Journal of the American Society for Mass Spectrometry

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Article

Quantitative Isomer Profiling of Synthetic Phosphatidylethanol by NMR and Advanced MS Techniques

Matthias Bantle,[▽] Lana Brockbals,[▽] Alvar D. Gossert, Ilche Gjuroski, Julien Furrer, Stefan Gaugler, Wolfgang Weinmann, and Marc Luginbühl*

Cite This: https://doi.org/10.1021/jasms.6c00154

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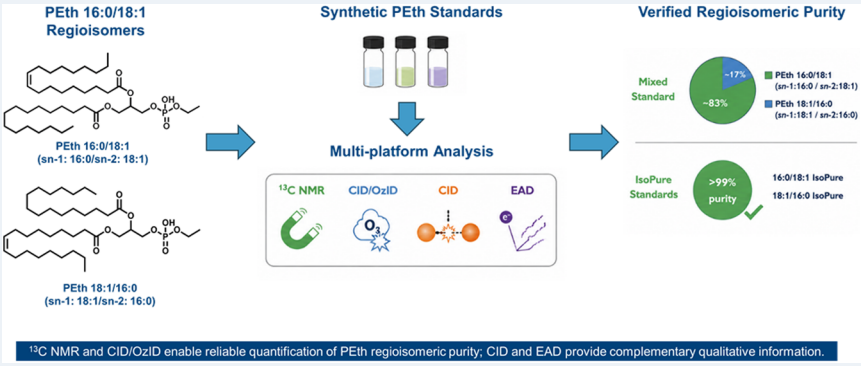

ABSTRACT: Phosphatidylethanols (PEths) are direct alcohol biomarkers of clinical and forensic importance. Their accurate quantification depends critically on the regioisomeric purity of reference materials, as deviations from the natural isomer distribution can bias measurement outcomes. In this study, we systematically evaluated the relative isomer abundance of synthetic PEth 16:0/18:1 standards using nuclear magnetic resonance spectroscopy (NMR), ozone-induced dissociation (CID/OzID), collision-induced dissociation (CID), and electron-activated dissociation (EAD). Three commercial preparations were investigated: a mixed isomer standard and two "IsoPure" materials claimed to contain exclusively the PEth 16:0/18:1 or 18:1/16:0 regioisomer, respectively. Quantitative ^{13}C NMR enabled unambiguous discrimination of the carbonyl resonances, revealing a mixture of ~83/17% in the non-IsoPure material and <1% cross-contamination in the IsoPure standards. CID/OzID provided complementary structural information, with isomeric compositions consistent with NMR results. CID and EAD (sodiated, positive ionization) yielded characteristic product ion ratios and homologue-specific product ions, respectively, that distinguished isomers, although precise quantification remained limited. In contrast, EAD spectra in negative-ion mode were dominated by nondiagnostic product ions and did not allow isomer differentiation. Together, our results demonstrate that ^{13}C NMR and CID/OzID are robust, quantitative approaches for assessing regioisomeric purity in synthetic PEth standards, while CID and EAD can support qualitative profiling. This multiplatform evaluation provides a framework for quality control of PEth reference materials and underlines the necessity of verifying regioisomeric composition to ensure reliable biomarker quantification.

KEYWORDS: *phosphatidylethanol, PEth, regioisomeric purity, CID, CID/OzID, NMR, EAD*

INTRODUCTION

Phosphatidylethanols (PEths) are a group of direct alcohol biomarkers with established applications in both clinical and forensic settings.[1] They are routinely employed in driving aptitude assessments, the evaluation of alcohol misuse in organ transplantation, assessments of maternal and neonatal alcohol exposure, and in the monitoring of alcohol withdrawal and rehabilitation therapies.[2−4] Among the various PEth species, PEth 16:0/18:1 (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol) emerged as the most relevant analogue, with established cutoff concentrations and reference concentrations for diagnosis of alcohol consumption during the previous weeks before blood sampling.[1] Quantification of PEths is typically performed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in negative-ion electrospray ionization mode, employing monitoring of multiple reaction monitoring (MRM) transitions based on the fatty acyl product anions formed upon collision-induced dissociation (CID).[5]

Received: April 30, 2026
Revised: July 24, 2026
Accepted: July 29, 2026

ACS Publications

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As observed for other classes of glycerophospholipids, such as phosphatidylcholine, the signal intensity of these transitions depends both on the identity of the fatty acyl chains and on their relative (*sn*-) position on the glycerol backbone.[6,7] Previous LC-MS/MS investigations of PEth in MRM mode have demonstrated the preferential formation of the fatty acyl product ion from the *sn*-2 position (central position) on the glycerol backbone and the sensitivity of the relative product ion abundance to collision energy (CE).[8,9]

Reference materials with regioisomeric compositions differing from that of biologically produced PEth in human blood can therefore result in significant quantification discrepancies.[8] Following an initial report highlighting the importance of regioisomeric purity, several reference material manufacturers responded by introducing new products specifically addressing this issue.[10]

In this study, we investigate several complementary analytical techniques for assessing the regioisomeric composition of commercially available synthetic PEth reference materials, including nuclear magnetic resonance spectroscopy (NMR), collision-induced dissociation (CID), ozone-induced dissociation (CID/OzID), and electron-activated dissociation (EAD). These techniques were selected because they provide complementary information regarding PEth structure and regioisomeric composition. Among these approaches, NMR provides direct structural information and enables determination of regioisomeric composition without relying on fragmentation behavior. In particular, NMR allows differentiation of fatty acyl chain positions on the glycerol backbone through characteristic chemical shift patterns and coupling relationships. However, the method generally requires purified analytes in milligram quantities, which may limit its applicability for low-abundance samples. To date, no established experimental workflows or reference data for the regioisomeric characterization of PEths by NMR have been reported in the literature. In contrast, CID in negative-ion electrospray ionization mode represents the conventional and routinely applied fragmentation approach for LC-MS/MS quantification of PEths.[5] Since CID-product ion abundances are strongly influenced by fatty acyl position and collision energy, it provides a direct means to evaluate the analytical consequences of regioisomeric composition for routine PEth assays.[8] Although CID primarily provides qualitative information in the form of characteristic differences in fragment ion ratios reflecting the fatty acyl chain distribution at the sn-2 position, upon calibration with reference standards of known regioisomeric purity, CID could also be used for quantitative analysis.[10] Additional structural information can be obtained by CID/OzID, which enables localization of double-bond positions within unsaturated fatty acyl chains through selective ozonolysis of carbon−carbon double bonds in the gas phase, generating characteristic product ions diagnostic for double-bond location (not applied in this project).[8,10,11] At the same time, the method retains information related to lipid structure and regiochemistry, thereby providing complementary structural characterization beyond conventional CID fragmentation. Finally, EAD was evaluated as an alternative fragmentation technique capable of generating structurally informative product ions while preserving labile functional groups.[12] Compared with CID, EAD may provide reduced dependence on preferential fatty acyl cleavage and thereby improve characterization of regioisomeric PEth species. To date, no established experimental workflows or reference data for the regioisomeric characterization of PEths by EAD have been reported in the literature.

Since most of the PEth reference material manufacturers do not provide sufficient quantities of reference material (>25 mg per material, particularly required for NMR analysis), our investigation was restricted to three selected, commercial materials: Material 1 (PEth 16:0/18:1), a mixture of both regioisomers; Material 2 (PEth 16:0/18:1 IsoPure); and Material 3 (PEth 18:1/16:0 IsoPure), the latter two claimed by the manufacturer to contain only a single regioisomer with less than 5% of acyl chain migration. Thereby, the aim of this work is to provide a framework for the determination of regioisomeric purity in commercially available PEth reference standards and authentic samples.

MATERIALS AND METHODS

Chemicals and Reagents

PEth 16:0/18:1 (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol (sodium salt) (MLOT: 5552PJF016)) as powder, PEth 16:0/18:1 IsoPure (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol (sodium salt)−IsoPure (MLOT: 6880CIB010)) as chloroform solution, and PEth 18:1/16:0 IsoPure (1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphoethanol (sodium salt)−IsoPure (MLOT: 6879CIA010)) as chloroform solution were purchased from Avanti Polar Lipids, Inc. (Alabama, USA). All solvents were of HPLC quality. Chloroform ($CHCl_3$, 99%, p.a) was obtained from Merck (Darmstadt, Germany). 2-Propanol (≥99.5%) was purchased from Fisher Scientific (Loughborough, UK). Methanol (≥99.9%) was obtained from Biosolve (Valkenswaard, NL).

NMR

For NMR experiments, PEth samples were dissolved in $CDCl_3$ (isomeric mixture, Material 1) or in a mixture of 10% $CDCl_3$/90% $CHCl_3$ (isomer-pure PEth, Materials 2 and 3, respectively). The resulting mixture from Material 1, containing 50 mg of PEth, was dissolved in 550 μL of $CDCl_3$, resulting in a nominal concentration of 125 mM. For the isomer-pure samples, 20 mg of each isomer delivered in $CHCl_3$ was spiked with 10% $CDCl_3$ as lock substance, resulting in samples of 630 μL at 44 mM nominal concentration. Samples were transferred into 5 mm NMR tubes (Norell). NMR experiments were performed on a Bruker Avance NEO 600 MHz spectrometer equipped with a TCI $^{1}H-^{13}C/^{15}N-^{2}H$ CryoProbe (Bruker). Measurements were carried out at 25 °C, where the temperature was calibrated using a 99.9% MeOD sample as an external standard.[13] Chemical shifts were calibrated to TMS. Standard ^{13}C NMR experiments are recorded with simultaneous ^{1}H broadband decoupling to increase the ^{13}C signal (by up to 200%) due to the nuclear Overhauser effect (NOE), which however changes signal intensities unevenly and was therefore avoided in these measurements. For quantification by ^{13}C NMR, it is important to have a sufficiently long interscan delay to allow full relaxation of the slowly relaxing ^{13}C signals.[14] In order to find the optimal interscan delay, T_1 of the signals of interest was determined. To this end, 1D ^{13}C inversion recovery spectra were recorded with ^{1}H decoupling during acquisition (1.7 s) using an interscan delay of 120 and 32 scans per spectrum.[15] No ^{1}H pulses were applied during the interscan delay in order to avoid alterations in signal intensity due to steady-state NOEs. T_1 relaxation delays of 0.01, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 s were measured. A spectral width of 250 ppm centered at 97 ppm was chosen, and 128k points were recorded. T_1 was extracted using a monoexponential fit of the data. For quantification, an interscan delay of 10 s plus an acquisition time of 1.7 s was chosen, fulfilling the rule of thumb of $d_1 \geq 7 \times T_1$ that ensures essentially full relaxation for quantification.[14] Again, no ^{1}H broadband decoupling was applied during the interscan delay in order to minimize steady-state NOEs. 1024 scans were recorded, resulting in a signal-to-noise ratio of 167 for the major component in material 1 (PEth 16:0/18:1), as determined by the sino routine in the software TopSpin 4.0.7, where the signal is the maximal intensity of the analyzed signal and

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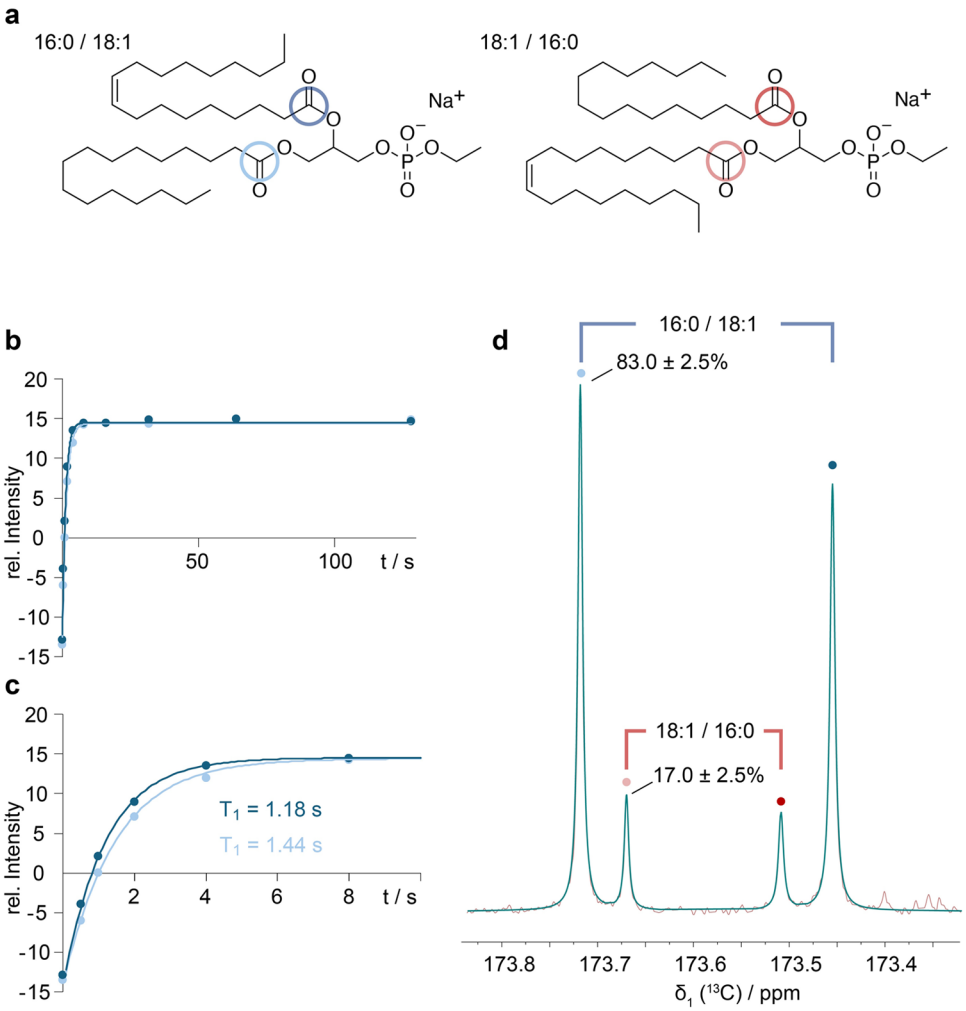

Figure 1. Quantification of isomer content by ^{13}C NMR. (a) Structures of PEth variants with the observed ^{13}C carbonyl resonances indicated with circles. The individual fatty acid chains within one PEth molecule can be tentatively assigned based on literature values, where glycerol C2 attached carbonyls have a lower chemical shift value than carbonyls at C1 and C3. The cis double bond additionally leads to a small upfield shift of the carbonyl resonance. (b, c) T_1-relaxation time measurement of PEth 16:0/18:1, where the two signals analyzed are identified in the ^{13}C NMR spectrum in (d) with blue spheres. (c) Initial 10 s of the data shown in (b) with the fitted T_1 values indicated. (d) ^{13}C NMR spectrum used for quantification (red), with the fitted spectrum in turquoise on top. Relative quantification based on integrals of the signals at 173.72 and 173.66 ppm is indicated in %. The error is based on the signal-to-noise ratio of the minor signal. Signals at 173.4 ppm stem from an impurity.[18] The spectrum was recorded on a 600 MHz spectrometer in 3 h 20 min.

the noise is defined as two times the standard deviation of a noise region. For analysis, spectra were processed in MestReNova v14.0.1 to 512k points with an exponential apodization of 0.2 Hz. Signal intensities were determined by the line fitting routine in the same program such that residual overlap was resolved.

CID/OzID

The materials were prepared in methanol at 100 μmol/L and mixed 1:1 (v/v) with 500 μM methanolic sodium acetate. A hybrid ion-trap orbitrap high resolution mass spectrometer (LTQ Orbitrap Elite, Thermo Fisher Scientific, San Jose, CA, USA) was equipped with an ozone generator (Titan UHC Absolute Ozone, Edmonton, Canada) for the introduction of ozone (O_3), as described by Paine et al.[16] CID/OzID analysis of these standards was executed using a procedure adapted from a method described by Luginbühl et al.[8] In brief, 15 μL of sample from a 96 well plate was introduced into the MS using a chip-based nanoelectrospray ionization (nESI) source (TriVersa Nanomate, Advion Interchim Scientific, Ithaca, NY, USA). The parameters for the analysis at MS^2 and MS^3 were defined using Xcalibur 4.7.102.25 (Thermo Fisher Scientific): The precursor ion ($[M + Na]^+$, m/z 725.5) was selected with an isolation width of 3 Da. The headgroup of the phospholipid was removed by CID using a normalized collision energy (NCE) of 45 and an activation time of 1 ms, yielding a product ion with m/z 599.5, which was subsequently isolated at an isolation width of 3 Da and trapped in the presence of O_3 for 500 ms without NCE. Diagnostic CID/OzID product ions specific for PEth 16:0/18:1 and PEth 18/1:16:0 (m/z 275, 291, 319, 345, 379, 395, 405, and 421) were extracted. The ion count was calculated based on the corresponding intensity values averaged over 100 scans. The relative composition of PEth 16:0/18:1 was calculated as the ratio of the sum of the abundance of ions specific for 16:0/18:1 (m/z 275, 291, 319, 379, and 395) and the sum of the abundance of all diagnostic ions for both regioisomers.

CID in Negative-Ion Electrospray Ionization Mode

After dissolution of the solid substances in each 1 mL $CHCl_3$, the solutions were diluted in 2-propanol, yielding concentrations of 50 ng/mL. The solutions were then analyzed on a LC-MS/MS system consisting of an UltiMate 3000 HPLC system (Dionex, Thermo Scientific Instruments, Reinach, Switzerland) coupled to a 5500 QTRAP with TurboIonSpray source (Sciex, Toronto, Canada) operated in negative ionization mode with an ionization voltage of −4500 V using a validated PEth method. The two most intense MRM transitions (m/z 701.3/255.3 and 701.3/281.2) were analyzed at

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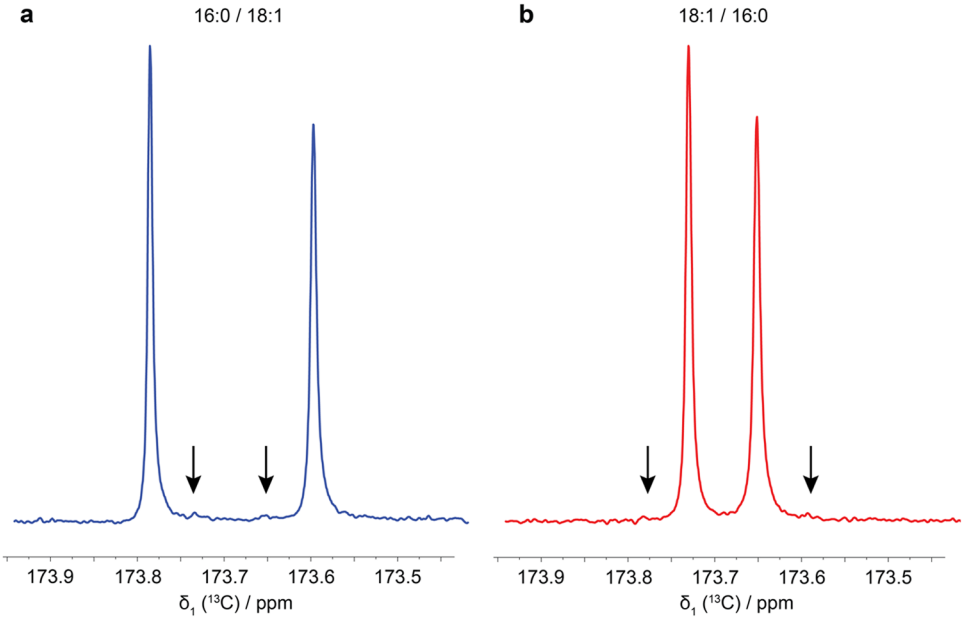

Figure 2. 1D ^{13}C NMR spectra of isomer-pure substances. Spectra of pure PEth 16:0/18:1 (a, blue) and PEth 18:1/16:0 (b, red) are shown, with signal positions of the respective other isomer indicated with arrows. Contaminations from the other respective isomer cannot be quantified, but an estimate of the maximal content of 1% can be given. Spectra were recorded as in Figure 1, except that the solvent was 90% $CHCl_3$/10% $CDCl_3$.

collision energies (CE) ranging from −5 to −70 V in steps of 5 V with dwell times of 5 ms per transition. 7.5 μL (50 ng/mL) of each reference standard was injected, and a short chromatographic run of 5.5 min was performed. A Kinetex C18 2.6 μm 100 Å 50 × 2.1 mm (Phenomenex, Torrance, CA, USA) was used at a flow rate of 0.46 mL/min and an oven temperature of 50 °C. Gradient elution was performed with MeCN/H_2O 30:70 with 5 mM NH_4FA (mobile phase A) and MeOH/MeCN/iPrOH 1:1:1 (mobile phase B): 0−1 min: 60% B, 1−2.6 min: 60−90% B (linear), 2.6−3.2 min: 90−100% B (linear), 3.2−4 min: 100% B, 4−4.1 min: 100−60% B (linear), 4.1−5.5 min: 60% B. For all transitions, declustering potential (DP) was set to −32 and −20 V (for m/z 701.3/255.3 and 701.3/281.2, respectively), entrance potential (EP) was −10 V, and cell exit potential (CXP) was −14 V. All substances were measured in triplicate.

EAD in Negative- and Positive-Ion and CID in Positive-Ion Electrospray Ionization Mode

Neat dilutions of 10 μg/mL in 2-propanol for all reference materials were analyzed on a Sciex 7600 zenoTOF (Sciex, Darmstadt, Germany) using direct infusion. A TOF MS2 experiment was conducted with electron-activated dissociation (EAD) in negative ionization mode using m/z 701.51 as the precursor mass. The MS was operated using the following parameters: ionization voltage: −4500 V, accumulation time: 100 ms, collision energy: −10 eV, electron beam current: 3500 nA, electron kinetic energy (KE): 25 eV, MS2 mass range: m/z 100−1000. Averaged MS/MS spectra were extracted across 1 min.

In addition, the same neat dilution samples were analyzed on a Sciex 8600 zenoTOF coupled to an Agilent LC, as this instrument allows fragmentation at higher maximum electron KE voltages (25 vs 50 eV). Data-dependent acquisition (top 4, exclude for 3 s after 1 occurrence) was carried out following gradient elution, using acetonitrile (mobile phase A) and 2-propanol (mobile phase B) at 0.6 mL/min, starting at 15% B, increasing to 30% B at 2 min, 48% B at 2.5 min, 82% B at 11 min, and 99% B at 11.5 min, hold for 0.5 min before re-equilibrating at starting conditions for 3 min (total run time: 15 min). EAD fragmentation was conducted in the negative ionization mode. MS parameters were set as follows: MS1 and MS2 mass ranges: m/z 50−1000, ionization voltage: −4500 V, CE: −10 eV (MS1)/-12 eV (MS2), electron beam current: 7500 nA, electron KE: 50 eV. MS/MS spectra were extracted at the retention time of 5.8 min.

Further, methanolic dilutions of the three reference materials were mixed with 500 μM methanolic sodium acetate to yield 50 μg/mL solutions. These were analyzed on a Sciex 7600 zenoTOF (Sciex, Darmstadt, Germany) using direct infusion. CID (CE: 40 eV) and EAD fragmentation (KE: 15 eV) were conducted in a TOF MS2 experiment using m/z 725.5 $[M + Na]^+$ as the precursor mass. MS parameters were set as above.

RESULTS AND DISCUSSION

NMR

Initial experiments were performed on a 400 MHz NMR spectrometer equipped with a room-temperature probe, which is typical for standard analytical instrumentation. Under these conditions, the two PEth regioisomers could not be resolved by 1D ^1H NMR due to extensive spectral overlap, necessitating the use of 1D ^{13}C NMR spectroscopy. In the carbonyl region, PEth 16:0/18:1 and 18:1/16:0 exhibit resolved resonances, enabling isomer discrimination (Supporting Information Figure 1).

At 400 MHz, partial separation of the carbonyl signals was observed (176.05 and 176.23 ppm), allowing preliminary quantification. However, the minor resonances (176.10 and 176.18 ppm) showed limited sensitivity (S/N ∼ 21 even after overnight acquisition). Integration after spectral deconvolution yielded an isomer ratio of 78:22 (16:0/18:1 vs 18:1/16:0). Uncertainty analysis indicated limited robustness, with combined contributions (S/N, reference material, and analytical variability) leading to an estimated error exceeding ∼6% for the minor component.

To improve resolution and quantitative reliability, measurements were subsequently performed on a 600 MHz NMR spectrometer using a cryogenically cooled probe, which, compared to a 400 MHz spectrometer, made it possible to approximately double the sensitivity in an acquisition time of just 3 h and 20 min (Figure 1). This enabled near-baseline separation of the carbonyl resonances and more reliable integration. Quantification based on line-shape-fitted signals at

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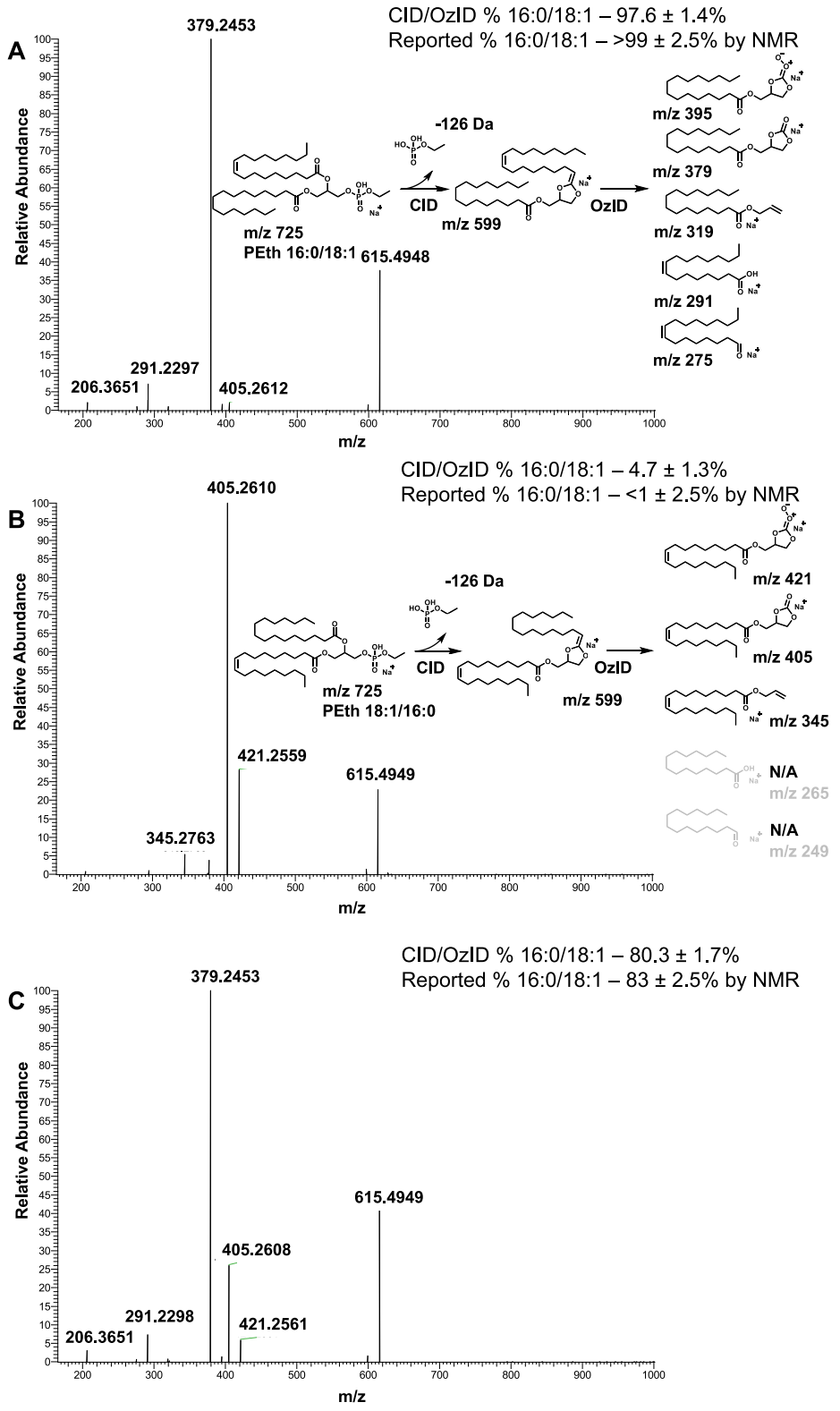

Figure 3. CID/OzID mass spectra of PEth reference materials, based on original data.[10] (A) Avanti 16:0/18:1 Isopure product showing characteristic ions for PEth 16:0/18:1 (m/z 275, 291, 319, 379, 395) and shared ions (m/z 275, 291, 319, 345, 379, 395, 405, 421), with a composition of 97.6 ± 1.4%. (B) Avanti 18:1/16:0 Isopure product, corresponding to a PEth 16:0/18:1 composition of 4.7 ± 1.3%. (C) Avanti 16:0/18:1 Mix product, composition 80.3 ± 1.7%.

176.23 and 176.18 ppm yielded an isomer distribution of 83% (16:0/18:1) and 17% (18:1/16:0), with the uncertainty reduced to ~2.5% for the minor component.[17] The difference relative to the 400 MHz result is attributed to improved spectral resolution and reduced integration bias.

T_1 relaxation times were determined to define the appropriate repetition delays. The measured value of 1.44 s

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Table 1. Comparison of the Reported Composition with the Composition Determined by CID/OzID, NMR, EAD, and CID[a]

material	reported or nominal [%]	CID/OzID [%]	NMR [%]	EAD [%]	CID [%]
Avanti 16:0/18:1 Isopure	>95%[b]	97.6 ± 1.4	>99 ± 2.5[e]	>99 ± 2.5	99 ± 2.1
Avanti 18:1/16:0 Isopure	<5%[b]	4.7 ± 1.3	<1 ± 2.5[e]	<1 ± 2.5	5 ± 2.1
Avanti Mix	not stated[c]	80.3 ± 1.7	83 ± 2.5[f]	84 ± 2.5[e]	83 ± 2.1[d]

[a]Percentages represent the relative abundance of [16:0/18:1] calculated as [16:0/18:1]/([16:0/18:1] + [18:1/16:0]). [b]Manufacturer states <5% acyl chain migration by ^{13}C NMR. [c]Manufacturer does not state regioisomeric purity. [d]Calculated based on a calibration-based model using CID/OzID as reference.[10] [e]Based on the observed signal intensities when measuring with EAD (signal ratio), replicates: n = 3. [f]Please see the Results and Discussion section for NMR uncertainty.

is comparatively short for small molecules in $CDCl_3$, suggesting the aggregation of PEth in solution, potentially involving higher-order assemblies such as reverse micelles. This interpretation is supported by spectra acquired at 900 MHz, which show pronounced line broadening relative to lower-field data, consistent with enhanced transverse relaxation (T_2) effects at higher magnetic field strength (data not shown here). On the basis of these findings, the high-field cryoprobe conditions at 600 MHz were used for quantitative measurements due to their optimal balance of resolution, sensitivity, and spectral separation.

Analysis of isomer-pure reference compounds under identical conditions confirmed minimal cross-contamination (Figure 2), with the nontarget isomer detected only at trace levels (<1%), supporting the selectivity of the carbonyl-based ^{13}C NMR quantification approach.

CID/OzID

Regioisomeric composition of PEth was further investigated using a combined collision-induced dissociation/ozone-induced dissociation (CID/OzID) approach. Although CID/OzID enables double-bond localization in phospholipids, the applied conditions in this study were optimized primarily for regioisomer characterization rather than double-bond assignment. Upon CID of the sodiated PEth 16:0/18:1 precursor ion (m/z 725.5), loss of the phosphate headgroup occurs via a cyclic acetal rearrangement of the glycerol backbone, consistent with previous reports for PEth and related glycerophospholipids.[8,10] This rearrangement generates a new carbon−carbon double bond at the acetal carbon of the fatty acyl chain located at the sn-2 position. Subsequent isolation of the resulting fragment ion (m/z 599.5) and reaction with ozone enables selective cleavage of this double bond, producing diagnostic fragments that report on the identity of the fatty acyl chain at the sn-1 position, while the complementary neutral loss corresponds to the sn-2 substituent. The intense peak observed at m/z 615.5 is an oxidated version of the 599.5 peak caused by the ozone treatment.

This CID/OzID workflow was applied to the three PEth materials, and the resulting diagnostic ion distributions are shown in Figure 3A−C. After normalization to the total ion chromatogram (TIC) and averaging over 100 scans, the relative abundance of PEth 16:0/18:1 was determined as 80.3 ± 1.7% (Material 1), 97.6 ± 1.4% (Material 2), and 4.7 ± 1.3% (Material 3). The corresponding MS/MS spectra exhibited distinct patterns of isomer-specific fragment ions (m/z 275, 291, 319, 345, 379, 395, 405, and 421), with relative abundances reflecting the underlying regioisomeric composition. Several diagnostic ion pairs displayed a characteristic 26 Da mass shift (m/z 395/421, 379/405, and 319/345), consistent with interconversion of the fatty acyl chains between the sn-1 and sn-2 positions. Fragment ions at m/z 395, 379, and 319 were characteristic of PEth 16:0/18:1, whereas the corresponding ions at m/z 421, 405, and 345 were indicative of PEth 18:1/16:0. These diagnostic fragments enabled unambiguous differentiation and assignment of the two regioisomers, demonstrating the utility of MS/MS fragmentation for structural characterization of PEth species.

Overall, the three materials showed clearly differentiated CID/OzID signatures consistent with varying proportions of PEth 16:0/18:1. The compositions obtained are in good agreement with the NMR-based quantification within experimental uncertainty, supporting the validity of both approaches for determining the regioisomeric purity of PEth reference materials (Table 1). Notably, CID/OzID requires substantially lower sample amounts compared with NMR, although access to specialized instrumentation limits its broader applicability.

During the course of this work, a related study by Ali et al. was published describing a comparable CID-based strategy for PEth analysis using sodiated precursor ions and a characteristic neutral loss of 126 Da, followed by MS^3 fragmentation for quantification.[19] In contrast to the mechanism proposed here, their interpretation does not invoke a cyclic acetal rearrangement of the glycerol backbone, highlighting ongoing mechanistic ambiguity in the gas-phase fragmentation behavior of PEth.

CID in Negative-Ion Electrospray Ionization Mode

The regioisomeric composition of PEth standards was assessed by collision-induced dissociation (CID) using electrospray ionization in negative-ion mode. Fragmentation of the deprotonated precursor ion preferentially occurs at the sn-2 position, yielding fatty acyl-specific product ions that enable differentiation of regioisomers.

Monitoring of multiple reaction monitoring (MRM) transitions revealed distinct sn-2−dependent fragmentation patterns. In materials predominantly containing PEth 16:0/18:1 (Materials 1 and 2), the transition corresponding to the oleic acid fragment (701 → 281, 18:1) exhibited higher absolute intensity than the palmitic acid fragment (701 → 255, 16:0). At a collision energy of − 40 V, the product ion ratio (701 → 255/701 → 281) was 0.340 ± 0.002 for the Avanti 16:0/18:1 Isopure standard. The Avanti Mix material showed an intermediate ratio of 0.523 ± 0.015, whereas a substantially higher ratio of 3.103 ± 0.046 was observed for the Avanti 18:1/16:0 Isopure standard (Figure 4).

The inverted ratio for the 18:1/16:0 isomer is consistent with preferential cleavage of the sn-2 ester bond, which in this regioisomer predominantly contains the palmitic acyl chain (16:0). Across all materials, the pronounced differences in product ion ratios reflect their distinct regioisomeric compositions, demonstrating that CID-MRM can differentiate between PEth isomers on a qualitative level.

The observed ratios are consistent with previous reports describing preferential sn-2 fragmentation behavior, although the collision energy dependence differed slightly from literature

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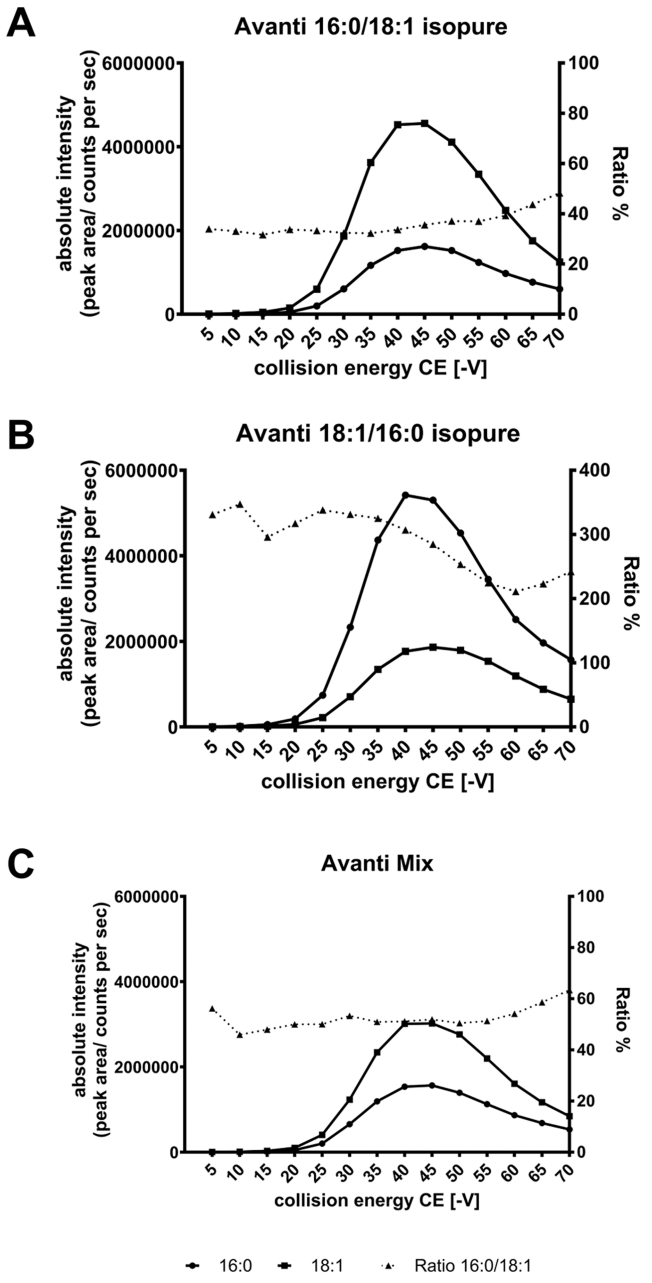

Figure 4. Absolute intensities for both MRMs 701 → 281 (18:1) and 701 → 255 (16:0) and product ion ratios for the Avanti 16:0/18:1 Isopure (A), the Avanti 18:1/16:0 Isopure (B), and the Avanti Mix (C) materials for collision energies ranging from −5 to −70 V. The data were acquired using electrospray ionization in negative-ion mode.

values, with the maximum response observed at −40 V rather than −30 V. Notably, the ratio for the PEth 16:0/18:1 Isopure standard remained in the range of 0.34, in good agreement with previously reported values for endogenous-like compositions (0.29−0.38; mean 0.33 ± 0.02).[8] However, despite its diagnostic value, CID alone does not enable absolute quantification of regioisomeric composition, as fragment ratios are influenced by instrumental conditions and energy settings.

For quantitative assessment of PEth regioisomeric purity by CID, external calibration is required, for example, via a calibration curve relating product ion ratios to known composition standards. This approach was explored in a previous study in which the Avanti Mix was estimated to contain 83% PEth 16:0/18:1 (Table 1). This value was obtained by referencing CID-derived product ion ratios from mixtures of Avanti Isopure standards prepared at defined compositions against independently determined CID/OzID-based purities.[10]

Compared with NMR and CID/OzID approaches, CID-MRM represents a more accessible method for assessing regioisomeric purity, as it requires only standard tandem mass spectrometry instrumentation. In practice, direct infusion measurements are sufficient to obtain a rapid estimate of isomeric composition, making this approach suitable for routine screening applications.

EAD in Negative- and Positive-Ion and CID in Positive-Ion Electrospray Ionization Mode

Using EAD fragmentation at 25 or 50 eV electron KE in negative ionization mode, noncharacteristic MS/MS spectra were produced (Figure 5). Besides the precursor ion (m/z 701.51), mainly product ions below m/z 250 were found that do not include the previously described characteristic CID-product ions (e.g., fatty acid side chains m/z 255.23 (16:0, $[M\text{-}H]^-$) and m/z 281.25 (18:1, $[M\text{-}H]^-$)).[8,20] No systematic regioisomeric differences in the MS2 spectra between PEth 16:0/18:1 and 18:1/16:0 could be identified. A reoccurring cluster with product ions between m/z 123.93 and m/z 126.90 can be observed in all negative ionization EAD spectra, likely attributed to the PEth headgroup.

For MS/MS analysis in positive ionization, PEth samples were sodiated. The most characteristic product ion observed for the sodiated PEth homologues under CID conditions was m/z 599.50. This ion was also selected as the precursor during the CID/OzID experiments; the product ion's formation mechanism is illustrated in Figure 3. The relative abundance of product ions m/z 443.25 and m/z 469.27 showed regioisomeric differences in CID fragmentation (ratio 443.25/469.27: 0.32 for PEth 16:0/18:1, 4.2 for PEth 18:1/16:0 and 0.54 for the mixture; see Figure 6A,C,E). These ions are best interpreted as regioisomer-specific glycerophosphate fragments formed after selective cleavage of the sn-1 or sn-2 ester bond. Their formation is analogous to the release of the characteristic fatty acyl carboxylate ions at m/z 255 (16:0) and m/z 281 (18:1) (which are traditionally observed in negative-ion CID experiments). However, the relative abundances of these fragment ions indicate that cleavage of the sn-1 ester bond is favored over cleavage of the sn-2 ester bond (see the Negative-IonCIDCID in Negative-Ion Electrospray Ionization Mode CID in Negative-IonElectrosprayIonizationMode section) under the applied, positive-ion electrospray CID conditions. This indicates that sodiation fundamentally alters the fragmentation pathway. Coordination of Na^+ to the phosphate group likely suppresses the charge-driven mechanism responsible for preferential sn-2 cleavage in negative-ion CID and instead promotes an alternative charge-assisted fragmentation pathway that favors cleavage of the sn-1 ester bond. Although the exact transition state remains to be elucidated, the observed product ion abundances consistently demonstrate preferential sn-1 cleavage under positive-ion CID conditions.

Similar results were found using EAD fragmentation (ratio 443.25/469.27: 0.92 for PEth 16:0/18:1, 1.7 for PEth 18:1/16:0 and 0.98 for the mixture; see Figure 6B,D,F). EAD fragmentation produced a wider variety of characteristic product ions, including homologue-specific occurrence of $m/$

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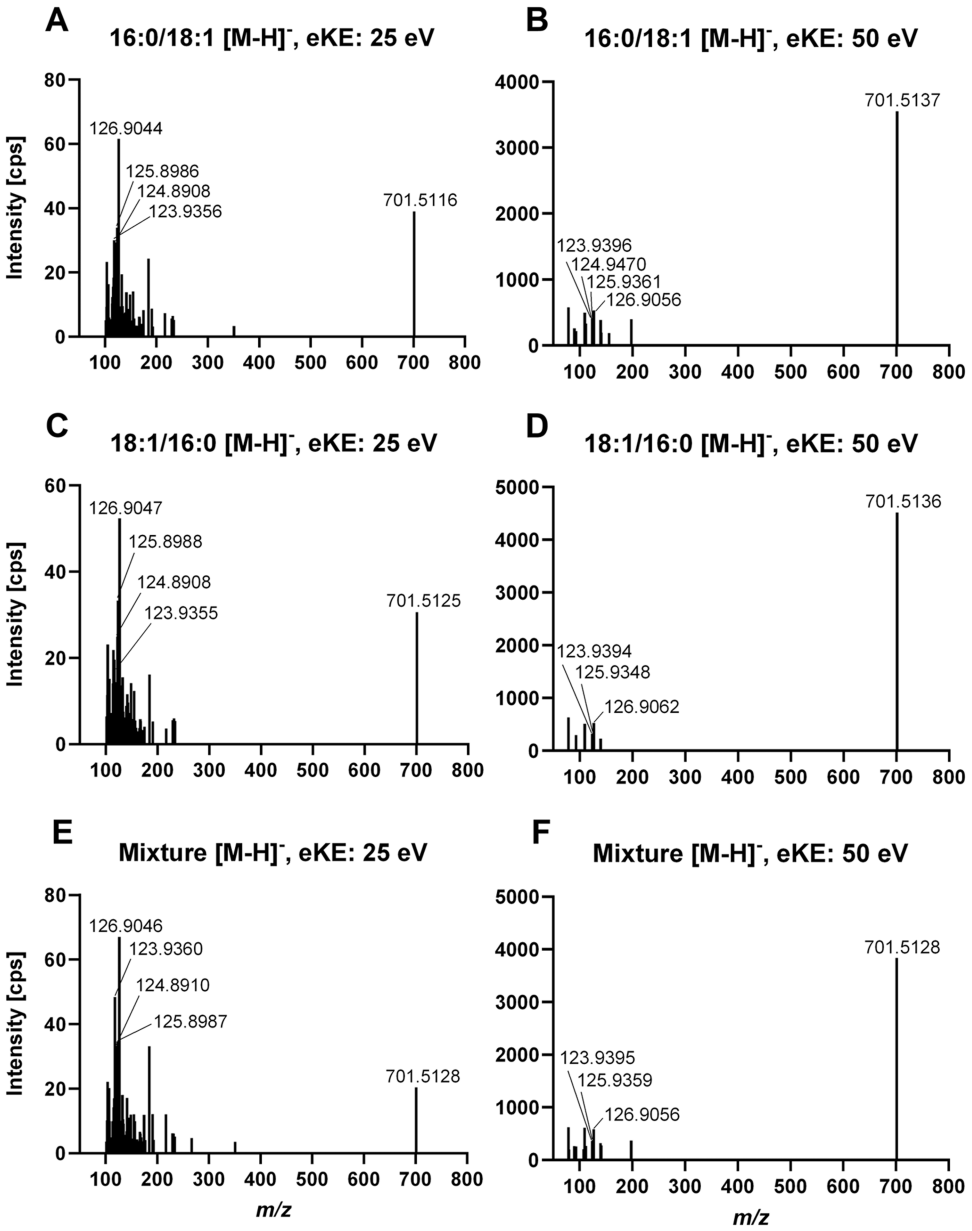

Figure 5. MS/MS spectra of the three analyzed reference materials (16:0/18:1, 18:1/16:0, and their mixture) using EAD fragmentation with an electron kinetic energy (eKE) of 25 eV (A, C, E) and 50 eV (B, D, F) in negative ionization mode $[M\text{-}H]^-$.

z 456.26 for the 16:0/18:1 homologue and *m*/*z* 430.25 for the 18:1/16:0 homologue, see Figure 6, similar to previous studies on phosphatidylcholine regioisomers.[12] Here, the exclusive formation of product ions *m*/*z* 456.26 and *m*/*z* 430.25 under EAD conditions demonstrates that electron-activated fragmentation also preferentially proceeds via loss of the sn-1 acyl chain. Although the mechanism of EAD differs substantially from positive-ion CID, both techniques consistently exhibit the same regioselectivity, indicating that the sodium-adducted PEth ion intrinsically favors fragmentation involving the sn-1 ester bond.

As previously observed in the CID/OzID experiments, the relevant EAD- and CID-derived product ions measured in positive-ionization mode also exhibited a characteristic 26 Da mass shift, consistent with an interchange of fatty acyl chains between the sn-1 and sn-2 positions.

If we compare the ratio of these homologue-specific occurrences (430.25/456.26) for the mixture's signal intensities, we get a ratio of 0.16 for the amount of PEth 18:1/16:0, which may translate into a purity of 84% for PEth 16:0/18:1.

Despite these promising results in positive-ion mode, no diagnostic fragmentation was obtained in negative-ion EAD, consistent with rapid cleavage of the PEth headgroup and consequent loss of informative MS2 pathways. The observed behavior was independent of electron kinetic energy, as lower energies also failed to produce meaningful improvements (data not shown). Conversely, positive-ion EAD of sodiated PEth at 15 eV consistently produced structurally informative spectra, and regioisomeric trends in product ion ratios were in line with CID behavior. Higher EAD kinetic energies (eKE 20 and 25) resulted in decreased intensities of the *m*/*z* 443.25 and *m*/*z* 469.27 product ions, while showing an increased number of

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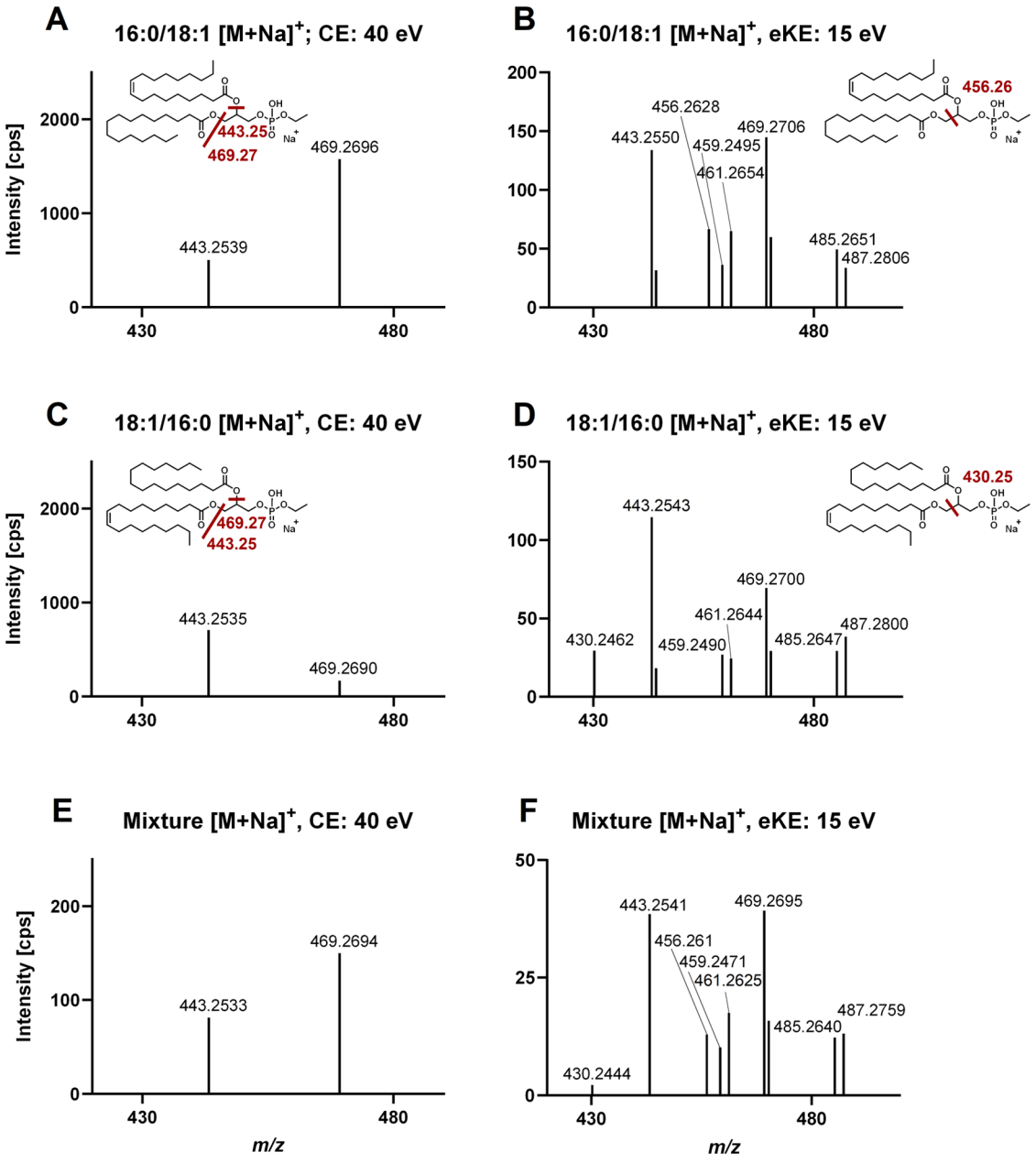

Figure 6. MS/MS spectra of the sodium adducts of the three analyzed reference materials (16:0/18:1, 18:1/16:0, and their mixture) using CID (A, C, E) and EAD fragmentation (B, D, F) in positive-ionization mode; the *x*-axis is zoomed to the *m/z* range 420−500.

small nondiagnostic (for regioisomeric differentiation) product ions in the range of $m/z < 200$.

Quantitative evaluation was, however, limited in highly pure standards, where no signal of the minor homologue was detected (m/z 456.26 was only detected in the 16:0/18:1 standard (Figure 6B), and m/z 430.25 was only detected in the 18:1/16:0 standard (Figure 6D)). This may reflect either the exceptionally high purity of the Isopure materials or insufficient sensitivity of the EAD approach under the applied conditions. Overall, these findings demonstrate that EAD performance for PEth is strongly ionization-mode-dependent: negative-ion EAD is dominated by noncharacteristic headgroup fragmentation, whereas positive-ion EAD of sodiated species enables regioisomeric discrimination through both CID-like fragments and additional homologue-specific ions suitable for rapid purity assessment.

CONCLUSION

This study provides a comprehensive comparison of analytical strategies for assessing the regioisomeric composition of the synthetic phosphatidylethanol (PEth) reference standards. Quantitative ^{13}C NMR proved to be the most definitive method, delivering unambiguous and reproducible isomer ratios. However, it requires larger amounts of reference material, prolonged acquisition times, and expensive equipment worth well over 1 Mio EUR per system and expensive maintenance due to the need for liquid helium and nitrogen. CID/OzID offered complementary structural insights with high sensitivity and yielded results consistent with NMR while requiring substantially less material. However, this method is still relatively uncommon in routine analytical practice. CID and EAD enabled reliable qualitative discrimination between isomers through characteristic product ion ratios and

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homologue-specific product ions, respectively, although they partly lacked the precision needed for absolute quantification. Given the widespread availability of tandem mass spectrometers worldwide, they represent a highly accessible approach. Taken together, these findings highlight NMR and CID/OzID as robust and quantitative approaches for quality control of PEth standards, while CID and EAD fragmentation may serve as supporting methods. In particular, EAD may merit further investigation to clarify its sensitivity for the quantitative assessment of regioisomeric composition. Overall, rigorous verification of isomeric purity remains essential to ensuring accurate biomarker quantification in clinical and forensic applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.6c00154.

^{13}C NMR measurement of the Avanti Mix performed at 400 MHz (Figure 1) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Marc Luginbühl − *Institute for Clinical Chemistry, University Hospital and University of Zurich, 8091 Zurich, Switzerland;* orcid.org/0000-0002-3111-0750; Email: marc.luginbuehl@usz.ch

Authors

Matthias Bantle − *Department of Chemistry, Biochemistry and Pharmacy, University of Bern, 3012 Bern, Switzerland; Institute of Forensic Medicine Bern, Forensic Toxicology and Chemistry, University of Bern, 3008 Bern, Switzerland*

Lana Brockbals − *Institute of Forensic Medicine Zurich, Department of Forensic Pharmacology and Toxicology, University of Zurich, 8057 Zurich, Switzerland;* orcid.org/0000-0001-7310-2671

Alvar D. Gossert − *Department of Biology, ETH Zurich, 8093 Zurich, Switzerland;* orcid.org/0000-0001-7732-495X

Ilche Gjuroski − *Department of Chemistry, Biochemistry and Pharmacy, University of Bern, 3012 Bern, Switzerland*

Julien Furrer − *Department of Chemistry, Biochemistry and Pharmacy, University of Bern, 3012 Bern, Switzerland;* orcid.org/0000-0003-2096-0618

Stefan Gaugler − *University of Applied Sciences and Arts Northwestern Switzerland, School of Life Sciences, Institute of Chemistry and Bioanalytics, 4132 Muttenz, Switzerland*

Wolfgang Weinmann − *Institute of Forensic Medicine Bern, Forensic Toxicology and Chemistry, University of Bern, 3008 Bern, Switzerland*

Complete contact information is available at: https://pubs.acs.org/10.1021/jasms.6c00154

Author Contributions

$^{\nabla}$M.B. and L.B. contributed equally to this work.

Funding

This study was supported by the German Society for Traffic Medicine (DGVM) through the awarding of the Scientific Young Investigator Award to Marc Luginbühl.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank Prof. Stephen Blanksby and his team from the Queensland University of Technology for their CID/OzID determinations. Furthermore, we would like to extend our gratitude to Prof. Zamboni and his team for Sciex ZENO TOF 8600 measurements.

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