Expanding Lipidomic Coverage in Multisegment Injection-Nonaqueous Capillary Electrophoresis-Mass Spectrometry via a Convenient and Quantitative Methylation Strategy

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Abstract

Orthogonal separation techniques coupled to high-resolution mass spectrometry (MS) are required for characterization of the human lipidome given its inherent chemical and structural complexity. However, electrophoretic separations remain largely unrecognized in contemporary lipidomics research as compared to various chromatographic and ion mobility methods. Herein, we introduce a novel derivatization protocol based on 3-methyl-1-*p*-tolyltriazene (MTT) as a safer alternative to diazomethane for quantitative phospholipid methylation (~ 90%), which enables their rapid analysis by multisegment injection-nonaqueous capillary electrophoresis-mass spectrometry (MSI-NACE-MS). Isobaric interferences and ion suppression effects were minimized by performing an initial reaction using 9-fluorenylmethyoxycarbonyl chloride with a subsequent back extraction in hexane. This charge-switch derivatization strategy expands lipidome coverage when using MSI-NACE-MS under positive ion mode with improved resolution, greater sensitivity and higher throughput (~ 3.5 min/sample), notably for zwitter-ionic phospholipids that are analyzed as their cationic phosphate methyl esters. Our method was validated by analyzing methyl-*tert*-butyl ether extracts of NIST SRM-1950 human plasma, which allowed for a direct comparison of 53 phosphatidylcholine and 30 sphingomyelin species previously reported in an inter-laboratory lipidomics harmonization study. The potential for reliable plasma phospholipid quantification by MSI-NACE-MS via a serial dilution of NIST-SRM-1950 was also demonstrated based on estimation of relative response factors using their reported consensus concentrations. Also, lipid identification was supported by modeling characteristic changes in the electrophoretic mobility for cationic phospholipids in conjunction with MS/MS. Overall, this work offers a practical derivatization protocol to expand lipidome coverage in CE-MS beyond the analysis of hydrophilic/polar metabolites under aqueous buffer conditions, which may also prove useful in shotgun and LC-MS lipidomic applications.

Introduction

The human lipidome comprises a vast number of lipid molecular species present in tissues, cells, exosomes and biofluids, which are defined by their specific polar head group, chemical linkage, fatty acid carbon chain length, number of double bond equivalents, oxygenated fatty acyls, and regio-/stereochemistry.[1,2] As lipid homeostasis plays an important role in energy metabolism, membrane structure, and cell signalling, dysregulation in lipid metabolism has long been associated with inflammation and the etiology of cardiometabolic disorders, including obesity, type 2 diabetes, cardiovascular and neurodegenerative diseases.[3,4] Lipidomic studies have also gained traction in nutritional epidemiology as objective indicators of food exposures since essential dietary fats and fat-soluble vitamins relevant to human health[5] are not accurately assessed from self-reports.[6] For these reasons, new advances in untargeted lipid profiling by high resolution mass spectrometry (MS)[7] provide a hypothesis-generating approach for gaining new insights into complex disease mechanisms.[8] However, several technical hurdles impede the progress in lipidomics given the lack of chemical standards and reference MS/MS spectra that limit comparative quantitative reporting and the unambiguous identification of unknown lipids of clinical significance.[9] Recent efforts have focused on developing consensus guidelines in lipid classification and annotation,[10,11] using internal standards for data normalization,[12] applying automated data processing with open-access software tools,[13,14] as well as implementing standardized lipidomic protocols and inter-laboratory ring trials using reference and quality control samples.[15–17] Nevertheless, a lipidomics data workflow requires careful method optimization to avoid bias and false discoveries depending on the specific biospecimen type and instrumental platform, including sample pretreatment protocols.[18]

Classical methods for lipid profiling of biological samples have relied on the analysis of esterified fatty acids from lipid hydrolysates using gas chromatography (GC)-MS.[19] However, comprehensive analysis of intact phospholipids was first achieved by MS when using soft ionization methods based on matrix-assisted laser desorption/ionization and electrospray ionization (ESI).[20] Although shotgun lipidomics enables the direct analysis of lipid extracts by direct infusion (DI)-ESI-MS,[21] high efficiency separations are often needed to improve method selectivity while reducing ion suppression effects, isobaric interferences and/or various other mass ambiguities.[22] To date, liquid chromatography (LC)-MS remains the instrumental platform of choice in lipidomics.[23] However, LC-MS protocols vary substantially in terms of operation conditions (*e.g.,* column types, elution conditions etc.) used to resolve different lipid classes primarily by reversed-phase, normal-phase and/or hydrophilic interaction chromatography (HILIC).[24,25] For instance, greater sample throughput, separation resolution and/or reproducibility can be achieved in reversed-phase LC-MS lipidomic analyses using core shell particles,[26] vacuum jacked columns,[27] capillaries operated under ultra-high pressure conditions,[28] and via multidimensional separations.[29] Alternatively, supercritical fluid chromatography-MS can resolve lipids that vary widely in their polarity with better robustness than HILIC-MS.[30] Also, ion mobility-MS enables the ultra-fast separation of phospholipids as compared to chromatographic methods with adequate selectivity to generate a lipidome atlas.[31] On the other hand, nonaqueous capillary electrophoresis-mass spectrometry (NACE-MS) is largely an unrecognized separation technique in lipidomics likely due to a paucity of published studies that have been limited to certain ionic lipids, such as saturated fatty acids[32] lipid A isomers[33] and glycerophospholipids.[34,35] Indeed, a lack of robust NACE-MS protocols, limited vendor support, and sparse method validation relative to existing chromatographic methods have deterred its use as a viable high efficiency separation platform in untargeted lipid profiling.

Recently, we have introduced multisegment injection (MSI)-NACE-MS as a multiplexed separation platform for the quantitative determination of fatty acids from blood specimens,[6,36,37] which can also resolve a broader range of anionic lipids under negative ion mode.[38] Serial injection of seven or more samples within a single capillary allows for higher sample throughput[39] together with temporal signal pattern recognition in ESI-MS[40] for rigorous molecular feature selection and lipid authentication when performing nontargeted screening.[38] However, separation resolution and selectivity is currently limited for phosphatidylcholines (PC) and other classes of zwitter-ionic lipids that migrate close to the electroosmotic flow (EOF). Pre-column chemical derivatization strategies have been developed to introduce or switch charge states on specific lipid classes to modify their chromatographic retention, reduce isobaric interferences, and improve ionization efficiency with lower detection limits in ESI-MS.[41] For instance, Smith *et al.*[42–44] have used diazomethane for charge inversion on modified cationic phospholipids via quantitative methylation. However, given the explosive and toxicity hazards of diazomethane that is generated *in-situ*,[45] safer methylating agents are required in routine MS-based lipidomic workflows without blast shields and other personal protective equipment. Herein, we introduce a novel two-step chemical derivatization strategy for the quantitative methylation of phospholipids based on 9-fluorenylmethyoxycarbonyl chloride (FMOC) followed by 3-methyl-1-*p*-tolyltriazene (MTT) that offers a practical way to expand coverage of zwitter-ionic phospholipids in MSI-NACE-MS. For the first time, we demonstrate that this procedure enables the rapid identification and quantification of phosphatidylcholines and sphingomyelins as their cationic methyl phosphoesters from reference human plasma samples.[15]

Experimental

Chemicals and Materials

Ultra LC-MS grade methanol, acetonitrile, water and 2-propanol were used to prepare the sheath liquid and the background electrolyte (BGE). Ammonium formate, formic acid, 1,2-distearoyl-d70-sn-glycero-3-phosphocholine (18:0 PC-d_{70}), 1,2-dipalmitoyl-d62-sn-glycero-3-phosphocholine (16:0 PC-d_{62}), methyl-*tert*-butyl ether (MTBE), MTT, FMOC and all other chemical standards were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) unless otherwise stated. All lipid standards purchased were either as a powder or dissolved in solution (1:1) of chloroform and methanol. Stock solutions for lipids were then diluted in chloroform and methanol and stored at -80 °C prior to further use. Reference material from the National Institute of Standards and Technology (NIST) SRM-1950 pooled human plasma was purchased from the NIST (Gaithersburg, ML, USA). While certified reference values for NIST SRM-1950 have been reported for several polar metabolites, plasma phospholipids measured in this study were compared to the median of mean concentrations reported for NIST SRM-1950 in an international study across 31 laboratories that adopted various LC-MS/MS lipidomic workflows, which required measurements from a minimum of 5 laboratories having a sample coefficient of dispersion (COD) < 40%.[15]

Plasma Lipid Extraction Using MTBE

Plasma samples and lipid calibrant solutions were extracted using a modified MTBE-based liquid extraction procedure previously described for fatty acids and anionic lipids using MSI-NACE-MS in negative ion mode.[36,38] Briefly, 50 µL of a NIST SRM-1950 plasma aliquot was mixed with 100 µL of methanol containing PC 16:0-d_{62} as a recovery standard and shaken for 10 min. Then, 250 µL of MTBE was added and the mixture was subject to vigorous shaking

for 10 min. To induce phase separation, 100 µL of deionized water was then added prior to centrifugation at 10 min at 4000 *g*. Next, 200 µL of the lipid-rich MTBE upper layer was transferred into another vial and dried down at room temperature using an Organomation MULTIVAP® nitrogen evaporator (Berlin, MA, USA). For underivatized lipids, dried plasma extracts were then reconstituted to a volume of 50 µL containing acetonitrile/isopropanol/water (70:20:10) with 10 mM ammonium formate containing internal standards PC 18:0-d_{70} (5 µM), benzyltriethylammoniumchloride (BTA) (1 µM), and of PC 16:0-d_{62} (5 µM) prior to analysis by MSI-NACE-MS.

Chemical Derivatization of Zwitterionic Phospholipids Using FMOC and MTT

All plasma ether extracts and phospholipid calibrants were subject to a two-step chemical labeling procedure using FMOC and MTT. In 2 mL amber glass vials, 100 µL of 0.85 mM FMOC in chloroform was added to dried ether plasma extracts and shaken vigorously for 5 min. Then, samples were blown down to dryness using nitrogen at room temperature prior to reconstitution in 50 µL of MTBE containing 450 mM of MTT. Vials were next sealed with Teflon tape and vortexed for 30 s prior to derivatization at 60 °C for 60 min (unless otherwise stated). Afterwards, 100 µL of MeOH, 250 µL of hexane and 200 µL of deionized water was added to back extract polar by-products of the reaction (*e.g.*, *p*-toluidine). After centrifuging for 10 min at 4000 *g*, 200 µL of hexane as the supernatant was transferred out to a separate glass vial and then evaporated to dryness under nitrogen. Lastly, derivatized extracts were then reconstituted in 50 µL of acetonitrile/isopropanol/water (70:20:10) with 10 mM ammonium formate containing internal standards PC 18:0-d_{70} (5 µM) and benzyltriethylammoniumchloride (BTA) (1 µM, and of PC 16:0-d_{62} (5 µM) prior to analysis by MSI-NACE-MS. Derivatization yields for methylated phospholipids from plasma extracts were calculated based on the integrated

relative peak area (RPA) for each native (unlabelled) phospholipid (PL) relative to PC 18:0-d_{70} as an internal standard using equation (1):

$$\%\ Derivatization\ Yield = 100 * (1 - \frac{FMOC\ \&\ MTT\ treated\ PL\ RPA}{Untreated\ PL\ RPA}) \qquad (1)$$

CE-MS Instrumentation and Serial Injection Configuration

An Agilent 6230 time-of-flight (TOF) mass spectrometer with a coaxial sheath liquid electrospray (ESI) ionization source equipped with an Agilent G7100A CE unit was used for all experiments (Agilent Technologies Inc., Mississauga, ON, Canada). An Agilent 1260 Infinity isocratic pump and a 1260 Infinity degasser were utilized to deliver an 80:20 MeOH-water with 0.1% vol formic acid at a flow rate of 10 μL/min using a CE-MS coaxial sheath liquid interface kit. For mass correction in real-time, the reference ions purine and hexakis(2,2,3,3-tetrafluoropropoxy)phosphazine (HP-921) were spiked into the sheath liquid at 0.02% vol to provide constant mass signals at *m/z* 121.0509 and 922.0098, which were utilized for monitoring ion suppression and/or enhancement effects. During sample introduction into the capillary, the nebulizer gas was turned off to prevent siphoning effects that may contribute to air bubbles and current errors upon voltage application.[36] This was subsequently turned on at a low pressure of 4 psi (27.6 kPa) following voltage application with the ion source operating at 300 ºC with a drying gas of nitrogen that was delivered at 4 L/min. The TOF-MS was operated in 2 GHz extended dynamic range under positive mode detection. A V_{cap} was set at 3500 V while the fragmentor was 120 V, the skimmer was 65 V and the octopole rf was 750 V. All separations were performed using bare fused-silica capillaries with 50 μm internal diameter, a 360 μm outer diameter, and 100 cm total length (Polymicro Technologies Inc., AZ). A capillary window maker (MicroSolv, Leland, NC) was used to remove 7 mm of the polyimide coating on both ends of the capillary to

prevent polyimide swelling with organic solvents in the background electrolyte (BGE) or aminolysis under alkaline nonaqueous buffer conditions.[46] An applied voltage of 30 kV was used for CE separations at 25 ºC together while using a forward pressure of 5 mbar (0.5 kPa). The BGE was 35 mM ammonium formate in 70% vol acetonitrile, 15% vol methanol, 10% vol water and 5% vol isopropanol with an apparent pH of 2.3 adjusted with the addition of formic acid. Derivatized plasma extracts and lipid standards were introduced in-capillary hydrodynamically at 50 mbar (5 kPa) alternating between 5 s for each sample plug and 40 s for the BGE spacer plug for a total of seven discrete samples analyzed within a single run.[38] Prior to first use, capillaries were conditioned by flushing at 950 mbar (95 kPa) with methanol, 0.1 M sodium hydroxide, deionized water, and BGE sequentially for 15 min each. The BGE and sheath liquid were degassed prior to use. For analysis of NIST SRM-1950 by MSI-NACE-MS in negative ion mode to verify acidic lipids not amenable by the FMOC/MTT labelling, an alkaline BGE with the same organic solvent composition was used, but with ammonium acetate and ammonium hydroxide as the BGE and pH modifier respectively as described elswhere.[36] In this case, the same MTBE extraction protocol was applied for the direct analysis of fatty acids and anionic lipids, but the extract was concentrated two-fold without FMOC/MTT chemical derivatization.

Results and Discussion

Separation Performance Enhancement After Phospholipid Methylation.

A two-step chemical labeling strategy using FMOC/MTT was first developed to generate a positive charge on methylated phospholipids to increase their electrophoretic mobility as depicted in **Figure 1a**. FMOC was first added as a protecting agent to rapidly react (< 5 min) with phosphatidylethanolamines (PEs) from plasma ether extracts since they can generate isobaric interferences with analogous phosphatidylcholines (PCs) following their permethylation.[44] In this

case, MSI-NACE-MS under negative ion mode can directly analyze native PEs and other acidic lipids without chemical derivatization.[38] FMOC not only reacts with PE species from plasma extract, but also with excess MTT by-product (*i.e.*, *p*-toluidine) to form a neutral adduct as shown in the proposed reaction mechanism (**Figure S1**). The reaction of *p*-toluidine with FMOC (**Figure S2**) contributes to a reduction of ion suppression on closely migrating methylated phospholipids in MSI-NACE-MS in conjunction with back extraction into hexane that was found to be superior to MTBE as organic solvent (**Figure S3**). Overall, methylation of acidic phosphoric acid moieties expands the separation window in MSI-NACE-MS by improving the resolution within phospholipid class species as shown in **Figure 1b**. Furthermore, methylated PC cations migrate with faster migration times and sharper peaks that enhances concentration sensitivity while avoiding ion suppression that occurs within the EOF region due to the co-migration of abundant and electrically neutral plasma lipids (*e.g.,* diacylglycerides, cholesterol esters). In all cases, a serial injection of seven independent plasma extracts were analyzed rapidly within a single run by MSI-NACE-MS (~ 3.5 min/sample) under positive ion mode with full-scan data acquisition. This method also analyzed methylated sphingomyelin (SM) species, which also undergo a distinct mobility and mass shift (+14 Da) as shown in their MS/MS spectra acquired under positive and negative ion modes (**Figure S4**). SMs have been reported to undergo methylation with a second equivalent on their hydroxyl moiety when using diazomethane, which leads to signal splitting and lower sensitivity gain.[42] In our case, dimethylated SM species were not detected likely due to the lower reactivity of MTT as compared to diazomethane that requires special safety precautions when handling given its explosive hazards and toxicity.[42-44]

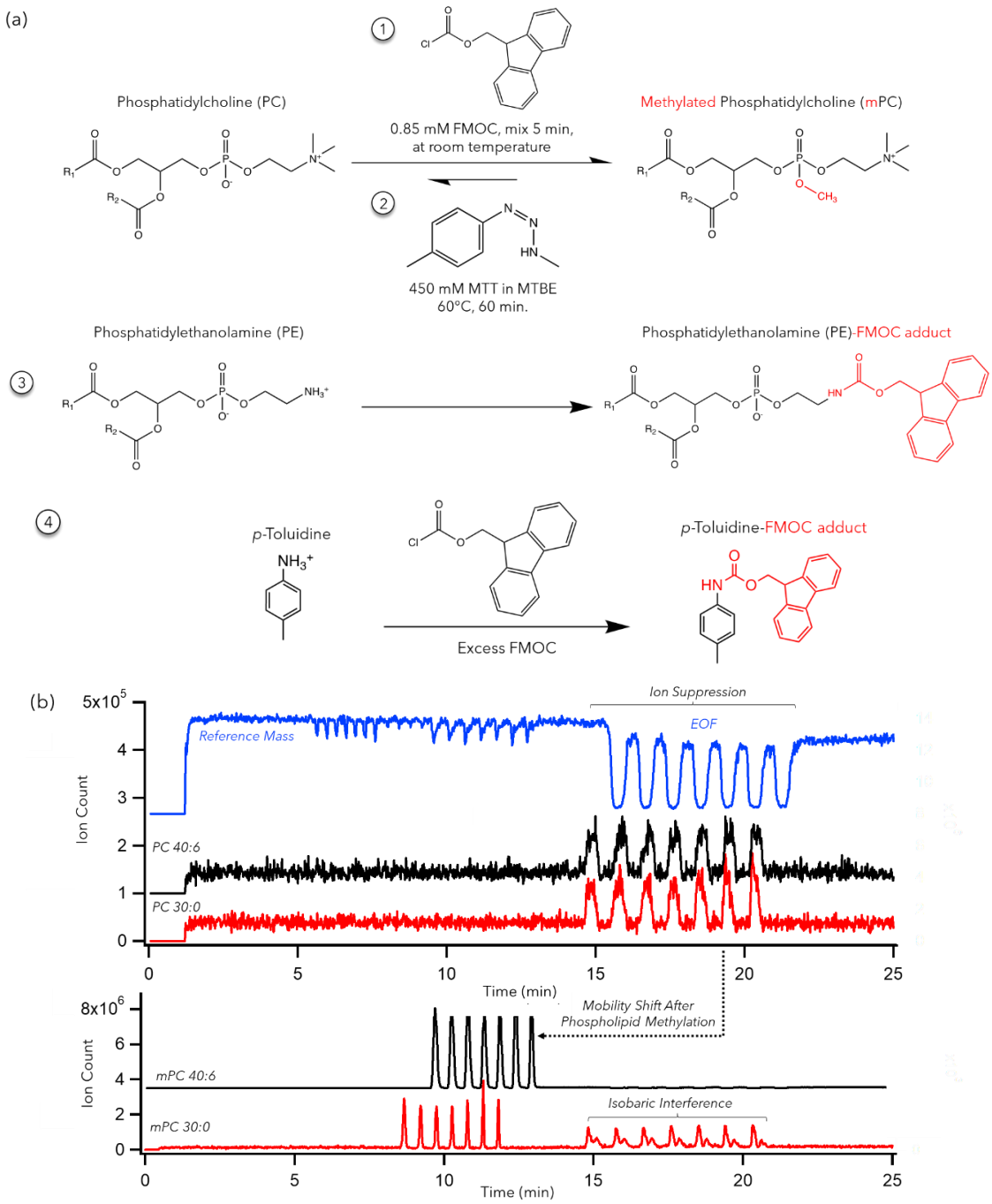

Figure 1 (a) Overview of FMOC/MTT derivatization scheme proposed as a safer alternative to diazomethane to render zwitter-ionic phospholipids with a net positive charge as their methylphosphate esters. The initial addition of excess FMOC reacts with interfering PEs that may become isobaric with PCs following methylation while also reacting with *p*-toluidine as major by-product in the reaction to reduce ion suppression prior to hexane back extraction. **(b)** Series of extracted ion electropherograms in MSI-NACE-MS under positive ion mode that highlight the large mobility shift following methylation, where modified cationic phospholipids migrate faster prior to the EOF with improved resolution and separation efficiency.

Optimization of FMOC/MTT Derivatization of Plasma Phospholipids

MTT was previously introduced as a methylation agent for esterification of carboxylic acids[47] that allowed for the analysis of acidic metabolites in urine by GC-MS.[48] Similarly, Furukawa *et al.*[49] reported using MTT to methylate oligosaccharides containing sialic acid residues in glycoblotting experiments prior to MALDI-MS analyses. However, this reagent remains largely unexplored to date with sparse information related to its reaction mechanism and applicability to routine lipidomic analyses. Initial studies were performed to optimize reaction conditions for the formation of methylated PCs as a function of three experimental factors, namely reaction time (0 to 180 min), MTT concentration (50 to 900 mM) and reaction temperature (20 to 100 ºC). A maximum yield for methylated PCs was achieved using 450 mM of MTT with a reaction time of 60 min at 60 ºC corresponding to an average yield of ~ 70%. This apparent reaction yield was lower than first anticipated without the use of FMOC due to the impact of ion suppression by *p*-toluidine when using excess MTT (data not shown). A kinetic study was next performed to determine the minimum reaction time needed when using a two-step chemical derivatization strategy based on FMOC/MTT, where the reaction progress was reflected by a more intense golden/amber hue color as shown in **Figure 2a**. Also, **Figure 2b** highlights that the reaction yield peaked overall at 60 min as shown for 16 representative plasma PCs species from NIST SRM-1950. Importantly, the use of FMOC and hexane back extraction alleviated the issues of isobaric lipid interferences and ion suppression effects, resulting in higher and more consistent quantitative yields (90.1 ± 6.4)% as demonstrated in **Figure 2c**. In some instances, this nearly doubled the conversion efficiency for certain methylated PCs (e.g., PC 36:5, PC 36:4, PC 40:6) as they only had a ~ 45% conversion when using MTT alone. The derivatization yield was assessed by taking the ratio of the normalized signal for each underivatized PC prior to and after FMOC/MTT treatment of NIST SRM-1950 human plasma (refer to equation 1) when

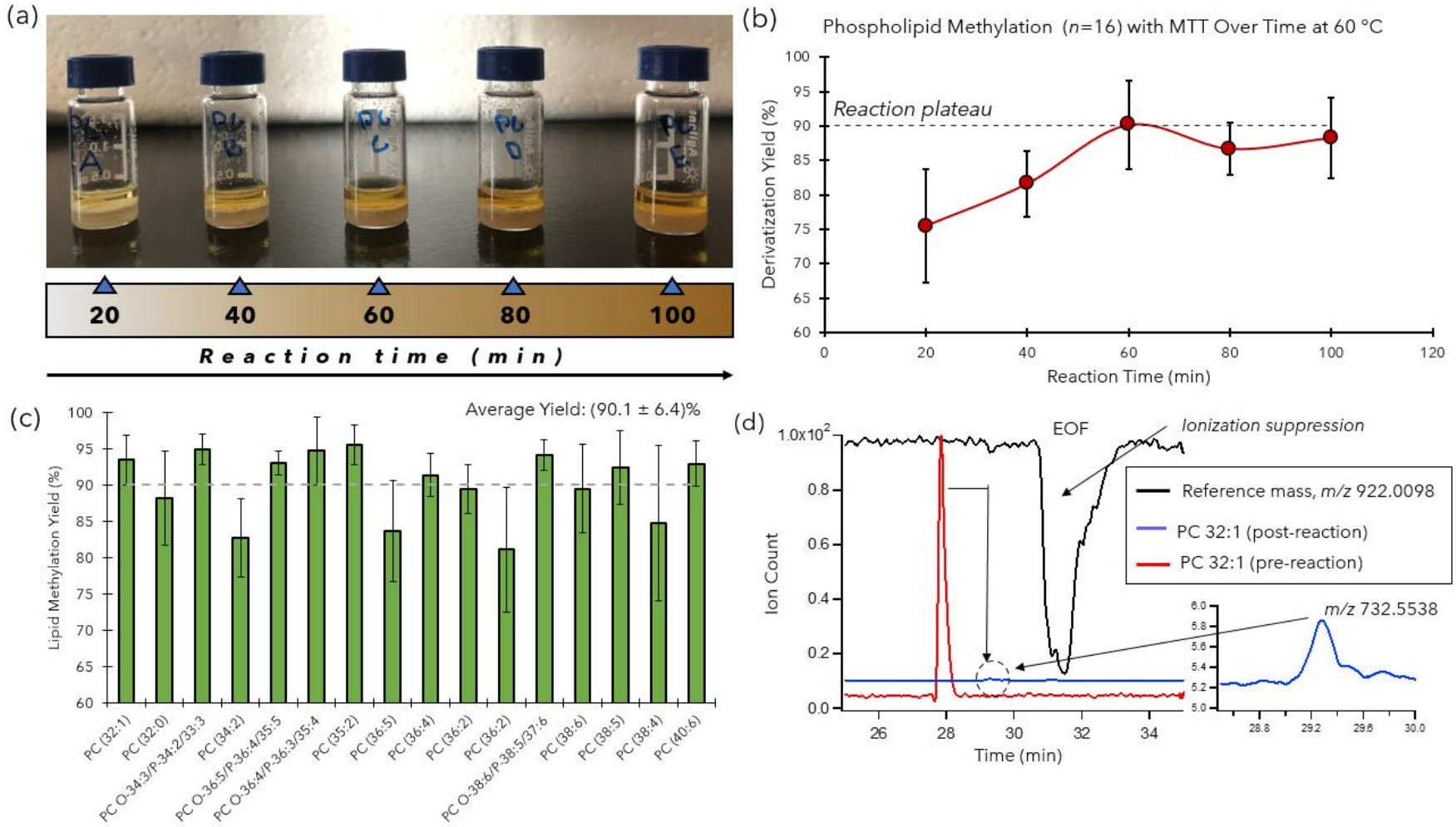

Figure 2 (a) Optimization of FMOC/MTT derivatization conditions as a function of reaction time that highlights a visible change in yellow color intensity with longer reaction times. **(b)** A minimum reaction time of 60 min at 60 ºC was determined to generate a quantitative and stable yield of mPCs from reference plasma extracts based on analysis of 16 representative plasma PCs from NIST SRM-1950. **(c)** Bar graphs that compare the average yield of methylated PCs (~ 90%) in plasma extracts, where errors bars represent standard deviation (± 1s, *n*=5). **(d)** Representative extracted ion electropherograms highlighting the quantitative yield of methylated PCs without ion suppression, where reaction yields were assessed on endogenous plasma PCs analyzed prior and after FMOC/MTT labeling using NACE-MS with a single sample injection to avoid co-migration with the EOF.

using a sample injection format in NACE-MS. This process ensured that native PCs were adequately resolved from the EOF to avoid ion suppression as highlighted for PC 32:1 in **Figure 2d**. One limitation of hexane back extraction following FMOC/MTT derivatization was that more polar lipid classes from plasma extracts were not adequately recovered, such as shorter chain PCs (< 30:0) and lysophosphatidylcholine (lysoPCs). However, most of these polar PC species can be analyzed by MSI-NACE-MS under negative ion mode detection as their acetate adducts without FMOC/MTT derivatization.[38] Indeed, previous plasma lipidomic protocols that rely on more polar organic solvent mixtures for single-phase extraction suffer from limited recovery and poor solubility for non-polar lipids that prevents their accurate quantification.[50]

Expanded Lipidome Coverage and Classification Via Mobility Maps

Similar to the use of collisional cross-section areas for classifying lipid structures as gas-phase ions in IMS,[31] the apparent electrophoretic mobility represents an intrinsic physicochemical parameter for classifying ionic lipids by MSI-NACE-MS.[38] Zwitter-ionic PC species that migrate close to the EOF under alkaline BGE conditions overlap substantially resulting in a more narrow separation window as compared to acidic lipid classes, such as PEs, phosphatidylinositols (PIs), lysophosphatidic acids (PAs), and free (nonesterified) fatty acids (FFAs). This scenario was suboptimal for PCs as it can contribute to false discoveries from isobaric interferences and ion suppression effects when performing untargeted lipidomics. In contrast, **Figure 3a** highlights that a large mobility shift with improved separation resolution occurred following FMOC/MTT derivatization for two major classes of phospholipids, namely methylated PCs (*n*=50) and SMs (*n*=27). These plasma phospholipids were annotated based on their sum composition with low mass error (< 5 ppm) and characteristic relative migration times (RMTs) or apparent electrophoretic mobilities (**Table S1, S2**) with select lipids further characterized by MS/MS.

Moreover, these modified cationic phospholipids also satisfied our selection criteria when using temporal signal pattern recognition in MSI-NACE-MS to reject spurious signals and background ions,[38] which were also independently verified as consensus plasma lipids in an inter-laboratory harmonization study using NIST SRM-1950.[15] In general, methylated SMs migrate with a slower mobility than PCs due to differences in their chemical linkage bonding that impacts their conformational size in solution. Among methylated PC and SM species having similar masses (*i.e.*, PC 32:1 ≈ SM 36:2), the SMs migrated later due to their longer acyl chains resulting in a slower overall electrophoretic mobility. Also, there were characteristic mobility shift patterns evident within both phospholipid sub-classes,[38] since a longer fatty acyl backbone (C30-C44) and greater degrees of unsaturation (n=0-8) predictably reduced or increased the apparent mobility for methylated PCs and SMs, respectively similar to acidic lipids and FFAs.[36,38] The separation resolution of native zwitter-ionic phospholipids under these conditions was otherwise poor in MSI-NACE-MS as they co-migrate close with the EOF. The steepness of the slope for underivatized phospholipids reflected their inadequate within-class separation, which were also more prone to ion suppression and isobaric interferences in human plasma ether extracts.

The benefit of methylation of phospholipids is more clearly illustrated in **Figure 3b**, which compares mobility changes among saturated PCs (including predicted mobility for non-detected PCs via extrapolation), as well as a homologous series of PC 36, PC 38 and PC 40 that demonstrate a linear increase in mobility as a function of greater degrees of unsaturation when using a least-squares linear regression model (R^2 > 0.930). **Figure 3c** confirms that the large mobility shift was a result of formation of a methylated phosphate ester head group as shown in the MS/MS spectra acquired for PC 40:6 under positive and negative ion mode. Annotation of MS/MS spectra under positive ion mode (at 40 V) for methylated PC 40:6 relative to native PC 40:6 confirmed a diagnostic product ion for its methylated phosphate headgroup (*m/z* 190.0982)

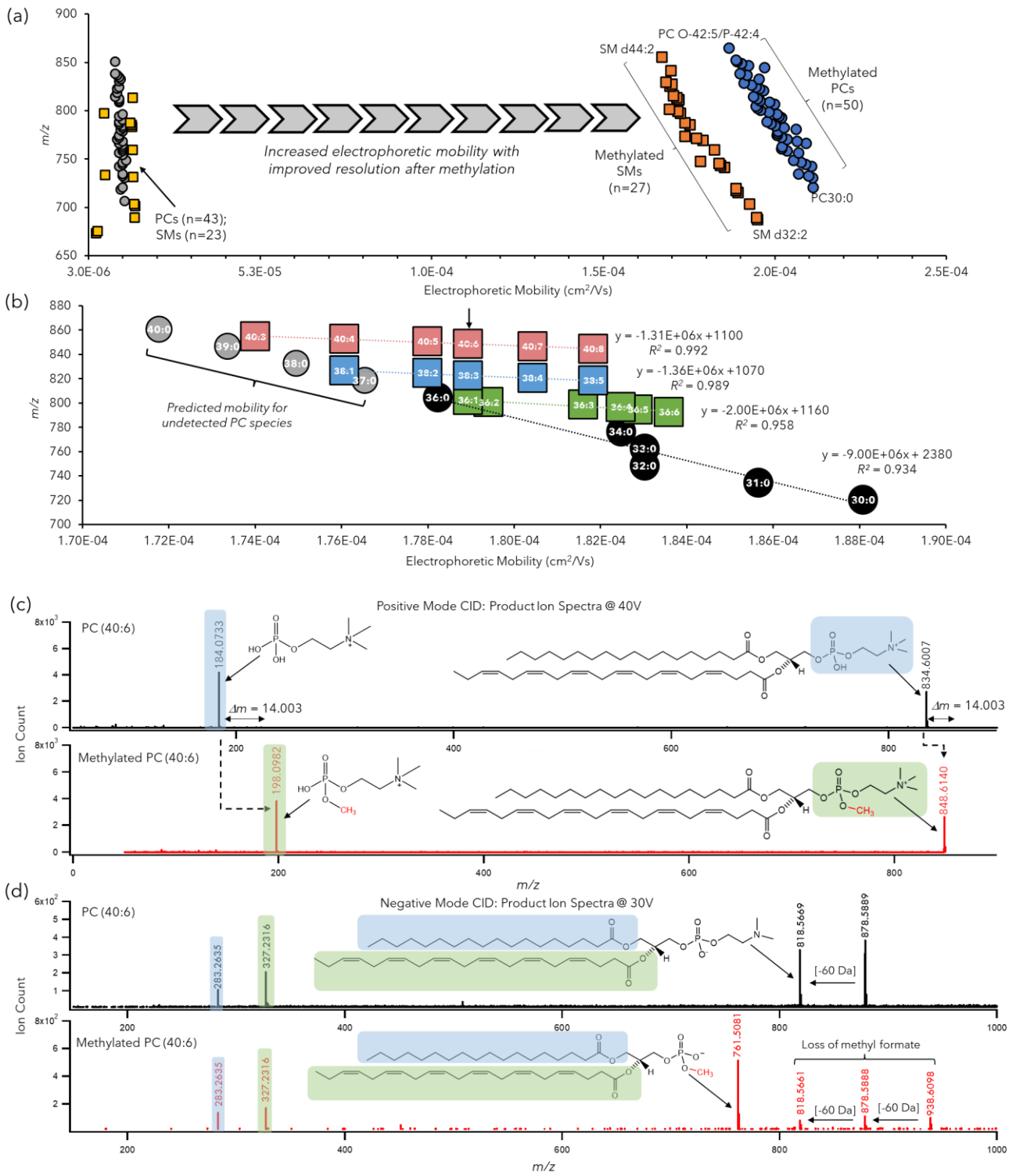

Figure 3 (a) Electrophoretic mobility plot as a function of the accurate mass for 76 phospholipids measured in NIST SRM-1950 plasma extracts by MSI-NACE-MS in positive ion mode. A large mobility shift occurs following quantitative methylation, resulting in better separation resolution of both methylated PCs and SMs that are dependent on their chemical linkage, total fatty acyl chain carbon numbers, and degrees of unsaturation. **(b)** Linear least-squares regression models can be used to predict changes in the apparent electrophoretic mobility for phospholipids as reflected by a homologous series of saturated PCs and unsaturated PCs with the same total carbon chain length as

a function of degrees of unsaturation. This approach can support phospholipid identification especially when chemical standards are lacking. A comparison of MS/MS spectra acquired after collision-induced dissociation experiments under **(c)** positive and **(d)** negative ion modes for methylated and native PC 40:6 from plasma extracts. This confirmed the methylation of the phosphatidylcholine head group as reflected by a characteristic methyl shift (+14 Da) when comparing the molecular ion and base peak/product ion under positive ion mode, whereas the fatty acyl chain backbone and their relative positioning under negative ion mode was consistent with PC 18:0/22:6.

corresponding to a mass shift of *m/z* 14 as compared to native phospholipid (*m/z* 184.0773). Also, annotation of MS/MS spectra acquired under negative ion mode (at 30 V) confirmed that both methylated PC 40:6 and native PC 40:6 contained a stearic acid (18:0) and docosahexaenoic acid (22:6, DHA) with the latter likely derived from a sn-2 position based on the signal fragment ratio for the two fatty acyl chains. Interestingly, a double formate adduct anion [M + 2Formate]- was detected as the molecular ion for methylated PC 40:6 when acquiring MS/MS spectra in negative ion mode since formic acid was included in the BGE and sheath liquid. This was reflected by a characteristic neutral loss of *m/z* 60 (methylformate) that occurred twice as compared to only once for native (zwitter-ionic) PC 40:6. Moreover, methylated PC 40:6 generated a unique base peak product ion at *m/z* 761.5081 under negative ion mode corresponding to a neutral loss of methylformate unlike native PC 40:6. However, not all methylated PC isomers from SRM-1950 plasma extracts were fully resolved by MSI-NACE-MS as highlighted for methylated PC 38:5 after acquiring MS/MS spectra by collision-induced dissociation under negative ion mode (**Figure S5**), which comprised a mixture of two co-migrating phospholipid species, namely PC 16:0/22:5 and PC 18:1/20:4. Distinctive MS/MS spectra were also acquired for methylated SM d34:1 under positive and negative ion mode conditions (**Figure S4**) that confirmed the same methylated phosphorylcholine head group, but lacked diagnostic fatty acyl chains, which may be better achieved as their lithiated adducts to lower the energy barrier in collision-induced dissociation.[51] Other approaches are needed to confirm the exact stereochemistry of methylated phospholipids and their potential isomers from human plasma extracts, such as the location of

unsaturation and/or geometric configuration when using MS/MS, such as ozone-induced dissociation experiments[52] or photochemical derivatization.[53] Nevertheless, mobility plots generated separately for methylated PCs and SMs provide complementary information to deduce the probable chemical structure of plasma phospholipids than relying on accurate mass (MS^1) alone (**Figure S6**) since NACE-MS combines the selectivity of HILIC (*i.e.*, polar head group/chemical linkage) and reversed-phase (*i.e.*, total carbon chain length) chromatography.[38]

Characterization of Consensus Phospholipids from Reference Human Plasma

Previously, Bowden *et al.*[15] reported the use of NIST SRM-1950 as a reference sample when comparing the performance of untargeted lipidomic platforms across 31 international laboratories, each using their own analysis data workflows, LC-MS methodology and hardware/software configuration. Although 1527 unique lipid features were measured quantitatively across all sites, only 339 of these plasma lipids were reported consistently from at least 5 or more laboratories with adequate precision based on a minimum coefficient of dispersion threshold (COD < 40%). We next aimed to validate our two-stage chemical derivatization protocol using MSI-NACE-MS for a panel of methylated PCs and SMs measured consistently from NIST-SRM-1950 plasma extracts as compared to various standardized LC-MS protocols. Overall, 83 eligible plasma phospholipids reported in the harmonization study were annotated based on their sum composition from NIST SRM-1950 ether extracts in a targeted manner, including 53 PCs and 30 SMs as their cationic methylated phosphoesters (**Table S1; Table S2**). Overall, MSI-NACE-MS was able to measure 94% (50 out of 53) and 90% (27 out of 30) of all consensus PCs and SMs from NIST SRM-1950, respectively.[15] We also performed an analysis of acidic lipids from plasma ether extracts when using MSI-NACE-MS under negative ion mode without chemical derivatization to expand lipidome coverage to include more polar

classes of lipids. [38] In this case, we were able to reliable measure 11/14 (79%) bile acids (BAs), 19/25 (76%) of LPCs, but only 24/35 (69%) PE and 7/13 (54%) PI species from the corresponding consensus plasma lipids reported in Bowden *et al.*[15] This sub-optimal coverage was likely due to the lower detectability of acidic lipids under negative ion mode while introducing much smaller sample volumes in-capillary (~ 10 nL) in MSI-NACE-MS than LC-MS methods. Although only 8 FFAs acyl species satisfied validation criteria in the lipidomics harmonization study, MSI-NACE-MS can quantify more than 20 FFAs species from blood extracts as described elsewhere.[6,53] **Figure S7** depicts a Venn diagram for consensus plasma lipids from NIST SRM-1950 that were measured by MSI-NACE-MS under both positive and negative ion mode. As expected, a larger fraction (~ 50%) of methylated PCs and SMs were measured consistently by MSI-NACE-MS in positive ion relative to negative ion mode without chemical derivatization. This was due to the improved separation resolution and greater ionization response achieved for cationic PCs and SMs following FMOC/MTT derivatization and hexane back extraction. Overall, our work highlights that > 150 ionic lipids can be measured in reference plasma when using MSI-NACE-MS under two complementary configurations, including phosphatidylserines (PSs) that were not reported as consensus plasma lipids.[15] For comparison, large-scale CE-MS metabolomic studies using aqueous BGE conditions typically measure less than 100 polar/hydrophilic metabolites consistently in blood specimens under positive and negative ion mode when using a coaxial sheath liquid flow interface.[39,54]

Semi-quantification of Phospholipids via Consensus Concentrations in Reference Plasma

A major challenge in contemporary lipidomic research remains the accurate quantification of blood lipids given the lack and/or high costs of lipid standards and stable-isotope internal standards. However, a key advantage of MSI-NACE-MS is that ionic lipids migrate with a

steady-state mobility under isocratic BGE conditions while using a continuous sheath liquid solution during ionization unlike LC-MS methods that rely on gradient elution for optimal separation performance. Multiplexed separations in MSI-NACE-MS not only improve sample throughput, but also enable versatile serial sample injection configuration to encode mass spectral information temporally within a separation,[38] which greatly reduces mass ambiguities when credentialing ionic lipids in an untargeted manner.[55] **Figure 4a** highlights that different serial injection configurations can be designed in MSI-NACE-MS within a single run, such as a spike recovery study for methylated PC 34:0 in NIST SRM-1950 human plasma, a serial dilution of NIST SRM-1950 to estimate the relative response ratio of methylated PC 40:6, and a serial dilution of a lipid standard for methylated PC 38:6 for generation of an external calibration curve. Spike and recovery experiments using four PC lipid standards were performed at three different concentration levels (low, medium, high) ranging from 1.0 to 20 μM (n = 5). In all cases, methylated PCs and SMs were normalized to a single deuterated internal standard given the lack of ion suppression or enhancement effects in MSI-NACE-MS after sample workup. The potential for reliable quantification of methylated PCs was evaluated by comparing relative response factors (*i.e.,* sensitivity) generated from the slopes of calibration curves for each lipid standard with those derived for the same lipid following a serial dilution of NIST SRM-1950 human plasma. **Figure 4b** depicts two representative calibration curve overlays for methylated PC 38:6 and PC 40:6, which highlights good mutual agreement in measured sensitivity (*i.e.,* slope of calibration curve) based on a least-squares linear regression with excellent linearity (R^2 > 0.980). This comparison also confirmed the lack of matrix-induced ion suppression in MSI-NACE-MS given minimal differences (bias < 2%) in the apparent sensitivity measured from calibrant standards and directly in reference plasma extracts.

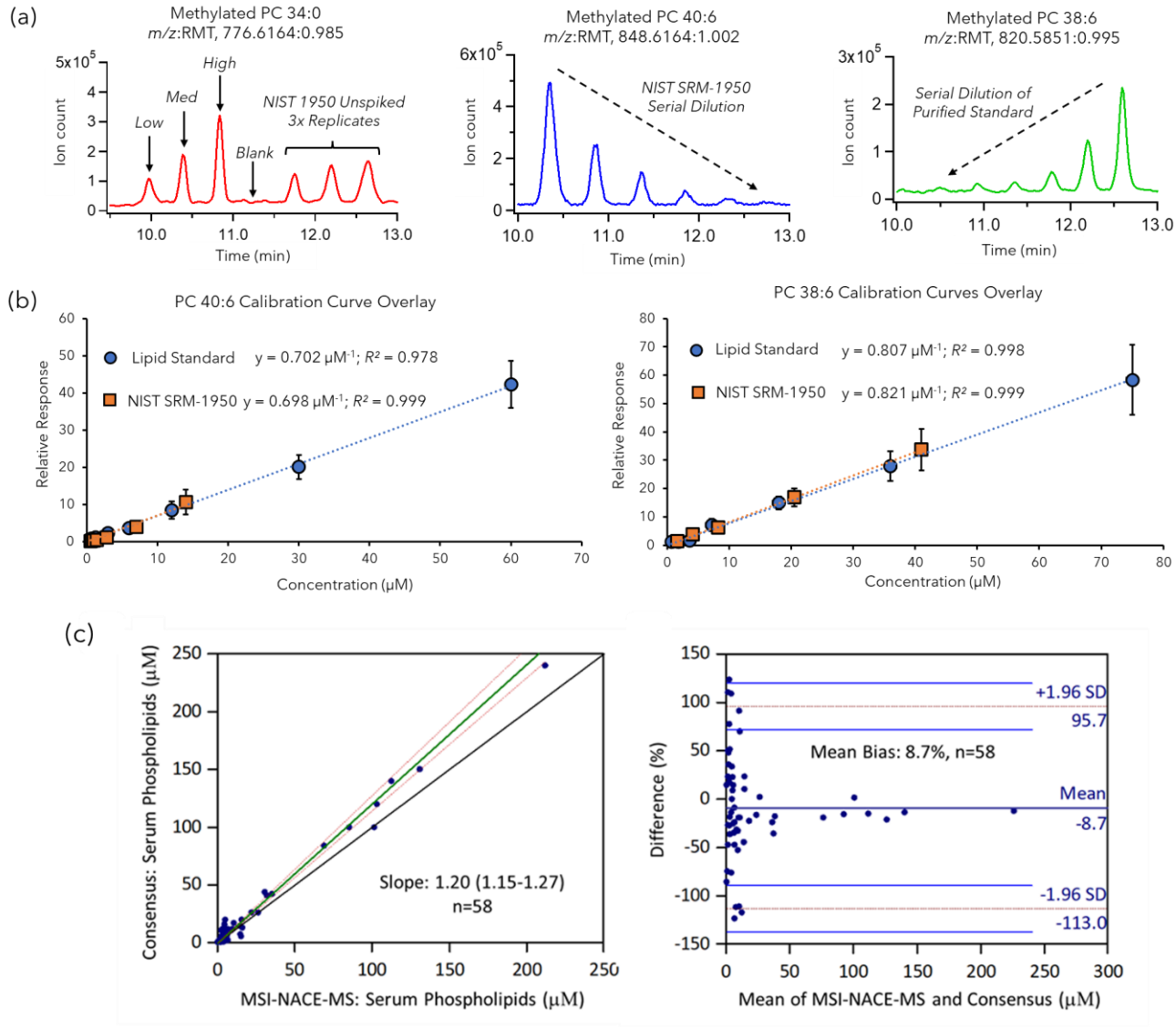

Figure 4 (a) Representative extracted ion electropherograms for methylated PC species when using distinct serial injection configurations in MSI-NACE-MS, including spike and recovery studies, serial dilution of NIST SRM-1950, and a serial dilution of phospholipid calibrants. **(b)** The lack of ion suppression effects for methylated PC 40:6 and PC 38:6 was evident based on the good mutual agreement of their relative response factors (*i.e.*, μM^{-1}) acquired from 5-point calibration curves after serial dilution of phospholipid standards or NIST SRM-1950. **(c)** Inter-laboratory method comparison of PCs (n=39) and SMs (n=19) as consensus phospholipids from NIST SRM-1950 reported by Bowden *et al.* relative to their mean concentrations measured by MSI-NACE-MS. Plasma phospholipid concentrations were estimated by performing a serial dilution of NIST SRM-1950 using their median of mean concentrations (> 0.5 µM, COV < 40%) to derive a response factor in MSI-NACE-MS among 28 quantifiable phospholipids (> 4 calibrants, **Table 1**). This strategy allows for the potential semi-quantification of plasma phospholipids by MSI-NACE-MS even when standards are lacking.

Table S3 summarizes the performance of MSI-NACE-MS for reliable quantification of four representative plasma PCs when using external calibration curves as compared to a serial dilution of NIST SRM-1950. As expected, good accuracy was achieved when quantifying methylated PC 34:0, PC 38:6, and PC 40:6 in both spike-recovery studies, as well as unspiked reference plasma (mean bias < 10%) when using calibration curves by MSI-NACE-MS when compared to untargeted LC-MS methods.[15] Slightly higher bias (< 25%) was found for PC 38:6 and PC 40:6 concentrations in reference plasma when compared to a targeted shotgun (separation-free) lipidomic inter-laboratory comparison study by DI-MS/MS using a commercial lipid kit under standardized operating conditions.[17] The latter discrepancy may arise due to isobaric interferences when high efficiency separations are not used in lipidomic analyses. However, lower precision and poor accuracy (mean bias ~ -50%) was noted for PC 30:0 after hexane sample cleanup since this procedure favors the recovery of more lipophilic lipid species (> C30). We also explored an alternative strategy for semi-quantitative estimation of various other plasma phospholipids lacking chemical standards using response factor ratios derived from the serial dilution of NIST SRM-1950 when using the median of mean consensus lipid concentrations reported by Bowden *et al.*[15] As expected, this strategy was better suited to abundant plasma phospholipids (> 10 μM) given the serial dilution process, whereas chemical standards permitted phospholipid quantification over a wider linear dynamic range. Overall, 28 plasma PCs (n=21) and SMs (n=7) were measured in at least 4 concentration levels with adequate precision (CV < 20%) and linearity (mean R^2 = 0.987) as summarized in **Table 1**. This in turn was used to estimate the response factors and corresponding concentrations for 58 annotated plasma phospholipids (> 0.5 μM), including 39 PCs and 19 SMs (**Table S3**). In cases where a direct measurement of a response factor was not feasible by MSI-NACE-MS due to inadequate

dynamic range, the closest phospholipid analog in terms of mass and chemical structure from **Table 1** was used to estimate its concentration sensitivity. **Figure 4c** demonstrates that this approach generated a good mutual agreement when estimating the concentration for most plasma phospholipids by MSI-NACE-MS as compared to their consensus concentrations by several LC-MS methods as reflected by a slope of 1.20 (95% CI: 1.15-1.27) and a mean bias of -8.7%. Greater bias was evident for lower abundance phospholipids where response factors were more difficult to assess with sufficient precision, however there was generally a normal data distribution over a 500-fold dynamic range in concentrations (0.5 to 200 μM). Further work is needed to further evaluate the quantitative accuracy and long-term analytical performance of plasma phospholipid determination by MSI-NACE-MS when using FMOC/MTT derivatization.

Table 1 Plasma phospholipids (PCs, n=21; SMs, n=7) from NIST SRM-1950 measured by MSI-NACE-MS following a serial dilution to estimate their relative response factor using consensus concentrations.[15]

Lipid Species[1]	**Methylated *m/z***	**Consensus Concentration[2] (μM)**	**# Labs Detected**	**Response Factor[3] (μM^{-1})**	**# Calibrant Data Points[3]**	**Linearity (R^2)**
PC 30:0*	720.5538	1.6	11	0.618	3	0.999
PC P-35:1/34:2	772.5850	240	18	1.114	6	0.992
PC 34:1	774.6008	120	19	1.451	6	0.989
PC 34:0	776.6164	2.1	12	1.438	4	0.979
PC O-36:5/ P-36:4/35:5	780.5902	6.9	11	0.899	4	0.987
PC O-36:4/ P-36:3/35:4	782.6058	12	17	1.006	5	0.989
PC 36:4	796.5850	150	19	0.862	6	0.993
PC 36:3	798.6008	100	17	1.176	6	0.992
PC 36:2	800.6164	140	18	1.495	6	0.990
PC 36:1	802.6320	26	17	2.170	5	0.990
PC O-38:6 / P-38:5/37:6	806.5694	3.6	12	1.463	4	0.986

PC O-38:5 / P-38:4/37:5	808.5850	11	16	1.399	5	0.989
PC O-38:4 / P-38:3/37:4	810.6008	7.4	12	1.800	5	0.990
PC 38:6	820.5850	41	18	0.821	5	0.999
PC 38:5	822.6008	42	18	0.896	5	0.990
PC 38:4	824.6164	84	18	1.093	5	0.990
PC 38:3	826.6320	26	14	2.010	5	0.981
PC O-40:5 / P-40:4/39:5	836.6164	1.7	12	2.198	4	0.992
PC 40:6	848.6164	14	17	0.702	4	0.978
PC 40:5	850.6320	6.7	18	1.695	5	0.989
PC 40:4	852.6476	2.9	18	1.961	5	0.993
SM d34:1	717.5904	100	21	0.241	5	0.984
SM d36:1	745.6218	20	22	0.320	4	0.992
SM d40:2	799.6688	12	15	0.714	4	0.975
SM d40:1	801.6844	20	17	0.740	4	0.983
SM d42:3	825.6844	17	12	0.625	4	0.971
SM d42:2	827.7000	44	18	0.579	5	0.970
SM d42:1	829.7156	20	21	0.720	4	0.982

[1]*Annotated lipid species/isobars from NIST SRM-1950 consistently measured by various LC-MS methods in an inter-laboratory lipidomics harmonization study by Bowden et al.*[15]
[2] *Reported consensus plasma phospholipid concentrations determined by a median of the means from at least 5 different labs having an overall COV < 40%.*
[3] *Relative response factors for each annotated plasma phospholipid species following a serial dilution of NIST SRM-1950 to derive a linear calibration curve by MSI-NACE-MS using consensus concentrations by Bowden et al.*[15]

In summary, expanded lipidome coverage was achieved in MSI-NACE-MS when using a two-step pre-column chemical derivatization strategy to charge switch zwitter-ionic phospholipids into their corresponding cationic methyl phosphoester adducts. This labeling procedure is less hazardous and more convenient to use than diazomethane for phospholipid methylation, which results in improved separation performance and ionization efficiency.

Overall, 77 methylated/cationic PCs and SMs were measured from reference plasma extracts with adequate precision when using MSI-NACE-MS following FMOC/MTT derivatization and hexane back extraction that were consistent with consensus lipids reported in an inter-laboratory harmonization study using LC-MS/MS methods. Additionally, 69 other acidic and polar PLs from NIST SRM-1950 plasma extracts can also be measured by MSI-NACE-MS under negative ion mode without chemical derivatization. This strategy greatly expands conventional CE-MS metabolomic protocols that rely on aqueous buffer systems and thus are limited to the analysis of hydrophilic/polar metabolites. Lipid annotation and structural classification was also supported based on predictable changes in the apparent electrophoretic mobility for methylated PCs and SMs that are dependent on polar head group/chemical linkage, total fatty acyl chain length and degrees of unsaturation. Advantages of MSI-NACE-MS include greater throughput (~ 3.5 min/sample) and minimal ion suppression effects that allows for unique data workflows for data acquisition and lipid authentication in comparison to chromatographic methods that utilize single sample injections. In fact, MSI-NACE-MS is more amenable to standardization since it operates using only a bare-fused silica capillary under an isocratic nonaqueous buffer system unlike other LC-MS methodologies that rely on different column types and gradient elution programs, including reversed-phase and HILIC-MS protocols. However, MSI-NACE-MS with a coaxial sheath liquid interface suffers from higher detection limits and lower concentration sensitivity for ionic lipids in human plasma as compared to LC-MS protocols due to the smaller sample volume introduced on-capillary (~ 10 nL). Furthermore, other electrically neutral lipid classes are not resolved or reliably measured by this method as they co-migrate with the EOF after methylation. Nevertheless, we anticipate that this two-stage methylation strategy and sample workup protocol may be more widely used than diazomethane and its derivatives when performing lipidomic studies by DI-MS/MS and LC-MS/MS, including the analysis of fatty acyl-coenzymes.[55] Future

studies are underway to better characterize other methylated PC lipid sub-classes, including isobaric plasmalogens when using MSI-NACE-MS, while applying this approach in larger-scale untargeted lipidomic studies.

Conclusion

In this work, we describe a two-step chemical derivatization strategy using FMOC/MTT for methylation of zwitter-ionic phospholipids to expand lipid profiling coverage by MSI-NACE-MS under positive ion mode conditions. FMOC was used as a compatible protecting agent to prevent generation of PE isobaric species that also reduced ion suppression effects from excess MTT by-products prior to hexane back extraction. We optimized the efficacy of this reaction to generate quantitative yields of 77 cationic methylated PCs and SMs authenticated in reference plasma ether extracts by MSI-NACE-MS under positive ion mode, which comprised 93% of consensus lipids reported in an inter-laboratory lipidomics harmonization study. Overall, methylation of phospholipids resulted in improved separation resolution, faster analysis times, reduced ion suppression, and allowed for better lipid structural classification based on changes in their electrophoretic mobility using a linear regression model. This method is optimal for large-scale lipidomic studies requiring higher sample throughput with stringent quality control, as well as lower sample volume requirements (~ 2 µL). Complementary analysis of other anionic or polar lipid classes can be achieved by their direct analysis using MSI-NACE-MS under negative ion mode without chemical derivatization. We also demonstrated good precision and accuracy when quantifying lipophilic methylated PCs (> 30) from reference plasma samples above their quantification limit, including the potential for use of serial dilution of NIST SRM-1950 human plasma to estimate relative response factors for lipids lacking standards. Overall, this work represents the first introduction of a hitherto unrecognized lipidomics platform based on MSI-

NACE-MS that takes advantage of a methylation strategy as a practical alternative to diazomethane without unwarranted hazards and safety precautions.

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