}).[90] It was reported that the low-energy CID spectra of all transition metal adducted GPL studied afforded the identification of lipid class, fatty acyl composition, and location of acyl chain esterification along the glycerol backbone. In particular, cobalt (II) was found to be the best cationization reagent, as the CID spectra of cobalt-adducted GPL were more straightforward than those derived from alternate transition metal complex cations due, in part, to the presence of a single naturally occurring isotope. Although, the $[M - H + Co]^+$ adduct ions can be applied to the structural elucidation of some lipid classes the approach does not significantly extend the information acquired from CID of these lipids in their native $[M - H]^-$ polarity.

Svane *et al.* report the utilization of digallium and dizinc complexes to facilitate positive ion mode analysis of various GPL classes, including PA, PE, PC, PG, and three different types of PI phosphates (*i.e.*, mono-, di-, and triphosphorylated PIs).[91] It was discovered that PI and PA lipids can undergo solution-based charge switch derivatization upon reaction with digallium complexes, while dizinc complexes could charge invert PG and PE lipids, although with low efficiency. However, PCs did not complex with either metal reagent likely due to the fixed, positively charged headgroup. Notably, the derivatization of PA and PI lipids with digallium complex cations was found to be highly efficient and resulted in up to a 100-fold increase in ion signals relative to the unmodified phospholipids in negative ion-mode. Moreover, the CID spectra of charge-switched lipids with digallium complex cations provided complementary information, and in the case of PI phosphates superior data, when compared to the structural information obtained from the dissociation of unmodified lipids in the negative ion mode. Briefly, interrogation of digallium complex-bound lipid ions allowed for the discrimination of different PI bisphosphate (PIP) regioisomers that differ only in the location of the phosphate moieties on the inositol sugar. Importantly, such discrimination cannot be achieved in the negative ion mode via CID of the unmodified lipid anions, as shown in Figure 9. Conversely, CID of PE or PG complexed with dizinc complexes did not provide additional information when compared to MS/MS experiments conducted on the underivatized lipid ions in negative ion mode.

While CID of metalated lipid ions has proven advantageous for lipid detection and identification, Yoo *et al.* investigated the utility of electron induced dissociation (EID) for FA characterization.[92] In short, EID involves interactions between singly charged analyte ions and free electrons. Metalated FA cations were first generated upon direct ESI of a FA solution doped with metal salts. Specifically, Li, Zn, Co, Ni, Mg, Ca, Fe, and Mn were used to derivatize the carboxylic acid moiety of FAs. The combination of metal-cationization with Mn (II) and EID proved to be particularly useful for the detailed structural elucidation of

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FAs. For example, the EID spectra of $[FA - H + Mn]^+$ adduct ions contains both even- and odd- electron product ions generated via charge-driven and charge-remote fragmentation. In turn, the resulting product ion spectra facilitated the identification of carbon-carbon double bonds in unsaturated FA, though the authors note structural assignments are restricted to FA structures containing three or fewer double bonds. However, this approach suffers from extreme spectral complexity and, due to limited fragmentation efficiency, diagnostic ions were only observed at low abundances. Despite its challenges, EID has been successfully extended to biological mixture analysis.[93–96]

2.1.2. Solution-based Covalent Derivatization—While the above examples of anion to cation charge-switching relied exclusively on the generation of lipid-metal adduct ions, an extensive body of work has been undertaken in employing covalent derivatization of lipid structures with novel chemical tags to enhance ionization in the positive ion mode. To date, most derivatization procedures report the introduction of a functional chemical tag that contains an amine group. The high proton affinity of amines enhances ionization with conjugation chemistries target the carboxylic acid group common to the FA lipid class that includes the low abundance lipid mediators such as the eicosanoids. In particular, the benefits of this type of charge-switching are twofold. First, the incorporation of easily ionizable moieties into the lipid structure provides enhanced detection sensitivities and dynamic range, as ionization efficiency is dramatically improved. Second, charge-switched lipids fragment to form characteristic product ions that can be exploited for increased selectivity.

Two such derivatization tactics targeted modification of FA structures, linking a chemical modifier to the FA carboxylic acid group via an ester bond. FAs esterified to picolylamine[97] and dimethylaminoether (DMAE)[98] modifiers integrated easily chargeable amine tags and have been successfully deployed for LC-MS/MS of FAs with positive ion mode ESI. Notably, Li and Franke established a fast, sensitive LC-orbitrap MS method utilizing fatty acid-picolylamine derivatives (FA-PA). When compared to DMAE derivatization, chemical modification of FAs with 3-picolylamine demonstrated enhanced sensitivity and improved chromatographic resolution.[97] Later covalent modification strategies redesign the readily ionizable tag so that an amide bond links the derivatization reagent to the carboxylic acid moiety. In these applications, an amide bond is preferred over ester linkage to the analytic carboxylic acid since the former is often more resistant to fragmentation upon collisional activation and can thus promote charge-remote fragmentation which is more characteristic of the FA structure. Explicitly, such strategies covalently link 5-(dimethylamino)naphthalene-1-sulfonyl piperazine (Dns-PP)[99], aminoxy tandem mass tags (aminoxyTMTs)[100], 4-aminomethylquinoline (AMQ)[101], and 3-hydroxymethyl-1-methylpyridium[102] to the carboxylic acid group of FAs, while 2-dimethylaminoethylamine (DMED) was coupled to both FAs and eicosanoids.[103, 104] In the abovementioned studies, chemical labeling coupled with LC-ESI-MS/MS analysis ensures the identification and quantitation of carboxyl-containing lipids in complex biological samples in the positive ion mode. Explicitly, the covalent modifiers have been found to enhance chromatographic resolution, increase ionization efficiency, and promote selective fragmentation. For instance, in a recent report, short-chain fatty acids (SCFAs) were converted into amide derivatives with AMQ.[101] Here,

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Liu and co-workers injected AMQ-derivatized fecal samples into an LC-MS/MS system for analysis. Notably, AMQ charge-switching improved chromatographic performance, SCFA ionization efficiency, and SCFA stability. In a final example based on carbodiimide chemistry, FAs labeled with 2, 4-dimethoxy-6-piperazin-1-yl pyrimidine (DMPP) were shown to provide a 1000-fold signal increase, providing excellent signal enhancement capacity and sufficient chromatographic separation by a C8 column.[105] This derivatization strategy is simple, fast, and highly sensitive, and in turn, offers an effective strategy to enhance MS detection signals and lipid identification.

To further improve ionization efficiency, fixed charge tags have been covalently linked to the carboxylic acid moieties of FAs. In particular, quaternary amines contain fixed cationic charge sites, and in turn, the unique chemical derivatization strategies featuring this functional group facilitate exceptional positive ESI efficiency. For example, the trimethylaminoethyl (TMAE) ester salts of FAs (*i.e.*, the quaternary ammonium analogue to DMAE-FA derivatives) enabled the precise and accurate identification and quantitation of FAs. Due to the fixed cation site, TMAE-derivatized FAs provided enhanced ion currents when compared to DMAE-FA derivatives, and in turn, this approach requires notably smaller sample volumes.[106] In an additional example, Kretschmer *et al.* utilized the bromine containing charge-switching reagent, (2-(4-(aminophenoxy)ethyl)4-bromophenethyl)-dimethylammonium bromide hydrobromide referred to as 4-APEBA to detect prostaglandins in positive ion mode.[107]

While the above-mentioned reagents incorporate quaternary amines, additional fixed-charge chemical tags have been crafted to include pyridinium motifs that enable efficient cation formation via positive ESI. Regnier and colleagues first described the development of an LC-MS/MS method for the identification and quantitation of FAs through derivatization with 3-acyl-oxymethyl-1-methylpyridinium (AMMP).[80] Here, esterification of FAs with AMMP increased detection sensitivity by a factor of 2500 when compared negative mode analysis of underivatized FAs. Importantly, the MS/MS spectra of AMMP-FA cations also allowed for the identification of carbon-carbon double bond position, a notable advantage to alternate covalent anion to cation charge switching strategies described above. However, the abundances of these structurally informative product ions are exceptionally low, as product ions reflective of fragmentation at the AMPP charge tag esterification site were predominantly observed. A similar strategy exploits a mild amidation coupling reaction to generate cholamine derivatives of targeted long-chain FAs (LCFAs) to quantify potential biomarkers in the asthma patient serum. Established by Bian *et al.*, LC-MS/MS analysis of chomaline-FA derivatives resulted in up to a 2000-fold increase in sensitivity with limits of detection at the low femtogram level.[108] Moreover, despite low intensities of relevant product ions, the MS/MS spectra of cholamine-derivatized FAs enable the localization of double bond positions and/or other functional groups (Figure 10).

Although the covalent charge-switching strategies described above afforded improved ionization efficiency and increased sensitivity, structure elucidation via these derivatization strategies was typically limited by fragmentation centralized at the charge-switch tag. Consequently, detailed lipid structural elucidation is significantly hindered. Recognizing these shortcomings, Bollinger *et al.* designed and synthesized the novel charge-switch

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derivatization reagent *N*-(4-aminomethylphenyl)pyridinium (AMPP).[109] Following a mild amidation reaction, AMPP is coupled to the carboxylic acid moiety of FA including lipid mediators. Positive ion ESI of the resulting pyridinium-derivatized lipids lead to an exceptional increase in sensitivity when compared to analysis of the underivatized lipid. Importantly, CID of the AMPP-FA cation induces fragmentation of carbon-carbon bonds within the FA hydrocarbon chain, affording structural characterization of FA structures, including the resolution of isomeric mixtures that are often incompletely resolved LC. The proposed dissociation mechanisms for AMPP-derivatized unsaturated FAs are highlighted with Scheme 1, utilizing FA 18:3(9,12,15) as an example.[110] Explicitly, the CID spectra of FA isomers derivatized with AMPP display significant spectral differences that permit the localization of subtle structural features like carbon-carbon double bond position(s), methyl chain branching, among others. Owning to the enhanced sensitivity and specificity, AMPP-derivatized lipids have been widely explored using both direct infusion and LC-MS/MS workflows for the identification and quantitation of nonesterified FAs from a number of biological samples (*i.e.*, mouse plasma, human plasma, and serum) as well as identification of unusual esterified FAs in extracts from bacteria.[110–114] In their report, Han and coworkers deployed a shotgun approach that exploits the charge-remote fragmentation patterns of AMPP-derivatized FA isomers to accurately determine the composition of the FA isomers extracted from human plasma.[112] In an additional example, Tatituri *et al.* adopted this charge-reversal strategy to profile the FAs in *Listeria monocytogenes*, a virulent food-borne pathogen. Here, alkaline hydrolysis of the lipid extract is first utilized to release FA substituents from a lipid extract, followed by conversion of the free FAs to the AMPP-derivative.[113] Positive ion mode mass spectrometry analysis of the liberated and charge-switched FA cations revealed that branched-chain FAs were the major fatty acid substituents found in all the lipid families analyzed. Despite successes, CID of FA-AMPP in complex isomeric or isobaric mixtures can be challenging to deconvolve. In response, a number of studies have combined AMPP with alternate mode of ion activation.

To mitigate limitations of low-energy CID of AMPP charge-switched FAs, novel ion activation methods such has ozone-induced dissociation (OzID) and photodissociation (PD) of fixed-charge pyridinium derivatives have been deployed. Briefly, OzID exploits gas-phase ion/molecule reactions between unsaturated lipid ions and neutral ozone within the mass spectrometer. The resulting OzID spectra reveal predictable neutral losses that can be used for assigning the position(s) of carbon-carbon double bonds and thus elucidation of lipid structure.[115–121] While effective, ion/molecule reactions can be slow, particularly regarding those conducted in negative ion mode, impacting sensitivity. Notably, alkali metal adducted lipids have been shown to be more reactive to ozone, thus greatly enhancing OzID reaction efficiency.[122] Importantly, the OzID spectra of metalated lipid cations also revealed the site(s) of unsaturation and relative positions of unsaturated acyl chains along the glycerol backbone. Poad *et al.* combined AMPP modification and OzID in an automated data-dependent workflow to both enhance the detection and structural elucidation of FAs.[123] Here, OzID of $[FA + AMPP]^+$ cations dramatically reduced spectral complexity when compared to those obtained with low-energy CID. In turn, OzID product ion spectra were straightforward and readily facilitated the unambiguous localization of unsaturation sites in AMPP-derivatized FAs (Figure 11). Application of this workflow to *vernix caseosa* revealed

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the presence of a wide range of isomers previously indistinguishable with conventional platforms, indicative of the broad coverage and dynamic range of the developed method.

Expanding upon the work of presented by Julian and coworkers[124], the Blanksby group has also utilized PD and radical-directed dissociation (RDD) to elucidate a wide array of FA structures, employing two separate charge-reversal strategies. In either approach, suitable radical initiators, explicitly those incorporating aryl-iodide motifs, are first covalently linked to the lipid of interest. Following ionization of even-electron FA precursor ions via direct positive ESI, subsequent irradiation of the charge-switched FA cation with a laser at 266 nm produces abundant radical FA cations, resulting from the loss of atomic iodine. The first charge-reversal strategy coupled with photochemistry relies on the generation and photodissociation of sodium-adducted FAs ions derivatized as 4-iodobenzyl esters (*i.e.*, $[FAIBE + Na]^+$).[125] Laser-photolysis of $[FAIBE + Na]^+$ results predominantly in the generation of $[M + Na - I]^{\bullet +}$ radical cations, yet low-abundance product ions formed by homolysis of carbon-carbon bonds allylic to the unsaturation site afford the identification of carbon-carbon double bond position(s) in unsaturated FAs. Moreover, $[M + Na - I]^{\bullet +}$ radical cations can be further activated via CID to discriminate between different types of isomeric FAs, as RDD provides evidence for hydroxylation and methyl chain branching localization. In a subsequent study, Narreddula *et al.* conceived 1-(3-(aminomethyl)-4-iodophenyl)pyridin-1-ium (4-I-$AMPP^+$) fixed-charge, photolabile tags to enhance the detection and characterization of FAs in LC-MS/MS workflows.[126, 127] Explicitly, the PD mass spectra of FA structures conjugated with 4-I-$AMPP^+$ reveal structurally diagnostic product ions that can be exploited to distinguish an array of isomeric FAs, including chain branching, unsaturation, hydroxylation, and cyclopropanation. Owing to the fixed charge, FA derivatization with I-$AMPP^+$ motifs provided increased sensitivity when compared to the previous approach that relied on 4-iodobenzyl alcohol esterification and metal cation adduction for positive ion mode FA analysis. Recent application of I-$AMPP^+$ charge switching was also deployed for the identification of ultralong *O*-acyl hydroxy fatty acids (OAHFAs).[128] Here, Hancock *et al.* achieve near-complete structural characterization of OAHFAs derived from human meibomian gland secretions, as the RDD mass spectra of 4-I-AMPP-derivatized meibum OAHFAs elucidated sites of unsaturation, the stereochemical configuration of carbon-carbon double bonds, and ester linkage regiochemistry.

Like OzID and RDD, the Paternò-Büchi (PB) reaction in conjunction with low-energy CID has been used to great effect for unsaturated lipid analysis, as this strategy permits the identification and quantitation of double bond positional isomers.[36, 129–131] In the earliest studies, the Xia group subjected unsaturated FAs to photochemical tagging with acetone, though this photochemistry can be readily applied to complex lipids as well.[129, 130] Ensuing CID of acetone-tagged unsaturated lipids provided predictable pairs of diagnostic ions exhibiting 26 Da spacing that are specific to the locations of unsaturation sites. However in the negative ion mode (*i.e.*, preferential ionization mode for FAs), low ionization efficiency of unsaturated FA-PB reaction product ions and non-specific neutral losses such as decarboxylation (− 44 Da) and the loss of the acetone tag (− 58 Da) hinder FA analysis, particularly those with more than three degrees of unsaturation. Once more, solution-based charge switching strategies proved advantageous for circumventing these obstacles. For example, Ma *et al.* demonstrated that PB-MS/MS on lithiated adduct ions, denoted $[FA^{PB}$ +

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Li]$^+$, generated the characteristic pairs of diagnostic ions indicative of double bond position.[131] In an additional example, Esch and Heiles replaced acetone as the PB reactive agent with 3-acetylpyridine (3-acpy), an easily protonated chemical modifier upon direct positive ESI.[132] Here, 254 nm ultraviolet (UV) light irradiates an emitter tip filled with the reaction mixture, resulting in the covalent attachment of 3-acypy to the carbon-carbon double bond of an unsaturated FA. Consequently, PB functionalization with 3-acypy enabled the detection of PB reaction products in the positive ion mode with ion signal enhancement factors of up to 631 and facilitated the localization of carbon-carbon double bonds in all unsaturated FAs examined, including those with more than three degrees of unsaturation. While effective, all PB functionalization strategies described above require the presence of at least one carbon-carbon double bond and are not amenable saturated lipid analysis. Recognizing this shortcoming, Wei and co-workers developed a double derivatization strategy for the enhanced detection and characterization of FAs.[133] In this study, the carbon-carbon double bonds in unsaturated FAs are first labeled with acetone using the photochemical PB reaction. Next, the entire FA pool comprised of acetone-labeled unsaturated FAs and unlabeled saturated FAs are derivatized with N,N-diethyl-1,2-ethanediamine (DEEA). This secondary chemical modification step results in the amidation of FA carboxyl groups and the incorporation of a tertiary amine group which is readily protonated in positive mode ionization, resulting in a remarkable increase in analytic sensitivity. Importantly, the novel double derivatization procedure has the primary advantage of being able to comprehensively profile FAs, including saturated and unsaturated FAs with a high degree of sensitivity and selectivity.

2.1.3. Gas-Phase Charge Inversion—In contrast with the above-mentioned charge-switching strategies utilizing solution-based derivatization, gas-phase ion/ion reactions to derivatize lipid anions within the mass spectrometer has been explored. For example, charge inversion ion/ion reactions have been utilized to convert FA anions into structurally informative, metalated cations.[134–137] These experiments are performed on mass spectrometers that have been modified to perform ion/ion reactions, as oppositely charged ions are mutually stored within a designated reaction cell.[138] Briefly, the process of charge inversion proceeds through the generation a long-lived electrostatic complex. In some cases, the noncovalent interactions within the complex are strong enough to yield adducted lipid ions, as the lipid and reagent ions remain electrostatically bound. However, if the noncovalent interactions are not sufficiently strong to generate adduct ions, multiple charges are transferred within the complex prior to the separation of the reagent and lipid ions, noting that the number of charges transferred must exceed the number required to neutralize the lipid ion in order to observe charge inverted product ions. Gas-phase charge switching offers the primary advantage of decoupling of the initial ionization event from lipid derivatization, and therefore, by conducting lipid derivatization in the gas phase, each reagent can be individually optimized with regards to solution and electrospray conditions.

Charge inversion has been particularly useful for lipid analysis, as conventional lipid ion types formed upon direct ESI can be altered into structurally informative ion types entirely within the mass spectrometer. Using a shotgun approach, gas-phase anionic to cationic charge switching chemistries were first developed for FA profiling.[134] Explicitly, tris-

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phenanthroline magnesium complexes have proven particularly advantageous for the selective derivatization of FA anions. Here, direct negative nESI of nonesterified (*i.e.*, free) FA or the decomposition of complex lipid precursors via solution-based hydrolysis or gas-phase collisional activation (broadband or single frequency) gives rise to $[FA - H]^-$ anions. Scheme 2 summarizes the ion/ion reaction. The resulting singly deprotonated FA anions are then transformed in the gas-phase following reaction with tris-phenanthroline magnesium reagent dications, yielding abundant $[FA - H + MgPhen]^+$ complex cations. The CID spectra of charge-inverted FA cations display isomer-specific and highly reproducible fragmentation patterns indicative of FA structure. For example, monounsaturated FA ions cationized with magnesium phenanthroline complexes dissociate to yield a characteristic spectral gap flanked by product ions arising from carbon-carbon cleavage allylic to the double bond, reminiscent of those spectral patterns observed with high-energy CID. While double bond localization in highly unsaturated FA was hindered due to rearrangements and congested CID spectra, discrimination amongst PUFAs can still be achieved. Explicitly, as the $[FA - H + MgPhen]^+$ CID spectra were both highly reproducible and isomer-specific, automated matching to spectra of standards in a FA library facilitated unambiguous isomeric distinction, and consequently the localization of carbon-carbon double bonds.[135] Importantly, the use of multiple linear regression analysis in conjunction with charge inversion ion/ion chemistry enables the relative quantitation of FA isomers over a broad dynamic range of molar ratios. Recent application of this charge-switch derivatization of fatty acid esters of hydroxy fatty acids (FAHFAs) via gas-phase ion/ion reactions facilitated the assignment of FA and HFA constituents, pinpointed unsaturation sites within the FA moiety, and elucidated ester linkage regiochemistry, demonstrating the versatility of charge inversion reactions for lipid identification.[139]

This gas-phase charge inversion chemistry was subsequently extended to intact GPL analysis. Importantly, this top-down approach afforded the near-complete elucidation GPL molecular structures.[136, 137] With this strategy, a single experiment facilitates assignment of the GPL headgroup, fatty acyl composition, carbon-carbon double bond position(s) in unsaturated fatty acyl chains, and, in some cases, fatty acyl *sn*-position and relative abundances for isomeric fatty acyl substituents. A notable advantage of conducting derivatization within the gas-phase (opposed to solution-based modification) is the ability to switch charge states and ion type on demand. In other words, ion/ion chemistry allows for facile, highly efficient, and structure-selective derivatization conducted independent of the ionization event. In turn, this platform offers a highly flexible approach to GPL characterization, as MS/MS events can be performed in the desirable polarity where structurally informative products ions are most readily observed. Briefly, the GPL was first ionized in negative ion mode and collisionally activated to liberate $[FA - H]^-$ anions via fragmentation of the ester bonds at the *sn*-1 and *sn*-2 positions of the central glycerol backbone. Subsequent ion/ion reactions with $[MgPhen_3]^{2+}$ reagent dications yield abundant $[FA - H + MgPhen]^+$ cations. As detailed above, the CID spectra of charge-inverted fatty acyl complex cations yielded isomer-specific product ion spectra that permit (i) unambiguous assignment of carbon-carbon double bond position(s) and (ii) relative quantitation of isomeric fatty acyl substituents (Figure 12).[136] A recent report combines a systematic MS^n platform with gas-phase charge switching to elucidate ether GPL structures,

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providing increased isomeric resolution as both *sn*-1 bond type and sites of unsaturation in the *sn*-2 fatty acyl were confidently assigned.[137] Applications of both workflows to proposed biomarkers found in human blood plasma extract revealed that, in fact, these structures exist as a mixture of isomers that are often left unresolved when employing conventional ESI-MS/MS.

2.2 Positive to Negative Charge Inversion

2.2.1 Solution-based Noncovalent Derivatization—Perhaps one of the most commonly exploited charge switching approaches involves the addition of millimolar concentrations of acetate, formate, bicarbonate, or chloride salts to lipid solutions to afford choline-containing lipid detection in negative ion mode.[75, 76, 78, 140–142] This approach also provides enhanced PC structural characterization, as charge-switching PC anions dissociate to yield structurally informative product ions. For example, due to the presence of the quaternary ammonium group in the phosphocholine headgroup, PCs readily ionize in the positive ion mode, yielding abundant protonated ions. However, CID of the protonated PC ion provides information that is restricted to the headgroup, as a dominant phosphocholine product ion is observed at *m/z* 184. Unfortunately, phosphocholine-containing lipids cannot be ionized in the negative polarity by deprotonation, and in turn, structural information regarding acyl chain composition cannot be readily accessed without solution-based derivatization. Explicitly, ionization in the negative ion mode is reliant on the formation of $[M + X]^-$ adduct ions, where X = Cl^-, HCO_2^-, HCO_3^-, or $CH_3CO_2^-$. CID of PC adduct anions produces a demethylated PC anion (*i.e.*, $[PC - CH_3]^-$), generated via combined losses of the counteranion and a methyl cation from the quaternary ammonium group, which fragments via subsequent collisional activation to provide product ions informative of acyl chain composition. Notably as highlighted with Figure 13, Zhao *et al.* reported that CID of bicarbonate-adducted PC anions exhibited nearly a 20-fold increase in ion signal compared to the $[PC + HCO_2]^-$ ion and can be beneficial for assigning *sn*-position, as CID of the $[PC + HCO_3]^-$ ion generated *sn*-specific diagnostic ions can be exploited for the identification and quantitation of *sn*-positional isomers.[141]

Lipids containing fixed cationic sites can also detected in negative ion mode by exploiting the noncovalent complexation of choline-containing target lipid analytes with the photocaged radical precursor 4-iodobenzoate (IB). Here, Pham *et al.* generated charge-switched $[PC + IB]^-$ adduct ions by direct negative ESI of a methanolic solution containing the PC analyte and IB.[143] Irradiation with 266 nm laser light cleaves the UV-labile carbon-iodine bond in the $[PC + IB]^-$ adduct ion, giving rise to a reactive phenyl radical. Subsequent CID of the PD-derived $[PC + IB - I]^{\bullet -}$ ion resulted in the formation of a dominant product ion corresponding to the neutral loss of methyl benzoate (*i.e.*, $[PC - CH_3 - H]^{\bullet -}$). The CID product ion spectra of $[PC - CH_3 - H]^{\bullet -}$ radical anions revealed a wealth of structural information and rich fragmentation chemistry. Contrary to conventional tandem MS spectra, RDD spectra facilitated the discrimination of isomeric lipids differing in double bond position and methyl chain branching, as product ions resulting from intrachain carbon-carbon cleavages were readily observed. Similar results were also obtained for SM. Moreover, alternate phospholipid classes like PS and PE were also found to noncovalently

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complex with IB, though in the absence of a fixed positive charge, complexation was not nearly as efficient.

2.2.2 Gas-Phase Charge Inversion—While the above approaches effectively transform cations into structurally informative anions by doping chemical additives into lipid solutions, similar strategies have been developed to charge-switch lipid cations in the gas phase. Recently, the McLuckey group[137, 144–148] and others[149] have explored the utilization of gas-phase ion/ion reactions for the charge inversion of lipid cations. For example, Stutzman *et al.* described the charge inversion of PC monocations via ion/ion reaction with doubly deprotonated 1,4-phenylenedipropionic acid (PDPA) anions in efforts to increase structural characterization of PCs.[146] Following the ion/ion reaction, the dominant product ion observed was a negatively charged complex anion comprised of the original PC lipid and PDPA (*i.e.*, $[PC + PDPA - H]^-$). Importantly, subsequent ion-trap CID of the $[PC + PDPA - H]^-$ adduct ion gave rise to a demethylated PC, which as described above, can be further interrogated via CID to produce abundant fatty acyl carboxylate anions, affording the identification of FA chain lengths and degree of unsaturation. Though outside the scope of this review, the Prentice group has recently utilized PDPA ion/ion reactions in MS imaging experiments to invert the polarity of PC ions generated by MALDI, alluding to the versatility of ion/ion approaches.[149] Charge inversion reactions with PDPA dianions have also been exploited to chemically separate isomeric PC and PE lipids in the gas-phase. Here, Betancourt *et al.* reported distinct reactivities of isomeric PC and PE ions with PDPA, attributing the unique charge inversion reactivities to differences in polar headgroup composition .[144] Specifically, $[PC + H]^+$ cations are transformed via ion/ion reactions with $[PDPA - 2H]^{2-}$ dianions to yield $[PC + PDPA - H]^-$ complex product anions, whereas protonated PEs undergo charge inversion via double proton transfer to the two carboxylate moieties, giving rise to $[PE - H]^-$ product ions.

Scheme 3 summarizes the reaction behaviors between $[PC + H]^+$ and $[PE + H]^+$ cations with $[PDPA - 2H]^{2-}$ reagent dianions. As highlighted with Figure 14, isomeric/isobaric PC and PE cations are effectively separated on the *m/z* scale. Importantly, the developed positive to negative ion charge inversion chemistry provides several benefits compared to conventional analysis conducted exclusively in a single ion polarity. First, this approach takes advantage of lipid ionization in the positive ion mode, which can be more efficient particularly for PCs. As the ionization and derivatization steps are decoupled, structural characterization can then be conducted in the negative ion polarity, where structurally informative product ions are readily observed. Second, when compared to direct negative ionization, the positive ion charge inversion approach was demonstrated to reduce chemical noise, minimize mixture complexities within an isolated precursor ion population, and offered lower detection limits.

Combinations of PDPA reactions with solution-based derivatization strategies have also been explored with the aims of further increasing isomeric resolution and mixture analysis performance. For example, Franklin *et al.* coupled PDPA charge inversion ion/ion reactions with photochemical derivatization of carbon-carbon double bonds in unsaturated GPLs via the Paternò-Büchi (PB) reaction.[145] Ultimately, this method provides the gas-phase separation of isomeric/isobaric PC and PE lipids while also affording the assignment of fatty acyl composition and the localization of carbon-carbon double bond(s) in unsaturated fatty

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acyl chains. In an additional example, gas-phase ion/ion reactions of ^{13}C-diazomethane-modified PE, PS, PC, and SM cations with PDPA reagent dianions were shown to charge-invert the positively charged GPL ions to the negative mode, where fatty acyl composition could be revealed.[147] Additional details regarding trimethylation enhancement using ^{13}C-diazomethane (^{13}C-TrEnDi) are provided below. The combination of ^{13}C-TrEnDi modification, PB derivatization, and PDPA reactions in a single workflow permitted enhanced structural identification at the carbon-carbon double bond positional level, particularly for PE lipids in complex biological samples.[148]

3. Charge Manipulation

The concept of charge manipulation is not inherently new to MS-based approaches. For example, there is an expansive body of literature regarding its applications the analysis of ionized biomolecules, especially peptide and protein characterization.[83] Therefore, it is not surprising that charge manipulation strategies have been adapted for lipid analysis. For example, chemical modification procedures and gas-phase chemistries that influence ion charging have been developed to aid in lipid ionization efficiency, enhance structural elucidation, and separate targeted lipid classes from co-existing lipid signals derived from alternate lipid classes.

3.1 Charge Manipulation of Cations

Many advancements in lipid detection and identification have been driven by the manipulation of lipid precursor cations charge states and/or ion types, achieved via complexation with metal cations or covalent modifications with novel derivatization reagents. Below, we will review the reported strategies for altering lipid structures that readily ionize in the positive ion mode upon ESI. Such lipids primarily include those with fixed, cationic charge sites like PCs. Although PE and PS lipids tend to yield deprotonated anions upon negative ESI, these anionic lipids can also be ionized in positive ion mode via protonation, though their ionization efficiency is substantially lowered in this modality. In turn, some charge manipulation strategies reviewed below include these lipid classes, though the majority of presented approaches will focus on choline-containing lipids.

In addition to charge-switching, metal cationization has been explored to transform ion-type of lipid precursors that already demonstrate a propensity to ionize in positive ion mode. Identical to the strategies for metal cationization described above, the formation of metal-lipid complex cations upon positive ESI again relies on the addition of metal salts to lipid solutions prior to ionization. In early studies, alkali metal-adducted PC cations were examined.[150] Sodium-cationized PCs were first investigated with ESI-MS/MS.[150, 151] However, the resulting CID spectra of sodium-PC adduct ions were largely uninformative. Turning to lithium as a cationization reagent, Turk and co-workers noted that CID of lithiated PC adducts offers advantages over analysis of protonated or sodiated PC ions.[152] Explicitly, the CID spectra of $[PC + Li]^+$ adduct ions contain abundant product ions generated via the loss of fatty acyl substituents, affording the assignment of acyl chain composition. In contrast to the CID spectra of lithium-lipid metal complexes described

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above with anionic phospholipid classes, this approach does not facilitate the localization of carbon-carbon double bonds.

Besides monovalent metal cationization, doubly charged PC-metal complexes have been examined by multiple groups. In the first example, O'Hair and colleagues investigated PC lipids complexed with Cu^{2+}, Co^{2+}, Mg^{2+}, and Ca^{2+} using electron capture dissociation (ECD).[153] Here, the ECD spectra of divalent metal-lipid complexes, notably those incorporating Ca and Mg dications, provided head group composition, fatty acyl composition, and in some cases, *sn*-position. In a recent example, Becher *et al*. explored the dissociation patterns of utilizing various activation methods, including CID, ultraviolet photodissociation (UVPD), and higher-energy collisional dissociation (HCD).[154] In this work, PC lipids were reported to complex with Fe^{2+} ions in solution, yielding doubly charged Fe-adducted PC cations upon positive mode ESI. The HCD, CID, and UVPD spectra of positive doubly charged PC-metal ion complexes revealed abundant carboxylate product ions created by ester bond cleavage at the *sn*-1 and *sn*-2 positions of the central glycerol backbone. Importantly, CID and UVPD of $[PC\text{-}Fe]^{2+}$ cations resulted in preferential loss of the *sn*-2 fatty acyl chain, enabling relative quantitation of *sn*-positional isomers. Unfortunately, neither approach described above facilitated detailed structural elucidation, as additional features like carbon-carbon double bond position were not revealed. Moreover, both strategies are limited to PC analysis, as alternate lipid classes were not effectively complexed with divalent metal cations. We note that this structure-selective reactivity could be beneficially exploited to enable the mass spectrometric separation of PCs from alternate phospholipid classes.

The advantages of cationic lipid covalent derivatization have been explored to alter lipid cation ion type, demonstrating the notable benefits of enhanced ionization efficiency and mass spectrometric separation of lipid classes. One such example involving trimethylation enhancement using diazomethane, commonly referred to as TrEnDi, was recently developed by the Smith group.[155] Here, phosphate moieties, carboxylic acids, and primary amines are exhaustively methylated following exposure to an solution of diazomethane and acid. Consequently, PC, PS, PE, and SM lipids are derivatized with a permanent, fixed positive charge. By doing so, the sensitivity of MS/MS experiments are markedly improved, as ionization efficiency was greatly enhanced. Moreover, TrEnDi modification resulted in the consolidation of ion fragmentation into at most two dissociation channels, generating class-specific product ions that readily identify polar headgroup composition. Employing ^{13}C-labeled diazomethane, Canez *et al*. demonstrated the effectiveness of ^{13}C-TrEnDi, noting that this approach has the notable advantage over derivatization with unlabeled diazomethane as isobaric PE and PCs are effectively resolved.[156] However, the CID spectra of TrEnDi-modified lipid cations in positive ion mode do not contain product ions that permit fatty acyl identification, though this limitation can be overcome when combined with additional gas-phase derivatizations as described above.[147, 148] We note an alternate methylation approach has also been developed to charge-switch anionic GPL species. Here, the phosphate moieties in PI molecular species from biological extracts are rapidly and completely methylated utilizing a one-step trimethylsilyl diazomethane (TMS-diazomethane) derivatization.[157–159] Later applications of TMS-diazomethane methylation

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permitted the quantitative analysis of isomeric bis(monoacylglycero)phosphate and PG lipids in tissue samples.[160, 161]

Alternate covalent derivatization of cationic lipids via modification with a fixed, net positively charged reagent have also been implemented by the Reid group.[162–164] In their work, Reid and co-workers developed a selective chemical derivatization strategy for the differentiation of phospholipid classes using a direct infusion positive ESI-MS/MS approach. Coupled with high-resolution MS, the aminophospholipids PS and PE are covalently modified with the d_6-S,S′-dimethylthiobutanoylhydroxysuccinimide ester (d_6-DMBNHS) reagent. As d_6-DMBNHS contains a fixed charge sulfonium ion, the developed derivatization strategy improved ionization efficiency, extending the dynamic range of aminophospholipid detection in crude lipid extracts. Furthermore, as d_6-DMBNHS-modified lipids display a 136.0829 Da shift from unmodified lipids, possible isobaric interferences, namely those attributed to PC, PA, and PG lipid ions, were minimized. In a subsequent report, Fhaner *et al.* improved the previous shotgun strategy by utilizing a combination of the ^{13}C-labeled version of the DMBNHS ester reagent (*i.e.*, ^{13}C-DMBNHS) and selective modification of the vinyl double bond within plasmenyl ether containing lipids using iodine and methanol to fully resolve isobaric aminophospholipids, including ether species, from choline-containing species.[162] Collectively, this approach enables the mass spectrometric resolution and molecular characterization of plasmenyl lipids and offers an attractive strategy to crude lipid extract analysis.

In a final shotgun approach, Pham and Julian achieved the separation of isomeric PE and PC structures via the selective, noncovalent complexation between PEs and 18-crown-6-ether (18C6).[165] Not only does this solution-based derivatization procedure eliminate overlapping PE and PC signals by shifting the PE cation signals into a higher *m/z* range, but it also improves the sensitivity of PE detection by nearly an order of magnitude, as the protonated primary amine is stabilized by the noncovalent binding of 18C6 This strategy is highlighted with Figure 15. Moreover, the incorporation of an IB motif into the 18C6 derivative enabled the generation of PE radical cations upon photoactivation and subsequent application of RDD experiments. The resulting RDD spectra of $[PE + H + IB - 18C6 - I]^{\bullet+}$ radical cations contained rich fragmentation patterns that allowed for the discrimination of isomeric lipids such as those varying in carbon-carbon double bond and methyl chain branching sites.

3.2 Charge Manipulation of Anions

Contrary to the many charge-manipulation strategies presented for lipid cation detection above, seemingly few parallel tactics have been applied for anion analysis. In a recent example, the McLuckey group applied proton transfer ion/ion reactions for the gas-phase separation, concentration, and identification of cardiolipins (CLs) from total lipid extract, relying exclusively on gas-phase chemistries.[166] Briefly, CLs are characterized by the presence of two phosphatidyl moieties linked to a central glycerol backbone and four fatty acyl substituents and are present at naturally low abundances within the cellular lipidome. In turn, analysis of this unique dimeric class of GPLs demands both high specificity and sensitivity, and thus, there are significant challenges associated with CL detection and structural elucidation, particularly when examining total lipid extracts. Here, $[CL - 2H]^{2-}$

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were reacted with proton transfer reagent monocations in the gas-phase, giving rise to charge-reduced $[CL - H]^-$ anions. Consequently, the CL class was effectively separated on the *m/z* scale from alternate acidic phospholipid classes, as charge-reduced CL anions were resolved from more abundant, isobaric GPL monoanions. Prior to MS^n experiments, multiple iterations of proton-transfer ion/ion reactions allowed for the selective accumulation of the desired CL product ions. The iterative process not only dramatically reduces the averaging time needed to achieve good S/N product ion ratios but also enhances sensitivity and improves mixture analysis performance. Application of this workflow to *Escherichia coli* extract afforded detailed characterization of low abundant CLs directly from total biological extract, as MS^n of charge-reduced $[CL - H]^-$ anions that have been concentrated in the gas-phase unveiled the structural complexities of CL molecular structures, revealing the presence of multiple CL isomers within a single mass-selected CL precursor anion population.

4. Conclusions

The importance of lipid analysis has become apparent when considering the relationships between altered lipid metabolism and the onset and progression of deleterious pathologies. Recent advances in ESI-MS/MS have largely facilitated the expansion of modern lipidomics, allowing for a deeper dive into the biochemical and functional roles of lipids. Nevertheless, there are significant challenges associated with conventional ESI-MS strategies and the reliance on native lipid ion types. As a consequence, a variety of charge and ion-type manipulation techniques have been developed with the dual aims of increasing ionization efficiency and enhancing structural characterization. Of these, solution-based derivatization methods have received the most attention, as these procedures can be readily utilized in conjunction with current commercially available mass spectrometers and can provide a number of benefits for lipid analysis. However, ion manipulation reliant on wet-chemical modification or alteration of solution conditions increase sample preparation timescales; promote undesired side-reactions that lead to complex mass spectra; and suffer incomplete derivatization leading to degenerate signals and impacting sensitivity. Post-source ion manipulation achieved through gas-phase ion/ion reactions thus offers unique advantages over solution-based counterparts. While it does not directly address ionization efficiency of lipids, benefits for structure elucidation and enhanced detection efficiencies have been demonstrated. By conducting lipid derivatization in the gas-phase, the ionization and derivatization events are decoupled, enabling the optimization of individual reagent solutions with regards to solution and electrospray conditions and enhanced control of reaction outcomes. Moreover, gas-phase ion/ion reactions offer the undeniable power to switch between charge states on-demand and undertake facile highly efficient and structure-selective derivatization. In some cases, a combination of both condensed-phase and gas-phase approaches have been shown to be particularly powerful. Though ion/ion reactions have been used to effectively manipulate the ion-type formed upon ionization, widespread adaptation of this strategy is primarily limited by the requirement of instrument modification. In total, regardless of derivatization method, charge manipulation, particularly charge inversion, in combination with ESI-MS/MS provides increased efficiency, access to alternate and desirable fragmentation channels, and enhanced detection and identification of

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lipids in complex biological samples. In total, due to the broad spectrum of lipid structures, mixture complexities, and associated issues of dynamic range, the answer to unlocking the intricacies of the cellular lipidome likely lies in a combination of techniques. Such a platform might call on a combination of advanced separation techniques (LC), condensed-phase chemistries, gas-phase ion/ion chemistries (*i.e.*, charge inversion), and alternative ion activation methods (*i.e.*, OzID).

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Highlights:

- Detection of subtle disturbances in lipid metabolism can provide key insights into many human pathologies.
- Modern electrospray ionization tandem mass spectrometry offers an unprecedented level of analytical power in lipid analysis.
- Manipulation of the nature and polarity of ionized lipids enhances detection and structural characterization.
- Contemporary solution- and gas-phase strategies to charge manipulation represent useful new tools for lipidomics.

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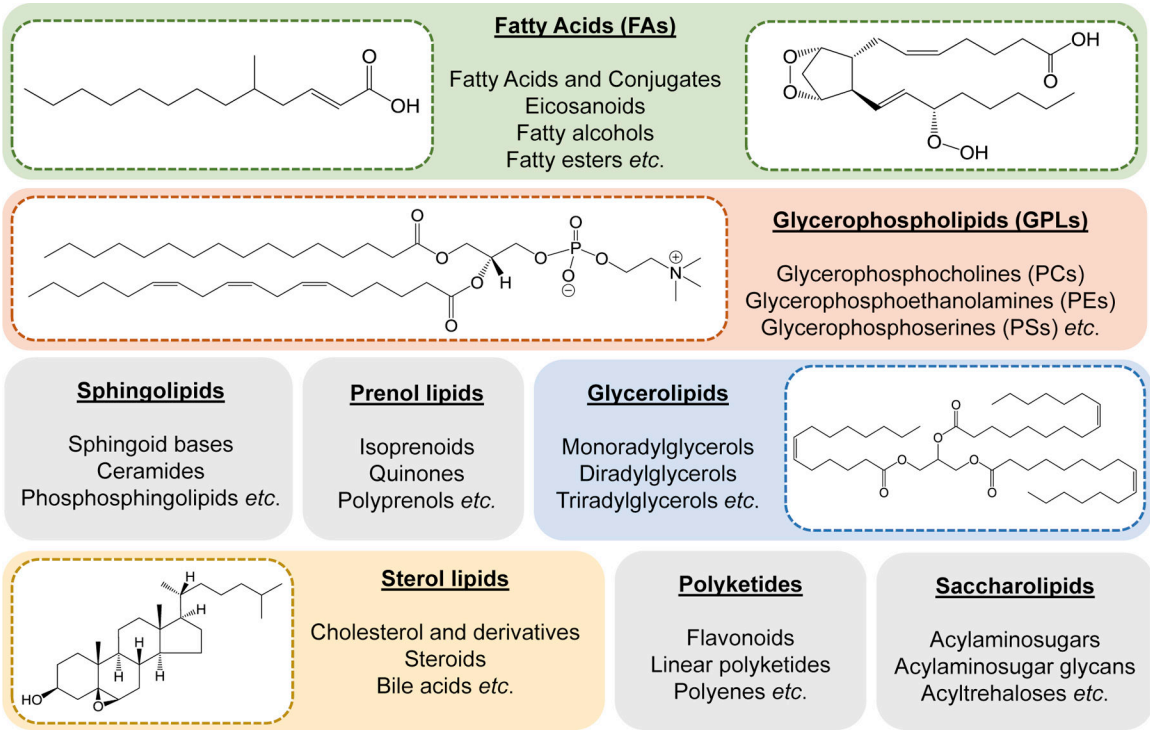

Figure 1.
Common lipid categories with structural examples.

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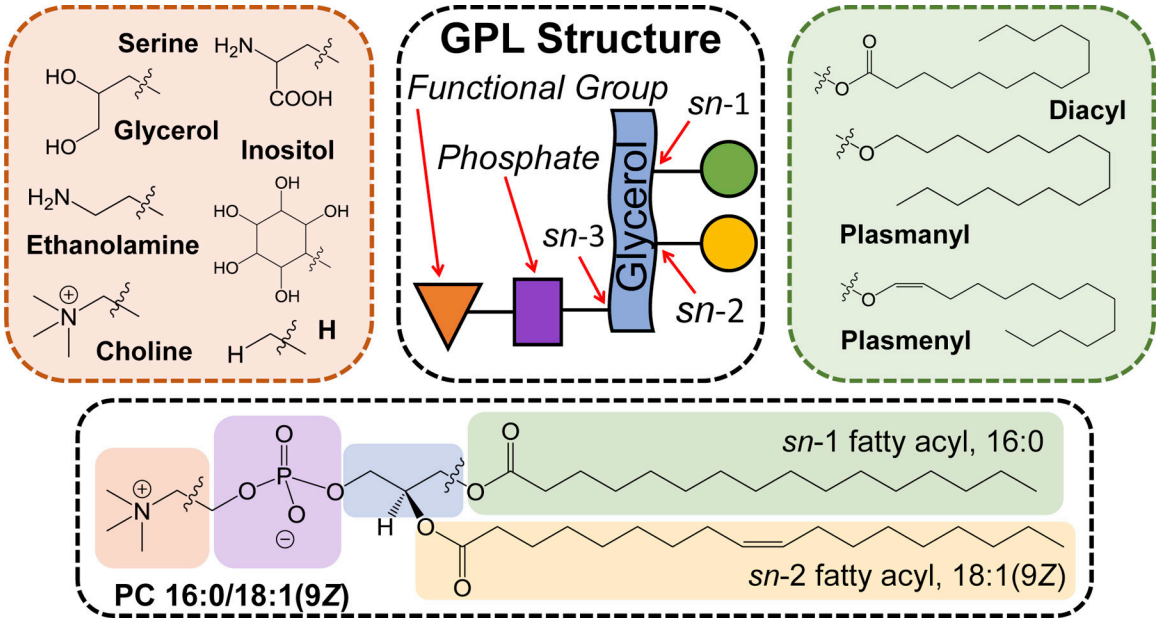

Figure 2.

General glycerophospholipid structure. Polar headgroup structures and names are given in the orange panel (top left corner). The headgroup composition determines GPL class. In the green panel (top right), example *sn*-1 radyl chains are portrayed, noting that the nature of the sn-1 linkage dictates GPL subclass. Explicitly, GPL contain either an acyl, 1-*O*-alkyl, or a 1-*O*-alk-1′-enyl group at the *sn*-1 position, indicating the diacyl, plasmanyl, or plasmenyl subclasses, respectively.

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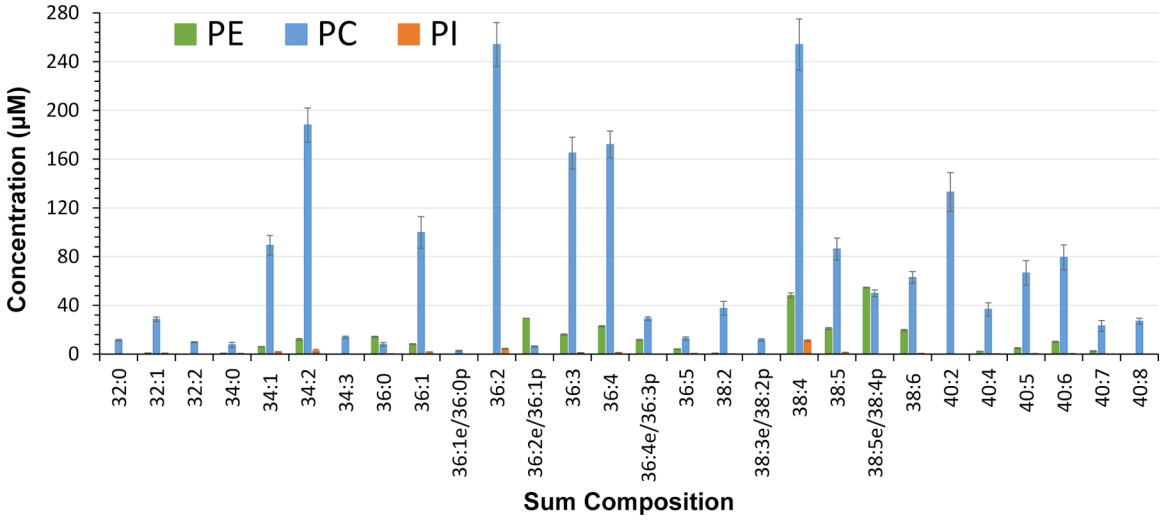

Figure 3.

GPLs in human plasma samples. The measurements for PE, PC, and PI species were performed in quintuplicate and reported as the mean ± standard error of the mean (SEM). Adapted with permission from Reference 14.

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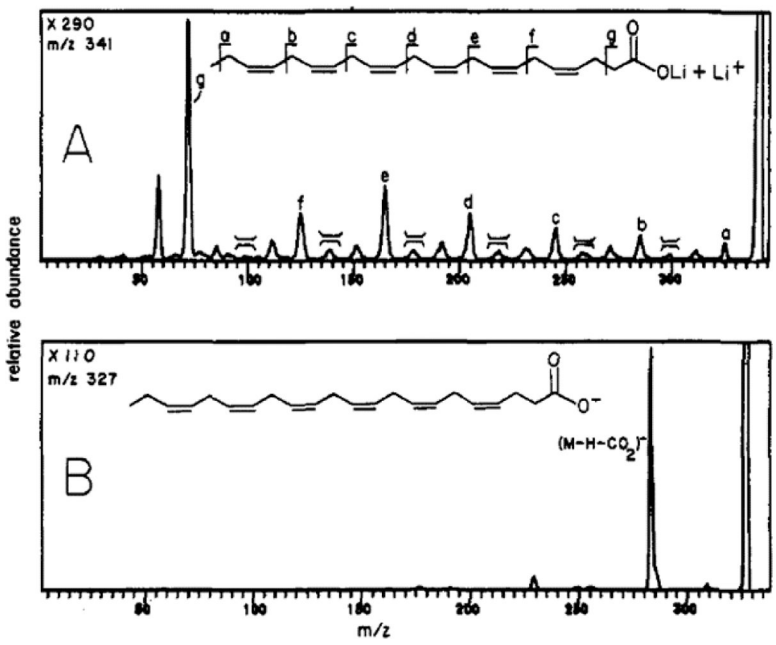

Figure 4.

FAB-MS/MS spectra of 22:6(4Z,7Z,10Z,13Z,16Z,19Z) **(a)** $[M - H + 2Li]^+$ and **(b)** $[M - H]^-$ ions. The product ions indicative of carbon-carbon double bond cleavage are indicated with double bond symbols. Reproduced with permission from Reference 52.

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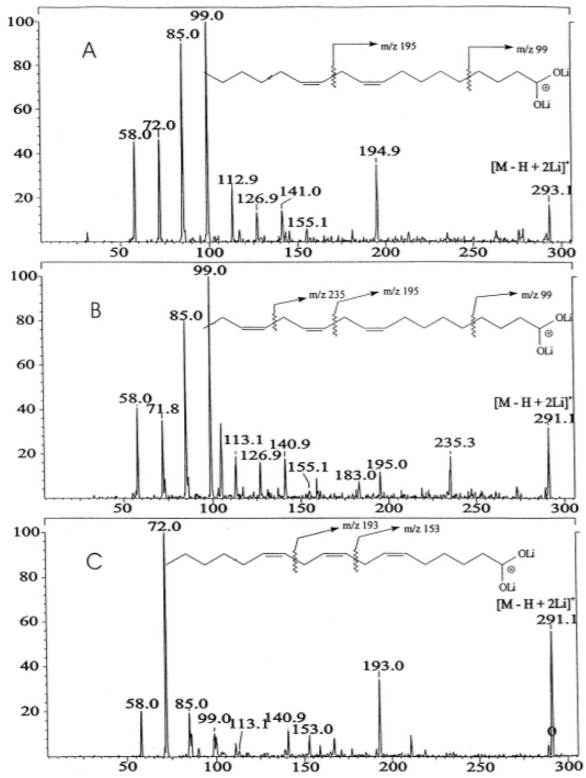

Figure 5.

CID spectra of $[FA - H + 2Li]^+$ adducts of **(a)** 18:2(9, 12), **(b)** 18:3(9,12,15), and **(c)** 18:3(6,9,12). Reproduced with permission from Reference 78.

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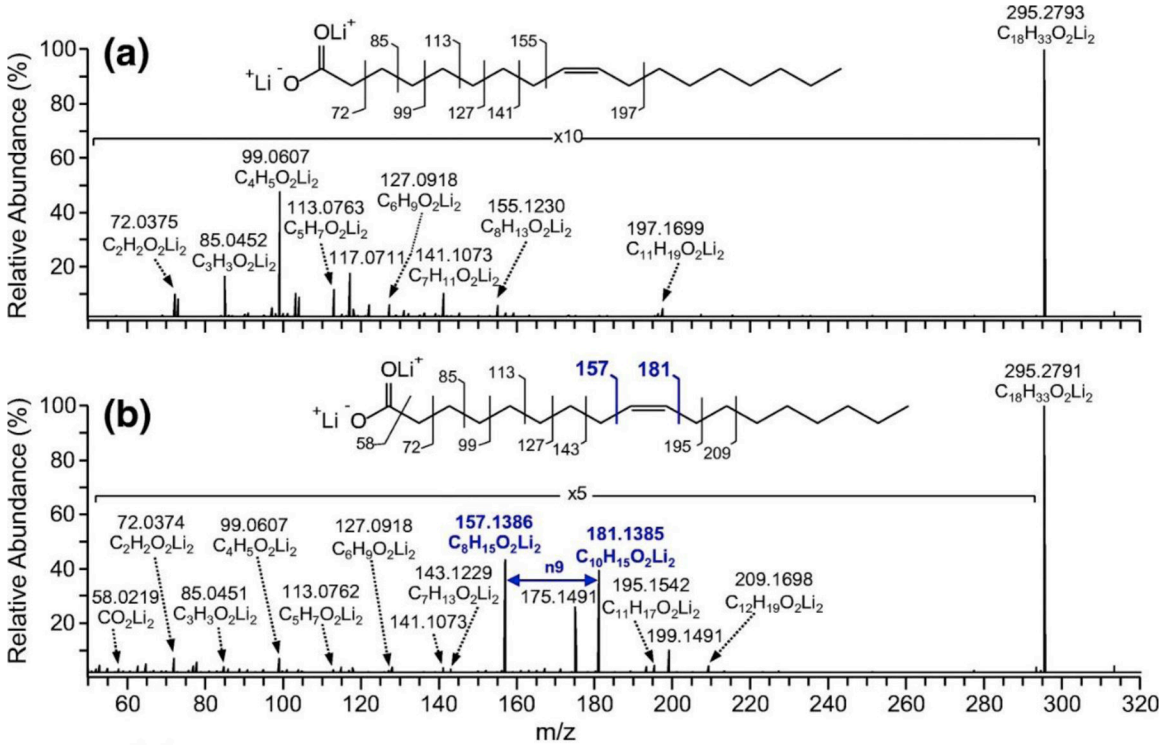

Figure 6.

Product ion spectra of the $[M - H + 2Li]^+$ precursor ion of FA 18:1(9*Z*) obtained utilizing collisional activation via **(a)** HCD (30 eV collision energy) and **(b)** 193 nm UVPD (100 laser pulses per scan were performed with the HCD collision energy set at 2 eV). Reproduced with permission from Reference 79.

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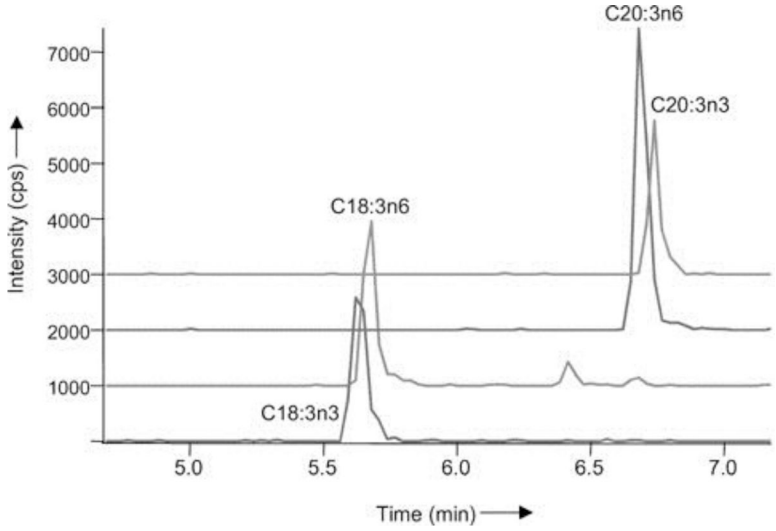

Figure 7.

UPLC MRM chromatograms for the double bond positional isomers of FA 18:3 and FA 20:3 The isomer-specific transitions used for 18:3(*n*-3), 18:3(*n*-6), 20:3(*n*-6), and 20:3(*n*-3) were $415^+ \rightarrow 317^+$, $415^+ \rightarrow 307^+$, $443^+ \rightarrow 335^+$, and $443^+ \rightarrow 345^+$, respectively. Reproduced with permission from Reference 81.

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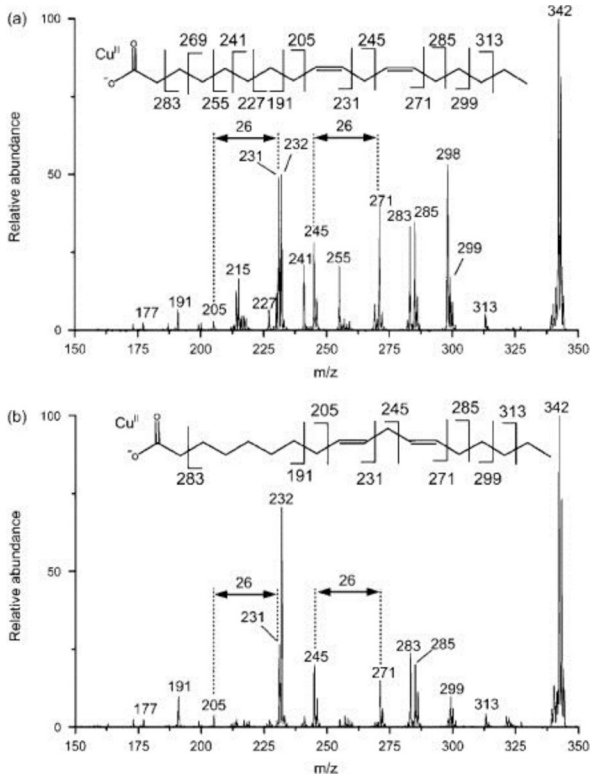

Figure 8.

Low-energy CID spectra of $[FA - H + Cu^{II}]^+$ ions for **(a)** 18:2(9*Z*,12*Z*) and **(b)** 18:2(9*E*, 12*E*). Reproduced with permission from Reference 83.

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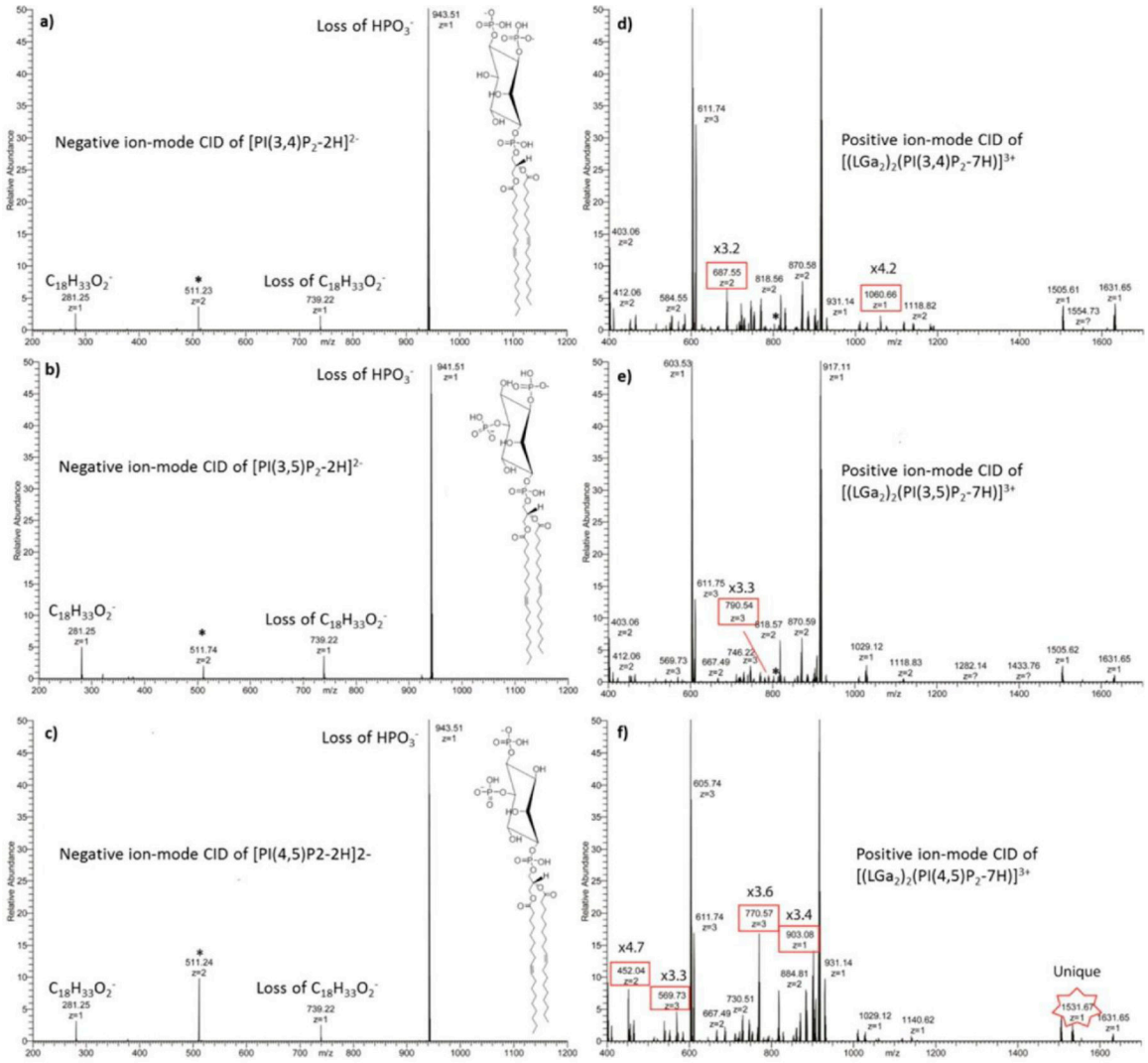

Figure 9.

Negative ion mode CID spectra of **(a)** $[PI(3,4)P_2 - 2H]^{2-}$ (*m/z* 510.2360), **(b)** $[PI(3,5)P_2 - 2H]^{2-}$ (*m/z* 510.2360), and **(c)** $[PI(4,5)P_2 - 2H]^{2-}$ (*m/z* 510.2360). Positive ion mode CID spectra of charge-inverted **(d)** $[(LGa_2)_2(PI(3,4)P_2 - 7H)]^{3+}$ (*m/z* 811.2594), **(e)** $[(LGa_2)_2(PI(3,5)P_2 - 7H)]^{3+}$ (*m/z* 811.2594), and **(f)** $[(LGa_2)_2(PI(4,5)P_2 - 7H)]^{3+}$ (*m/z* 811.2594). The precursor ion subjected to CID are marked with an asterisk (*), and product ions that display at least a 3-fold increase abundance relative to corresponding identical ions in the CID spectra of the other two isomers are indicated with a red box. Reprinted with permission from Reference 85.

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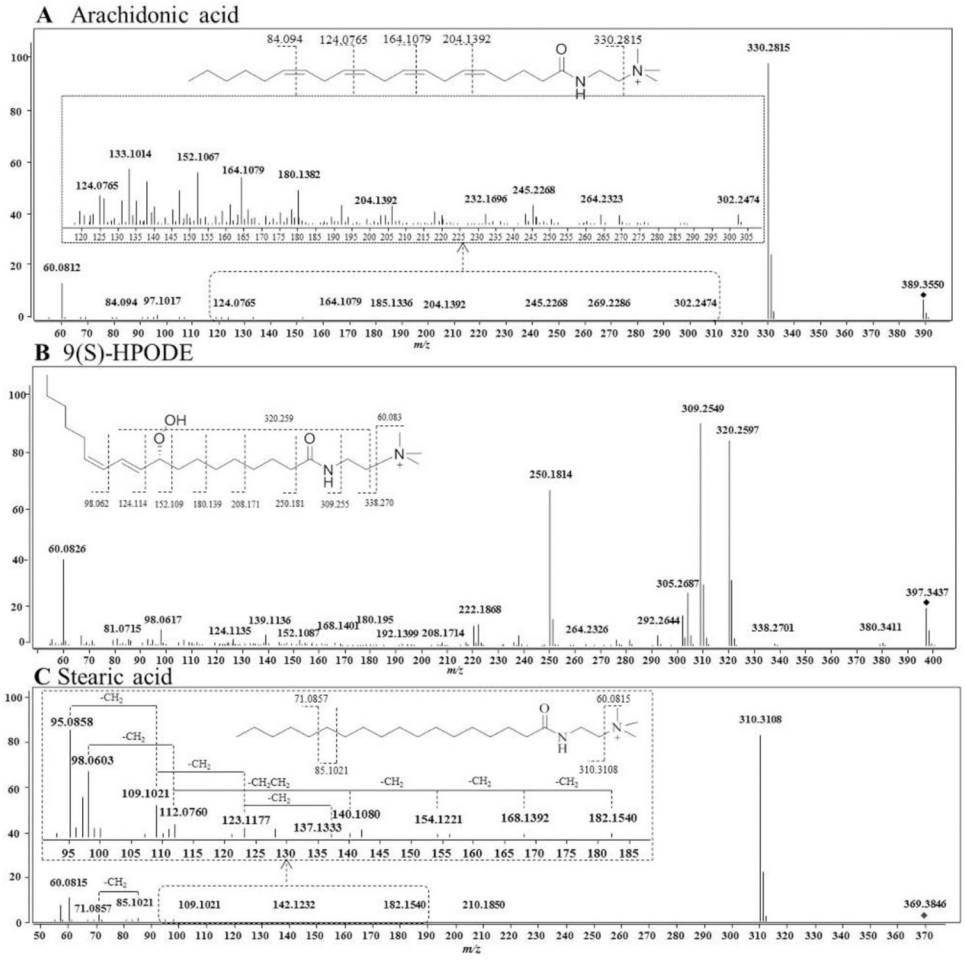

Figure 10.

Positive ion mode CID spectra for **(a)** arachidonic acid (*i.e.*, FA 20:4(5*Z*,8*Z*,11*Z*,14*Z*), **(b)** 9(*S*)-Hydroperoxyoctadecadienoic acid (HPODE), and **(c)** stearic acid (*i.e.*, FA 18:0) derivatized with cholamine. Reprinted with permission from Reference 98.

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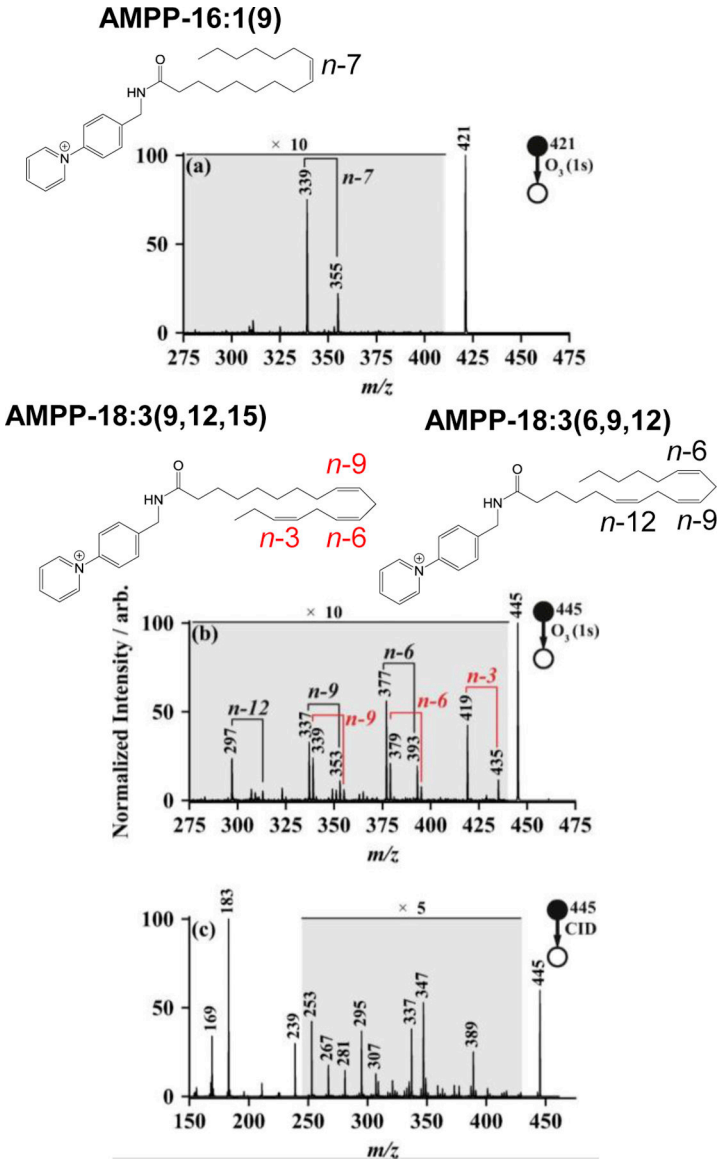

Figure 11.

Analysis of a commercially available FAME mixture using OzID in combination with charge-switch derivatization. OzID spectra of **(a)** AMPP-FA 16:1 (*m/z* 421) and **(b)** AMPP-FA 18:3 (*m/z* 445). Diagnostic product ions highlighted in **(a)** reveal the presence of FA 16:0(*n*-7). The red lines indicate peaks diagnostic for polyunsaturated FA 18:3(*n*-3, *n*-6, *n*-9), and black lines indicate peaks characteristic of FA 18:3(*n*-6, *n*-9, *n*-12) in **(b)**. **(c)** CID product ion spectrum of AMPP-FA 18:3 (*m/z* 445). Adapted with permission from Reference 113.

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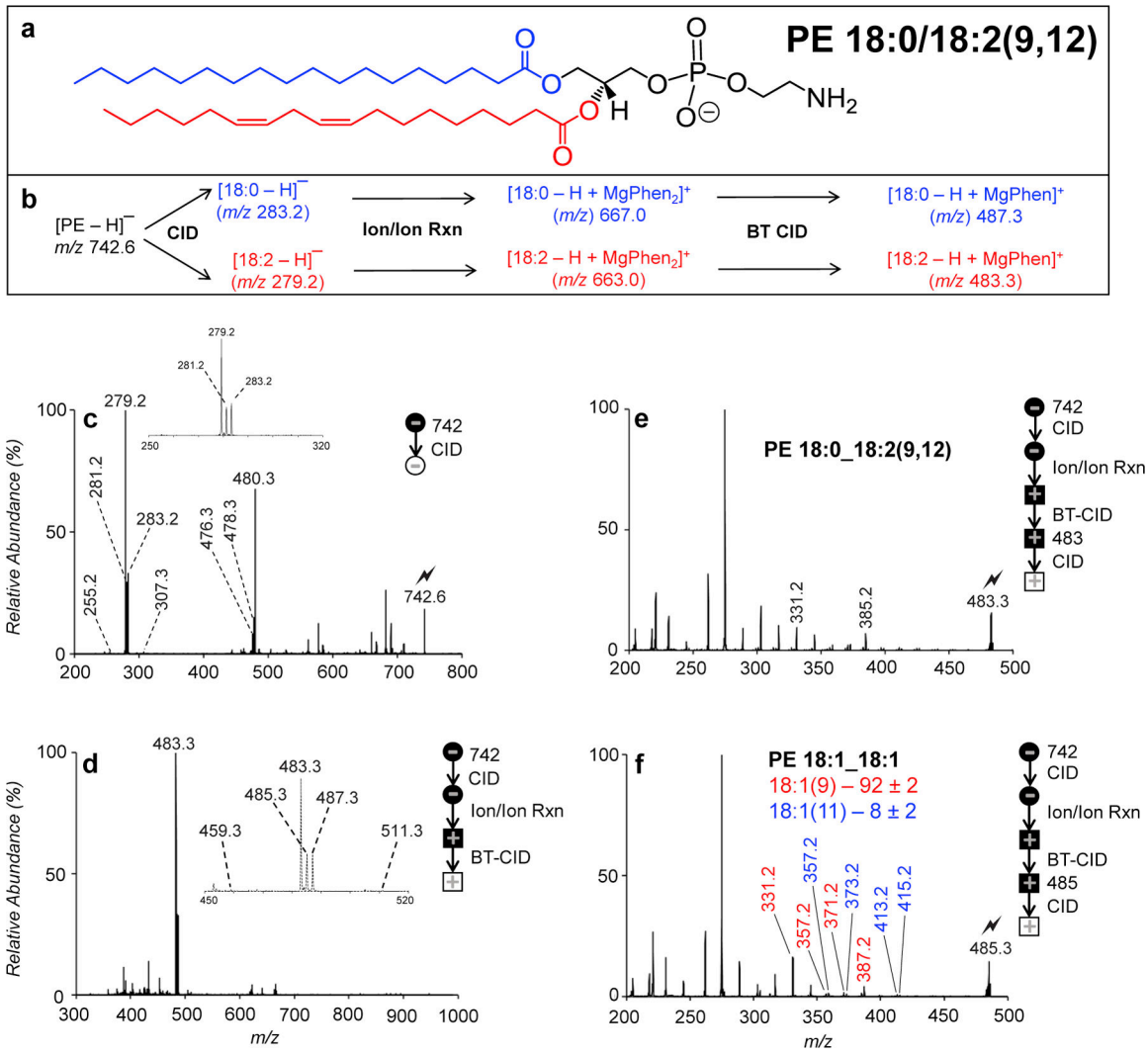

Figure 12.

Demonstration of gas-phase charge inversion ion/ion chemistry for the analysis of PE 36:2 in human plasma extract. **(a)** Structure of ionized PE 18:0/18:2(9,12). **(b)** Reaction scheme detailing identification procedure for the PE 18:0/18:2(9,12) anion. **(c)** Ion-trap CID spectrum resulting from activation of $[PE\ 36:2 - H]^-$. **(d)** Product ion spectrum following ion/ion reaction of fragment ions generated via activation of $[PE\ 36:2 - H]^-$ and $[MgPhen_3]^{2+}$ dications and subsequent beam-type CID. **(e)** CID spectrum of $[18:2 - H + MgPhen]^+$. **(f)** CID spectrum of $[18:1 - H + MgPhen]^+$. Adapted with permission from Reference 126.

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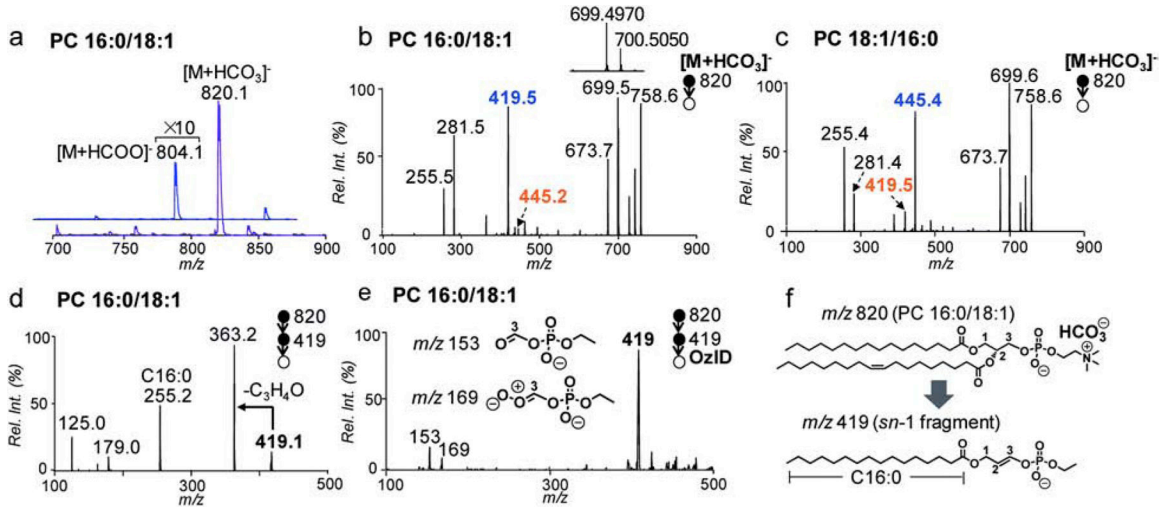

Figure 13. (a)

(a) Negative ion mode mass spectra of PC 16:0/18:1 from solutions containing 5 mM ammonium bicarbonate (NH_4HCO_3) and ammonium formate (NH_4COOH). **(b)** CID spectra (CE = 30 eV) of $[M + HCO_3]^-$ of PC 16:0/18:1 (*m/z* 820) and **(c)** $[M + HCO_3]^-$ of PC 18:1/16:0 (*m/z* 820). MS^3 product ion spectra obtained via **(d)** CID and **(e)** OzID of the product ions at *m/z* 419 from **(b)**. The proposed structure of the product ion observed at *m/z* 419 derived from CID of $[M + HCO_3]^-$ of PC 16:0/18:1 is given in **(f)**. Reprinted with permission from Reference 131.

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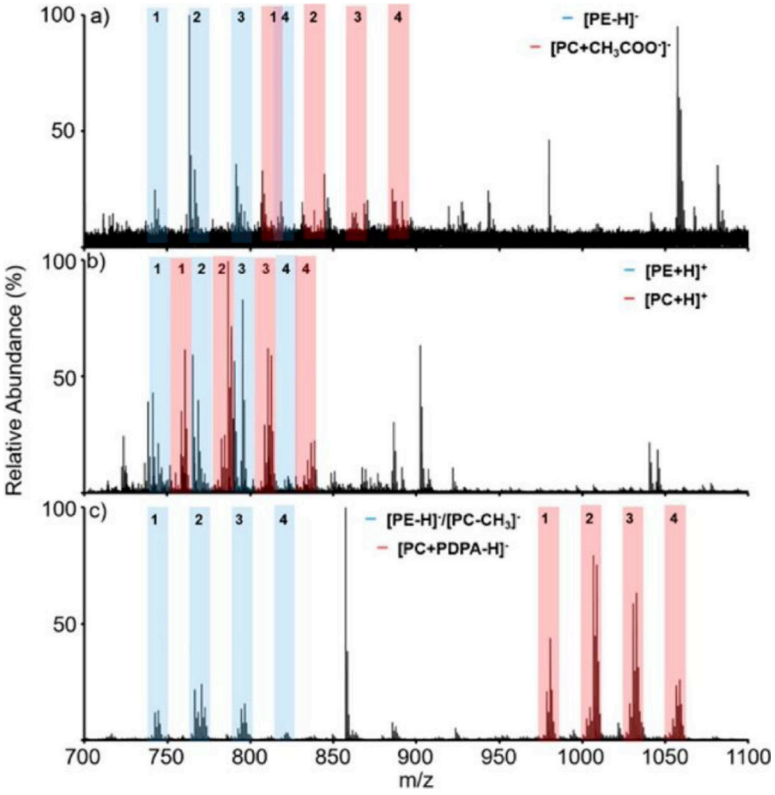

Figure 14.

Mass spectra of a bovine liver extract (0.5 μM total lipid concentration) obtained via **(a)** direct negative nano-ESI, **(b)** direct positive nano-ESI, and **(c)** charge inversion of the lipid cations shown in **(b)** with $[PDPA - 2H]^{2-}$ reagent dianions. Reprinted with permission from Reference 135.

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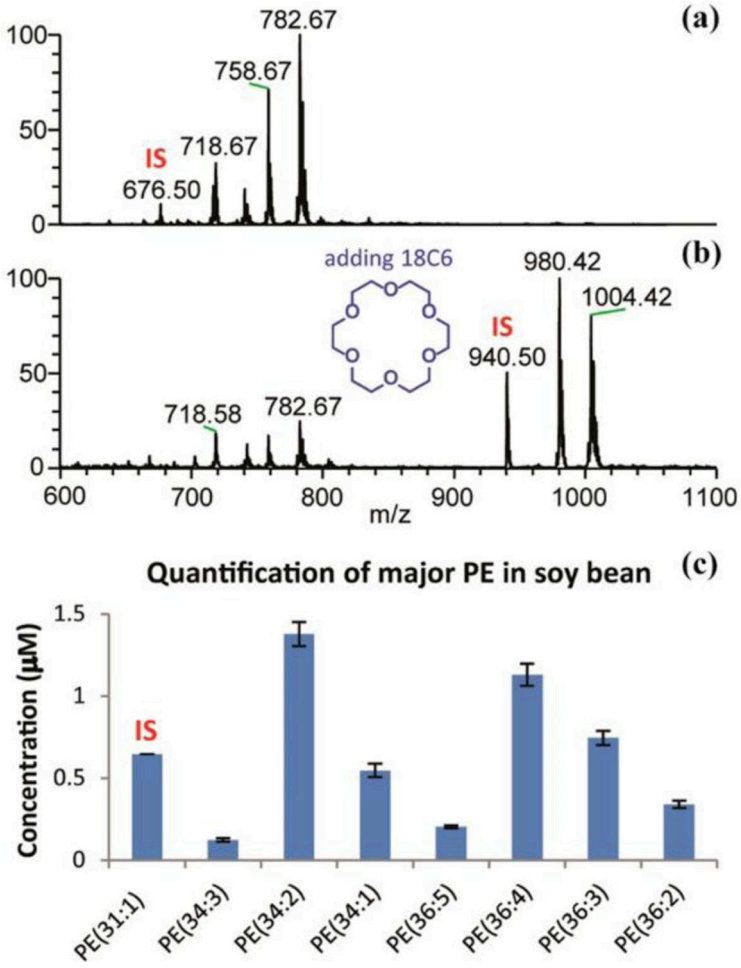

Figure 15.

Positive ion ESI-MS spectra of soybean asolectin solutions containing PE 31:1 as an internal standard (IS) within known concentration in methanol **(a)** without and **(b)** with the addition of 18C6. **(c)** The concentrations (μM) of major PEs found in soy asolectin (including isotopic corrections). Reprinted with permission from Reference 150.

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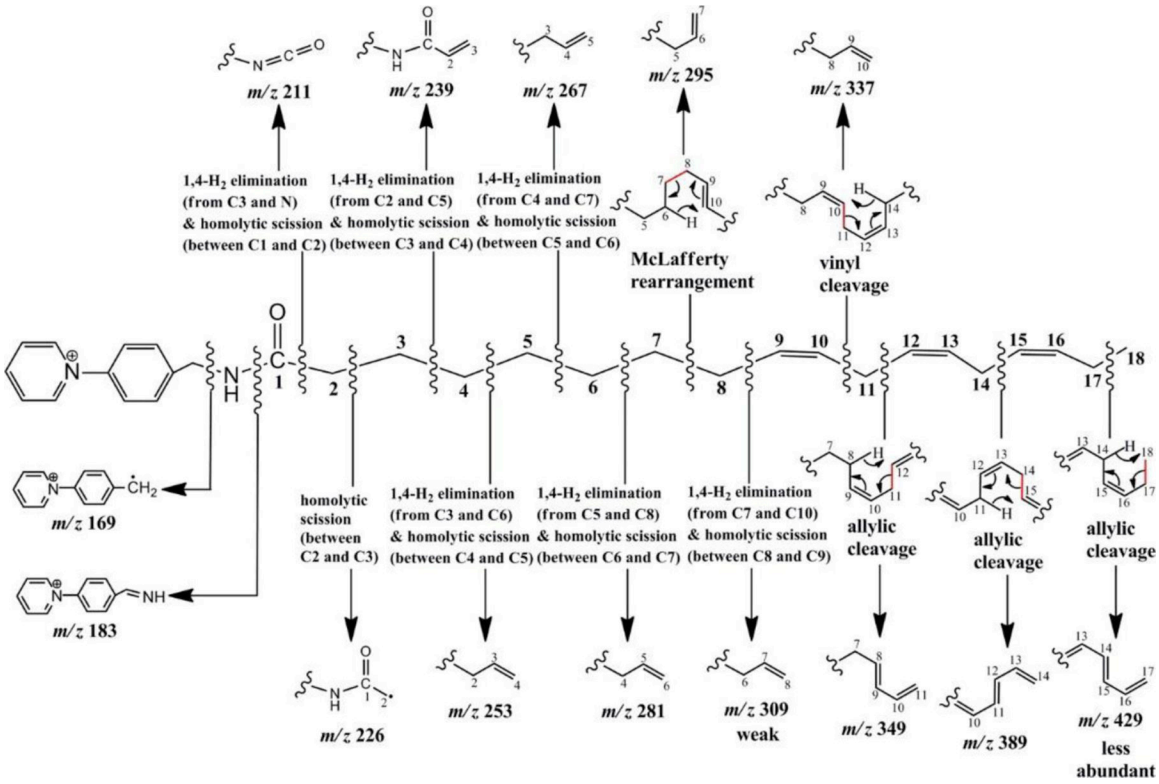

Scheme 1.

Proposed fragmentation channels for unsaturated FA-AMPP cations. The fragmentation pathway for AMPP-derivatized 18:3(9,12,15) is presented. Note that carbon-carbon bonds highlighted in red indicate the site of cleavage. Reprinted with permission from Reference 100.

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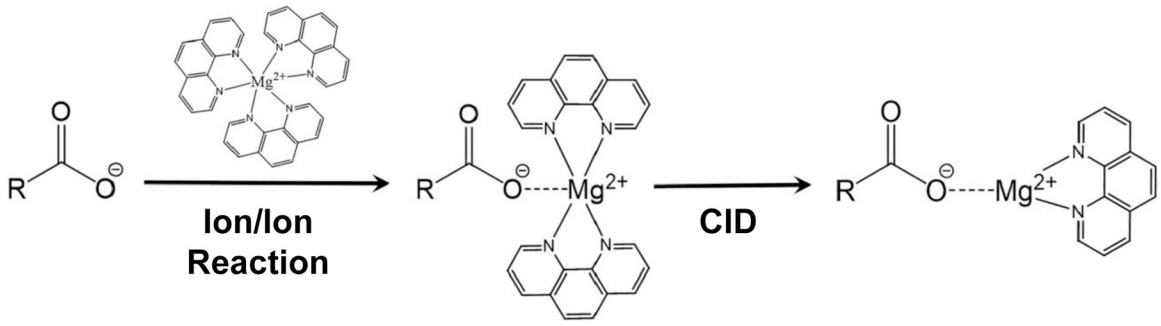
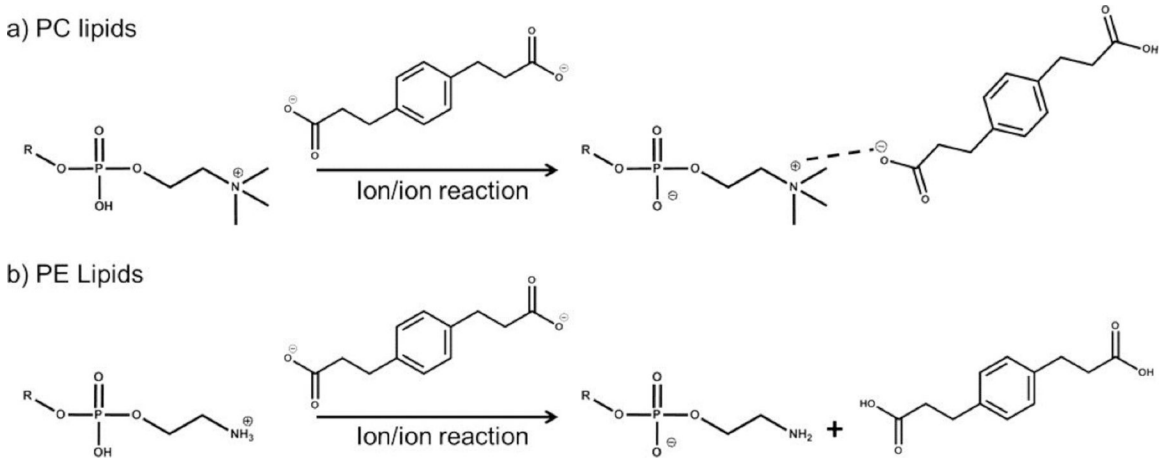


Scheme 2.

Gas-Phase Charge Inversion Ion/Ion Reaction between $[FA - H]^-$ and $[MgPhen_3]^{2+}$ for the Generation of the $[FA - H + MgPhen]^+$ Ion. Adapted with permission from Reference 125.



Scheme 3.

Charge inversion ion/ion reactions of (a) $[PC + H]^+$ and (b) [PE + H]+ cations with doubly deprotonated PDPA reagent dianions. Reprinted with permission from Reference 135.

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