

# Identification of Monomethyl Branched-Chain Lipids by a Combination of Liquid Chromatography Tandem Mass Spectrometry and Charge-Switching Chemistries

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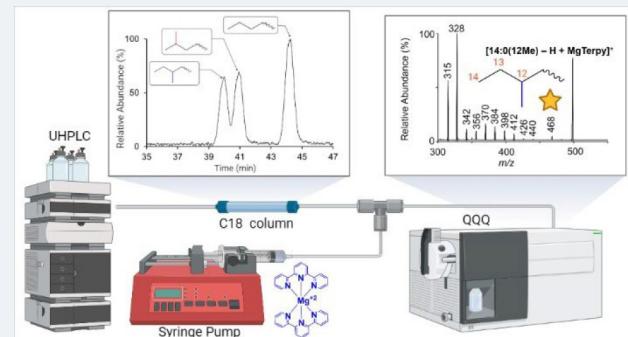
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**ABSTRACT:** While various mass spectrometric approaches have been applied to lipid analysis, unraveling the extensive structural diversity of lipids remains a significant challenge. Notably, these approaches often fail to differentiate between isomeric lipids—a challenge that is particularly acute for branched-chain fatty acids (FAs) that often share similar (or identical) mass spectra to their straight-chain isomers. Here, we utilize charge-switching strategies that combine ligated magnesium dications with deprotonated fatty acid anions. Subsequent activation of these charge inverted anions yields mass spectra that differentiate *anteiso*-branched- from straight-chain and *iso*-branched-chain FA isomers with the predictable fragmentation enabling *de novo* assignment of *anteiso* branch points. The application of these charge-inversion chemistries in both gas- and solution-phase modalities is demonstrated to assign the position of *anteiso*-methyl branch-points in FAs and, with the aid of liquid chromatography, can be extended to *de novo* assignment of additional branching sites *via* predictable fragmentation patterns as methyl branching site(s) move closer to the carboxyl carbon. The gas-phase approach is shown to be compatible with top-down structure elucidation of complex lipids such as phosphatidylcholines, while the integration of solution-phase charge-inversion with reversed phase liquid chromatography enables separation and unambiguous identification of FA structures within isomeric mixtures. Taken together, the presented charge-switching MS-based technique, in combination with liquid chromatography, enables the structural identification of branched-chain FA without the requirement of authentic methyl-branched FA reference standards.



## INTRODUCTION

Fatty acids (FAs) constitute an essential lipid class in all living systems, serving pivotal biological roles in various physiological functions and during inflammation, affecting immune response and apoptosis.<sup>1–3</sup> Moreover, FAs are building blocks of many complex lipid structures where they are coupled *via* an ester, ether, or amide linkage to structural backbones including glycerol, sphingosine, and carbohydrates. The general FA structure consists of a carboxylic acid group and a hydrocarbon chain. Variations in carbon-chain length, degree of unsaturation, location and stereochemistry of unsaturation site(s), chain branching, and functionalization (e.g., nitration, hydroxylation, etc.) lead to a vast array of potential FA structures. The modification of lipid structure impacts both chemical and physical properties. Furthermore, these structural variations inform the physiological role of individual lipid molecules.<sup>1,4,5</sup> Importantly, recent research implicates alterations in FA structure and/or composition associated with lipid metabolism dysregulation in numerous pathologies, including cancer, cardiovascular diseases, neurodegeneration, and diabetes.<sup>6–13</sup> For example, long-chain saturated fatty acids

were identified as the toxic factor killing injured neurons and oligodendrocytes in the brain during inflammation.<sup>14</sup> Consequently, analytical methods capable of accurately identifying and quantifying FAs in complex biological samples are needed in order to unravel their roles in health and disease.

Branched-chain fatty acids (BCFAs) are a unique structural subset of the fatty acyl lipid class. BCFAs are known to dominate many bacterial lipid profiles.<sup>15–20</sup> For example, BCFAs have been suggested to constitute more than 75% of the total fatty acid profile of *Bacillus subtilis*.<sup>20,15</sup> While BCFAs are minor lipid components of internal mammalian tissues, they have been detected as major components of meibomian gland secretions (within the eyelid) and vernix caseosa, the

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sebaceous wax coating human newborns.<sup>21–23</sup> Specific short

BCFAs, such as valproic acid, are used as a mood-stabilizing and antiepileptic drug but also cause neurotoxic effects and anticonvulsant effects related to neural tube defects, congenital defects, and fetal growth.<sup>24–28</sup> Among the most common BCFAs are saturated FAs with a methyl group at either the antepenultimate or penultimate carbon, known as the *anteiso*- or *iso*-position, respectively. Traditionally, gas chromatography–mass spectrometry (GC–MS) is deployed to resolve BCFAs from their straight-chain isomers and to facilitate identification and quantitation in complex biological samples.<sup>1,18,29–31</sup> In general, GC–MS analysis first requires the conversion of FA structures to fatty acid methyl esters (FAMEs) to increase analyte volatility. Unfortunately, branched-chain fatty acid methyl esters (BCFAMEs) often coelute with other FAMEs, even with the use of long, highly polar columns.<sup>31</sup> The problem of resolution and unambiguous identification is further compounded by a high degree of similarity in the conventional (70 eV) electron ionization (EI) mass spectra of isomeric FAMEs. Thus, the presence of structurally distinct FAs can be masked in both chromatographic and mass spectrometric dimensions that further hinders the detection, identification, and quantitation of BCFAs. Moreover, BCFAME identification is often accomplished using spectral matching or *via* the use of retention time alignment, both of which require the utilization of reference standards that are often costly and rarely available. To circumvent these issues, Murphy and co-workers combined electron-ionization with collision-induced dissociation, demonstrating that the activation of the molecular ions of FAMEs gives rise to mass spectra that were much more sensitive to molecular structure; including the position(s) of methyl-chain branching.<sup>32</sup> Brenna and co-workers have utilized this approach to provide detailed structure elucidation of branched-chain fatty acids in lipid extracts from human cell lines and food.<sup>18,31</sup> Notably, this approach enables the mass spectrometric resolution of coeluting GC peaks and is thus capable of both quantitative and qualitative analyses of BCFAMEs without reference standards. One challenge with this approach is that the molecular ion abundance resulting from EI of FAMEs is low and can impact sensitivity.

Alternative GC–MS approaches that can increase the sensitivity for the detection and identification of BCFAs rely upon wet-chemical derivatization strategies prior to analysis that are designed to promote sensitive detection and structurally diagnostic fragmentation upon EI. Two of the most successful and widely adopted examples include 4,4-dimethyloxazoline (DMOX) and 3-pyridylcarbinol (historically and incorrectly referenced as “picolinyl”) ester derivatives of FAs.<sup>29,33–35</sup> Both derivatives produce EI mass spectra with fragmentation patterns that can identify structural motifs including branching points in the acyl chain. Both methods require offline wet-chemical preparation prior to GC–MS. However, complex lipid hydrolysis of fatty acyl chains from complex lipid precursors results in the loss of valuable information regarding origin and function of BCFAs. Due to recent advances in electrospray-ionization mass spectrometry (ESI-MS) and liquid chromatography (LC) protocols, LC–MS is gaining wider acceptance as an alternative approach to the identification and quantification of FAs in biological extracts.<sup>1,10,14,36,37</sup> In these protocols, free

FA (or those liberated by hydrolysis) can be subjected to LC–MS directly or analysis of complex lipids can be analyzed by

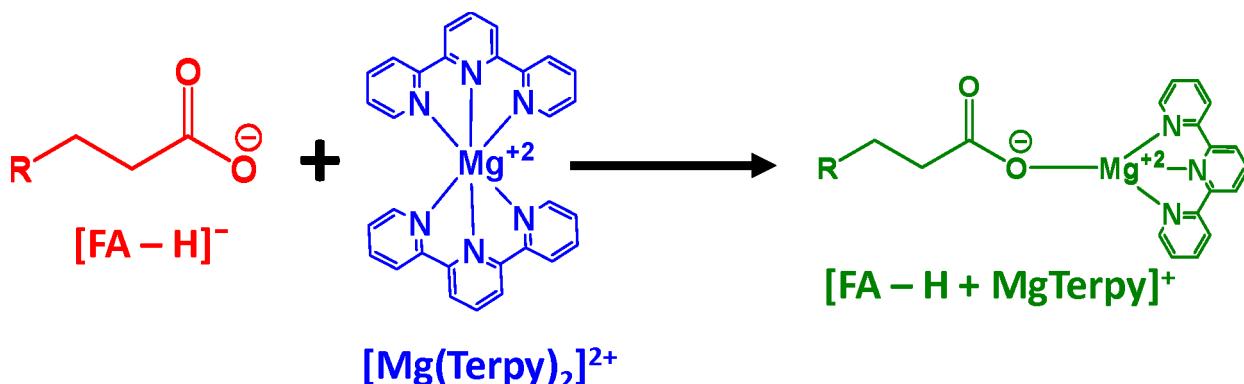
LC–MS with subsequent interrogation of the FA building blocks enabled by fragmentation of the precursor lipid in tandem mass spectrometry (MS/MS) modalities. When subjected to conventional low energy collision-induced dissociation (CID), singly deprotonated FA anions (denoted  $[FA - H]^-$  and formed directly or as fragmentation products ions from complex lipids) typically results in very little fragmentation from dissociation of the carbon–carbon bonds in the acyl chain, as the dominant product ions observed correspond to decarboxylation ( $-CO_2$ ) and dehydration ( $-H_2O$ ) of the FA precursor anion.<sup>38,39</sup> To increase the generation of structurally informative product ions, charge-inversion of FAs has been explored, often in concert with novel laser-based dissociation strategies like photodissociation (PD) and ultraviolet photodissociation (UPD).<sup>40–45</sup> Extending the radical-directed dissociation approaches for complex lipids developed by the Julian and Blanksby groups, the Xia group has identified the location(s) of methyl branching in bacterial glycerophospholipids, including sphingomyelins, phosphatidylcholines, and phosphatidylethanolamines.<sup>42,46–49</sup> Recently, we have described the efficacy of gas-phase ion/ion charge inversion reactions for the identification of FA both in nonesterified forms and also when esterified within complex lipids.<sup>50–57</sup> This strategy has significant advantages as it can exploit the preferred ionization and fragmentation of many lipids in negative ion mode while taking advantage of structurally selective charge-remote fragmentation in charge-inverted positive ions. Herein, we demonstrate the ability of charge-inversion ion/ion reactions combined with CID to differentiate isomeric BCFAs. Additionally, we present the development of a LC–MS/MS method that deploys postcolumn charge-switch derivatization to provide online and unambiguous discrimination of (1) nonbranched and branched acyl chains from isomeric methyl-branched lipid structures and (2) the identification of methyl chain branching site in saturated BCFAs.

## EXPERIMENTAL SECTION

**Nomenclature.** Here, we adopt the shorthand notation recommended by Liebisch et al.<sup>58</sup> Briefly, fatty acyl chains are described by the total number of carbons, as indicated before the colon, and the total number of double bonds, as indicated after the colon. When present, the methyl branch functional group is abbreviated as “Me”. Identified positions of methyl branching are shown within parentheses after the integer indicating the number of double bonds. For example, 13-methyltetradecanoic acid, commonly referred to as isopentadecylic acid (*iso* 15:0), can be represented as FA 14:0(13Me). Similarly, 12-methyltetradecanoic acid, commonly referred to as anteisopentadecylic acid (*anteiso* 15:0), can be represented as FA 14:0(12Me).

**Materials.** HPLC-grade methanol, water, and acetic acid solution were purchased from Fisher Scientific (Pittsburgh, PA). Magnesium chloride and 2,2':6',2"-terpyridine (Terpy) were purchased from Millipore-Sigma (St. Louis, MO). The following lipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL): 12-methyltetradecanoic acid (FA 14:0(12Me) or *anteiso* 15:0), 13-methyltetradecanoic acid (FA 14:0(13Me) or *iso* 15:0), 1-palmitoyl-(12S-methylmyristoyl)-*sn*-glycero-3-phosphocholine (PC 16:0/*anteiso* 15:0 or PC 16:0/14:0(12Me)), and 1-palmitoyl-(13S-methylmyristoyl)-*sn*-glycero-3-phosphocholine (PC 16:0/*iso* 15:0 or PC 16:0/

Scheme 1. Generation of  $[FA - H + MgTerpy]^+$  Complex Cations *via* Ion/Ion Reaction of Singly Deprotonated FA Anions and Reagent Bis-Terpyridine Magnesium Dications



14:0(13Me)). Pentadecanoic acid (FA 15:0) was purchased from Cayman Chemical (Ann Arbor, MI).

**Gas-Phase Ion/Ion Experiments.** All gas-phase charge inversion data were collected on a Sciex QTRAP 4000 hybrid triple quadrupole/linear ion trap mass spectrometer (SCIEX, Concord, ON, Canada) with modifications analogous to those previously described.<sup>59</sup> Alternately, pulsed nanoelectrospray ionization (nESI) allows for the sequential injection of lipid anions and charge inversion reagent bis-terpyridine magnesium dications,  $[MgTerpy_2]^{2+}$ .<sup>60</sup> Mass spectra were produced *via* the analysis of ions using mass-selective axial ejection (MSAE).<sup>61</sup>

**Scheme 1** illustrates the generation of  $[FA - H + MgTerpy]^+$  complex cations. Briefly, singly deprotonated FA anions are transformed *via* gas-phase ion/ion reaction with bis-terpyridine magnesium dications. First,  $[FA - H]^-$  were generated *via* direct negative nESI of either (1) a methanolic solution of fatty acid standard or (2) from the liberation fatty acyl carboxylate anions from a phospholipid precursor anion utilizing collisional activation of lipid anions in q2 *via* single frequency resonance excitation, which results in the cleavage of *sn*-1 and *sn*-2 ester bonds and production of singly deprotonated FA anions.<sup>52</sup> Following the generation of  $[FA - H]^-$  anions, reagent dications were mass-selected during transit through Q1 and transferred to q2. Together in q2, reagent  $[MgTerpy_2]^{2+}$  dications and  $[FA - H]^-$  anions were permitted to react, yielding charge-inverted  $[FA - H + MgTerpy]^+$  complex cations. Next, monoisotopic isolation and ion trap CID of charge-inverted FA complex cations permit unambiguous isomeric *anteiso*-branched- and straight-chain lipid discrimination *via* exploitation of reproducible spectral patterns.

**LC-MS/MS Experiments.** Lipid samples dissolved in methanol at  $\sim 1 \mu M$  were placed in the autosampler of an Agilent 1290 Infinity II LC System (Santa Clara, CA). One microliter of lipid solution was injected onto an Ascentis Express C18-HPLC column (150 mm  $\times$  3 mm, 2.7  $\mu m$ ) at 25 °C. The mobile phase A was methanol, and mobile phase B was 97/3 Water/MeOH with 15 mM acetic acid (v/v). To achieve separation, a gradient beginning with 80% B and increasing to 88% B over 73 min at a flow rate of 0.2 mL min<sup>-1</sup> was used. At 74 min, the mobile phase composition was returned to and held at initial conditions for a total of 11 min to re-equilibrate the column. The total run time was 85 min. The postcolumn eluent was combined with a methanolic solution of  $[MgTerpy_2]^{2+}$  (100  $\mu M$ ) *via* a syringe pump and T-junction prior to infusion into the mass spectrometer to

facilitate the formation of  $[FA - H + MgTerpy]^+$  cations. To make the solution of  $[MgTerpy_2]^{2+}$ , equimolar amounts of magnesium chloride and 2,2':6',2"-terpyridine were first combined and dissolved in methanol, before dilution prior to analysis.

Mass spectra were recorded using an Agilent 6495C triple quadrupole mass spectrometer (Santa Clara, CA) equipped with a Jet Stream ESI source. All mass spectra reported here are the result of 30–50 scan averages. The instrument was operated in positive ion mode. Product ion scans were collected using a normalized collision energy (CE) of 55 eV. Nitrogen served as the sheath, drying, and collision gases. MS parameters were optimized and set as drying gas temperature at 150 °C, dry gas flow rate at 15 L min<sup>-1</sup>, sheath gas temperature at 225 °C, sheath gas flow rate at 11 L min<sup>-1</sup>, nebulizer pressure at 30 psi, capillary voltage at 3500 V, and nozzle voltage at 1500 V.

## RESULTS AND DISCUSSION

**Saturated Straight-Chain and Branched Lipid Analysis Using Ion/Ion Chemistry.** Saturated and branched FA anions undergo charge inversion when subjected to mutual storage with  $[MgTerpy_2]^{2+}$  reagent dications in the high-pressure collision cell q2. As a result of the ion/ion reaction, singly deprotonated FA anions are transformed to  $[FA - H + MgTerpy]^+$  cations (Supporting Information Figure S1). Figure 1 shows the ion trap CID spectra of a series of  $[FA - H + MgTerpy]^+$  ions derived from straight-chain FA 15:0 and two branched-chain variants, 13-methyltetradecanoic acid (FA 14:0(13Me)) and 12-methyltetradecanoic acid (FA 14:0(12Me)). The CID spectrum of  $[FA 15:0 - H + MgTerpy]^+$  displays an uninterrupted series of product ions between *m/z* 315 and 468 with 14 Da spacings, noting that product ion relative abundance generally decreases as carbon–carbon fragmentation approaches the methyl end of aliphatic chain (Figure 1A). Collisional activation of the charge-inverted FA 14:0(12Me) ion, as shown in Figure 1B, contains a series of product ions spaced at 14 Da apart and generated *via* carbon–carbon bond cleavage beginning at C2–C3 (*m/z* 315) and ending at C12–C13 (*m/z* 468). Importantly, as fragmentation approaches the methyl branching site, a dramatic suppression in product ion relative abundance is observed, as highlighted with the low abundance product ion observed at *m/z* 454. Flanking the methyl branching site, carbon–carbon bond cleavage generates more highly abundant product ions, as indicated with the product ions observed at *m/z* 440 and *m/z*

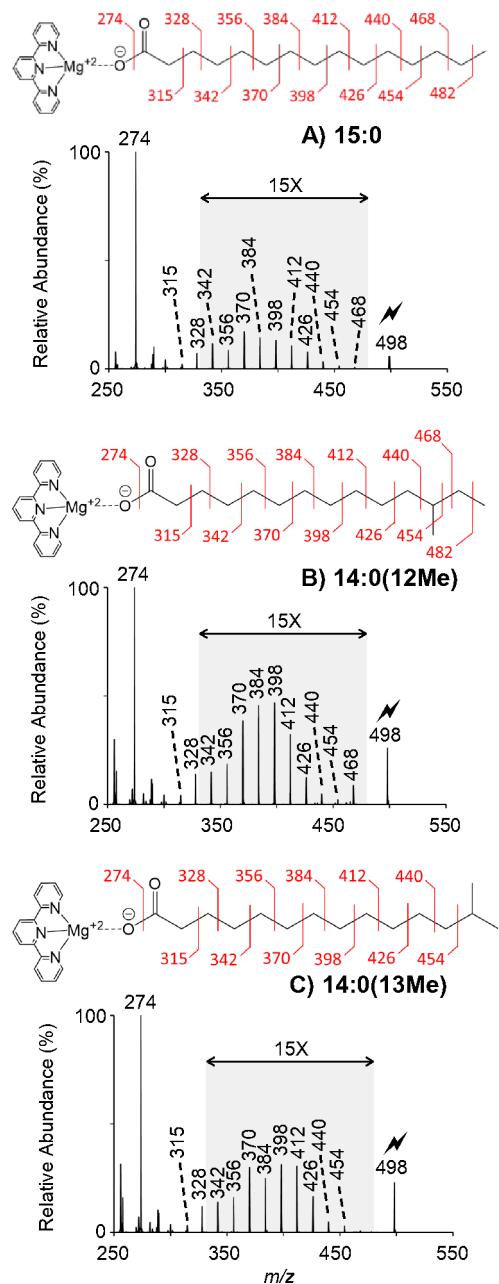


Figure 1. Ion-trap CID of  $[M - H + \text{MgTerpy}]^+$  derived from the gas phase ion/ion reaction of  $[\text{MgTerpy}_2]^{2+}$  with the  $[M - H]^-$  anions, where  $M =$  (A) FA 15:0, (B) FA 14:0(12Me), and (C) FA 14:0(13Me).

468 (Figure 1B). In comparison, Figure 1C displays the CID spectrum of  $[\text{FA 14:0(13Me)} - H + \text{MgTerpy}]^+$ . Notably absent in the product ion spectrum of the charge inverted FA 14:0(13Me) ion is an abundant product ion at  $m/z$  468. However, the product ion spectra of  $[\text{FA 14:0(13Me)} - H + \text{MgTerpy}]^+$  (Figure 1C) and  $[\text{FA 15:0} - H + \text{MgTerpy}]^+$  (Figure 1A) are nearly identical, with only subtle differences in relative product ion abundances observed. Thus, the *anteiso* isomer can be distinguished from straight-chain and *iso* isomers, but the latter cannot be distinguished from each other. Therefore, other methods must be used to distinguish the latter two isomers.

The development of a top-down shotgun-MS method utilizing gas phase ion/ion charge inversion chemistry provides

near-complete glycerophospholipid (GPL) structural identification as described previously.<sup>52</sup> To date, few approaches are capable of discerning methyl-branching site in complex lipid structures. We report an  $\text{MS}^n$  experiment involving ion/ion chemistry to facilitate the assignment of the GPL headgroup and fatty acyl composition, identification of the presence of *anteiso* methyl branching position in saturated acyl chains, and, in some cases, assignment of fatty acyl *sn*-position. To demonstrate this approach, we examined synthetic phosphatidylcholines (PCs), including PC 16:0/14:0(12Me) and PC 16:0/14:0(13Me). Briefly, as ionization of PCs in negative ion mode relies on the formation of an adduct ion (e.g.,  $[M + X]^-$  where  $X = \text{Cl}$  or  $\text{CH}_3\text{CO}_2$ ), direct negative nESI of PC 16:0/14:0(12Me) resulted in the generation of abundant  $[\text{PC} + \text{CH}_3\text{CO}_2]^-$  precursor anions. Next, CID of mass-selected  $[\text{PC 16:0/14:0(12Me)} + \text{CH}_3\text{CO}_2]^-$  ( $m/z$  778) generated demethylated PC product anions (i.e.,  $[\text{PC 16:0/14:0(12Me)} - \text{CH}_3]^-$ ) detected at  $m/z$  704. The product ion spectrum of  $[\text{PC 16:0/14:0(12Me)} + \text{CH}_3\text{CO}_2]^-$  ( $m/z$  778) displayed in Figure S2 ultimately permits the identification of the polar headgroup. To release FA anions from the GPL precursors, subsequent ion-trap CID of the  $[\text{PC 16:0/14:0(12Me)} - \text{CH}_3]^-$  was employed, as detailed in Figure 2A. The greater abundance of the *sn*-2 [14:0(12Me) - H]<sup>-</sup> fragment ion ( $m/z$  241) relative to the *sn*-1 [16:0 - H]<sup>-</sup> ( $m/z$  255) is in good agreement with previous observations and suggests preferential formation of the carboxylate anion from the *sn*-2 acyl substituent.<sup>62</sup> Importantly for unknown lipids, fatty acyl

chain regiochemical assignments based only on relative abundances of the carboxylate anions alone are insufficient without calibrating to standards and should be made with caution.<sup>63</sup> However, it can provide a useful guide to the dominant regiochemistry, i.e., the most abundant regioisomer. To generate charge-inverted FA complex cations, all  $\text{MS}^3$  product ions, including  $[\text{FA} - \text{H}]^-$  anions, were allowed to react with  $[\text{MgTerpy}_2]^{2+}$  dication. The ion/ion reaction mostly resulted in the formation of  $[\text{FA} - \text{H} + \text{MgTerpy}]^+$  complex cations (Figure 2B). Subsequent interrogation of the

charge-inverted 14:0(12Me) complex cation permits the unambiguous assignment of methyl branching site in the same fashion as described above (Figure 2C). Employing an identical  $\text{MS}^n$  and ion/ion approach, isomeric PC 16:0/14:0(13Me) was examined, noting that the  $\text{MS}^2$ ,  $\text{MS}^3$ , and ion/ion product ion spectra are identical to those described for PC 16:0/14:0(12Me). However, in the case of PC 16:0/14:0(13Me), collisional activation of the MgTerpy derivative of FA 14:0(13Me) generates a CID spectrum distinguishable from that of FA 14:0(12Me) derived from PC 16:0/14:0(12Me) (c.f. Figure 2C,D). Importantly, the CID spectra of charge-inverted-branched FAs derived from nonesterified FAs or from complex GPL precursor anions are identical, meaning that this workflow can be utilized to examine a diverse

range of branched lipid species. Moreover, as all GPL classes can be ionized in negative ion mode, fatty acyl anions can be liberated from any GPL precursor anion regardless of headgroup composition using low-energy CID. Thus, the developed gas-phase ion/ion chemistry can be applied to unravel GPL structure, including methyl branching position, independent of the polar headgroup present, further highlighting the versatility of ion/ion platforms.

**Charge-Switching and LC-MS/MS of Synthetic-Branched Fatty Acids.** While effective, the inherent complexity of the cellular lipidome can complicate lipid

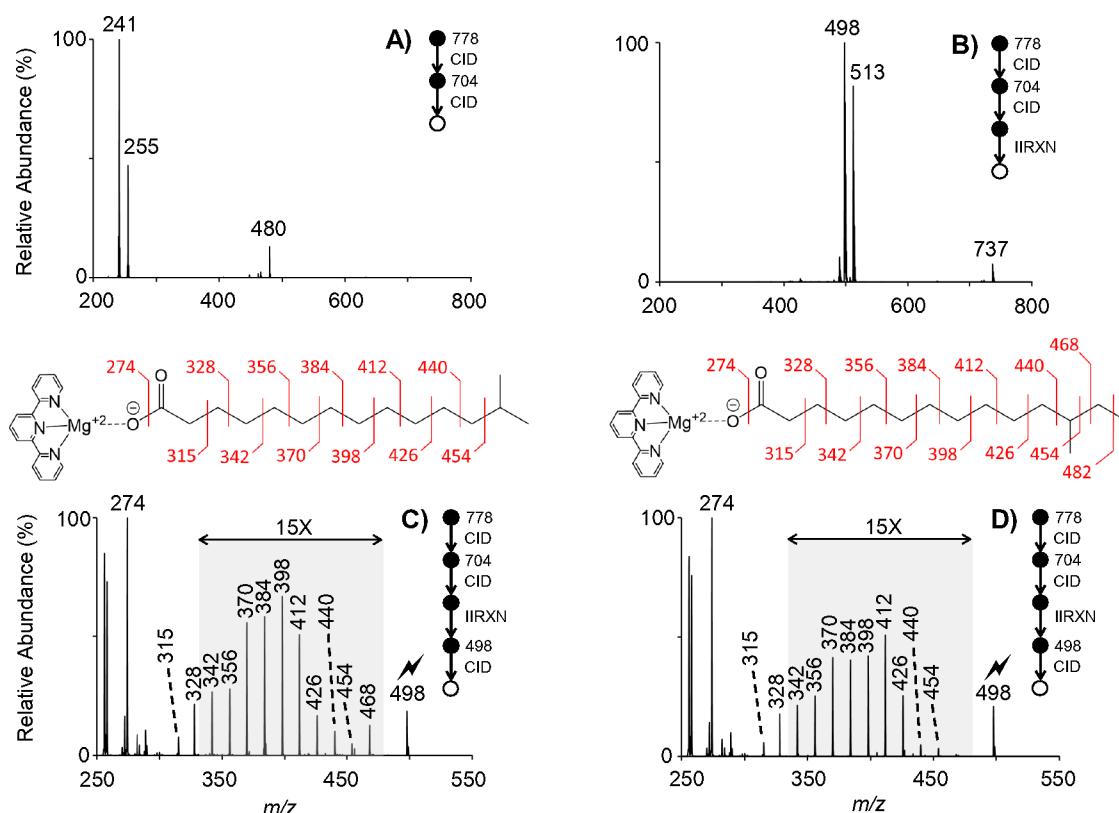


Figure 2. Sequence of MS events used to interrogate the molecular structures of the phosphatidylcholine isomers, PC 16:0/14:0(12Me) and PC 16:0/14:0(13Me). (A) Ion-trap CID spectrum of  $[PC\ 16:0/14:0(12Me)\ - CH_3]^-$  ( $m/z\ 704$ ). (B) Charge inversion spectrum generated via the gas-phase ion/ion reaction between product ions generated from CID of the  $[PC\ - CH_3]^-$  precursor ion as shown in (A) and  $[MgTerpy_2]^{2+}$  reagent dications. Ion-trap CID product ion spectrum following monoisotopic mass selection and collisional activation of the branched FA complex cation at  $m/z\ 498$ , derived from (C) PC 16:0/14:0(12Me) and (D) PC 16:0/14:0(13Me).

identification, especially when utilizing a shotgun approach, as multiple isomers can be present in a single sample. Moreover, the extensive fragmentation pattern observed upon CID of charge-switched FAs only further complicates accurate identification, particularly in the case of complex mixture analysis. While the ion/ion approach described above can confidently distinguish *anteiso*- and *iso*-branched lipid isomers, this approach is useful only when the straight-chain isomer is not present. As the CID spectra of charge-switched *iso*- and straight-chain lipid isomers are nearly identical, structural assignments in the absence of an *anteiso*-branched lipid isomer can be ambiguous. To circumvent the shortcomings of the shotgun ion/ion approach, an alternate LC-MS/MS strategy utilizing postcolumn wet-chemical charge-switch derivatization was developed. Importantly, the presented LC-MS/MS charge-switching strategy not only enhances mixture analysis performance but also eliminates ambiguities in the assignment of isomeric saturated FA structures. Specifically, this platform includes reversed phase liquid chromatography (RPLC) interfaced with a commercially available triple quadrupole mass spectrometer. The experimental set up is depicted in Figure 3A. As a first step,  $[FA\ - H + MgTerpy]^{+}$  complex cations were first generated in solution. To do so,  $MgCl_2$ , 2,2':6',2"-terpyridine, and a synthetic FA standard were combined in a methanolic solution at room temperature. Next, the methanolic solution was infused directly into the mass spectrometer. Upon ESI, abundant charge-switched FA anions were observed. Notably, the MS/MS spectra of  $[14:0(12Me)\ - H + MgTerpy]^{+}$  ( $m/z\ 498$ ) and  $[14:0(13Me)$

$- H + MgTerpy]^{+}$  ( $m/z\ 498$ ) obtained on the triple quadrupole mass spectrometer following condensed-phased FA derivatization (Figure S3) are identical to those obtained utilizing the gas-phase ion/ion approach described previously for nonesterified FA structures.

Chromatography coupled with mass spectrometry is a well-established means of overcoming the associated challenges with shotgun-MS experiments, providing improved resolution of molecular species. In our approach, the lipid sample was first separated by RPLC. Using a C18 column with the above-described gradient (see the LC-MS/MS Experiments section), a synthetic standard of 14:0(12Me) eluted ca. 1 min earlier than isomeric 14:0(13Me), as shown in Figure 3B. Importantly, excellent chromatographic resolution of straight-chain FA 15:0 from the two branched-chain variants was achieved, as FA 15:0 eluted nearly 3 min after isomeric 14:0(13Me) (Figure 3B). Although branched-chain FAs are known to elute earlier than their straight-chain isomers on reversed-phase columns, the elution order may not be sufficient for structural assignment in a complex mixture, especially if unknown branched points are present. As described before, the MS/MS spectra of underderivatized (i.e., singly deprotonated FA anions) saturated and branched FA isomers are identical, leading to ambiguous FA identification in the absence of authentic reference standards.

In turn, postcolumn charge-switch derivatization was utilized to enhance structural information obtained from fragmentation experiments. Briefly, the introduction of bis-terpyridine magnesium complex dications as the derivatization reagent

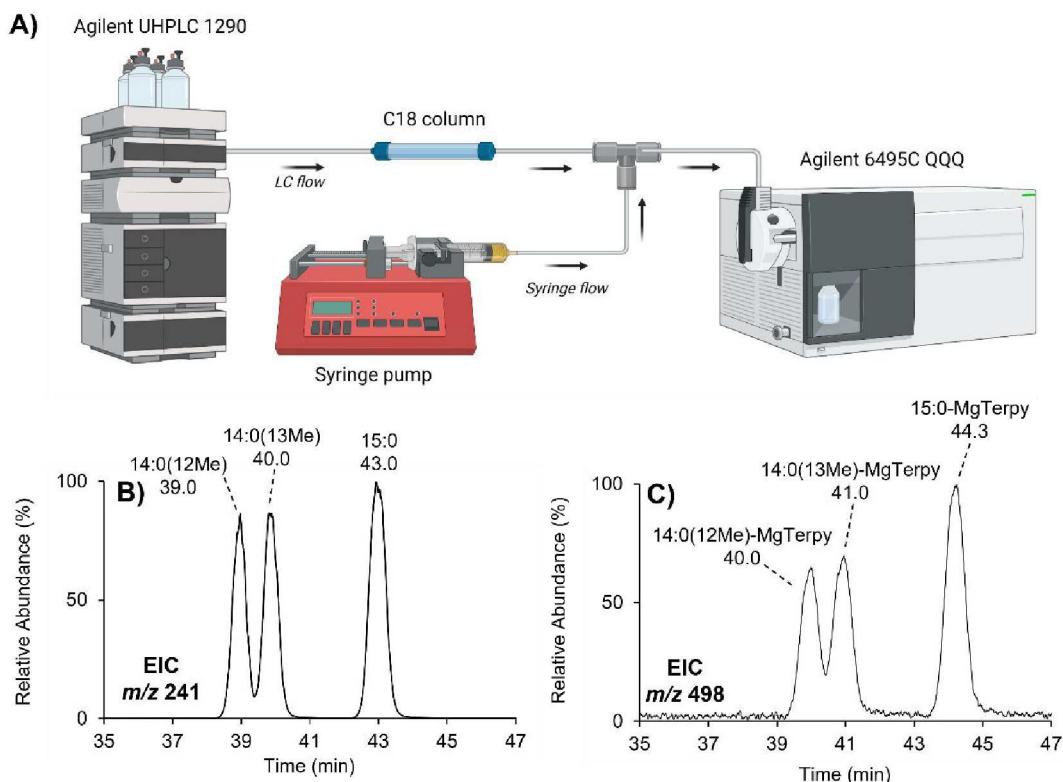


Figure 3. (A) Illustrative schematic of postcolumn charge-switching LC-MS/MS method. (B) EIC of  $m/z$  241 without FA derivatization (i.e., negative ion mode). (C) EIC of  $m/z$  498 with FA derivatization (i.e., positive ion mode).

via a postcolumn tee injunction (Figure 3A) facilitated the formation of charge-switched [FA – H + MgTerpy]<sup>+</sup> ions from chromatographically resolved FA structures. The extracted ion chromatograms (EICs) of the charge-switched FA products ( $m/z$  498) in Figure 3C denote that the slight shift in retention time (c.f. Figure 3B,C) is attributed to the increase in dead time associated with the tee junction setup. Figure 3C displays three abundant peaks, corresponding to the charge-switched postcolumn derivatization FA products. The first two eluting analytes correspond to the charge-switched FA 14:0(12Me) (RT = 40.0 min) and 14:0(13Me) (RT = 41.0 min) products. Last to elute is the straight-chain FA 15:0 (RT = 44.3 min). Based on these results, methyl-branched FA isomers can be distinguished from not only each other but also their straight-chain counterparts based on retention time.

The LC-MS/MS spectra of postcolumn derivatized FAs 15:0, 14:0(12Me), and 14:0(13Me) are shown in Figure 4 and display key diagnostic fragmentation patterns analogous to those described before utilizing the gas-phase ion/ion approach. Specifically, the product ion observed at  $m/z$  468 serves as a diagnostic signature of *anteiso*-methyl-chain branching. The indicator product ion at  $m/z$  468 is readily observed in the LC-MS/MS spectrum of charge-switched FA 14:0(12Me), as shown with Figure 4A, and is notably suppressed in the LC-MS/MS spectra of charge-switched FA 14:0(13Me) (Figure 4B) and FA 15:0 (Figure 4C) structures, respectively. We note that, while the product ion spectra of the saturated straight-chain FA 15:0 and *iso*-branched FA 14:0(13Me) are nearly identical, excellent chromatographic separation of these isomers prior to MS/MS affords unambiguous FA identification *via* retention time matching utilizing authentic straight-chain FA reference standards, which are readily available from a surplus of

commercial vendors. Therefore, the combination of LC and postcolumn charge-switch derivatization can readily distinguish saturated straight-chain and methyl-branched FA isomers with detailed structural information on methyl branching sites for respective FA structures. Lastly, noting that fragmentation patterns observed utilizing the presented online LC charge-switching approach are predictable, reproducible, and dependent on saturated FA structure, methyl branching assignments could be made algorithmically, supporting high-throughput FA identification.

## CONCLUSIONS

We have demonstrated two separate charge-switching approaches for the detailed structural identification of BCFAs. The first approach utilizes an entirely gas-phase strategy in which FA anions are transformed *via* charge inversion ion/ion reactions with magnesium bis-terpyridine reagent dications, yielding abundant [FA – H + MgTerpy]<sup>+</sup> complex cations. Upon collisional activation, charge inverted BCFA complex cations fragment provide distinct diagnostic product ions indicative of methyl-chain branching site. Importantly, the presented MS<sup>n</sup> gas-phase charge inversion strategy can readily be extended to identify methyl-chain branching site on saturated fatty acyl chains esterified in glycerophospholipid structures. However, for shotgun approaches, the existence of multiple isomeric species within a complex mixture may become problematic and hinder accurate BCFA identification. In addition, the gas-phase ion/ion reactions require specialized instrument modifications and highly trained personnel to conduct these analyses. Thus, to improve isomeric mixture analysis performance and ease of use, an LC-MS/MS method employing postcolumn charge-

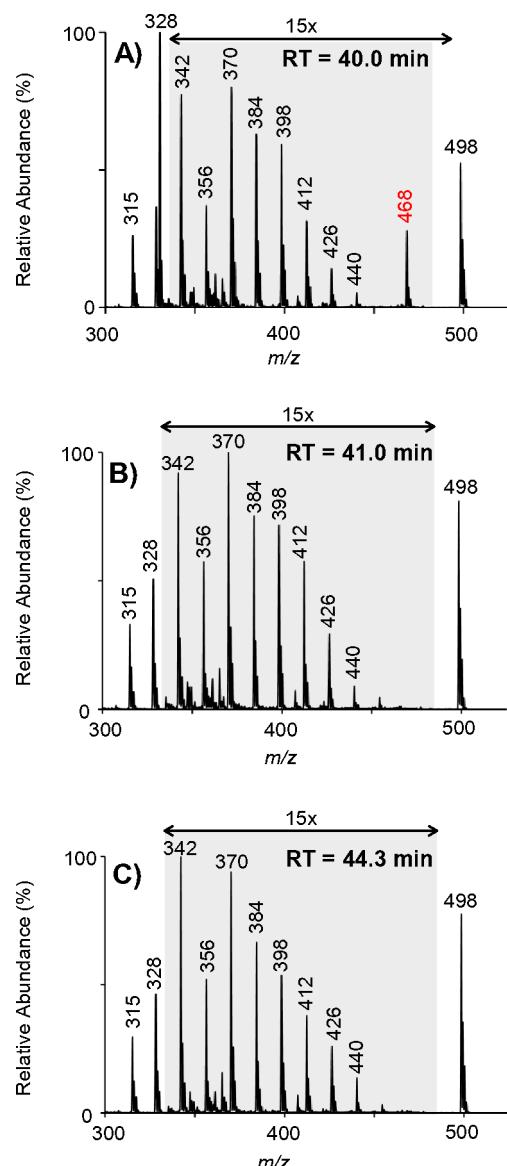


Figure 4. LC-MS/MS spectra of charge-switched (A) FA 14:0(12Me), (B) FA 14:0(13Me), and (C) FA 15:0.

switching derivatization was established based on conventional LC and MS platforms. In this approach, BCFA isomers are effectively separated from each other and from their straight-chain counterparts. Excellent chromatographic resolution alone affords the accurate identification of saturated FA isomers based on retention time matching with authentic reference standards. However, given that several BCFA reference standards are not readily or commercially available, post-column charge-switch derivatization facilitated by a continual infusion of magnesium bis-terpyridine reagent solution and subsequent MS/MS affords facile identification of methyl branching site by exploiting the generation of significant indicator product ions influenced by acyl chain branching. Consequently, postcolumn generation of MgTerpy-adducted FAs permits the localization of methyl-chain branching site in the absence of authentic reference standards. The analysis of intact methyl-branched complex lipids could not be achieved using this configuration, as it would first require condensed-phase hydrolysis of ester bonds linking acyl chains to the

glycerol backbone of complex lipids and subsequent loss of information on the intact structure.

LC-based separations of branched-chain and straight-chain isomers of complex lipids have been demonstrated using long gradients.<sup>64</sup> Future efforts will focus on combining such LC methodologies with ion/ion chemistries for near-complete structure elucidation of complex lipids with branched-chain structures. Developing a chemical conjugation toolbox combined with LC-MS/MS promises to empower further discovery of structural complexity within the lipidome.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jasms.2c00225>.

Figures of product ion spectrum resulting from the mutual storage ion/ion reaction between  $[\text{FA} - \text{H}]^-$  anions and  $[\text{MgTerpy}]^{2+}$  reagent cations, CID spectrum of  $[\text{PC } 16:0/14:0(12\text{Me}) + \text{CH}_3\text{CO}_2]^-$ , and MS/MS spectra of BCFA-MgTerpy complex cations obtained on the triple quadrupole mass spectrometer following condensed-phased charge-switch derivatization (PDF)

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### Notes

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