Phospholipids are well known as the building blocks of cellular membranes, but their role in biochemistry is only partially understood. In addition to forming the physical boundary of cells and compartmentalizing the subcellular components, phospholipids are involved in many key regulatory functions within mammalian cells. For example, membrane phospholipids are the source of arachidonic acid (AA), which is the precursor of lipid mediators such as leukotrienes and prostaglandins. Given the importance of phospholipids, there is great interest in monitoring these species and observing the changes that occur as a result of stimulation of a cell to initiate a biological response.

A powerful method to identify and quantitate these phospholipids present in a cell extract is electrospray tandem mass spectrometry (ESI-MS/MS). MS/MS spectra possess unique product ions and neutral losses in both the positive and negative ion mode that are indicative of the polar head group and fatty acyl substituents, respectively. While the use of mass spectrometry and the stable isotope dilution strategy has been a powerful qualitative and quantitative approach to address changes in biomolecules, there are few sources for stable isotope phospholipid internal standards for such studies.

Previously, a method was developed using isobaric iTRAQ® reagents and direct infusion MS3/IDA to monitor the changes in Glycerophosphoethanolamine (GPEtn) lipids after calcium ionophore stimulation and subsequent cleavage of arachidonic acid from phospholipids. This method was successful in providing some insight into the changes of arachidonate containing GPEtn lipids of stimulated samples compared to control samples. Useful quantitative information was obtained in the positive ion mode through the iTRAQ reagent reporter ions formed during CID, however these are not present in negative ion mode. The use of direct infusion MS3 was absolutely necessary in the analysis of these changes, but the ability to observe changes in the detail necessary for our studies was hindered due to the inability to couple this method with liquid chromatography and to obtain useful negative ion mode data.

The mTRAQ® reagents (non-isobaric amine labeling reagents, similar to the iTRAQ reagents) were developed to enable LC-MRM (Multiple Reaction Monitoring) analysis of amine-containing biomolecules. Analysis of mTRAQ reagent labeled GPEtn lipids can be achieved by LC-MRM strategies in both the positive or negative ion mode, providing significant improvement in quantitation over the previous iTRAQ reagent strategy. The non-isobaric nature of these reagents (4 Da mass difference) eliminates the need for MS3 analysis of GPEtn lipids. The ability to use MRM transitions in the negative ion mode for mTRAQ reagent labeled GPEtn lipids allows for more specific transitions to be selected, providing better specificity for the fatty acids esterified to the glycerol backbone. This method allowed one to monitor changes specifically in arachidonate containing GPEtn lipids.

Figure 1. 3-Dimensional Model of Glycerophosphoethanolamine (GPEtn) Lipid.
Methods and Materials

**Samples:** Human polymorphonuclear leukocyte experiments with A23187 and acrolein: Human polymorphonuclear leukocytes (neutrophils) were obtained from the whole blood of volunteers using the Percoll gradient centrifugation technique as previously described\(^3\). The neutrophils (20x10\(^6\) cells) were spun down and resuspended in HBSS at a concentration of 10x10\(^6\) cells/mL. The cell suspension (1 mL) was then aliquotted into separate tubes for the control and A23187 treated experiments. CaCl\(_2\) (final concentration 2 mM) and MgCl\(_2\) (final concentration 500 μM) were added to the control and A23187 treated samples. A23187 is a compound that elevates calcium levels and thereby activates cPLA2, which releases AA from membrane phospholipids. A23187 was added to the A23187 treated sample for a final concentration of 2 μM. The control and A23187 treated samples were allowed to incubate at 37°C for 10 min and the reaction was stopped by the addition of one volume of ice-cold methanol. The cell membranes were pelleted by centrifugation and saved for mass spectrometric analysis.

**Sample Preparation:** Lipids were extracted from the cell pellets of the control and A23187 treated samples by addition of chloroform-methanol-water according to the method of Bligh and Dyer\(^4\). Additionally, 14:0a/14:0-GPEtn (2 μg) was added as an internal standard to each sample. The phospholipid extract was labeled with mTRAQ\(^\text{®}\) reagent using the same procedure as outlined for iTRAQ\(^\text{®}\) reagent labeling of GPEtn lipids\(^2\). The control GPEtn lipids were labeled with mTRAQ reagent Δ4 and the A23187 GPEtn lipids were labeled with mTRAQ reagent Δ0. Each sample was separately introduced onto the SPE column.

**Chromatography:** A C18 5 μm Phenomenex Gemini (2.0 x 150 mm) column (Phenomenex, Torrance, CA) was used. The HPLC was operated at a flow rate of 200 μL/min with a mobile phase of methanol-acetonitrile-water 60:20:20 (v/v/v) with 1 mM ammonium acetate (solvent A) and 1 mM methanolic ammonium acetate (solvent B). The initial mobile phase was 75% solvent B which was held for 2 min. A gradient was started at 2 min from 75% solvent B to 100% solvent B in 5 min, followed by isocratic elution at 100% solvent B for 23 min.

**Mass Spectrometry:** All of the mass spectrometry experiments were performed on a QTRAP\(^\text{®}\) system in both the positive and negative ion mode. MRM triggered IDA on a QTRAP system was used initially to determine the mTRAQ reagent labeled GPEtn species present in the human neutrophil in positive ion mode. Specific MRM methods in both positive and negative ion mode were developed for mTRAQ reagent labeled GPEtn species.

![Figure 2](image-url) Positive Ion Mode MS/MS Characterization of 18:0p/20:4- GPEtn. Enhanced product ion spectra of the [M+H]\(^+\) of 1-O-1'-(Z)-octadecenyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine (18:0p/20:4-GPEtn) labeled at the primary amine group with (a) mTRAQ reagent Δ0 and (b) mTRAQ reagent Δ4 with a collision energy of 30V. The origins of the ions that resulted from fragmentation by collisional activation are indicated in the structures of these molecules.

**Positive Ion MRM Analysis of mTRAQ Reagent Labeled GPEtn Lipids**

GPEtn lipids from neutrophils were labeled with either Δ0 or Δ4 versions of the mTRAQ reagent. The +EPI of mTRAQ reagent Δ0 labeled 18:0p/20:4-GPEtn revealed an mTRAQ reagent specific ion at m/z 282.2 (Figure 2a), whereas the mTRAQ reagent specific ion for mTRAQ reagent Δ4 labeled 18:0p/20:4- GPEtn was at m/z 286.2 (Figure 2b). The fragment ions at m/z 282 and 286 result from cleavage at the phosphate-glycerol bond with the site of protonation being the mTRAQ reagent modified polar headgroup of the GPEtn lipid.
An MRM method was built using 34 MRM mTRAQ® reagent transitions for all 16 GPEtn lipids that were identified from previous iTRAQ® reagent experiments¹ as well as the 14:0a/14:0- GPEtn internal standard (Figure 3). The MRM transitions (Q1→Q3) of mTRAQ reagent labeled GPEtn molecular species corresponded to Q1→m/z 282 for mTRAQ reagent Δ0 labeled and Q1→m/z 286 for mTRAQ reagent Δ4 labeled, where Q1 corresponds to the [M+H]+ ions for each molecular species. MRM triggered IDA was used to verify the structure of GPEtn lipids identified from the MRM survey scan.

For some mTRAQ reagent labeled GPEtn lipids, this data revealed the many isobaric compounds under these conditions, highlighting the complexity of GPEtn lipids in neutrophils. The extracted MRM of 894→282 (Figure 4a) and 918→282 (Figure 4b) are two examples that highlight the presence of multiple isobaric compounds of some of the GPEtn species in the human neutrophils, which demonstrates the complexity of these samples. MRM transition m/z 894→282 could represent mTRAQ reagent labeled 18:0p/20:3-GPEtn, 18:0e/20:4-GPEtn, 20:1p/18:2-GPEtn, or 16:0e/22:4-GPEtn, which are isobaric species that contain different fatty acids. In addition, the MRM transition m/z 918→282 could represent mTRAQ reagent labeled 18:0p/22:5-GPEtn, 20:1p/20:4, or 18:1p/22:4-GPEtn, which are isobaric species that contain different fatty acids. All of these isobaric species which contain different fatty acids cannot be differentiated by their +EPI spectrum due to no characteristic fatty acid fragment ions in the positive ion mode.

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¹ Note that there are multiple chromatographic peaks for both MRM transitions, which indicates the presence of isobaric species in the control GPEtn lipids, where Q1 corresponds to the [M+H]+ ions for each molecular species.
Negative Ion MRM Analysis of mTRAQ® Reagent Labeled GPEtn Lipids

GPEtn lipids have characteristic negative ion MS/MS spectra, containing rich structural information about the fatty acids esterified to the glycerol backbone. For this reason, the negative ion LC-MRM approach for the GPEtn lipids previously identified by iTRAQ® reagent labeling1 was investigated. The negative MS/MS spectrum (-EPI) of mTRAQ reagent Δ0 labeled 18:0p/20:4-GPEtn (Figure 5a) and the mTRAQ reagent Δ4 labeled 18:0p/20:4-GPEtn (Figure 5b) both contain a major fragment ion at m/z 303 which corresponds to arachidonic acid.

The EPI spectra obtained in negative ion mode contain specific fatty acid fragment ions but do not contain mTRAQ reagent and/or iTRAQ reagent specific fragment ions. The corresponding negative MRM method provided specificity for each mTRAQ reagent labeled GPEtn lipid, which could not be achieved in the positive ion mode. In addition to GPEtn lipids positively identified in the original iTRAQ reagent work1, several other GPEtn lipids thought to be important were added to the final MRM method of 64 MRM transitions for all 32 GPEtn mTRAQ reagent labeled lipids (Figure 6). The MRM transitions of mTRAQ reagent labeled GPEtn molecular species corresponded to predicted m/z Q1→Q3, where Q1 corresponds to the [M-H]- ion for each molecular species and Q3 corresponds to the fatty acids esterified to the glycerol backbone.
The specificity to distinguish between different isobaric GPEtn lipids in negative mode as opposed to positive mode is shown in Figure 7. In positive ion mode, the MRM transition m/z 918→282 was monitored and showed three different chromatographic peaks at 16.5, 17.8, and 18.2 min (Figure 7a). These peaks are isobaric and could not be further distinguished in positive ion mode by MRM or MS/MS analysis. In negative ion mode, these isobaric peaks could be differentiated by monitoring the MRM transitions m/z 916→303 (20:1p/20:4), 916→329 (18:0p/22:5- GPEtn or 18:1e/22:5-GPEtn), and 916→331 (18:1p/22:4-GPEtn) (Figure 7b). The negative ion mode MRM data reveals that the first chromatographic peak at 16.5 min in Figure 7a is the 18:1p/22:4-GPEtn lipid (Figure 7b, gray). The two chromatographic peaks at 17.1 and 17.8 min (red and green, Figure 7b), which corresponded to 20:1p/20:4 and 18:0p/22:5-GPEtn, contribute to the second chromatographic peak in Figure 7a that is centered at 17.8 min. Finally, the last chromatographic peak at 18.2 min was identified as 18:1e/22:5-GPEtn from the negative ion mode data (second green peak, Figure 7b).

Figure 7. Improved Specificity in Negative Ion Mode. The MRM transition of (a) m/z 918→282 was extracted from Figure 3. Note the multiple chromatographic peaks for this particular [M+H]+. The MRM transitions of (b) m/z 916→303 (red), 916→329 (green), and 916→331 (gray) were extracted from the same data shown in Figure 6. This negative MRM data reveals the identity of the isobaric GPEtn lipids in the positive MRM data.

This specificity in the negative ion mode allowed for the observation of changes of specific phospholipids after stimulus. For example, 20:1p/20:4-GPEtn decreases much more (red to green, Figure 8) than 18:1p/22:4-GPEtn after A23187 stimulus (blue to pink, Figure 8), which indicates that arachidonate containing GPEtn lipids are more affected by A23187 stimulus.

Using this negative ion MRM method, eight arachidonate containing mTRAQ® reagent labeled GPEtn species were monitored and all showed decreases after treatment with A23187 as compared to the 24 non-arachidonate containing GPEtn species monitored. This negative ion MRM workflow using mTRAQ reagent to label GPEtn lipids will allow for the monitoring of changes of specific phospholipids after biological stimulation which has not been achieved previously.
Conclusions

• The non-isobaric mTRAQ® reagents provide an excellent solution for the quantitative monitoring of Glycerophosphoethanolamine (GPEtn) lipids in human neutrophils.

• This example illustrates a pairwise comparison of treated vs. control samples, however this workflow is extensible to multiple sample comparisons through use of an mTRAQ reagent-labeled Reference Internal Standard strategy, where the labeled control sample is added to multiple treated lipid samples in parallel.

• Ability to use both positive and negative ion polarities with the mTRAQ reagent labeling strategy provides more qualitative and quantitative information.

References

2. K.A. Zemski Berry, R.C. Murphy, J.Lipid Res. 46 (2005) 1038-1046