

## Supporting Information:

# Trimethylation Enhancement using Diazomethane (TrEnDi) II: Rapid In-Solution Concomitant Quaternization of Glycerophospholipid Amino Groups and Methylation of Phosphate Groups via Reaction with Diazomethane Significantly Enhances Sensitivity in Mass Spectrometry Analyses via a Fixed, Permanent Positive Charge

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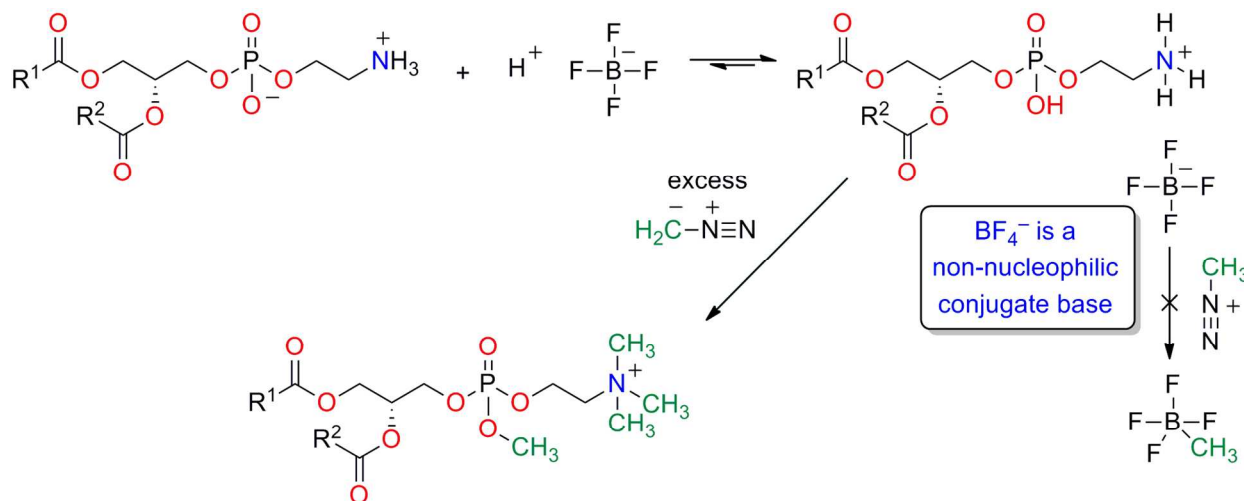
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**CONTENTS:** Additional figures and mass spectra, as referenced in the Article.

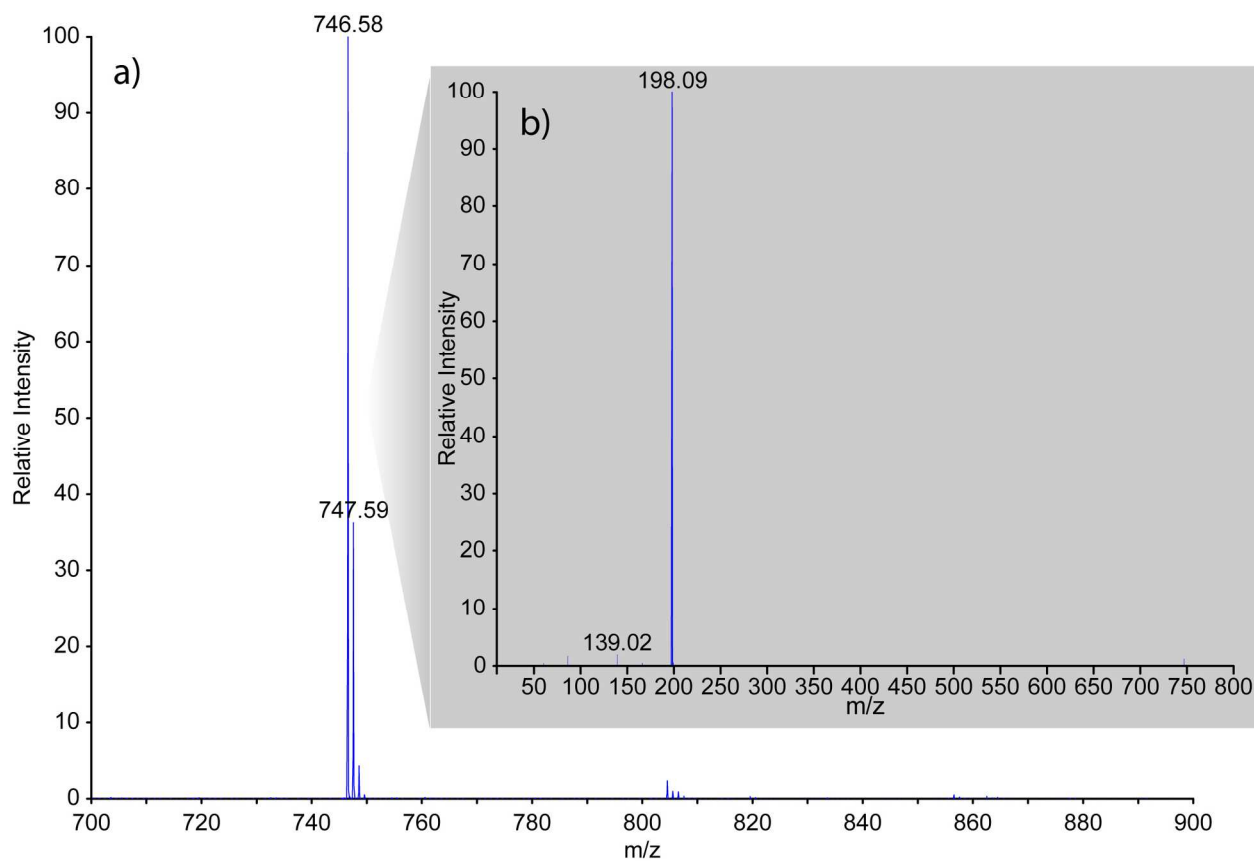
The method we have developed is successful at fully methylating lipids (such as PE, PC, SM and PS) when they are subjected to very small amounts of diazomethane in the presence of tetrafluoroboric acid (HBF<sub>4</sub>). HBF<sub>4</sub> is the key to the success of in-solution lipid-based TrEnDi because it is extremely acidic, enabling the protonation of the phosphate groups in the lipids of interest, but also produces a non-coordinating, non-nucleophilic counterion. This ultimately prevents diazomethane from methylating the conjugate base of the acid, thereby directing all methylation to the lipids (Figure S1). Figure S1 illustrates the reaction of diazomethane with a primary amine; the acidic ammonium ion protons protonate diazomethane, creating an unstable diazonium cation, which carries out an S<sub>N</sub>2 reaction, methylating the amine while releasing N<sub>2</sub> gas. Critically, this reaction produces a fixed positive charge on the lipid via the formation of a quaternary ammonium ion while also neutralizing the negative charge of the phosphate group via the formation of the phosphate methyl ester.



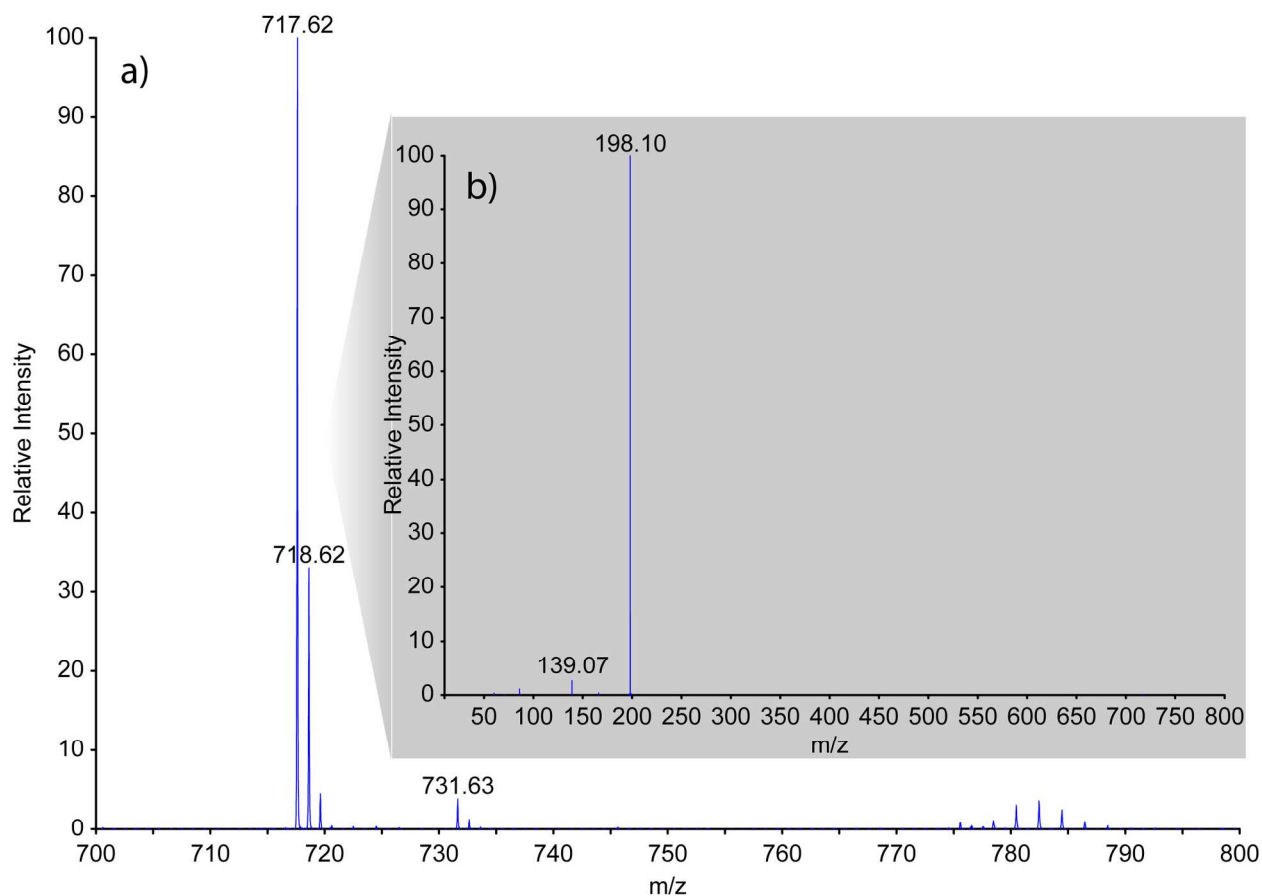
**Figure S1.** Methylation of a phosphoethanolamine lipid head group with diazomethane.

To determine the reactivity of diazomethane with more frequently studied lipids, PC and SM standards were purchased and subjected to our methylating strategy. While other studies have shown the characteristic fragments formed in MS/MS analysis of PC and SM lipids, here we

observe the methylation of the phosphate group, adding a mass of 14 Da to the loss of the phosphocholine head group during MS/MS (Figure S2 and Figure S3). The results of the treatment of each lipid with diazomethane and HBF<sub>4</sub> in solution are summarized in Table 2 of the Article.



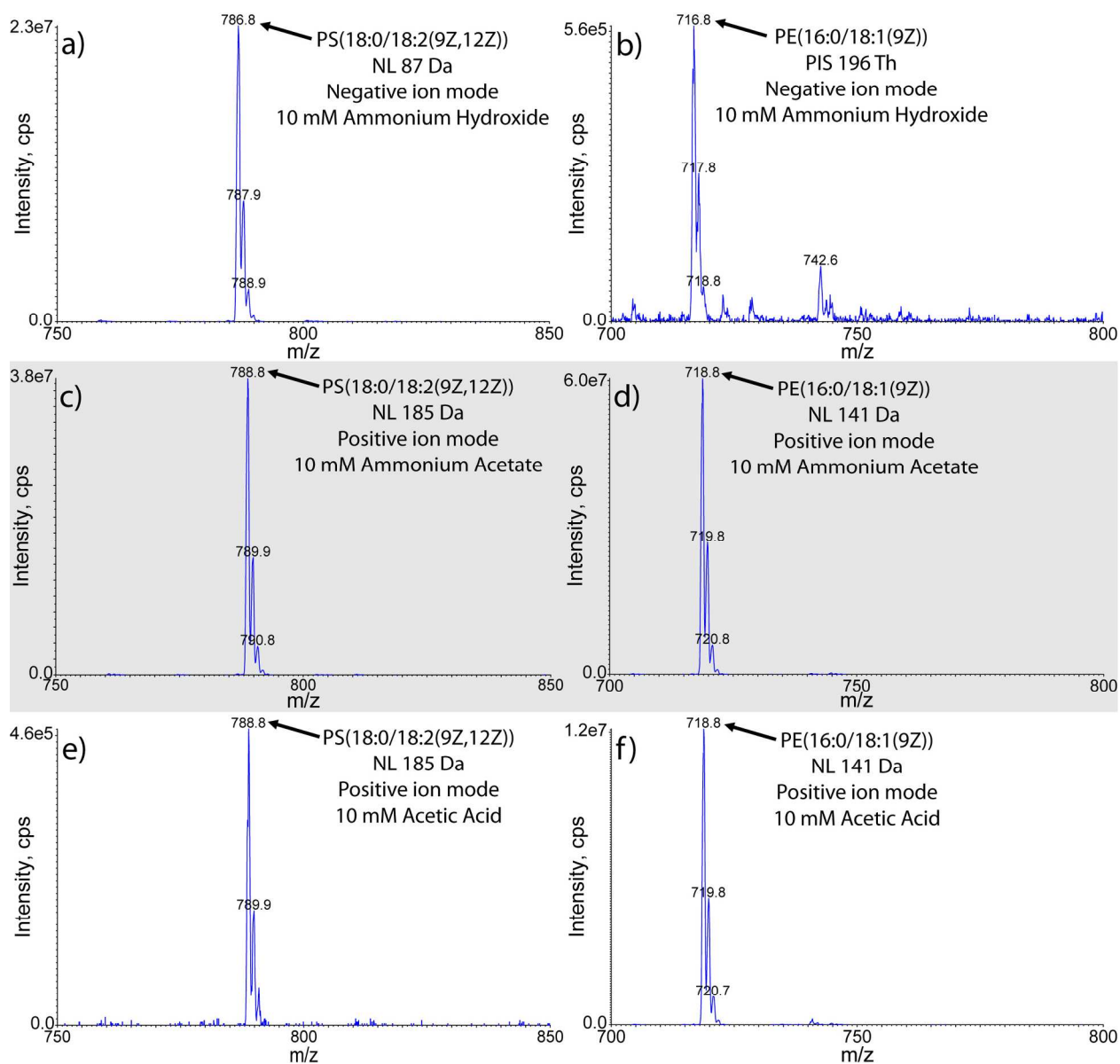
**Figure S2.** a) PC treated with diazomethane ( $[\text{PC}^{\text{Tr}}]^+$ ) reveals addition of one methyl group (m/z 746.58). b) Collision-induced fragmentation of  $[\text{PC}^{\text{Tr}}]^+$  revealed a single fragmentation channel (m/z 198.09) homologous to that known and observed for unmodified PC.



**Figure S3.** a) SM treated with diazomethane ( $[\text{SM}^{\text{Tr}}]^+$ ) reveals addition of one methyl group (m/z 717.62). b) Collision-induced fragmentation of  $[\text{SM}^{\text{Tr}}]^+$  revealed a single fragmentation channel (m/z 198.10) homologous to that known and observed for unmodified SM.

In order to demonstrate that TrEnDi-modified lipids show greater signal intensity than non-modified lipids and are useful in real analytical scenarios, it was important to ensure that non-modified lipids were being electrosprayed in a buffer and using a scanning mode that maximized intensity. It was known that PC and SM electrospray most efficiently in positive ion mode in an acidified buffer; however, PE and PS are known to ionize in positive and negative ion modes and may benefit from volatile reagents such as  $\text{NH}_4\text{OH}$ ,  $\text{CH}_3\text{CO}_2\text{H}$  or  $\text{NH}_4\text{OAc}$ . We could not find a consistent trend in the literature regarding which solvents/reagents promoted ion sensitivity best for each lipid type, nor could we find a consistent pattern that indicated which MS/MS ion

transition was in fact the most sensitive for each lipid type. As a result, these variables were tested to determine the optimal ESI conditions for PS and PE to which we could compare our TrEnDi signal intensity. The results of these experiments are indicated in Figure S4 and Table S1, where it is demonstrated that 10 mM  $\text{NH}_4\text{OH}$  provides the most intense ions in negative ion mode for PE and PS while 10 mM  $\text{NH}_4\text{OAc}$  provides the most intense ions for both PE and PS in positive ion mode. These buffers were therefore used to generate Figure 4 and Table 5 in the Article (see Article for more details).



**Figure S4.** Tandem mass spectra of 10 pmol of PS and PE in various buffers. Lipid, scan type, instrument polarity and buffer type and concentration are indicated in each panel. Panels c) and d) indicate that 10 mM ammonium acetate provides the greatest signal strength in positive ion mode for both PE and PS.

**Table S1.** Areas generated from electrospraying 10 pmol of PE or PS in various buffers in both positive and negative ion modes.

Lipid		10 mM NH <sub>4</sub> OH	10 mM NH <sub>4</sub> OAc	10 mM CH <sub>3</sub> CO <sub>2</sub> H
	m/z (Th) Identity	Area (counts)	Area (counts)	Area (counts)
PE(16:0/18:1(9Z))	718.8 [M+H] <sup>+</sup>	N/A	3.75x10 <sup>8</sup> (100%)	7.48x10 <sup>7</sup> (19.94%)
	716.8 [M-H] <sup>-</sup>	3.96x10 <sup>6</sup> (1.05%)	N/A	N/A
PS(18:0/18:2(9Z,12Z))	788.8 [M+H] <sup>+</sup>	N/A	2.38x10 <sup>8</sup> (100%)	5.90x10 <sup>7</sup> (24.76%)
	786.8 [M-H] <sup>-</sup>	1.71x10 <sup>8</sup> (71.57%)	N/A	N/A