Development and Application of Isobaric Labeling Strategy for Multiplexed Relative Quantification of Biomolecules in Complex Biological Samples

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Abstract

Biomolecules are essential for the survival and growth of all living organisms, serving as building blocks for various cell structures and performing various physiological functions. Four major types of biomolecules exist in organisms, including carbohydrates, proteins, lipids, and nucleic acids, each performing unique functions in the body and oftentimes working together with other biomolecules to orchestrate complex biological processes. To fully understand the mechanisms and significance of biomolecules, comprehensive characterization and quantification of these molecular players are crucial, requiring the development of sensitive and informative techniques such as mass spectrometry (MS) for analyzing diverse types of biomolecules. To further enhance the information content and overcome technical limitations of MS-based approaches, chemical derivatization is often employed, with the goal to improve ionization efficiency, enable isomer differentiation, enrichment, and quantitative analysis. Isobaric labeling is an example of chemical derivatization used for multiplexed quantitative analysis. This strategy was initially developed for collision-induced dissociation-based quantitative proteomics, but has recently been extended to multiplexed quantitative analysis of other essential biomolecules, such as carbohydrates and lipids. The development and application of isobaric labeling strategy for multiplexed quantitative analysis

is a promising approach to high-throughput bioanalysis of multiple samples, which will help to expand our understanding of biomolecule functions, biological processes, potential pathogenesis, and ultimately lead to the discovery of biomarkers or therapeutic targets. This dissertation focuses on the development and application of isobaric labeling strategy for multiplexed quantitative analysis of several major classes of biomolecules. A portion of this work provides a comprehensive overview of isobaric tagging approaches and their recent advancements, current limitations, and multiple applications, including biomarker identification, structural studies, single-cell examination, and the application to diverse molecules such as neuropeptides, glycans, metabolites, and lipids. Furthermore, this dissertation described several novel isobaric labeling strategies coupled with additional chemical derivatization to expand this technique to quantitative lipidomics, enabling multiplexed quantification of phospholipids and fatty acids with double bonds localized. Besides lipidomics, a new set of DiLeuPMP and DiLeu isobaric mass tags with different isotopic configurations were also designed to achieve isobaric labeling quantitation for O-glycomics and electron-transfer dissociation (ETD)-based proteomics. This dissertation showcases the evolution and implementation of advanced MS techniques and isobaric labeling strategies for quantitative biomolecule analysis, demonstrating their potential applications in pharmaceutical and biomedical research. These developments will further expand the application of isobaric labeling strategies in biomolecule analysis and have great potential in pharmaceutical and clinical research.

Chapter 1

Introduction and Research Summary

Introduction

Biomolecules are essential components of life that are necessary for the survival and growth of all living organisms.[1] These molecules serve as the building blocks of various cell structures, including the cell membrane and nucleus. They also perform various physiological functions such as metabolism and growth. Additionally, they serve as the primary source of energy that fuels all cell activities.

In organisms, there are four major types of biomolecules, namely carbohydrates, proteins, lipids, and nucleic acids. Carbohydrates are sugars that can be classified as monosaccharides, disaccharides, or polysaccharides.[2] They serve as the primary structural components of plants, fungi, and prokaryotes, and as an energy source for many organisms. Carbohydrates also play a vital role in post-translational modifications (PTMs) of proteins, such as glycosylation. Proteins and peptides are complex molecules made up of different amino acids.[3] They perform a wide range of functions, including forming the structure of organisms, maintaining biological environments, transmitting signals, and catalyzing biochemical reactions. In addition to their amino acid sequence, proteins are often modified by other biomolecules or inorganic molecules to alter their states and perform various biochemical reactions. Lipids are unique biomolecules with distinct physiochemical properties.[4] They are hydrophobic and insoluble in water, enabling them to form lipid bilayer membranes that separate intracellular and extracellular environments. These membranes are fundamental to many biomolecules' evolution. Additionally, some lipids are important for producing energy, hormones, and other signaling molecules. Nucleic acids make up the DNA and RNA that are the basis of all life.[5] DNA carries genetic information, and RNA, which is derived from DNA, is responsible for protein synthesis.

Each biomolecule performs unique functions in the body and interact with other biomolecules through covalent or non-covalent bonds to create more complex processes that are essential for sustaining life.[6] A comprehensive investigation of biomolecules and their interactions are crucial for fully understanding the mechanisms and physiological state of living organisms. These understandings and discoveries can contribute to a better elucidation of the molecular mechanism underlying a disease or facilitate studies of aging process and could lead to development of novel therapeutics, ultimately promoting human health and well-being.

Various techniques have been developed to investigate biomolecules, including polymerase chain reaction (PCR),[7] nuclear magnetic resonance (NMR) spectroscopy,[8] and X-ray crystallography.[9] In recent years, mass spectrometry (MS) has emerged as one of the most powerful methods for analyzing diverse types of biomolecules.[10] Among the MS techniques, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are two more common soft ionization techniques. These ionization methods are coupled with various mass analyzers to separate and analyze ionized biomolecules in the gas phase based on their mass-to-charge ratios (*m/z*), often followed by tandem mass fragmentation such as collision-induced dissociation (CID) to produce fragment ions from precursor ions for structural elucidation.

Due to its sensitivity and accuracy, MS is particularly suited for biological sample analysis, as it requires only small sample amounts. Coupling MS with separation techniques such as liquid chromatography (LC) and computational algorithms that can process large datasets empowers MS-based analysis for the large-scale investigation of highly complex systems.[11,12] This approach enables the simultaneous identification of hundreds or thousands of proteins, lipids, and metabolites within a single experiment, making it an invaluable tool in the study of biomolecules.

As LC-MS and software programs become essential tools in MS analyses, chemical derivatization is gaining attention for overcoming specific MS limitations. This pre-MS modification of analytes addresses several MS limitations. First, mass spectrometers only analyze charged molecules, and the ionization efficiency, which depends on molecular properties and structures, is crucial for detection. Derivatization can improve the ionization efficiency of compounds, resulting in higher ionization yields and more intense signals in the mass spectrum, leading to improved detection sensitivity.[13] This enhancement is achieved by introducing functional groups that increase or change the ionization potential of the analyte. Second, differentiating isomeric compounds can be challenging in MS analysis, as isomers share identical molecular weights and exhibit the same signals in MS spectra. Isomer differentiation can be more feasible by selectively reacting with one isomer, resulting in a distinguishable mass difference in the mass spectra, or producing unique MS2 patterns for identification.[14] Third, enrichment is an essential step where the analyte of interest is present at low concentrations in a complex matrix. Introducing an affinity chemical structure with a functional group that can selectively interact with the analyte enables the enrichment of the analytes for specific studies.[15] Lastly, derivatization supports quantitative goals by employing stable isotopes with similar physicochemical properties and ionization efficiency that generate comparable MS responses.[16] This strategy is commonly utilized in quantitative MS analysis to achieve accurate results.

Chemical derivatization has emerged as a valuable approach to address current mass spectrometry limitations, including ionization efficiency, isomer differentiation, enrichment, and quantitative analysis, enabling comprehensive and deeper characterization of various biomolecules. Among these strategies, the use of chemical derivatization for achieving quantitative analysis has been extensively explored.[17,18] Quantification enables accurate measurement of analytes in

complex mixtures, allowing monitoring of changes in different biological states. This facilitates the understanding of biomolecule functions, biological processes, potential pathogenesis, and discovery of biomarkers or therapeutic targets, contributing to the fields of biology, medicine, and pharmaceutical sciences.[19]

In this dissertation, I will discuss the development and application of isobaric labeling strategy for multiplexed quantitative analysis. Isobaric labeling was initially developed for collision-induced dissociation-based quantitative proteomics, here I will explore its applicability to other essential biomolecules by developing novel labeling strategies, MS acquisition methods, or designing novel isobaric mass tags to further expand the multiplexed relative quantification scheme to multiple research areas.

Research summary

Chapter 1 provides an introduction to biomolecules and their significance in the biological systems as well as the studies of these biomolecules by MS-based approaches coupled with separation techniques, computational algorithms, and chemical derivatization. Furthermore, it provides an overview of quantitative analytical strategies and highlights the main focus of this dissertation.

In **Chapter 2**, the concept, the advances, and the application of isobaric labeling strategy are discussed. Isobaric tagging approaches, which are among the chemical isotopic labeling techniques, depend on the analysis from MS2-based quantification rather than MS1-based quantification. A comprehensive overview of several isobaric tags, recent advancements such as complementary ion tags, enhancements in the sensitive quantification of lower-abundance analytes,

tactics to augment multiplexing capabilities, and targeted analysis approaches is presented. Additionally, the constraints of isobaric tags and the methods to mitigate these limitations using other techniques are discussed. Finally, this chapter highlights multiple applications of isobaric tags, encompassing biomarker identification and validation, thermal proteome profiling, cross-linking for structural studies, single-cell analysis, top-down proteomics, as well as the application to diverse molecules like neuropeptides, glycans, metabolites, and lipids, while offering insights and assessments for each application.

Chapter 3 describes an innovative isobaric labeling strategy of phospholipids and sulfated glycolipids for multiplexed quantitative lipidomics. Phospholipids and glycolipids are essential biomolecules that are involved in many biological processes, including membrane fusion, apoptosis, and the regulation of membrane proteins. Disturbance in the homeostasis of lipids is associated with many diseases, such as cancer. To understand the roles of phospholipids in these disease progressions, it is desirable to measure the changes in phospholipids during the progressions. However, the studies of quantification in high-throughput manners were still limited, and the current methods were not able to target all phospholipid classes simultaneously due to the high structural diversity of phospholipids. Herein, a novel diazobutanone reagent was designed to enable the coupling of phosphate and sulfate group-containing lipids and isobaric mass tags, representing the first study to achieve multiplexed quantification of all central phospholipid classes simultaneously on a global scale from multiple complex biological samples.

Chapter 4 introduces a multiplexed quantitative approach by combining isobaric carbonyl-containing compound tags (SUGAR tags) and *m*-CPBA epoxidation strategy to simultaneous identify and quantify fatty acid positional isomers. Fatty acids are essential biomolecules that not only comprise a wide range of lipid species but also involved in many biological pathways. The

functions of fatty acids are greatly determined by their conformational differences in the length of aliphatic chains, the numbers of carbon-carbon double bonds, double bond positions, and cis-trans geometry, etc. Despite unclear mechanisms of fatty acids, numerous diseases are associated with disturbances in the fatty acid metabolism. Comprehensive identification and quantification of fatty acid positional isomers will facilitate the understanding of their functions and roles in disease development. Therefore, the method to combine C=C bond differentiation strategy and isobaric mass tags was explored in this chapter.

Chapter 5 seeks to expand the applicability of collision-induced dissociation (CID)-based isobaric labeling to electron transfer dissociation (ETD)-based analysis. Although LC-MS/MS with CID approaches has become a widely used method for investigating protein post-translational modifications (PTMs). However, many PTMs are unstable under this fragmentation technique, resulting in a loss of PTM localization. ETD fragmentation is an alternative technique for preserving labile PTMs on peptide backbone fragment ions, enabling accurate PTM localizations, while due to its unique fragmentation mechanism, no existing isobaric tags have been able to effectively generate reporter ions. A novel method using complementary ion has been developed to achieve the application of isobaric labeling to ETD based analysis.

Chapter 6 reports on the development of isobaric mass tags for quantitative O-glycomics. Glycosylation is an essential and highly complex PTM. N-glycosylation is the attachment of glycans to amine groups of asparagine residues in proteins, and release of which is achieved via enzymatic treatment. O-glycosylation is the attachment to the hydroxyl groups of serine or threonine residues, chemical release is common methods for releasing O-glycans, while glycan degradation occurs during chemical releasing leading to the loss of glycan structural information. Derivatizing glycans with 1-phenyl-3-methyl-5-pyrazolone (PMP) in alkaline conditions has been

reported to release O-glycans effectively with minimal degradation and improve the detection of O-glycans. In this chapter, we designed an isobaric mass tag, N,N-dimethyl leucine containing pyrazolone analogue (DiLeuPMP), for one-pot releasing and 4-plex labeling of O-glycans from O-glycopeptides.

The concluding chapter provides the overall conclusions and future directions of various subsequent projects. In summary, this dissertation showcases the evolution and implementation of advanced mass spectrometry techniques and isobaric labeling strategies for quantitative biomolecule analysis including peptides, phospholipids, fatty acids, and O-glycans. This research highlights the utility of MS-based analytical platforms for various biomolecule quantitative analysis and illustrates their applications in pharmaceutical and medical research.

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Chapter 2

Recent advances in isobaric labeling and applications in quantitative proteomics

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Abstract:

Mass spectrometry (MS) has emerged at the forefront of quantitative proteomic techniques. Liquid chromatography-mass spectrometry (LC-MS) can be used to determine relative abundances of proteins and peptides in complex biological samples. Several methods have been developed and adapted for accurate quantification based on chemical isotopic labeling. Among various chemical isotopic labeling techniques, isobaric tagging approaches rely on the analysis of peptides from MS2-based quantification rather than MS1-based quantification. In this review, we will provide an overview of several isobaric tags along with some recent developments including complementary ion tags, improvements in sensitive quantitation of analytes with lower abundance, strategies to increase multiplexing capabilities, and targeted analysis strategies. We will also discuss limitations of isobaric tags and approaches to alleviate these restrictions through bioinformatic tools and data acquisition methods. This review will highlight several applications of isobaric tags, including biomarker discovery and validation, thermal proteome profiling, cross-linking for structural investigations, single-cell analysis, top-down proteomics, along with applications to different molecules including neuropeptides, glycans, metabolites, and lipids, while providing considerations and evaluations to each application.

Introduction

Mass spectrometry (MS) is a highly sensitive analytical tool that can be used to determine abundance levels of proteins and peptides in complex biological mixtures. Quantification results can be used to understand different biological and pathological processes. Quantitative proteomics combined with liquid chromatography-mass spectrometry (LC-MS) has been a leading method to quantify proteins and peptides. Existing proteome-wide quantification methods can be classified into label-free proteomics and label-based proteomics.[1-4]

Label-free proteomics does not involve any derivatization with chemical isotopic labeling of a sample and requires each sample to be separated in individual LC-MS or LC/LC-MS/MS runs.[5,6] Relative quantification of proteins is performed either by comparison of chromatographic peak area or peptide peak areas, or via spectral counting of identified protein spectra.[7] Label-free quantification (LFQ) has been found to be effective for high identification of proteins and peptides based on advancements in data processing and acquisition strategies such as delayed normalization, maximizing peptide ratio extraction, utilization of programs like MaxQuant or OpenMS.[8,9] LFQ does inherently suffer from several limitations compared to chemical labeling methods due to variability in reproducibility between technical replicate injections. Such variation can arise from differing sample preparation between samples, variation in sample injection, and time limitations due to running only one sample at a time, thus creating long run times for larger quantities of samples. This may lead to sample degradation, thus artifactually affecting quantitation results. It also is prone to sample loss, ionization efficiency variation, and retention time shifts between runs.

Chemical isotope labeling was first introduced through the isotope-coded affinity tag (ICAT) strategy and expanded to other methods, including stable isotope labeling by amino acids

in cell culture (SILAC), dimethyl labeling, ^{18}O labeling, and neutron encoding (NeuCode) SILAC.[10-13] These methods introduce small mass differences via heavy isotopologues either at the protein or peptide level that can be distinguished from one another at the MS1 precursor spectrum. Multiple samples can be simultaneously analyzed as respectively labeled peptides can be resolved via MS. Furthermore, pooling of samples prior to LC-MS decreases sample variation across the workflow, signal variation, and overall analysis time.[14,15] To determine abundance changes, ratios of peptide signal intensities are compared between heavy/light peptide pairs with further protein statistical evaluation. Chemical isotope labeling has limitations in multiplexing capabilities as the higher the number of labeled samples analyzed at the MS1 level; spectral complexity increases due to each analyte contributing multiple peaks.[16] Different methods generally also require a minimum mass shift of 4 Da to prevent overlap of isotopic envelopes, thereby requiring a tag with larger mass that could inherently reduce the overall identification numbers or requiring a high-resolution mass spectrometer that can distinguish small mass differences.[17,18]

MS2-based isobaric quantification overcomes some of the limitations to the MS1-based quantification methods by labeling the same peptides with isobaric tags. Instead of labeling via mass difference methods such as SILAC and dimethyl labeling, isobaric tags are structures that have similar physical and chemical properties and identical masses with different isotopic configurations in each channel to label peptides from various samples. A standard design for an isobaric tag consists of three elements: (1) a reactive group to target specific functional groups or residues on peptides, (2) a balance group that contains isotopes to ensure the same overall mass of the tag based on the reporter group ion mass, and (3) a reporter group that contains discrete isotopes for different channels that allows for relative quantification between samples. Peptides of the same *m/z* values at the MS1 level will appear as one composite peak, which upon fragmentation will

produce peptide fragment ion peaks and reporter ion peaks. Relative intensities of reporter ions are distinguished at small *m/z* values to not interfere with peptide backbone fragment ions at higher mass and allow for determination of relative quantitative information. The peptide fragment ion peaks are utilized to resolve amino acid sequences, which are then assigned to a protein identification based on sequences. A typical workflow for isobaric labeling is illustrated in **Fig. 1.** Isobaric tags allow for high multiplexing capabilities that increase throughput and reproducibility due to pooling of samples prior to running on LC-MS and decreasing variability with no increase of spectral complexity.[19] We discuss notable examples of isobaric tags in the following sections and summarize their varieties in Table 1.

TMT and iTRAQ.

The first example of an isobaric tag was proposed by Thompson et al. as Tandem Mass Tag (TMT).[20] The concept of TMT was initially designed as a duplex tag with fragmentation occurring at the proline residue on the N-terminus. This paradigm allowed for subsequent acquisition of peptide backbone and reporter ions via collision-induced dissociation (CID) for relative quantification by labeling at free N-termini of peptides along with ε-amino functions of lysine residues. The 2-plex tag had two different generations with the major difference including a proline enhancement group in the second version but maintaining the same reporter ion group and amine reactive group at the C-terminus. The 2-plex was increased to a 6-plex by reducing the size of the reporter ion to a dimethylpiperidine and balance group to be more compact, while maintaining the same N-hydroxysuccinimide (NHS) moiety as the reactive group.[21] The 6-plex also removed the use of deuterium ions with 13C and 15N isotopes to remove the deuterium effect causing retention time shift. TMT was further extended to a 10-plex system with neutron encoding

that took advantage of neutron binding energy differences between C and N isotopes to create mass shift differences of 6.3 mDa, which can be resolved at a resolution of 50k with reporter masses ranging from m/z 126 to 131,[22,23] The 10-plex was also modified to accommodate electron-transfer dissociation (ETD) via substitution of heavy carbons for heavy nitrogen. 11-plex is currently the highest commercially available multiplexing capacity due to synthetic capabilities and cost restrictions, but it would be possible for 18-plex capabilities if every N and C in the structure are replaced with isotopic variants.

Recently, a 16-plex proline-based tandem mass tag (TMTpro) was established employing an isobutyl-proline as the reporter ion along with two β-alanine residues on the extended balance group instead of glycine-based tags.[23] Neutron encoding was incorporated into the structure's initial 9 tag channels to increase to a 16-plex with 6.3 mDa differences and 1 Da differences between reporter ions. When compared to the 10/11-plex, TMTpro had similar identification numbers for total proteins and peptides with high labeling efficiency, but TMTpro required less time for MS3 ion injection times. At lower collisional energies, TMT10/11-plex outperformed TMTpro with quantitative accuracy, but at higher energies, TMTpro outperformed the former.[24] Currently, TMTpro has the capacity of an 18-plex with recent developments.

Isobaric tag for absolute and relative quantification (iTRAQ) was published not long after the first example of TMT with a 4-plex tag design.[19] Similar to TMT, iTRAQ isobarically labels peptides at N-termini and lysine side chains, with reporter ions being distinguishable after CID fragmentation at low m/z values. iTRAQ retains the same amine reactive NHS moiety as TMT but opts for a smaller carbonyl balance group of m/z 28 to 31 and a N-methylpiperazine reporter ion group of m/z 114-117 for an overall total mass of 145. It also does not utilize any deuterium ions on the structure but does place ^{18}O on the balance group compared to TMT and TMTpro which

only utilizes 13C and 15N isotopes. Eventually iTRAQ was increased to an 8-plex system with reporter ion masses ranging from m/z 113 to 121.[25] Comparison later showed that lower identification numbers of protein and peptides result from the 8-plex compared to the 4-plex iTRAQ and the 6-plex TMT, which may be due to internal fragmentation of the tag structure.[26-28] The findings of the initial study were questioned by Pottiez et al. This study compared 8-plex iTRAQ to the 4-plex iTRAQ with the 8-plex providing more accurate quantitation compared to the 4-plex version.[29] All three of the tags (4-plex iTRAQ, 6-plex TMT, and 8-plex iTRAQ) though had similar dynamic ranges and precision with peptide-spectrum matches. **Fig. 2** illustrates the structures of iTRAQ, TMT 10/11-plex and TMTpro side by side.

DiLeu:

N,N-Dimethyl Leucine (DiLeu) is an isobaric tag developed by our lab as an alternative to the commercially available TMT and iTRAQ reagents. DiLeu was originally proposed as a 4-plex tag by Xiang et al. for MS2 isobaric labeling with reporter ions ranging from m/z 115 to 118 and a mass shift of 145.[30] The structure consists of a dimethylated leucine as the reporter group, a carbonyl balancing group and a triazine ester amine reactive group at the C-terminus. One of the major differences compared to other isobaric tags is the inclusion of a triazine ester for the reactive group. DiLeu labeling has been reported to generate more intense reporter ions in comparison to iTRAQ-labeled peptides due to the dimethylated reporter ion structure. DiLeu can also achieve better fragmentation of peptide backbones with reduced overall collision energy compared to other tags. Other benefits of DiLeu compared to TMT and iTRAQ include the lower cost to synthesize different channels, high yield percentage of tag over 80 to 90%, and do not require custom reagents to synthesize, while still maintaining high labeling efficiency and accurate quantification of

proteins and peptides. A current limitation to DiLeu tagging strategy has been the relatively lower coverage due to sample loss from additional strong cation exchange (SCX) cleanup step for the need to remove excess tagging reagents. The leftover tagging reagents could also cause signal suppression of lower abundance proteins and peptides. The multiplexing capability of DiLeu was expanded upon via neutron encoding to increase multiplexing from 4-plex to 12-plex with subtle mass differences of ~6 mDa between different reporter ions.[31] These differences could be distinguished at baseline separation of resolving power (RP) 30K via Orbitrap HCD tandem MS, though the most accurate quantification occurred at RP of 60K and greater. Reducing the mass differences to 3 mDa via neutron encoding and a stepwise mono-methylation increased the multiplexing capabilities to a 21-plex system as shown in **Fig. 3**.[32] All 21 reporter ions are resolvable at RP 60K (m/z 400) via HCD LC-MS/MS with reporter ions ranging from m/z 115 to 118. A key difference with 21-plex DiLeu is the implementation of a stepwise N-monomethylation strategy to protect leucine with benzyl chloroformate to allow derivatization of a single N-methyl group, rather than reductive dimethylation which was previously utilized. This strategy allows for incorporation of an odd number of deuterium isotopes onto the reporter ion to develop new channels. One feature of DiLeu tags is the incorporation of deuterium ions that affect retention time of labeled peptides. DiLeu, however, also takes advantage of placing deuterium proximal to the amines mitigating interactions with reversed-phase stationary phases during LC separation.

DiLeu multiplexing has also increased with incorporation of a β-Alanine group in the balance group and 1 Da spacing between reporter groups, which increased multiplexing to an 8-plex isobaric tag.[33] The 8-plex DiLeu varied from the 4-plex, 12-plex and 21-plex DiLeu tags due to the NHS moiety being more stable with the DiLeuAlaOH molecule.[31,34] 8-plex DiLeu exhibits greater retention time shifts from deuterium ions being placed on the balance group, in comparison

to the more compact structures, but can be alleviated with utilization of non-deuterated isotopologues of alanine.[35] DiAla and DiVal have also been synthesized by our lab as a variation on the dimethylated amino acid structure of DiLeu. We found that DiAla produced more abundant backbone peptide fragmentation allowing for higher protein identification and quantification numbers but resulted in lower reporter ion intensity compared to DiLeu.[36,37] DiAla was still able to produce accurate quantitative analysis of peptides and complete labeling. The combinatorial usage of different demethylated amino acid tags may offer enhanced quantitation accuracy and protein identification coverage.

DiART and IBT:

Another alternative to commercially available isobaric tags is deuterium isobaric amine reactive tag (DiART).[38,39] DiART contains an NHS amine reactive group similar to TMT and iTRAQ, a β-alanine balancer, and a *N,N*-dimethyl leucine reporter group with m/z between 114 to 119 similar to DiLeu with the capability of 6-plex. DiART has been compared to iTRAQ previously and reported to have a stronger reporter ion that enhanced signal to noise ratio (S/N) along with less ratio compression.[40] The isobaric tags (IBT) represent another alternative to other isobaric tags.[41] Structurally, IBT is similar to DiART, although the β-Alanine balance group is replaced with alanine due to lower costs of isotopically labeled alanine. It is structurally identical to DiLeu, though it does not incorporate ^{2}H and ^{18}O isotopes to avoid deuterium chromatographic shifts. IBT also is activated in a different manner utilizing TSTU (1,1,3,3-tetramethyl-O-(N-succinimidyl) uronium tetrafluoroborate) for an NHS amine reactive moiety.

Factors/Disadvantages:

Isobaric tags offer many advantages compared to chemical isotopic labeling and label-free quantification strategies with multiplexing, which not only increases sample throughput, quantitative accuracy, and reproducibility but also reduces run-to-run variability, instrument time, and missing values, yet there are some disadvantages that must be considered. Ratio distortion is a common issue with isobaric tags when peptides of similar mass co-isolate with each other in an isolation window that then distorts the reporter ion intensity in the corresponding MS2 spectra.[17] Several methods have been developed with the intention of mitigating or removing this distortion from occurring including narrowing the precursor isolation window, addition of a gas-phase reaction, delaying fragmentation at the apex of LC peaks, MS3 fragmentation, and estimation of redundant MS2 spectra from precursor ions.[42-47]

Isotopic impurities cause contamination in reporter ion peaks due to isotopes contributing to adjacent reporter ions. Corrections must be made to account for this isotopic overlap to ensure accurate quantification. We recommend readers to view a previously published procedure that describes how to calculate peak areas that account for these contributions via reagent purity values.[48]

The accuracy of quantification can also be affected by a mass detector's saturation point.[49,50] Reporter ion intensities have upper intensity limits and intensities may be underestimated due to saturation effects of the detector, which is instrument dependent. Intensity can be influenced by increasing MS/MS acquisition while diminishing the number of possible identifications and also by spiking in samples of known concentration to confirm expected protein and peptide ratios.[51]

Another point of emphasis to mention is that variability can occur across batch from batch of different isobaric tags. This was apparent when a large-scale study was conducted on TMT that identified significant missing values of proteins across different batches of tags, that influences the high precision of quantitation.[52] Although normalization methods could mitigate batch-to-batch variations, it is important to remember that variations could potentially occur from any isobaric tag, whether commercial or synthetically made.

Unconventional Labeling Methods:

Besides the above-mentioned tags, there are other novel isobaric tags that have been developed that could be called unconventional compared to the standard ideology of reporter, balance, and amine reactive groups. One such example is the combinatorial isobaric mass tag (CMT).[53] The concept of CMT is that every isobaric tag will produce two reporter ions from fragmentation that are independent from one another with *m/z* values ranging from 126 to 128 and 172 to 175, respectively. This tag has the capacity of a 28-plex utilizing mass shift differences of ~6 mDa, even though the published results showed a 6-plex, perhaps due to a constraint of RP or due to the amount of time and resources necessary to synthesize a 28-plex.

Another set of examples involves peptide backbone fragmentation, where to circumvent the issue of reporter ion distortion, specific fragment ions are employed from the backbone to mitigate this effect. The first reported method was published in 2009 by Koehler et al. known as isobaric peptide termini labeling (IPTL).[54] Derivatization occurs at lysine residues followed by succinylation with variants of succinic anhydride, which will generate different fragment ions that can be compared via b and y ion intensities from proteins and peptides. IPTL has since been expanded to several new strategies.[55-58] Initial IPTL strategies are limited compared to labeling reagents due to minimal

capacity to multiplex and complexity of MS2 spectra from multiplets of fragment ions. Since the initial report, new methods have been developed that increase multiplexing to a 7-plex system and minimize spectral complexity via data software optimization.[42,43,59-63]

Recent developments include chemical tagging strategy that will contain complete peptide sequences coupled to the balance and reporter ion groups referred to "peptide-coupled reporter-ion based quantification." Instead of producing the same set of reporter ions for various peptides after fragmentation, reporter ions are peptide-specific. The first example of this was complement TMT (TMT^C).[42] Designed as an alternative to replacing any MS3 scans or additional purification steps, TMT^C relatively quantifies differences in samples from the complementary TMT fragment ion cluster rather than the reporter ions of the same masses. This method is limited due to the isotopic envelope of reporter ions from a precursor isolation window of 2 Th, and overall efficiency due to peptide ion charge state and sequence. To reduce these problems, a modified version was developed called TMT^{C+} that reduces the isolation window down to 0.4 Th, which also mitigates the long instrumental duty cycles from TMT^C.[43]

Another peptide-coupled reporter ion tag was developed in 2018 by Winter et al. called easily abstractable sulfoxide-based isobaric (EASI) tag.[64] The tag design includes an NHS amine reactive group, a balance group, and a neutral loss group that fragments at the sulfoxide group to enhance dissociation efficiency. This combined with an asymmetric isolation window to suppress adjacent peaks and lower collision energy needed for fragmentation makes it an attractive replacement for accurate and sensitive quantification as a 6-plex. Similar to EASI tag, the acetyl-alanine-glycine (Ac-AG) tag was developed as a new peptide-coupled reporter-ion-based tag with improvement via ionization efficiency but is also versatile for DIA.[65]

Increase in Multiplexing Capabilities:

Hybrid methods have been developed that combine MS1 and MS2 based quantification from isotopic mass differences and isobaric labeling to increase throughput and multiplexing capabilities. The initial idea of "hyperplexing" was developed by coupling 6-plex TMT with triplex SILAC to achieve 18-plex quantification.[66,67] A recent method combined 16-plex TMTpro along with 3-plex mass tags for absolute and relative quantification (mTRAQ) to achieve 48-plex capabilities for a novel NHS-ester tandem labeling in a one-pot (NETLOP) workflow.[68] A targeted proteomics approach has also been developed by combining 3 distinct TMT reagents and 6-plex TMT tags with 3 mass variation of targeted peptides to achieve 54-plex quantification.[69] Another technique made called combined precursor isotopic labeling and isobaric tagging (cPILOT) combining duplex stable isotope dimethyl labeling and 6-plex TMT labeling permitting 12-plex quantification of N-termini and lysine residues.[70] We developed a similar approach known as DiLeu cPILOT combining stable isotope dimethyl labeling with 12-plex DiLeu isobaric tags for 24-plex quantification utilizing a synchronous precursor scan (SPS)-MS3 acquisition method.[71] For deeper discussions of higher order multiplexing, we refer the reader to an excellent review published elsewhere.[72]

Targeted Analysis Approaches:

Absolute quantification is generally accomplished with targeted mass spectrometry with methods such as selected reaction monitoring (SRM) and parallel reaction monitoring (PRM).[73-76] These methods combine stable-isotope encoded peptides standards that are spiked in at known concentrations to determine absolute abundance of target peptides via signal intensity (AQUA).[77,78] A method known as triggered by offset, multiplexed, accurate-mass, high-resolution

and absolute quantification (TOMAHAQ) was developed with synthetic trigger peptides being utilized as an offset mass to trigger quantification.[79] These methods do depend on single point calibration, however, increasing the degree of potential inaccuracy due to the wide quantitative span of peptides across orders of magnitude and rely on expensive isotopically encoded peptide standards and isotopic labeling reagents. Recently our lab has developed a strategy similar to TOMAHAQ called hybrid offset-triggered multiplex absolute quantification (HOTMAQ) strategy that combined isobaric 12-plex DiLeu tags with 5-plex isotopic DiLeu (iDiLeu) tags that utilize 3 Da mass differences between channels to enable accurate absolute quantification or targeted peptides in higher throughput.[80,81]

Improvements for Identification of Lower Abundance Species:

Efforts have been made to improve on identification of lower abundance peptides and proteins. Due to poor MS signal intensity and the suppression from higher abundant species, a recent influx of new methods has been developed to mitigate these effects.[82-84] Several of these techniques utilize match between runs (MBR) to align retention time measurements of lower abundant peptides to their corresponding *m/z* to a library or reference run to yield new identification. A recent method called BoxCar combines MBR with a new acquisition method to boost the precursor MS1 signal with a library to identify over 10,000+ proteins in a 100 min gradient LC-MS/MS analysis.[85] One strategy that has caught the attention of the field is boosting to amplify signal with isobaric labeling (BASIL).[86] This approach works by using isobaric tags with one of the channels being significantly higher in sample amount compared to other channels. This strategy enhances detectability of precursor signal intensities of peptides and identifiability from fragment spectra. The workflow for BASIL is illustrated in **Fig. 4.**

Post Translational Modification (PTM) Specific Tags:

Isobaric tagging has been widely utilized in PTM quantitative analysis. For example, phosphoproteomics coupled with high-specificity enrichments has been shown to be a well-studied PTM with the use of isobaric tags.[92] In our lab, we are also developing a strategy to couple DiLeu isobaric tagging with BASIL to enhance the analyses of glycoproteomics. Besides isobaric tags targeting amine groups, other tags have been developed that are specifically designed to target sub-classes of proteins and peptides. Initial concepts began with targeting cysteine residues with the cleavable isobaric labeled affinity tag (CILAT).[87] CILAT takes advantage of an isobaric tag structure with affinity enrichment that utilizes a biotin affinity tag and acid-labile linker. Another tag that targets cysteine residues is called cysteine TMT (cysTMT).[88,89] cysTMT was designed to be an alternative N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio) propionamide (biotin-HPDP) to detect S-nitrosylation with a similar Cys reactive group, a smaller balance group and a mass reporter. Subsequent development of iodoacetyl TMT (iodoTMT) has since replaced cysTMT with the principle remaining constant of quantifying protein S-nitrosylation.[90] Compared to cysTMT, iodoTMT irreversibly labels Cys thiols that improves overall labeling efficiency.

Several tags have been developed to detect carbonyl groups for the purpose of quantification. The first of these tags developed was the carbonyl-reactive tandem mass tag (glycoTMT) with a broader application of the tag being applied with aminoxy reactive TMT (aminoxyTMT).[93] Another tag known as quaternary amine containing isobaric tag for glycan (QUANTITY) was developed for quantification of N-glycosylation.[94] Isobaric aldehyde reactive tag (iART) was created by Yang et al. as another alternative for glycan analysis.[95] Further development led to isobaric tag for absolute and relative quantification hydrazide (iTRAQH).[96] Our lab has developed an alternative for analysis of carbonyl groups with isobaric multiplex

labeling reagents for carbonyl-containing compound (SUGAR) tags.[97] SUGAR features efficient multistep synthesis at a lower cost with high labeling efficiency of N-glycans. Recently our lab developed the first isobaric tag aimed to quantify O-glycans in the form of 4-plex dimethyl leucine containing pyrazolone analogue (DiLeuPMP).[98]

Applications of Isobaric Tags:

Biomarker Discovery

Quantitative proteomics has been combined in several facets to biomarker discovery in a variety of different diseases.[99,100] One example used iTRAQ 8-plex tags to label serum protein digests and identify biomarkers for acute myocardial infarction.[101] iTRAQ 8-plex was also utilized to quantify targets for breast cancer from tissue samples.[102] Several examples have been used to identify cancer related targets including biomarkers for tumor metastasis and dysregulated proteins found in leukemic stem cells.[103-105] TMT has also been integrated into biomarker discovery by Tokuoka et al. to discover lipid biomarker candidates related to Alzheimer's Disease.[106] A point of emphasis in biomarker studies to consider is potential variance when it comes to the type of sample being utilized and the type of quantification with tags. Isobaric tags can heavily underestimate fold changes of different disease states and, given that biomarkers need extensive orthogonal methods for verification and validation, this can be detrimental for potential false positives and negatives. Several strategies we have mentioned previously can be implemented to mitigate these effects. It is necessary, however, to have targeted approaches and absolute quantification methods for validation and verification of biomarkers.

Study of Neuropeptides and Isobaric Tags

Neuropeptides comprise a large class of signaling molecules with prominent roles in various body functions including growth and metabolism.[107] Neuropeptides are very challenging to analyze due to their variety in functions, sequences, and sizes.[108-111] They are also difficult to analyze due to their trace level in vivo. Several examples show it is possible to quantify neuropeptides at the MS1 level, however it is necessary to have MS2 quantification strategies to mitigate the spectral complexity at the precursor level.[112-115] In one example, we quantified neuropeptides impacted from the gut microbiome with label-free quantification and 10-plex DiLeu isobaric tags with 282 labeled species exhibiting changes in regulation.[115] Another work performed relative quantification of neuropeptides from American lobsters using 4-plex isobaric DiLeu tags at different stages of lobster brain development.[116] Most recently Sauer & Li performed relative quantification of neuropeptides in response to copper toxicity with 4-plex DiLeu tags.[117]

Isobaric Tags and Metabolomics:

Metabolomics is a rapidly growing field of research in omics-based studies. Metabolites are small molecules that are either intermediates or products of metabolic processes with typical molecular weights lower than 2000 Da. Challenges of metabolomics include the wide range of biological variance in metabolite levels and the structural variety of metabolite molecules that prevents the existence of a universal analysis method. Furthermore, some metabolites are very labile and may degrade quickly, thus making their analyses more difficult.[118] Typically, metabolomics can be studied in a targeted manner with MRM via tandem MS for an increase of sensitivity and selectivity of metabolites for absolute quantification, or from an untargeted approach for global detection of metabolites via relative quantification.[119] Several examples show

absolute and relative quantification of metabolites utilizing isobaric tags.[119-122] A study by Hao et al. featured the first use of mass defect DiLeu (mdDiLeu) tags to study metabolites from pancreatic cancer cells.[122] The 12-plex DiLeu tags were also utilized to quantify urinary metabolites.[123] Furthermore, a software tool called Metandem has been developed to facilitate MS-based isobaric labeling metabolomics.[124]

Thermal Proteome Profiling with Isobaric Tags:

Drug-targeting methods to understand protein-ligand interactions are becoming increasingly popular. Targeted approaches have proteins or peptides that are either activated or inhibited from chemical processes to measure different activities of large arrays of proteins at once. This is different compared to typical phenotypic approaches that rely on observable traits.[125-127] Approaches range from affinity purification, activity profiling, protein painting, studying of stabilities and enrichment strategies.[126-129] Many of these approaches are limited due to requiring chemical modifications and are limited to cell extracts, making them impractical for in vivo studies.

One approach that would not require chemical modifications is based on ligand binding. An increasingly popular method based on biophysical alterations to study protein abundance changes within recent years is from the cellular thermal shift assay (CETSA),[130] based on the principle that ligand binding will affect protein thermal stability. This method treats cells either with a control or drug of interest and perturbs them with subtle temperature differences via a thermal cycler to influence conformational state changes of protein-ligand complexes. A thermal shift curve can then be built to distinguish differences in regulation of proteins based on drug-ligand interactions.

Thermal proteome profiling (TPP) is based off CETSA where cells are treated either in vivo or extracted with subsequent heat perturbation and then labeled with isobaric tags for multiplexed quantitative mass spectrometry as illustrated in **Fig. 5**.[131] Thermal shift curves are created from normalization of reporter ion intensities and complex programming to determine protein regulation differences. This method offers an unbiased approach towards studying drug target interactions in a high-throughput manner. Nonetheless, it suffers from limited information related to the binding site.

Since TPP's inception, there have been several new methods developed to improve and expand its applicability.[132] TPP has been expanded to monitor membrane proteins, phosphorylated proteins and peptides via phosphoproteomics combined with TPP (phospho-TPP), bacteria, plants, plasmodium, yeast, viruses, tissue samples, and plasma membrane proteins.[133-146] Other protocol based methods include a two dimensional approach that studies protein abundance and regulation changes simultaneously, a proteome integral solubility alteration (PISA) to increase throughput and reduction in data analysis, and utilization of a vacuum manifold to increase throughput.[147,148] Data analysis approaches have been developed as well including nonparametric analysis of response curves procedure (NPARC) and detection of ligand-protein interactions from thermal profiles (DLPTP).[149,150]

Cross-Linking Approaches

Cross-linking mass spectrometry (XL-MS) is a powerful tool to provide spatial details about proteins and protein-protein interaction information complementary to current predominant techniques for protein structure determination, such as X-ray crystallography. The advantages to

this method include a requirement of small sample sizes, application to highly complex samples, and providing data on dynamic conformations and transient states in the solution.[151]

The concept of quantitative cross-linking with mass spectrometry (qXL-MS) has emerged recently enabling the assessment of changes in proteins under different conditions. As in other quantitative proteomics studies, the application of isobaric labeling would be a major player in permitting large-scale comparative analyses. An analytical platform proposed by Yu et al. involved an MS-cleavable crosslinker DSSO and labeled cross-linked peptides after enzymatic digestion.[152] In the MS2 scan, S-C bonds in DSSO crosslinker were cleaved preferentially by CID to generate single peptide chains that still contain TMT tags. The peptide sequencing and quantification were then done at the MS3 level. The coupling of cleavable crosslinker and isobaric labeling not only facilitates the identification of cross-linked peptides but also enables comparative analysis. An accurate quantitative result has been achieved to demonstrate the compatibility of isobaric labeling in cross-linking. However, despite the use of TMT tags, only binary comparison has been conducted in this study where the advantage of the multiplexed capacity of isobaric tags was not presented.

Recently, a novel crosslinker has been introduced called isobaric quantitative protein interaction reporter (iqPIR), where stable isotopes were incorporated in different regions of crosslinker structures, eliminating the need for additional isobaric tagging.[153] Upon fragmentation, reporter ions were generated at the MS2 level with high sensitivity along with complementary fragment ions to provide additional quantitative information. This approach allows samples to be pooled once crosslinking has been done, therefore minimizing the variations which might be introduced during sample preparation. With the design using amino acids as building blocks, iqPIR can offer a higher multiplexing capacity and has been extended to 6-plex iqPIR recently.

Single-cell analysis

The study of single cells enabling elucidation of many fundamental cellular processes and investigation of biological systems with single-cell resolution offers tremendous value in biomedical research.[154] MS methods capable of analyzing thousands of proteins in a single experiment provide a promising tool towards this goal. However, despite the high sensitivity of MS, the losses arising from sample preparation and delivery of proteins to MS analyzers confined their development of single-cell analysis.[155] Recently, advances in many aspects of workflows have favored analysis of small samples and made the profiling of considerable number of proteins from a single mammalian cell possible. Among those advances, isobaric labeling is one of the innovative strategies to alleviate several common difficulties in the single-cell analysis field.[156]

The first application of isobaric labeling to mammalian single-cell analysis was published in 2018.[157] Single Cell ProtEomics by Mass Spectrometry (SCoPE-MS) strategy labeled single-cell samples with distinct channels of TMT reagents and pooled them with carrier channels which labeled 200 cells with one of isobaric channels. As shown in **Fig. 6**, the key feature of a second-generation method (SCoPE2) is the isobaric carrier concept where different channels of TMT tags are employed to label proteins from a carrier sample, a reference sample, and single cells, with defined ratios.[158] For example, sample losses occurring throughout sample preparation due to nonspecific absorption over large surfaces have been reported to have a substantial impact on small samples, while the introduction of carrier samples significantly disperse the surface adhesion from single-cell samples. Moreover, the signals of peptides that might fall below the detection limits would be detected with the enhancement from carrier samples and thus trigger MS2 fragmentation for sequencing peptides.

This strategy has been further improved by Tsai et al. to achieve reliable and precise quantitation and optimal experimental conditions by evaluating different carrier-to-sample ratios and MS acquisition settings, such as automatic gain control (AGC) and injection time (IT).[159] However, there are some potential pitfalls for using isobaric labeling in single-cell analysis. According to the previous reports, experimental designs need to be carefully optimized for each instrument and sample type to achieve reliable quantification and maximum protein identification. Since additional fractionations and MS3 are not favorable to small samples, ratio compression presents a more serious problem for single-cell analysis than bulk proteomics leading to skewed measurement. The choices of carrier samples that determine the sets of peptides to be enhanced and detected are also crucial for getting meaningful results.

Data-independent acquisition (DIA)

DIA features higher reproducibility, fewer missing values, and better quantitative comparisons between runs compared to data-dependent acquisition (DDA).[160] The execution of fragmentation is not based on signals in MS1 scan, so DIA is more likely to detect proteins in low abundance. To achieve higher sample throughput, the applicability of isobaric labeling has been studied. Since DIA co-isolates multiple precursor ions in a wider m/z window for MS2 fragmentation, all reporter ions from different precursor ions will be present in the same spectrum, meaning that those reporter ions no longer represent the abundance of specific peptides. Therefore, the unconventional mass tags that use neutron encoding or produce complementary reporter ions are often employed for DIA analysis. Our lab demonstrated a strategy using mdDiLeu to analyze human cerebrospinal fluid for biomarker discovery using DIA mode.[161] As shown in **Fig. 7**, to ensure the labeled analytes to be isolated in the same DIA window and minimize MS1 spectral

complexity, millidalton differences between channels were introduced to analytes, by which precursor ions remained to exhibit single ion peaks using a lower resolving power in the MS1 scan. Upon HCD fragmentation, multiplets of b- and y-ions would be generated and detected under a higher resolving power for multiplexed quantification. This method showed a higher reproducibility and more quantifiable proteins compared to DDA while maintaining high quantitative accuracy.

The use of complementary reporter ions in DIA mode has been proposed to circumvent ratio distortion resulting from co-isolation.[162] Although this method seems compatible with DIA mode, additional fragments originating from tag labeling further complicate DIA MS2 spectra which are already convoluted, where the interpretation of spectra will become more challenging. On top of that, compared to high-throughput methods without labeling empowered by simplifying sample complexities or comprehensive spectral libraries, the elevated spectral complexities and the requirement of ultra-high resolving powers which increases instrument cycle time hinder isobaric labeling methods in DIA mode.[163,164]

Top-Down Proteomics:

In contrast to bottom-up proteomics, top-down methods analyze intact proteins without using proteolytic digestion. This strategy preserves protein modifications to a higher extent and characterizes proteoforms that might be unexpected or not detectable using bottom-up approaches. Owing to the nature of intact proteins, the development of chemical labeling encounters many challenges, including protein precipitation, incomplete labeling, and inherently lower signal-to-noise ratio.[165] The earliest report of isobaric labeling of intact proteins from 2007 described the derivatization of standard proteins using iTRAQ, followed by gel electrophoresis separation and

in-gel tryptic digestion before MS analysis.[166] A later report took a similar approach using serum from patients who later developed pancreatic ductal adenocarcinoma. Following immunodepletion, serum proteins were labeled with 6-plex TMT, separated using gel electrophoresis, then enzymatically digested.[167]

Further pilot studies have demonstrated proof-of-principle for labeling-based top-down proteomics (without subsequent enzymatic digestion) using TMT reagents, albeit partially suffering from the previously mentioned limitations.[168,169] IodoTMT reagents were applied to complex samples by Winkels et al.[170] The use of thiol-reactive TMT instead of amine-reactive reagents, which is more sensitive to aqueous solutions, allowed a lower amount of organic solvent for labeling conditions, so that the depletion of larger proteins was not necessary to avoid protein precipitation. IodoTMT showed quantitative derivatization and accurate quantification in a 6-plex analysis. This workflow performed isobaric labeling right after protein reduction, which minimized the variation between samples introduced during sample preparation. Although this method was restricted to cysteine-containing proteoforms, simple and flexible experimental designs showed promising applicability for multiplexed top-down proteomics.

Conclusions and Future Directions:

Relative quantitation via isobaric labeling is a powerful technique for quantitative proteomics studies because of the higher throughput and reproducibility from high multiplexing capabilities while mitigating variation from run to run. In this review, we have discussed a number of isobaric tagging reagents and their multiplexing and quantitation capabilities. We further explored a plethora of applications that isobaric tags can be applied to along with different molecules that can be studied. In recent years, the number of applications of isobaric labeling in

MS-based studies has skyrocketed. In parallel, the number of publications describing new labeling reagents and strategies, even beyond just proteomics, has also increased.

Based on recent trends in the field, we speculate that isobaric tagging technology will continue to grow with increasing multiplexing capabilities. The commercial TMTpro 16plex reagent set is currently available, though recent reports have described multiplexing from 21-plex up to 48-plex.[32] One limitation to increasing multiplexing capability is the necessity of higher MS2 resolution for ensuring quantitative accuracy, which could result in lower identification rates due to increased duty cycle. This limitation can be overcome with improving scanning speeds in newer high-resolution instruments. For further increases to multiplexing, development of complementary ion tagging strategies will provide promising routes to higher sensitivity and numbers of quantified proteins in complex samples.

Concerning future isobaric labeling applications, we speculate that as single-cell analyses become more widespread, so will isobaric labeling in these analyses. The advantages of sample pooling from multiplexing make quantitative comparisons between single cells more accessible.[171,172] In studies of cancer for example, quantitative interrogation of cell-to-cell heterogeneity using isobaric labeling can elucidate changes that are masked when performing analyses from homogenized, bulk samples.

Quantitative proteomic information derived from studies using isobaric labeling have proven useful in guiding more targeted studies toward protein functions and disease pathologies. Given that technologies and strategies for isobaric labeling continue to evolve, it is certain that isobaric labeling will continue to remain at the forefront of quantitative proteomics.

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Table 1. Advantages/Disadvantages of Isobaric Tags Details at some of the major pros and cons with each general tag mentioned in the article.

Isobaric Tag	Multiplexing Capacity	ETD-compatible	Reporter ion yield	Deuterium	Cost-effective	References
iTRAQ	8-plex	✓				20-24
TMT	18-plex (TMTpro)	✓				17, 25-27, 29
DiLeu	21-plex		High (less ratio compression)	✓	✓	30-35
DiART	6-plex		High (less ratio compression)	✓	✓	38-40
IBT	10-plex		High (less ratio compression)		✓	41

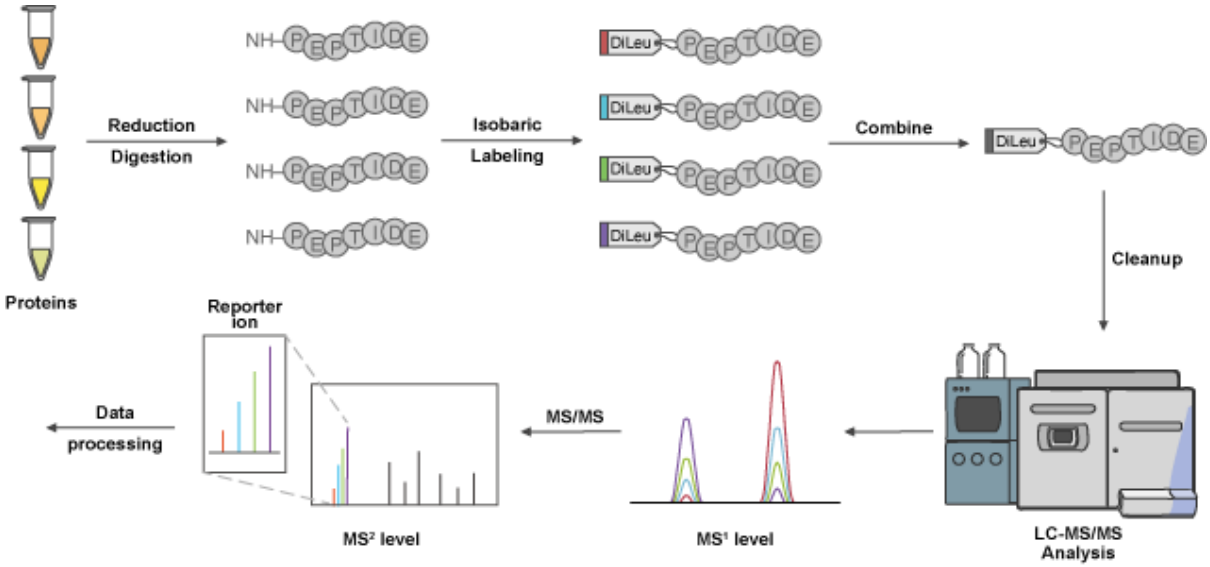


Fig. 1. Overview of Isobaric Tag Workflow. The standard protocol involves proteins that undergo reduction, alkylation, and digestion to generate peptides. For the case of DiLeu 4-plex, up to four samples are labeled with different channels, and are combined at equal concentrations prior to clean up steps. DiLeu-labeled samples are analyzed via LC-MS/MS where at the MS1 level, peptides from the pooled samples will appear as a single composite peak, which after fragmentation will show distinct reporter ion masses between *m/z* 114-118. The intensity of the reporter ion will indicate the relative amount of peptide in the mixture. All LC-MS/MS data will undergo further data processing for downstream analysis.

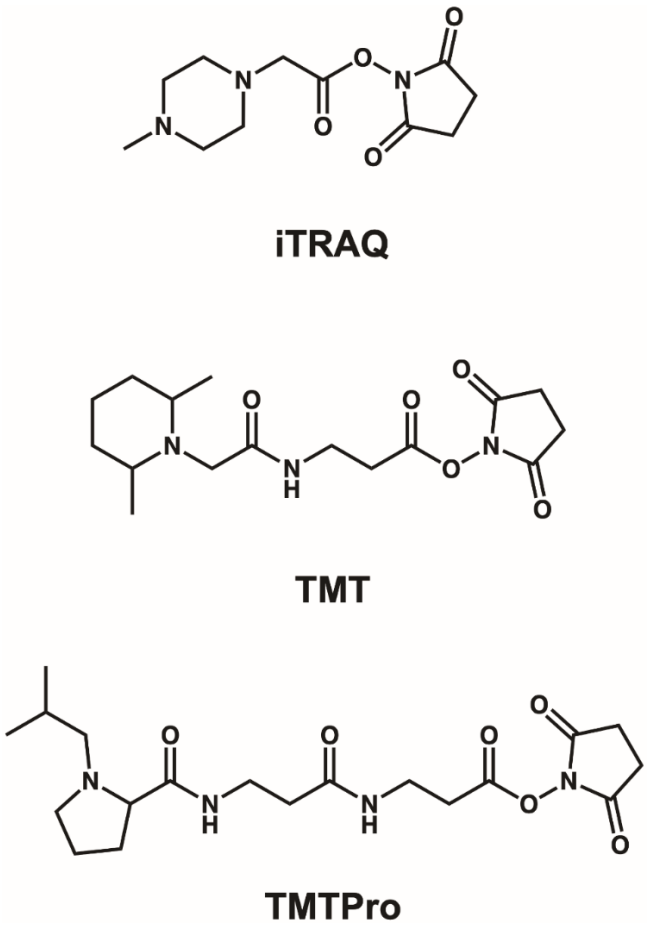
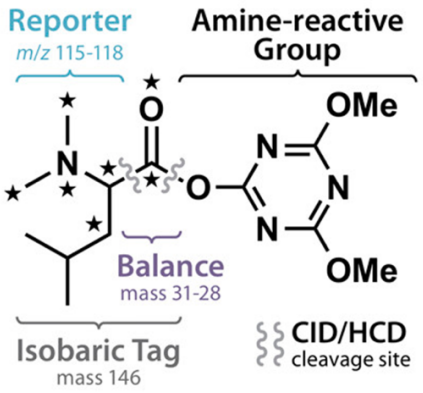


Fig. 2. Chemical Structures of iTRAQ, TMT and TMTPro Each molecule consists of a reporter group, a mass balance group, and a peptide-reactive group. iTRAQ contains a distribution of ^{13}C, ^{15}N, and ^{18}O isotopes across the balance and reporter groups, while TMT and TMTPro consist of ^{13}C and ^{15}N only. iTRAQ consists of a N-methylpiperazine reporter group, TMT with a dimethylpiperidine reporter and TMTPro contains an isobutyl-proline reporter ion. Each tag carries an NHS reactive group, while mass normalization groups vary across each structure.



21-plex DiLeu Isobaric Tags			
DiLeu label	Stable isotopes		Reporter mass
	Reporter	Balance	
115a	●	●●	115.12476
115b	●	●●	115.13108
115c	●	●●	115.13400
116a	●●	●	116.12812
116b	●●	●	116.13104
116c	●●	●	116.13444
116d	●●	●	116.13736
116e	●●	●	116.14028
117a	●●●	●	117.13147
117b	●●●	●	117.13439
117c	●●●	●	117.13731
117d	●●●	●	117.14071
117e	●●●	●	117.14363
117f	●●●	●	117.14656
118a	●●●●		118.13483
118b	●●●●		118.13775
118c	●●●●		118.14067
118d	●●●●		118.14359
118e	●●●●		118.14699
118f	●●●●		118.14991
118g	●●●●		118.15283

● ^{13}C ● ^{2}H ● ^{15}N ● ^{18}O

Fig. 3. Chemical Structure of DiLeu and Multiplexing Chart. DiLeu isobaric tag structure with multiplexing capability chart illustrating the isotopic configurations across reporter ion and balance groups for each channel along with reporter ion masses.

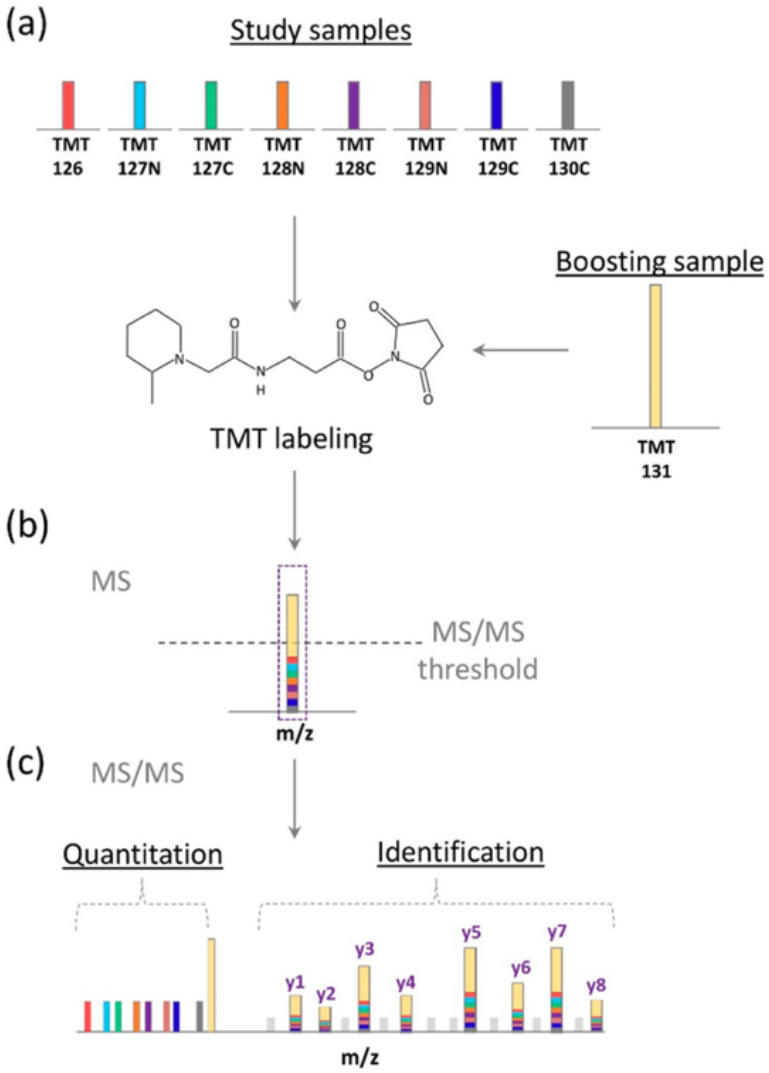


Fig. 4. **Illustration of BASIL Strategy.** Adapted from Yi. L et al. (2019) with permission. (a) Study samples are labeled with a smaller amount of TMT tag, while the boosting sample is labeled with a larger amount of TMT tag. (b) Peptides will appear as a single composite peak at the MS1 level as a sum of all intensities from the study and boosting samples. (c). Tandem MS fragmentation of peptide backbones reveal the intensities of the TMT reporter ions along with quantification of the study samples.

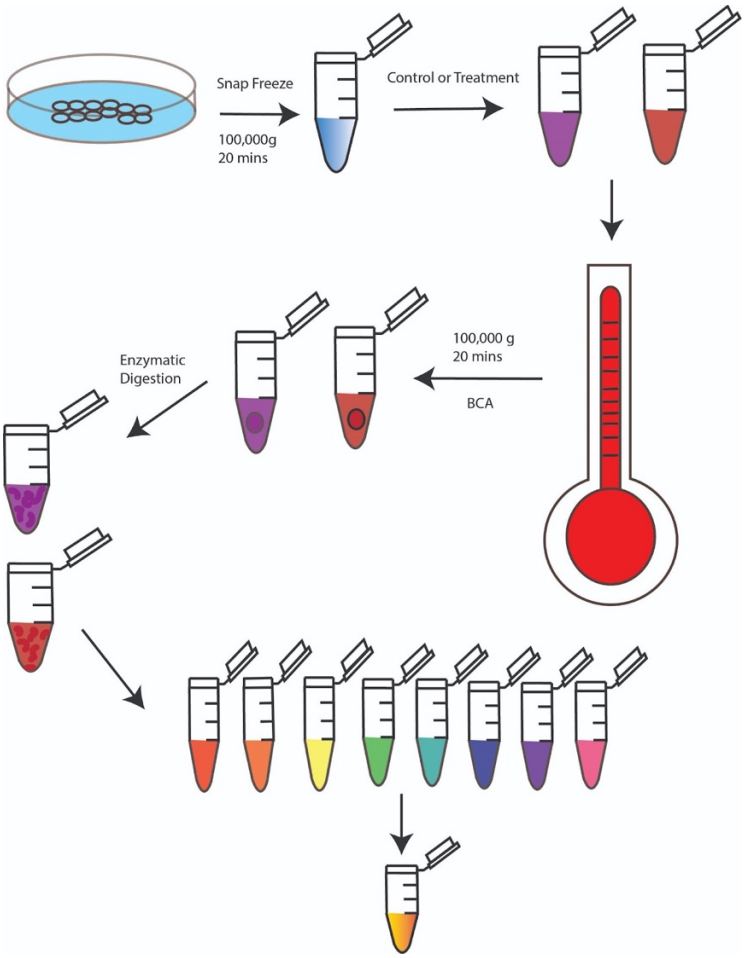


Fig. 5. Thermal Proteome Profiling Workflow. Cells are snap frozen via liquid nitrogen for cell extraction, followed by ultra-centrifugation. The supernatant is then subjected to treatment either with a control or drug with further exposure via a thermal cycler at varying temperatures. The heated samples are ultracentrifuged again with enzymatic digestion of the supernatant. Once peptides are formed, they are labeled with isobaric tags and combined at equal concentrations prior to cleanup steps and LC-MS/MS analysis.

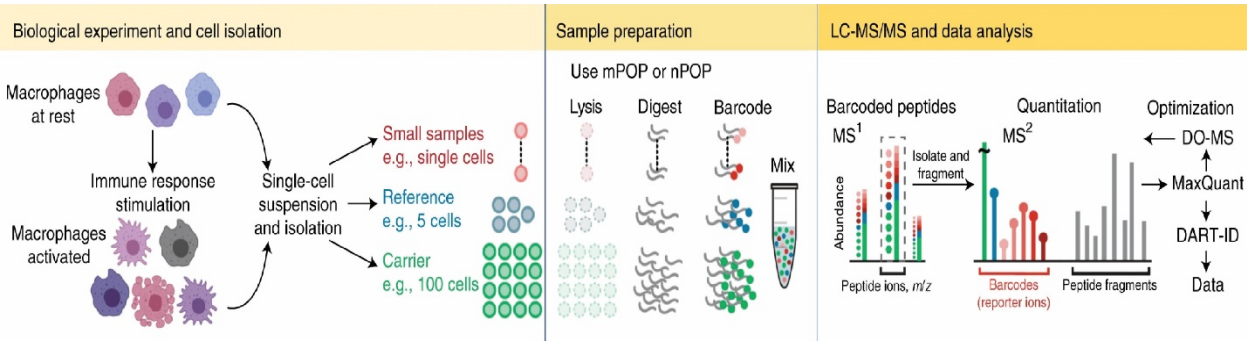


Fig. 6. **Single cell quantitative proteomics workflow via isobaric tagging**. Adapted from Petelski A. et al. (2021) with permission. Individual cells are isolated from single-cell suspensions, which are then lysed into proteins, digested into peptide chains, and labeled with isobaric tags for LC-MS/MS analysis with subsequent data processing.

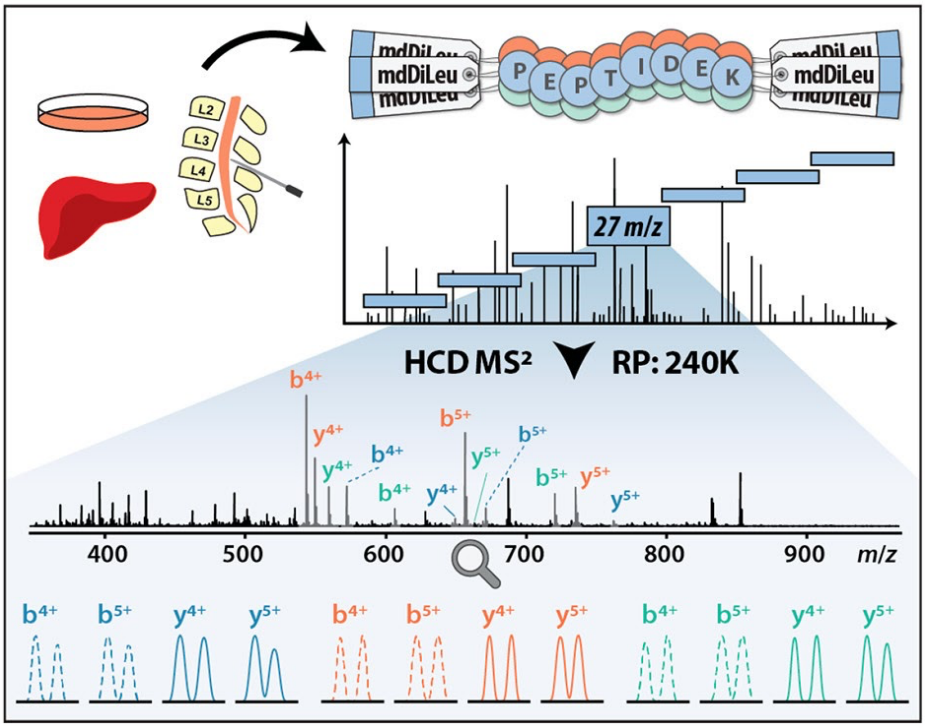


Fig. 7. **mdDiLeu DIA Workflow**. Adapted from Zhong X. et al (2020) with permission. Either cerebral spinal fluid or samples were labeled with mdDiLeu tags, with LC-MS/MS analysis using a *m/z* 27 scan range for DIA isolation windows. These were analyzed using HCD tandem MS with further data processing to illustrate the different b and y ions for differentially labeled samples.

Chapter 3

Diazobutanone-assisted isobaric labeling of phospholipids and sulfated glycolipids enables multiplexed quantitative lipidomics using LC-MS/MS

Adapted from: **Gu, T. J.[#],** Liu, P.K.[#], Wang, Y. W., Flowers, M. T., Xu, S., Lui, Y., Davis, D. B., & Li, L. Diazobutanone-assisted isobaric labeling of phospholipids and sulfated glycolipids enables multiplexed quantitative lipidomics using LC-MS/MS. *Under revision*.

Author contribution: Study was designed by **Gu, T. J**., Liu, P.K, and L. Li; experiment was performed by **Gu, T. J.** and Liu, P.K,; data was analyzed by **Gu, T. J**. and Liu, P.K,; manuscript was written by **Gu, T. J**. and edited by L. Li.

Abstract

MS-based quantitative lipidomics is an emerging field for investigating the intricate relationship between lipidomes and disease development. However, comprehensive quantification of lipidomes in high-throughput manners remains a significant challenge due to the highly diverse lipid structures. Here, we present a diazobutanone-assisted isobaric labeling strategy that provides a rapid and robust platform for multiplexed quantitative lipidomics across a broad range of lipid classes, including various phospholipids and glycolipids. The design of the diazobutanone reagent achieved bioconjugation of phosphodiester or sulfate groups while accommodating various functional groups on different lipid classes. Therefore, this method enables subsequent isobaric labeling for high-throughput multiplex quantitation. This diazo-assisted method features remarkable performance, including labeling efficiency, detection sensitivity, quantitative accuracy, and broad applicability to various biological samples. Finally, we performed a 6-plex quantification analysis of lipid extracts from lean and obese mouse livers to demonstrate the utility of this new strategy. In total, 245 phospholipids were successfully identified and quantified in a high-throughput manner to reveal the lipidomic changes in mouse obesity.

Introduction

Lipidomics is a rapidly expanding field that characterizes the lipidome in biological systems to elucidate lipid functions and their roles in the pathogenesis of diseases[1]. Phospholipids (PL) and glycolipids, the major compositions of lipidomes, regulate cell membrane dynamics, serve as storage depots of energy, and precursors of bioactive metabolites[2–4]. The regulation is determined by lipid chemical structures and relative abundance between each[5]. Recently, the associations between lipidomes and diseases have stimulated the development of quantitative lipidomics for discovering disease biomarkers and therapeutic targets[6–8]. Yet, the high-throughput approaches for quantifying lipidomes fall behind genomics and proteomics mainly due to the high diversity in chemical structures and physiochemical properties of lipids. Therefore, a high-throughput strategy that can identify and quantify broad range of lipids accurately is vital for addressing critical biological questions relevant to lipidomes and lipid regulation.

Liquid chromatography linked with tandem mass spectrometry (LC-MS/MS) has become powerful techniques to profile lipidomes in complex samples[9], and several strategies coupled with chemoselective reactions were developed to further facilitate the detection of lipids or the differentiation of lipid isomers[10–12]. However, the quantitative strategies for lipidomics remain limited. Currently, quantification of lipids mainly relies on label-free methods[13], which might suffer from run-to-run variations, long instrument time, and the difficulty in the preparation of numerous isotope-incorporated standards. Alternatively, stable isotopic labeling that introduces light and heavy isotopic reagents into analytes to achieve the relative quantification at the MS1 level[14] is still restricted to low-plexed analysis due to the increased spectral complexity and the limited availability of isotopic reagents.

Isobaric labeling is a powerful technique that allows for quantitative analyses of multiple samples in one experiment[15]. Featuring higher quantification accuracy, reproducibility, and sample throughput, this technique has been extensively utilized in proteomics and glycomics for high-throughput quantification using tandem mass tags (TMT) [16], *N,N*-dimethyl leucine (DiLeu)[17], or aminooxy tandem mass tags (aminoxyTMT)[18]. Although several studies have tried to utilize isobaric labeling for lipidomic quantification typically via amine, carboxylate, or carbon-carbon double bonds[19,20], it has been difficult to target a wide range of PL classes in the complex biological milieu or develop a rapid and highly efficient approach that is crucial to the sensitive, reproducible, and large-scale analysis of diverse lipid classes in complex biological samples.

Unlike peptides or glycans that have well-established bioconjugation via shared functional groups, the diversity of chemical structures of lipids brings challenges for targeting all PL classes. For the functional groups shared among PL classes such as phosphodiester and aliphatic groups, limited reactions have been examined for direct conjugation for biological applications[21]. Diazo reagents have been reported to have broad and tunable reactivity to alkylate oxygen, nitrogen, and even carbon, which show a great potential to conjugate phosphodiesters. However, selective O-alkylation of phosphodiester for subsequent labeling is a difficult task considering their poor nucleophilicity and the presence of more reactive nucleophiles on PL structures[22].

In this work, we designed a diazo compound, diazobutanone, that enables the first Diazo compound-assisted isobaric labeling strategy for multiplexed quantification of all phosphate and sulfate-containing lipid classes simultaneously, including the classes that have not been previously reported to achieve isobaric labeling on the global scale. Diazobutanone, which can be synthesized in two steps and easily removed by vacuum after reactions, enables O-alkylation of PL and specific reactions with aminooxy isobaric reagents. With an optimized condition, all lipids can be

isobarically tagged under a mild condition, and the final products can generate reporter ions upon tandem MS for relative quantification, diagnostic ions indicating lipid classes, and acylium ions for differentiating acyl chains. With aminoxyTMT that is currently available up to 6 plex, we successfully profiled and quantified six major classes of phospholipids in liver tissues from three healthy lean mice and three insulin resistant obese mice in a high throughput fashion. Altogether, we quantified 245 phospholipids in liver tissues, offering a universal and highly efficient method to quantify broad classes of phospholipids in complex biological samples.

Results

Design of diazobutanone and aminoxyTMT labeling:

Aiming to develop a simple and applicable high-throughput method, we designed diazobutanone compound that is incorporated with a diazo group for phosphodiester O-alkylation and a ketone group for subsequent isobaric labeling (**Fig. 1, Supplementary Fig. 1 and Supplementary Methods**). The oxime bond formation was selected for the labeling step in our study because the oxime bond formation between carbonyl groups and aminooxy groups is highly specific and efficient, leaving other functional groups on PL intact[23]. Additionally, we observed that the final products, phosphotriesters, are prone to hydrolysis under alkaline or reductive conditions while tolerating mild acidic conditions where the oxime formation occurs.

Critical to the properties of diazobutanone, we designed the compound to be volatile and placed carbonyl groups next to the diazo groups. Diazoalkanes, such as diazomethane, are typically toxic and explosively reactive, while diazo groups could be stabilized by delocalizing the electrons on the α carbon[24]. Therefore, to make the compound stable and compatible with biomolecules, the

use of carbonyl groups to diminish the reactivity of diazo groups allows diazobutanone to be stored at -20°C for months without an observed decrease in reaction efficiency (**Supplementary Fig. 2**). The volatility of diazobutanone allows the removal of an excess amount of reagent by vacuum, which simplifies the cleanup steps and reduces sample loss, making it compatible with subsequent mass spectrometry (MS)-based analyses.

To demonstrate the wide applicability to various lipid classes, we tested diazobutanone using representative lipid standards from 9 phosphate-containing lipid classes including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI), lysophosphatidylcholine (LysoPC), ether-phosphatidylcholines (ePC), and sphingomyelin (SM). The lipid structures for each class were shown in **Fig. 1.** Besides PL, we also found sulfate groups might hold similar reactivity to phosphate groups. Sulfated glycolipids (SGL) are enriched in the nervous and immune systems to exert unique functions from their non-modified glycolipids due to the negative charges. Thus, we included SGL in our method development[25]. In the initial tests, we found fluoroboric acid (HBF_4) as the catalyst to be the most efficient in our reactions, probably due to the generation of non-coordinating counterion during the reaction[26]. However, higher concentrations of HBF_4 caused severe side reactions on multiple functional groups on lipids, such as the alkylation of amine, amide, or hydroxyl groups, while lower amounts of HBF_4 failed to reach enough reaction yields. In order to promote efficiency without generating unwanted reactions, several condition factors were examined, such as temperature and solvent systems. We found that the solvent effect was a key to the reactions[27]. We observed that diazobutanone reacted with hydroxyl groups of PG in chloroform or showed low reactivity under ether systems. After testing all common reaction solvents, ethyl propionate, which was not commonly used for diazo

compounds, was found to generate minimum side reactions and obtain the highest signal intensity of lipid derivatives. With careful optimizations (**Supplementary Fig. 3**), a mild and lipid condition of 75 mM diazobutanone and 0.24 mM fluoroboric acid in ethyl propionate at room temperature for 35 min was used for subsequent experiments. An average of 98% conversion yields were achieved for all lipid standards with minimum side-products (**Table 1**). The optimized reaction conditions exhibited high reaction efficiency and easy clean-up by vacuum leading to maximum conversions of PL derivatives for quantitative analysis. It is worth noting that the selectivity of diazobutanone toward phosphate and sulfate groups under the optimized condition leaves other functional groups on lipids intact such as ester, hydroxyl, amine, amide, carboxylate, carbon-carbon double bond, and quaternary amine groups and thus avoids multiple side-products resulting from single lipid at different reactive sites, which might complicate the analysis and lower sensitivity of target lipids.

In the second step of the labeling, aminoxyTMT isobaric mass tags that are developed for targeting carbonyl-containing biomolecules were used to conduct isobaric labeling. Aminooxy groups of the tag reagents could specifically react with the ketone groups on the lipid derivatives that were introduced by diazobutanone. A lipid-to-aminoxyTMT ratio of 1:2 and reaction solvent of 10% isopropanol/methanol with 0.1% acetic acid were used. We achieved near-complete labeling efficiencies for all lipid standards after reacting for 15 minutes and did not observe the hydrolysis of PL products. The excess tag reagents can be removed by extraction using water and ethyl acetate. The whole labeling procedure could be completed within 2 hours, enabling fast and cost-effective experiments for high throughput quantification.

Characterization of labeled lipids

After labeling (**Fig. 2a**), 372 Da mass increments were introduced to lipid standards, which comprised of butanone plus aminoxyTMT. PA had a 744 Da mass increment resulting from 2 units of tags incorporated, while some single-labeled PA product was also observed in low yields. Unlike previous reports in which amino or other functional groups would be alkylated using diazomethane, we did not observe products with alkylation on amine groups. A possible explanation is that the lower reactivity of diazobutanone and milder reaction conditions were used compared to the previous works[24].

To assess the sensitivity of this method, we compared the quantitation limit of lipids before and after labeling (**Table 1**). Despite the additional reactions and clean-ups, labeled lipids showed comparable quantification limit to that of unlabeled lipids, suggesting that our method could achieve multiplexed quantification without sacrificing the sensitivity. Furthermore, the PA class, which usually has lower ionization efficiency and is present in low abundance, displayed significantly enhanced detection sensitivity after labeling that could benefit the analysis of PA class in complex biological samples.

We next investigated the characteristics of the labeled lipids. During LC-MS analysis, chromatography behaviors of labeled lipids were similar to unlabeled lipids. Labeled lipids with longer aliphatic chains and lower unsaturated degrees tended to have longer retention times during C18 reversed phase separation. In terms of MS characteristics, the amine groups of aminoxyTMT tags, which are favorably protonated under acidic mobile phases, caused PA and lipids with choline or ethanolamine to primarily exhibit charged 2+ species. Labeled lipids displayed different fragmentation patterns from their original counterparts (**Table 1, Supplementary Fig. 4**). **Fig. 2b** shows representative MS2 spectra of labeled PE 15:0_18:1(d7) and PG 16:0_18:1. First, the peaks

at *m/z* 128 were the reporter ions generated from the aminoxyTMT tags that would be used for multiplexed quantification. Next, the peaks at *m/z* 513 and 544 were the fragments of PE and PG head groups with oxime-formed aminoxyTMT, indicating the lipid classes of the precursor ions. Lastly, important information on acyl chains for differentiating lipid molecular species was also provided. The peaks located at 570 and 577 were the neutral loss of polar head groups, which can further undergo MS3 fragmentation to elucidate fatty acid chains (**Supplementary Fig. 5**). Furthermore, some single acyl chain-related fragments, which were not generated efficiently before labeling, would appear in the MS2 level despite lower signal intensities, eliminating the need for MS3 analysis for differentiating acyl chains. For example, according to the acylium ions ($[RCO]^+$) or acyl chains with glycerol backbones ($[RCOO + C_3H_5O + H]^+$), we could identify the PE species as PE 15:0_18:1(d7) and the PG 34:1 as PG 16:0_18:1. The corresponding neutral loss product ($[M + H - RCOO]^+$) was also observed in some lipid species (**Supplementary Fig. 6**). In order to identify lipids in high confidence, all fragmentation patterns and retention time of labeled lipids were considered to validate the identification of lipids in addition to accurate mass matching.

Isobaric labeling of phospholipid extract from human cells

In mammalian cells, PC is the most abundant PL and comprises about 50% of the total lipids, followed by PE, and PI. Each PL class distributes differently among cell types and exerts its functions[28], and all lipid classes also contribute collectively to the homeostasis and maintenance of cellular environments. Here, we applied our method to profile six central PL classes (PC, PE, PI, PS, PG, and PA) simultaneously in the human pancreatic cancer PANC-1 cell line to further demonstrate the applicability of our approach using complex biological samples (**Fig. 3**).

From the MALDI MS spectra (**Supplementary Fig. 7**), signal peaks existing in the previous steps were consumed completely by each reaction as we observed on lipid standards, suggesting that this two-step labeling procedure was highly compatible with complex systems. The labeled mixtures were then subjected to LC-MS/MS analysis using the positive ion mode. The labeled PLs were identified by accurate mass matching to the database and the diagnostic ions for class identification using an in-house developed script in R program. Quantitative information was acquired by extracting the abundances of reporter ions from six aminoxyTMT channels. All MS2 fragmentation patterns, charges, and retention time were considered to validate their identification. In triplicate analysis, we identified an average of 337 PL species per replicate using modified LipidBlast database[29] (**Fig. 4a**). Approximately 70% of identifications were found in two of three replicates. Among them, PC accounts for 31% of identifications, PE for 23%, PI for 15%, PG for 12%, PS for 11%, and PA for 7%. The lipid composition is consistent with previous label-free lipidomics [30] analysis of PANC-1 cells with higher percentages in PG and PA (**Fig. 4b**) probably due to the enhanced detection sensitivity, demonstrating a high reproducibility and the ability to profile lipidome reliably using our new method.

To evaluate the quantitative accuracy for human cell samples, we conducted 6-plex isobaric labeling of six aliquots of PANC-1 PL extracts. PL extracts were each separately subjected to diazobutanone reaction and 6-plex aminoxyTMT labeling. The mixtures were then combined with molar ratios of 1:1:1:1:1:1 and 1:1:2:4:6:8 prior to MS analysis to represent different dynamic ranges. Additionally, we added the deuterated PL standards with a ratio of 1:2:4:4:2:1 to the mixtures to further assess the quantitative accuracy. The reporter ions of the deuterated PL standards showed an expected ratio of 1:2:4:4:2:1 (**Fig. 4c**) without ratio distortion. The retention time extracted for 6 channels showed no retention time shift among each channel, ensuring

simultaneous MS2 analysis of PLs with different tag variants (**Fig. 4d**). All quantified PLs were plotted against each other (**Fig. 4e, f**). The median coefficient of variation (CV) less than 10% for both theoretical ratios of 1:1:1:1:1:1 and 1:1:2:4:6:8 were obtained, demonstrating excellent quantitative performance.

Relatively quantify phospholipids in liver tissues from lean and obese mice

Obesity has become a serious health problem over the past few decades that contributes to an increased risk for various diseases including non-alcoholic fatty liver disease, type 2 diabetes, and cancer. Aberrant lipid accumulation in the liver is a hallmark of those diseases[31]. Given the critical roles of lipids in biological systems, lipidomic analyses of liver tissues have the potential to not only decipher lipid functions in metabolic pathways, but also identify potential biomarkers for disease diagnosis. Several studies have reported the mechanisms of lipids to cause the development of diseases. For example, a decreased PC/PE ratio was found responsible for steatohepatitis and liver failure progressions by affecting membrane integrity[32,33]. Additionally, the aliphatic chains on high-density lipoprotein PLs are correlated to the capability of the efflux of cellular cholesterol[34,35].

Here, we performed a 6-plex quantitative analysis of liver tissues from three healthy, lean male mice and three insulin resistant, obese male ob/ob mice. Equal amounts of liver tissues were collected and homogenized. Deuterated lipid standards were added to the mixtures before lipid extraction. Next, extracted lipids underwent 6-plex diazobutanone-assisted isobaric labeling. PLs were identified by examining their exact masses and diagnostic ions. The abundance of reporter ions was extracted from 6 channels and normalized using deuterated lipid standards to correct the lipid recovery of lipid extraction.

In total, 161 PLs including 245 PL species with acyl chain compositions were quantified in liver tissues from lean and obese mice (**Fig. 5a**). Our methods have revealed 86 PLs and 122 PL molecular species enriched in obese mice, and 23 PLs and 23 PL molecular species enriched in lean mice (adjusted p-value < 0.05). The ratio of PC/PE decreased in obesity (**Fig. 5b**), which was associated with nonalcoholic fatty liver disease[32,33]. PLs with relatively long acyl chains (>38 carbon atoms) were up-regulated, and PLs with short acyl chains (< 34 carbon atoms) decreased in obese mice (**Fig. 5c, d**), which might be related to a long-chain fatty acid elongase, *Elovl6*, that has a crucial role in obesity-induced insulin resistance[36,37]. From hierarchical clustering analyses (**Fig. 5d and Supplementary Fig. 8**), these data showed remarkable reproducibility within groups and produced strong clustering of the two groups. Four main clusters of lipids can be visually identified, including one cluster enriched in the lean mice group, two clusters enriched in the obese mice group, and one cluster without obvious enrichment. In addition to PCs, many lipid species in other classes that are not commonly studied also displayed strong correlations with obesity. Overall, these results suggest that obesity and possibly insulin resistance led to a severe disturbance in lipid homeostasis. It is possible that the results from these lipidomic studies on mouse livers may not be representative of results from human livers with metabolic diseases. Therefore, further investigations are needed to explore the functions of these key identified lipids and to ensure their relevance to human studies. Nonetheless, these distinct PL profiles between lean and obese mouse livers increase our understanding of the relationship between lipids and diseases. The ability of this method to analyze a broad range of lipid classes will facilitate quantitative lipidomics in comprehensive and high throughput manners.

Discussion

Lipidomic reports often provide conflicting results with regard to lipid compositions, likely due to the non-standardized lipidomic procedures and variability occurring in label-free workflows. Here, we report a novel diazobutanone-assisted isobaric labeling strategy that empowers accurate multiplexed quantification and structure elucidation of phosphate and sulfate-containing lipids. We present here the rationale for the design and optimization of diazobutanone reaction. The property of diazobutanone enables easy, mild, and MS-compatible sample preparation protocols. The chemical reactivity of diazobutanone exhibits efficient bioconjugation of phosphodiesters and compatibility with various functional groups under optimized reaction conditions, rendering the method broadly applicable to complex biological sample types. Additionally, the fast procedure and cost-effectiveness of our isobaric tagging strategy are favorable to large-scale lipidomics. In this work, phospholipids extracted from healthy and obese mouse livers were analyzed and quantified in a high-throughput manner. The alterations of phospholipid expression levels from all major classes were observed in the obese mouse models, indicating the alteration of enzyme activity involved in lipid metabolism for obesity conditions. For example, the significant up-regulation of phospholipids with relatively long carbon chains might result from fatty acid elongases (*Elovl-5,6*). However, the current quantitative studies mainly focus on fatty acids or certain phospholipid classes but lack the ability to comprehensively interrogate the lipidome. We envision this new method can be applied to investigate multiple classes of lipids and their dynamic interplay occurring in various diseases and facilitate the discovery of lipid biomarkers in different diseases and physiological states, in a non-target fashion. Diazobutanone-assisted isobaric labeling strategy provides a strong starting point for lipidomics to leverage the benefits of isobaric labeling,

including higher quantitative accuracy, reproducibility, fewer missing values, elimination of matrix effect, and the capability to conduct analysis of replicates or multiple test groups within the same experiment. Some advanced strategies that involve isobaric mass tags could also be achieved, such as adding boosting channels to enhance the detection of target analytes or low-abundance lipids, or bridge channels to normalize the quantification across multiple sample sets. In summary, this diazobutanone-assisted isobaric labeling will drive the field of quantitative lipidomics to unprecedented higher throughput analysis with enhanced coverage and improved sensitivity.

Methods

Materials and Reagents

All Lipid standards were purchased from Avanti Polar Lipids (Alabaster, Al). Optima LC/MS grade acetic acid (AA), acetonitrile (ACN), ammonium formate, formic acid (FA), isopropanol (IPA), Methanol (MeOH), and water were purchased from Fisher Scientific (Pittsburgh, PA). ACS grade chloroform, dichloromethane (DCM), ether, ethyl acetate (EA), ethyl propionate, 3,5-heptanedione, tetrafluoroboric acid diethyl ether complex (HBF4.Et2O), p-toluenesulfonyl azide (TsN3), and triethylamine (NEt3) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) was purchased from Crystalgen (Commack, NY). AminoxyTMTsixplex™ label reagent was purchased from Thermo Fisher Scientific (Waltham, MA). All reagents were used without additional purification.

Pancreatic Cancer Cell (PANC1) Culture

The commercially available pancreatic cancer cell line PANC-1 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and was maintained in DMEM: F12 (Hyclone, GE Healthcare Life Sciences, Logan, Utah, USA) containing 10% fetal bovine serum (FBS) (Gibco, Origin: Mexico) and 1% penicillin-streptomycin solution (Gibco, Life Technologies Corporation, Grand Island, NY, USA). Cells were cultured in a 37°C moisture incubator filled with 5% CO2. Cells were trypsinized at 70%-90% confluence using 0.25% trypsin EDTA solution (Gibco, Life Technologies Corporation, Grand Island, NY, USA). The cell suspension was centrifuged at 300 g for 5 minutes, and the medium was discarded. Cells were resuspended in phosphate-buffered saline (PBS) (Gibco, Life Technologies Europe B.V., Bleiswijk, The Netherlands) and washed twice with PBS, and stored at −80°C freezers un l use.

Obese mouse model and liver collection

Animal care and experimental procedures were performed with approval from the Institutional Animal Care and Use Committees from the University of Wisconsin-Madison and William S. Middleton Memorial Veterans Affairs to meet acceptable standards of humane animal care. All experiments were carried out in accordance with their guidelines and regulations. Mice were housed in facilities with a standard light-dark cycle and fed ad libitum. Breeders were fed Teklad 2919 (Envigo), and at weaning mice were fed Teklad 2920x (Envigo) until transfer from the Biomedical Research Model Services breeding core to the Wisconsin Institute for Medical Research vivarium, at which time mice were fed Teklad 2018 (Envigo). Thirteen- to fourteen-week-old obese male C57BL/6N ob/ob mice were generated by first backcrossing the C57BL/6J ob allele (Jackson Laboratory Strain #000632) to C57BL/6NTac (Taconic) mice, and then

breeding heterozygous C57BL/6N ob/+ mice to produce ob/ob mice. Mice were genotyped for the ob allele by PCR as previously described (Ellett JD et al. 2009). Sixteen-week-old lean male C57BL/6NTac mice, generated in our breeding colony, were used as controls. The liver was isolated from non-fasted mice after euthanasia by carbon dioxide inhalation and flash frozen in liquid nitrogen.

Sample preparation and PL extraction

Approximately 20 mg of liver tissues from lean and obese mice was weighed and dissolved in 0.35 mL PBS buffer. Tissue was homogenized with a probe sonicator in an ice water bath at 50% power with pulse 12s on and 12s off for 12 cycles. The homogenates were transferred to glass tubes for lipid extraction.

Lipid extraction from cells or liver tissues began by adding 1.5 ml methanol/chloroform (2:1 v/v) and vortexing for 15 min followed by adding 0.6 ml chloroform and 0.6 ml H2O. The mixture was vortexed for another 15 min and then centrifuged at 4000 rpm for 10 min to separate liquid layers. The bottom layer was collected. The extraction of the aqueous layer was repeated twice with 1 mL chloroform. The organic layers were combined and dried under a nitrogen stream. Subsequently, lipid extraction was added with 1.8 ml hexane/methanol/ddH2O (1:1:0.1). The mixture was vortexed for 5 min and then centrifuged at 4000 rpm for 10 min. The bottom layer was collected to obtain phospholipid extract, dried under a nitrogen stream, and stored at -20°C until use.

Diazobutanone reaction of phospholipids and isobaric labeling

The synthesis of diazobutanone is given in Supplementary Methods. Approximate 5 μg PL standards or PL extracts were dissolved in ethyl propionate at a concentration of 50 μg/mL. The reaction was conducted with 75 mM diazobutanone and 0.24 mM fluoroboric acid in ethyl propionate at room temperature for 35 minutes, followed by quenching with 0.7% FA in IPA. Subsequently, the reaction mixture was dried *in vacuo*. For 6-plex AminoxyTMT isobaric labeling, the optimized labeling protocol was modified according to the manufacturer's instructions (Thermo Fisher Scientific). The derivatized PL sample was reconstituted in a solution containing AA/IPA/MeOH (0.1:10:90) to a concentration of 250 μg/mL and was labeled under 3 mM aminoxyTMT. The reaction mixture was incubated at room temperature for 15 minutes and dried *in vacuo*. Then, 20 μL IPA/MeOH (1:9) was added and vortexed for 10 min and dried *in vacuo*. Next, the mixture was extracted with 15 μL of 0.05% acetic acid in H_2O and 100 μL EA twice. The collected organic layers were dried and stored at -20 ^oC until MS analysis.

LC-MS/MS analysis

Sample was analyzed using a binary nanoAcquity UPLC system (Waters, Milford, MA) coupled with a Q Exactive mass spectrometer (Thermo Scientific, San Jose, CA) to monitor the product of derivatives and labeled PLs. Labeled PLs were dissolved in 30% phase B solution. The samples were loaded onto a self-fabricated microcapillary column packed with C18 beads (Waters Bridged Ethylene Hybrid, 1.7 μm, 130 Å, 101.3 μm × 15 cm). For mobile phases, phase A consisted of ACN:H_2O:MeOH:IPA (2:2:2:1) with 10 mM ammonium formate and 0.1% formic acid. Phase B consisted of IPA:ACN (9:1) with 10 mM ammonium formate and 0.1% formic acid. PLs were separated using a gradient elution of 5–60% B over 10 min and 60-95% B over 60 min

at a flow rate of 300 nL/min. For precursor MS scans, 440–1440 *m/z* were collected at a resolving power of 70k (at 200 *m/z*) with automatic gain control (AGC) target of 1×10^6 and a maximum injection time of 200 ms. Targeted tandem MS analysis was performed using HCD with 30% normalized collision energy at a resolving power of 17.5k, and the top 15 precursors were selected for HCD analysis. The AGC, maximum injection time, resolution (at *m/z* 200), and lower mass limit for tandem mass scan were 1×10^6, 200 ms, and 17.5k, 120 *m/z* respectively. Precursors were subjected to dynamic exclusion for 5 s.

Data analysis

The fatty acid list was obtained from LIPID MAPS. The PL database was modified from LipidBlast according to the mass increments resulting from the labeling. PL identification was based on the retention time, precursor mass accuracy, and fragmentation patterns. Accurate mass tolerance for identification was 5 ppm for both MS full scans and MS/MS. Diagnostic ions were used for class identification, while since diagnostic ion intensity for some PG species was low, $[M + H - RCOO]^+$ was used for PG identification. Acyl chain elucidation was based on at least one acylium ion found in the spectra. Retention time was manually revised for correct assignment. Identification of labeled PLs and quantitative analysis were performed in the R statistical computing environment. To identify lipids that are enriched in either group of mice, t-tests were conducted on the log-ratio per PL and PL species. FDR-adjusted p-values (< 0.05) were calculated to account for multiple testing. Logged ratios of PL and PL species were subtracted by their logarithmic mean and used for hierarchical clustering analyses. Bioinformatics analyses including hierarchical clustering were achieved using R packages.

Acknowledgements

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Table 1. Information on diagnostic ions, reaction efficiency and sensitivity for the labeled PLs from 10 lipid classes.

Lipid Class	Diagnostic Ion (*m/z*)	Unlabeled LOQ (pM) (S/N = 10)	Labeled LOQ (pM) (S/N = 10)	Change of LOQ	Diazobutanone efficiency (%)[a]	Labeling efficiency[b]
PC	278.18	73.21	19.23	3.81	99.78	
PE	513.30	48.75	30.38	1.60	98.63	
PG	544.30	37.69	22.84	1.65	99.68	
PS	557.29	81.68	329.62	0.25	99.24	
PI	632.31	45.53	15.55	2.93	97.04	
PA	841.53	1251.81	23.66	52.91	99.93[c]	>99%
Lyso PC	278.18	692.62	41.25	16.79	96.78	
Ether PC	278.18	42.40	22.51	1.88	99.48	
SM	278.18	53.37	54.28	0.98	97.38	
SGL	534.33	71.62	676.69	0.11	93.02	

a Conversions of PC, lyso PC, ether PC, and SM were monitored in the positive ion mode, and the others were in the negative ion mode. Internal standards were added to unreacted and reacted mixtures. The amounts of remaining lipids were calculated by their relative abundance to the internal standards.
b Labeling efficiency was monitored in the positive ion mode. No remaining lipid derivatives were observed.
c Single-alkylated PA was included, while double-alkylated PA was the main product.

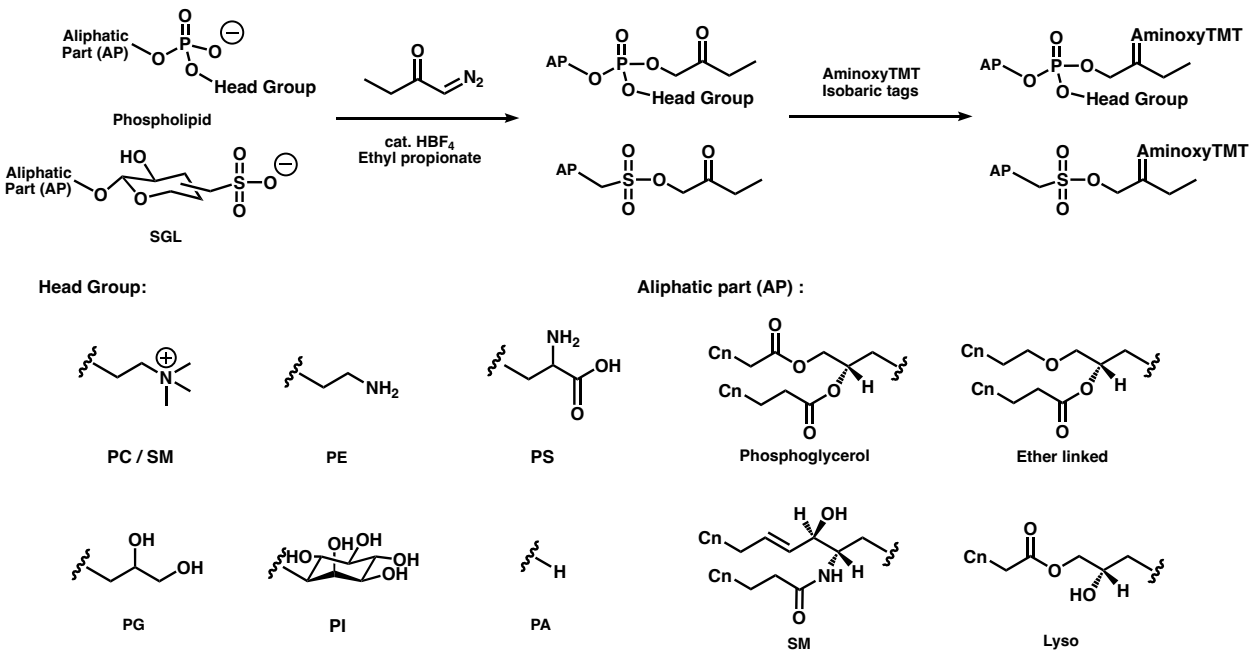


Fig. 1. Structures of lipid classes and diazobutanone-assisted isobaric labeling scheme.

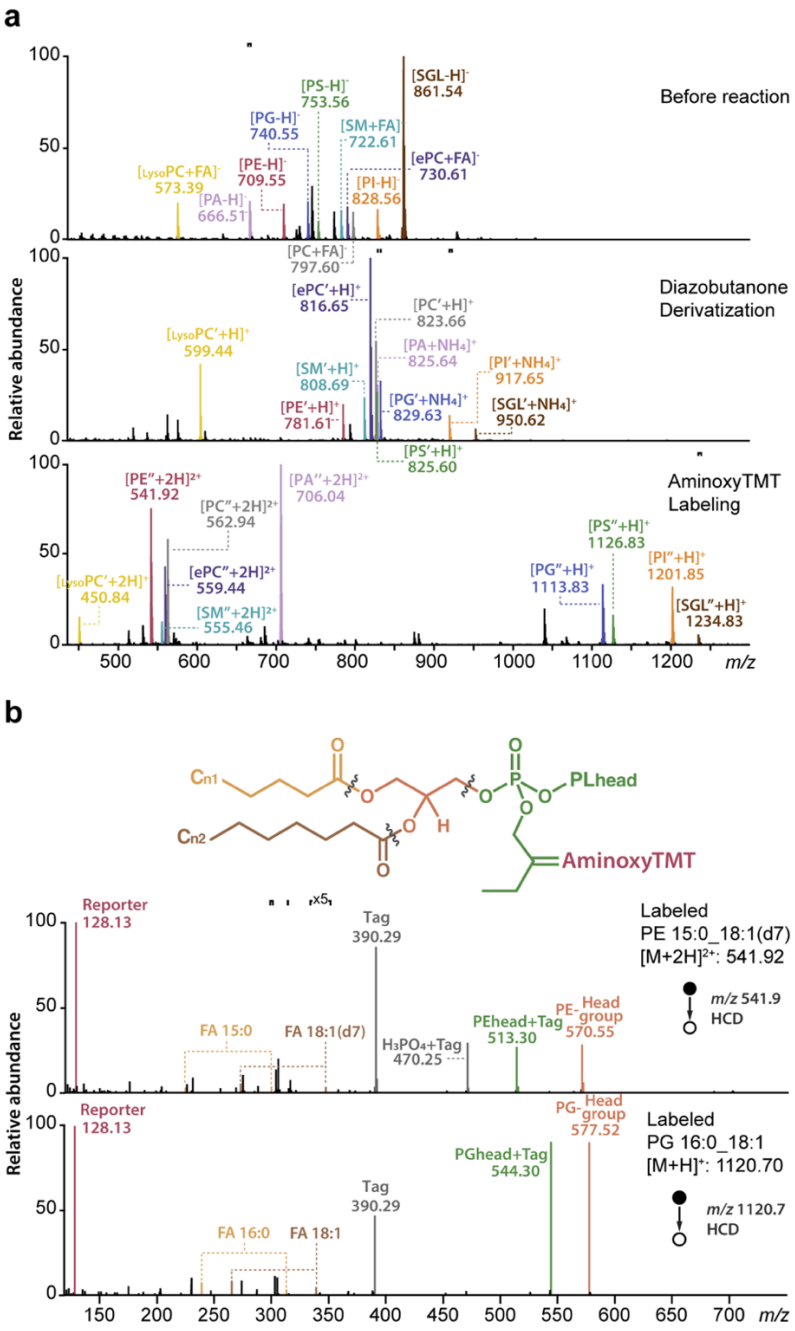


Fig. 2. Diazobutanone-assisted isobaric labeling of lipid standards and MS2 fragmentation of labeled lipids. a. Spectra of unreacted lipids in the negative ion mode (top panel), derivatized lipids by diazobutanone in the positive ion mode (middle panel), and AminoxyTMT-labeled lipids (bottom panel). **b**. MS2 spectra of representative labeled lipids, PE 15:0_18:1(d7) and PG 16:0_18:1. FA ions represent single acyl chain-related fragment ions.

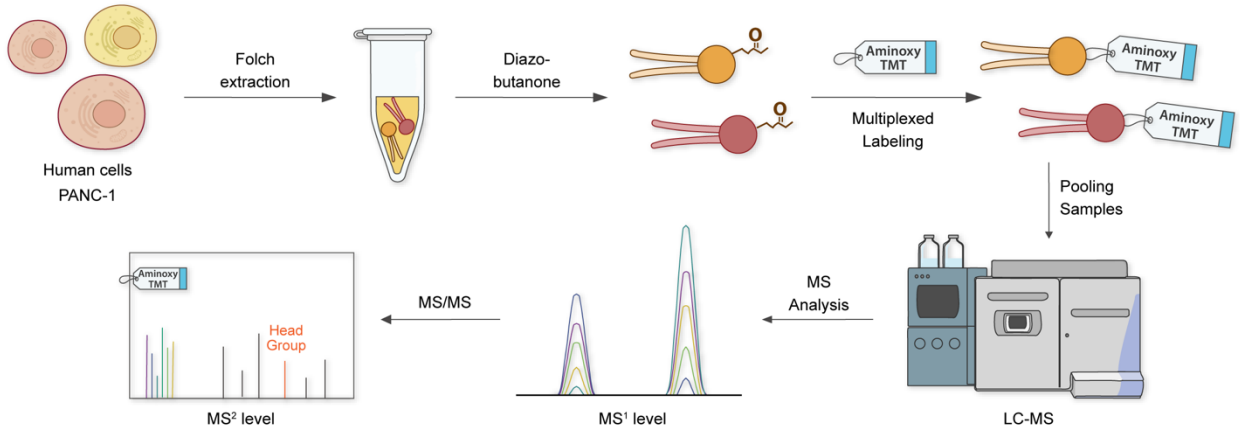


Fig. 3. Diazobutanone-assisted two-step isobaric labeling and mass spectrometry workflow.

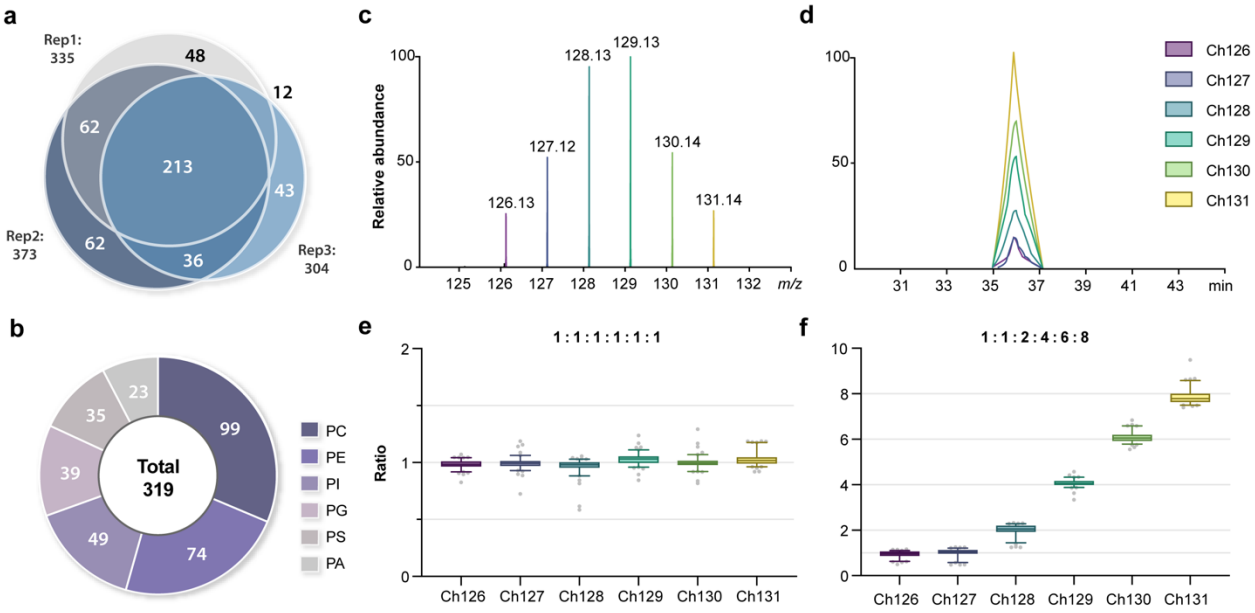


Fig. 4. Performance of diazobutanone-assisted isobaric labeling. a. Venn diagram of identified PL species from triplicates. **b**. Identified PL species from 6 central PL classes. **c**. Reporter ions of deuterated PL with a ratio of 1:2:4:4:2:1 in the mixture of the labeled cell lipid extracts. **d**. Extracted LC chromatography of the labeled PL, PG 18:1_18:1, from the mixture with the ratio 1:1:2:4:6:8. 6 channels showed no retention time shift among each other. **e.** and **f.** Reporter ion ratios for 1:1:1:1:1:1 mixture (**e**) and 1:1:2:4:6:8 mixture (**f**). Box plots demarcate the median (line), the 25th and 75th percentile (box), and the 5th and 95th percentile (whiskers).

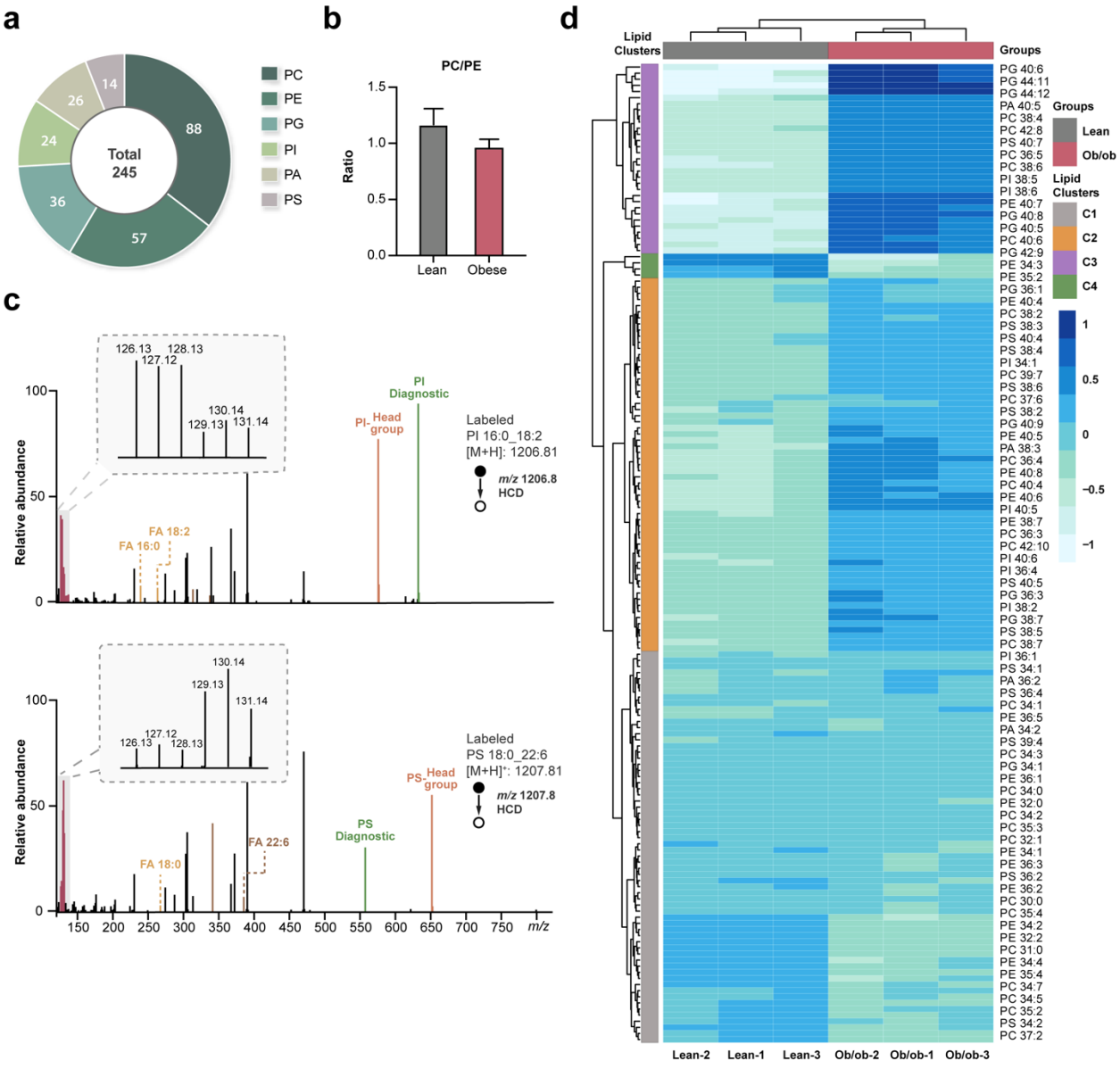
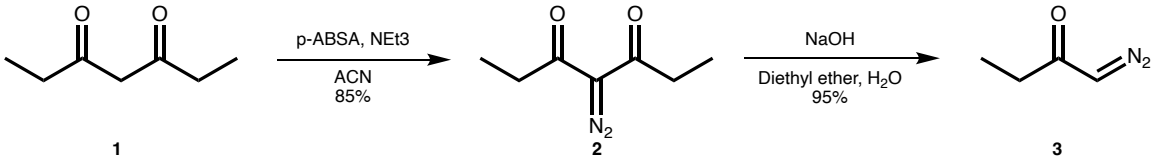
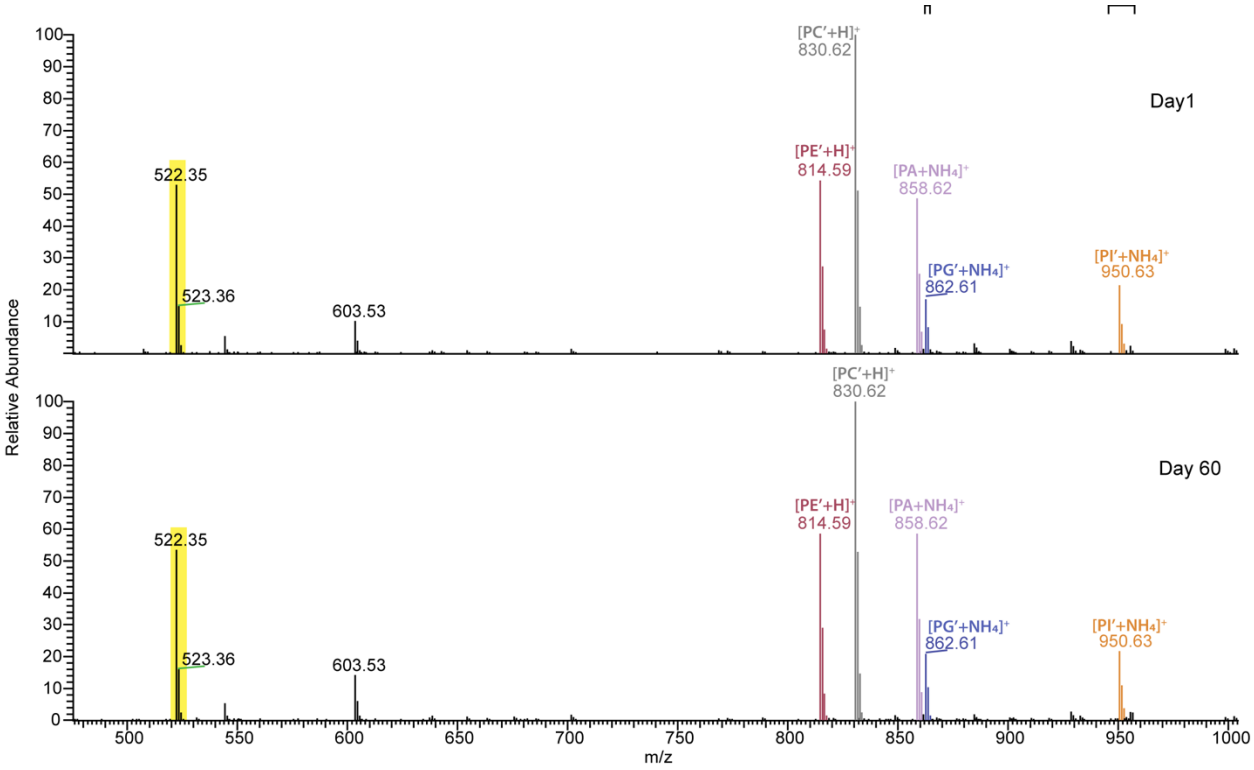


Fig. 5. Quantitative lipidomics of lean and obese mouse liver tissues. a. Identified PL species from liver samples. **b**. PC/PE ratios in lean and obese mice. **c**. Tandem MS spectra of representative PLs with shorter acyl chains (PI 16:0_18:2; upper panel) and longer acyl chains (PS 18:0_22:6; lower panel). **d**. Hierarchical cluster analysis discriminated healthy and obese mice by quantitative analysis of PLs. The complete PL list is provided in **Supplementary Data**. Colors represent relative amounts as indicated by the color bar.



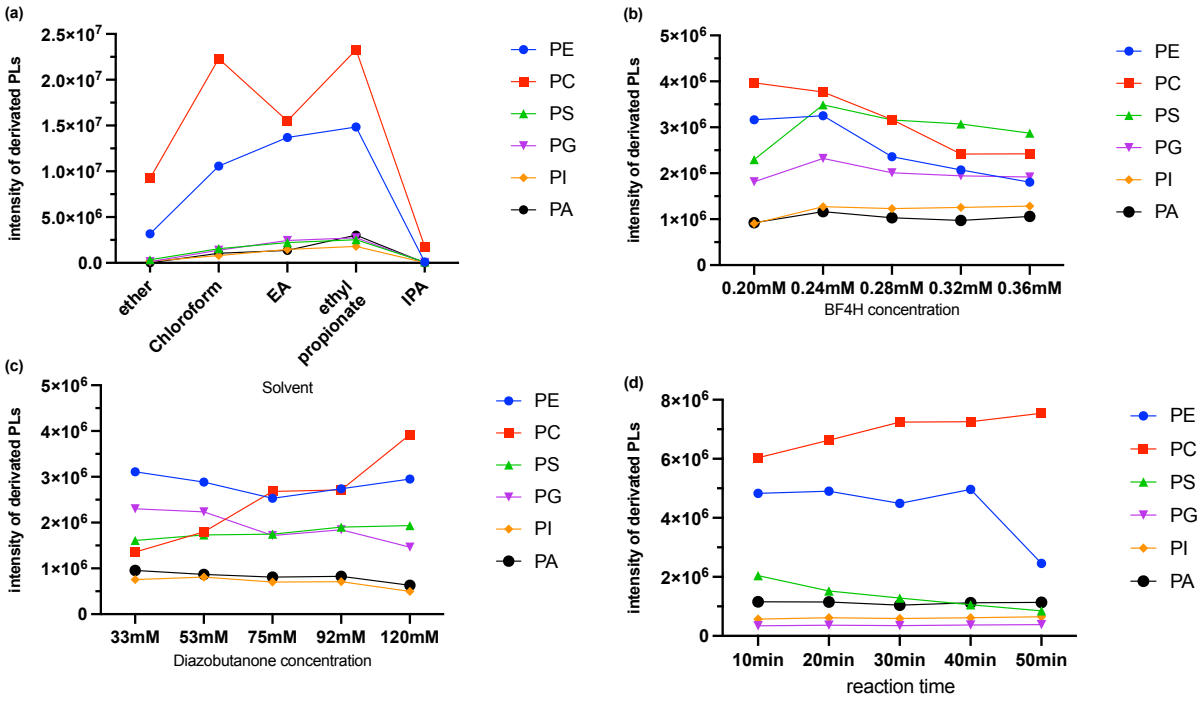
Supplementary Figure 1.

Synthesis scheme of Diazobutanone. The detailed synthetic methods were described in **Supplementary Method.**



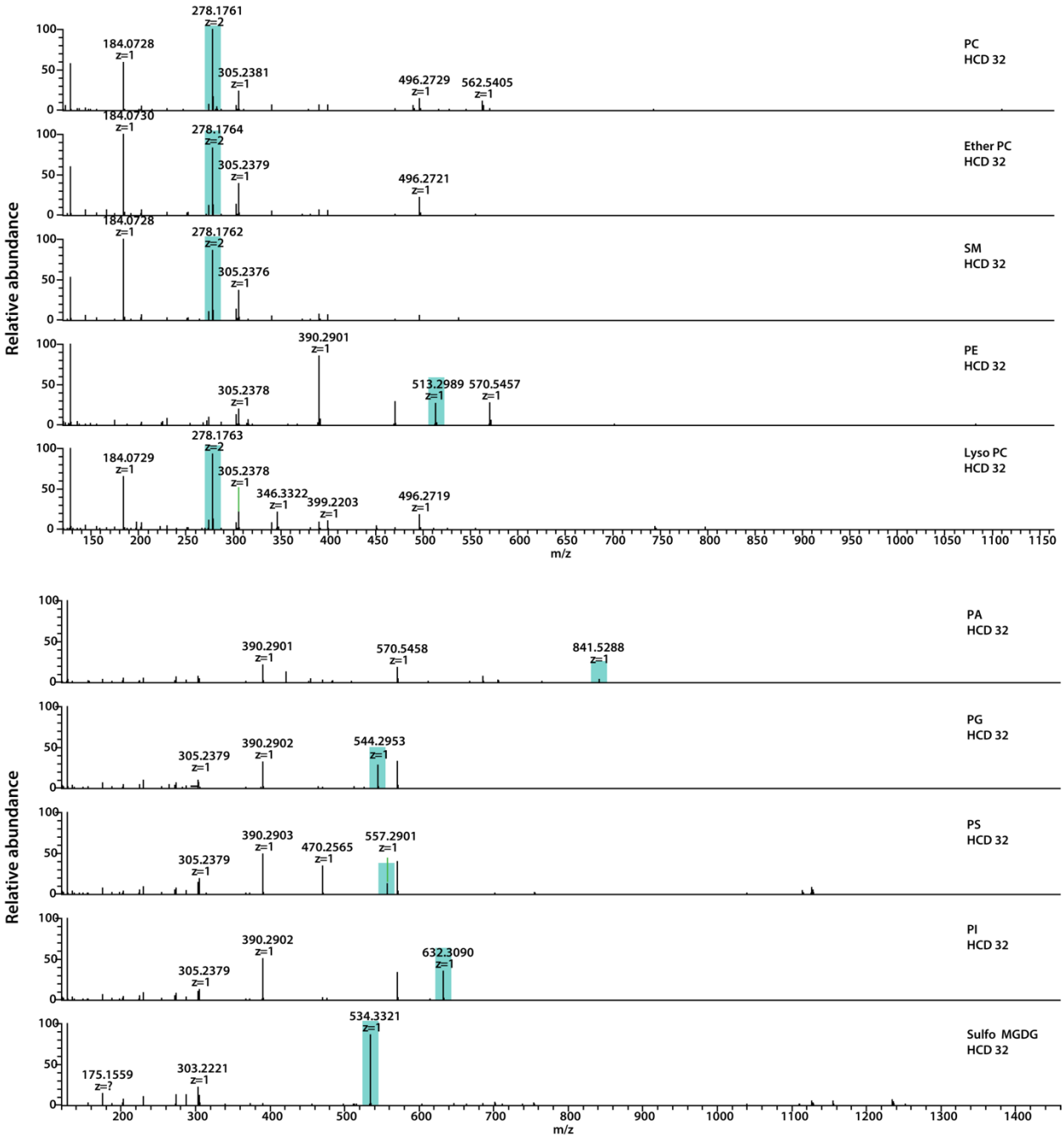
Supplementary Figure 2.

Full MS scan spectra of derivatization of PL mixtures by new and 60-day-old diazobutanone. The peaks highlighted in yellow are the internal standards. Nearly no differences were shown between the two spectra, indicating good stability of diazobutanone.



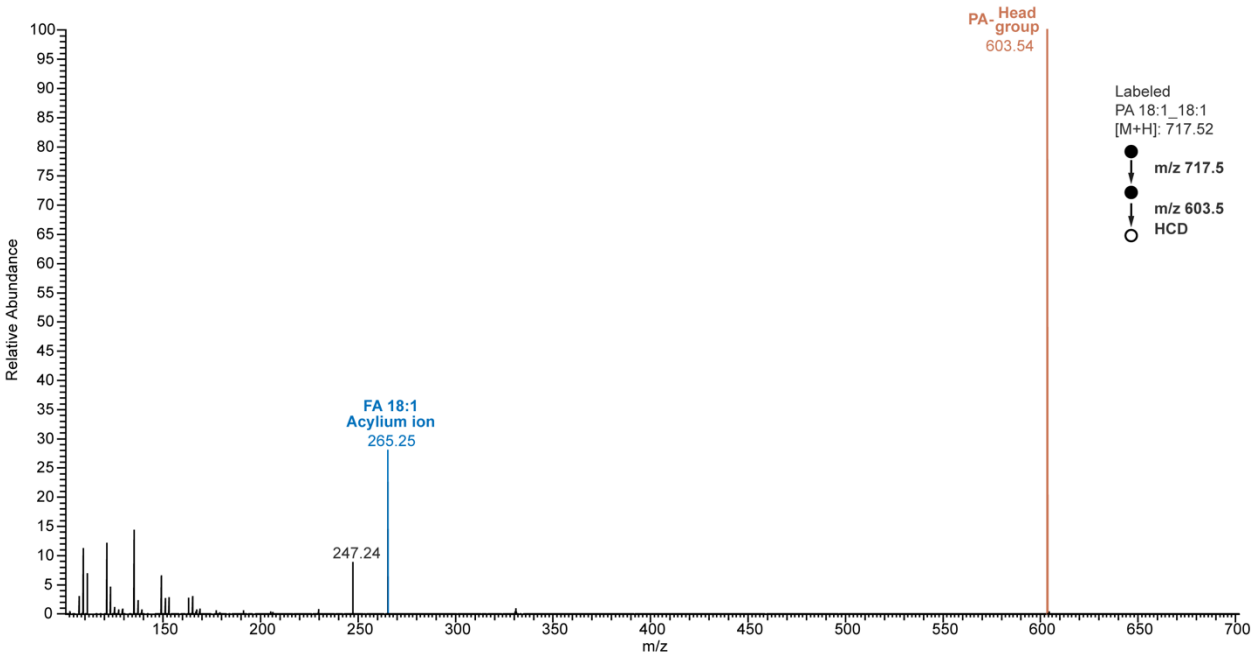
Supplementary Figure 3.

The derivatization condition was optimized by (a) reaction solvents, concentrations of (b) HBF_4 and (c) diazobutanone, and (d) reaction time using a lipid mixture consisting of PE 18:1_18:1, PC 16:0_18:1, PS 18:1_18:1, PG 18:1_18:1, PI 18:1_18:1, and PA 18:1_18:1.



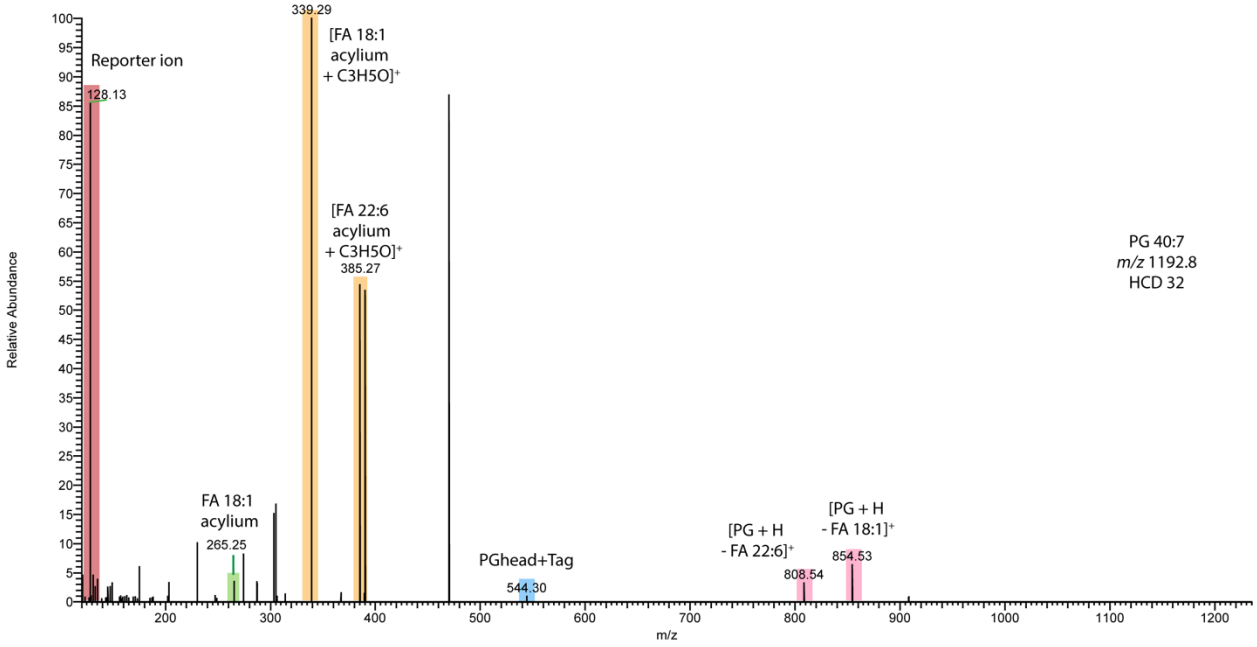
Supplementary Figure 4.

MS2 spectra of all lipid standards from 10 lipid classes by HCD (NCE 32%). Diagnostic ions for class identification were highlighted in green. Lipid standards included PC 15:0-18:1(d7), ether PC 16:0-18:1, SM d18:1-18:1(d9), PE 15:0-18:1(d7), Lyso PC 18:1(d7), PA 15:0-18:1(d7), PG 15:0-18:1(d7), PS 15:0-18:1(d7), PI 15:0-18:1(d7), and 3-O-Sulfo MGDG 18:1 representing SGL.



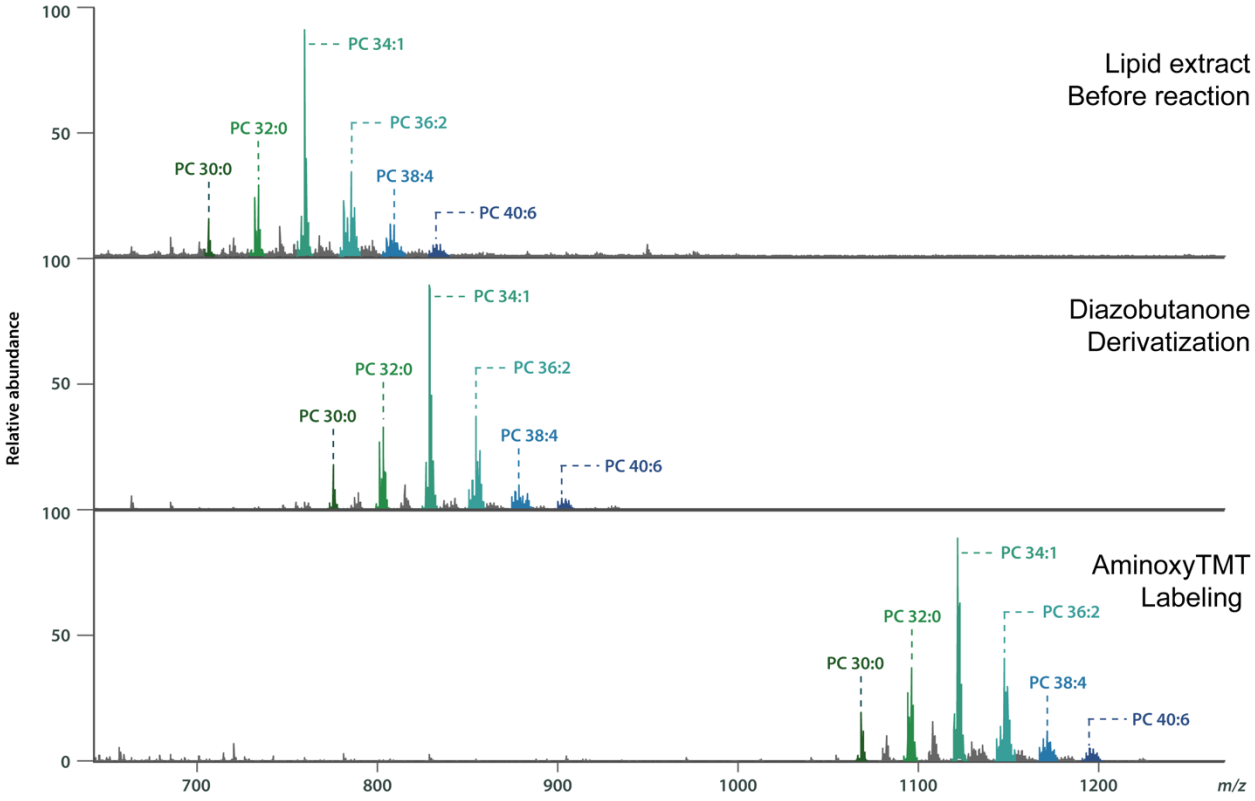
Supplementary Figure 5.

MS3 spectrum of PA 18:1_18:1. The peak highlighted in blue represents FA 18:1 acylium ion, indicating the acyl chain information of the precursor ion.



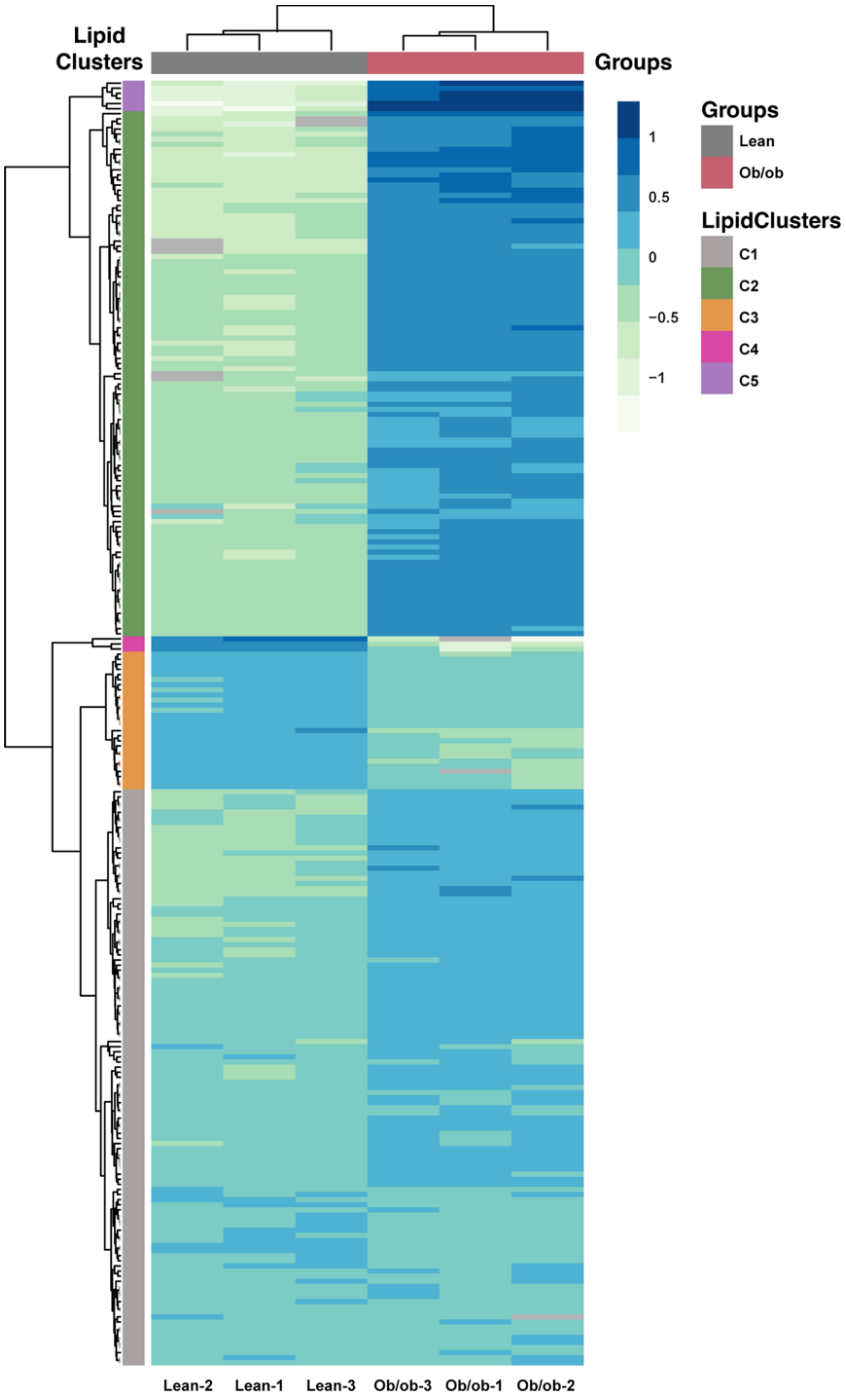
Supplementary Figure 6.

MS2 spectrum of PG 40:7. The peaks highlighted are diagnostic ions for class identification or acyl chain elucidation. PG species tend to generate $[PG + H - RCOO]^+$ and show less efficiency on $[PGhead+Tag+H]^+$.



Supplementary Figure 7.

MALDI MS spectra of unreacted PL extract (top panel), derivatized PL extract (middle panel), and labeled PL extract (bottom panel). Representative peaks are highlighted and marked. Due to the high ionization efficiency of PC species on MALDI, PCs are indicated.



Supplementary Figure 8. Hierarchical cluster analysis discriminated health and obese mice by quantitative analysis of PL species. The complete PL species list is provided in **Supplementary Data**. Colors represent relative amounts as indicated by the color bar with darker blue showing higher abundance and lighter green showing lower abundance.

Supplementary method.

Synthesis of diazobutanone

3,5-heptanedione (250 mg, 1.95 mmol) and TsN_3 (514 mg, 2.61 mmol) were dissolved in 4 mL ACN at 0 °C. NEt_3 (326 µL, 2.34 mmol) was added to the cooled solution and the resulting reaction was stirred for 4 h. The reaction was filtered through Celite and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (0% → 20% EA in hexane) to afford 3,5-dione-4-diazoheptane (256 mg, 85%) as a yellow liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.15 – 2.28 (m, 4H), 1.13 – 1.09 (m, 6H). ESI-MS: m/z calcd for $C_7H_{10}N_2O_2$; 154.07423 found 155.0819 $(M + H)^+$.

3,5-dione-4-diazoheptane (40 mg, 0.26 mmol) was dissolved in 530 µL ether at 0 °C. NaOH (530 µL, 3M) was slowly added to the reaction mixture and the resulting reaction was stirred at 0 °C for 3 h. The aqueous phase was extracted with ether (3 × 500 µL). The combined organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure (T = 20 °C, P ≥ 250 mbar) to afford diazobutanone as a volatile yellow liquid (24 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.22 (s, 1H), 2.89 – 2.04 (m, 2H), 1.09 (td, 3H). ESI-MS: m/z calcd for $C_4H_6N_2O$; 98.04801 found 99.0559 $(M + H)^+$.

Chapter 4

Simultaneous multiplexed quantification and C=C localization of fatty acids with LC-MS/MS using isobaric multiplex reagents for carbonyl-containing compound (SUGAR) tags and C=C epoxidation

Adapted from: **Gu, T. J.,** Feng, Y., Wang, D., & Li, L. (2022). Simultaneous multiplexed quantification and C= C localization of fatty acids with LC-MS/MS using isobaric multiplex reagents for carbonyl-containing compound (SUGAR) tags and C=C epoxidation. *Analytica Chimica Acta*, *1225*, 340215

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Abstract

The fatty acid (FA) category contains highly diverse structures and can be divided into saturated and unsaturated classes. For unsaturated FAs, both the numbers and positions of the carbon-carbon double bond (C=C) determine their biological functions. Abnormal levels of FA isomers have been reported to be involved in some disease development, such as cancer. Despite the advances in lipidomics, simultaneously quantifying and pinpointing C=C bond positions in a high-throughput manner remain a challenge. Here we conducted epoxidation of C=C bonds of unsaturated FAs followed by the conjugation with isobaric SUGAR tags. With the assistance of LC-MS, FA isomers with the same masses were separated on the C18 column and individually subjected to MS/MS fragmentation. Upon higher-energy collisional dissociation, both reporter ions for multiplexed quantification and diagnostic ions for C=C localization were generated at the same time, allowing quantitative analyses of different unsaturated FA isomers in the samples. This approach was optimized in all aspects including epoxidation, labeling efficiency, quantitative accuracy, and capability to pinpoint the C=C bond position. To further evaluate our method, free FA extracts from healthy human serum were used to benchmark the feasibility of this method to complex biological samples. Finally, this method was also applied to investigate the changes of unsaturated FA isomers in serum samples between healthy human and Alzheimer's disease (AD) patients.

Introduction

Fatty acids (FAs) consist of carboxylic acids and long aliphatic chains with different numbers of carbon atoms. FAs are not only the building blocks forming diverse lipid species but also play essential roles in a range of biological processes and metabolism [1–3]. The fluidity and functions of FAs are regulated by their relative abundance and structural configurations, including the locations, cis/trans geometry, and numbers of C=C double bonds [4–6]. New advances in the lipidomic analysis have revealed that disturbance in the anabolism and catabolism of FA species is strongly associated with some diseases, such as cancer, Alzheimer's disease, and cardiovascular disease [7–9]. Furthermore, the changes in FA isomers with different C=C bond positions in cancerous cells were also reported [10,11]. Therefore, the quantitative analysis and structural investigation of FAs provides a deeper understanding of disease mechanism involving the changes of FA positional isomers and facilitate the finding of potential therapeutic candidates.

Owing to its high sensitivity and accuracy, mass spectrometry (MS) coupled with chromatographic separation techniques has been extensively exploited in lipidomic studies to identify substantial lipids [12–14]. Several derivatization strategies have been developed to assist the characterization or quantification of FAs in MS analysis, where FAs are derivatized with chemical reagents to improve ionization efficiency or fragmentation patterns to enhance the detection of FA species. For example, FAs are methylated or silylated to facilitate ionization in gas chromatography-mass spectrometry (GC-MS) [15,16]. In liquid chromatography−mass spectrometry (LC−MS), chemical reagents carrying a permanent positive charge were used to promote the analysis of FAs in the positive ion mode [17].

Traditional analysis of FA quantification involves spiking in internal standards or stable isotopic labeling [18–20]. In the former methods, FAs are calibrated with internal standards and quantified one by one. Although this strategy does not require additional labeling, samples are prepared and analyzed separately leading to tedious experimental procedures and long instrument time. Stable isotopic labeling with light and heavy isotopic reagents allows FAs to be quantified simultaneously at the MS1 level where FAs from multiple samples exhibit distinguishable mass-to-charge (*m/z*) signals. However, the increase in spectral complexity and interference from analytes with close *m/z* ratios negatively affect the spectral interpretation and limit the sample throughput.

Instead, isobaric labeling is a powerful technique that can quantify analytes in high-throughput manners [21–23]. Isobaric tags consist of three components, reporter, balance, and reactive groups. In the reporter regions, each variant only differs in its isotopic configurations, while mass differences between each tag are normalized by balance regions. When samples are labeled separately with multiple variants of isobaric mass tags, the masses and physiological properties of labeled analytes are kept the same. Labeled analytes appear as a single precursor ion in the full MS scan without increasing spectral complexity. Upon fragmentation through collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), or electron-transfer dissociation (ETD), reporter ions with unique *m/z* values are generated from mass tags and represent the relative abundance of the analytes [24]. This technique has been shown to enhance quantification accuracy, reproducibility, and sample throughput. Given these advantages, isobaric labeling for multiplexed quantitative analysis has been widely utilized in proteomic and glycomic analyses [25,26]. In the field of glycomics, commercially available aminoxy tandem mass tag (aminoxyTMT) reagent is used to target carbonyl groups on carbohydrates [27,28]. Previously, our group also developed SUGAR

isobaric tags containing hydrazide groups to label reducing ends of glycans, where labeled glycans produced higher intensity of reporter ions compared to aminoxyTMT-labeled glycans [29]. Although the application of isobaric tags has been well demonstrated in proteomic and glycomic studies, this technique is underexplored in lipid and FA analysis [28,30]. Recently, aminoxyTMT was applied to quantitative FA analysis where FAs were first activated by 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) followed by aminoxyTMT labeling [31]. A promising quantitative analysis of cell FA extracts has been achieved by using LC-MS.

Besides quantification, localizing C=C double bond position is a challenging task since the efficiency of generating fragment ions that contain C=C double bond information is low by using CID or HCD. In order to generate structurally informative fragments, various methods, including metal-adduction, electron-ion reaction-based dissociation, and ultraviolet photodissociation have been developed to enable the generation of diagnostic ions to specify double bond positions [32–34]. Alternatively, derivatization methods prior to MS analysis using ozonolysis, Paternò−Büchi, and epoxidation have been also explored to elucidate lipid structures [35–41]. Recently, meta-Chloroperoxybenzoic acid (*m*-CPBA) epoxidation has been reported to be capable of localizing double bond positions on lipids [42,43]. The production of three-member epoxide rings could be fragmented readily upon CID or HCD fragmentation and generate a pair of diagnostic ions with a 16 Da mass difference for double bond localization.

Though the hydrazide group of SUGAR tags was designed to label carbonyl groups on glycans originally, it also has an ability to react with activated carboxylate groups. Here, we developed a method that couples *m*-CPBA epoxidation with SUAGR mass tags followed by LC-MS/MS analysis to enable high-throughput quantification of FAs and localizations of double bond positions simultaneously. *m*-CPBA can derivatize C=C bonds offline with high production yield

and produce epoxide rings that are stable for a period of time [43]. By combining these two strategies, the labeled FAs with epoxide rings were able to generate reporter ions for quantification and diagnostic ions for double bond localization upon HCD fragmentation. To further validate the feasibility of this method to complex biological samples, it was applied to free fatty acids (FFAs) extracts from human serum samples to evaluate the change of FFA levels between healthy people and Alzheimer's disease (AD) patients.

Results and Discussion

Optimization of epoxidation conditions

m-CPBA is a common reagent in organic synthesis for oxidizing C=C double bonds. By reacting with *m*-CPBA, each C=C double bond will be added with one oxygen atom and converted to an epoxide ring leading to a 16 Da mass increment to FA. The epoxide ring can be fragmented easily upon CID or HCD (**Fig. 1A**). When testing *m*-CPBA epoxidation, we found that FAs with fewer double bonds were able to be derivatized in high yields by treating with 60 mM *m*-CPBA at room temperature, while C=C bonds on polyunsaturated FAs might not be converted completely.

To achieve complete derivatization for all FA species, here a polyunsaturated FA 20:4 was used to optimize the reaction (**Fig. 2**). At room temperature, products with only 2 and 3 epoxide rings still existed at a certain amount. In such case, variations might be introduced among samples, and the resulting products from the same FAs would complicate LC chromatograms. When the reaction temperature was increased to 35 °C, a complete conversion of all four C=C bonds to epoxide rings was achieved without observing any side products. Therefore, the condition of 60 mM m-CPBA at 35 °C was used for the epoxidation of FAs.

SUGAR labeling of FA standards

Besides C=C bonds, *m*-CPBA is also known to react with tertiary amine groups to form N-oxide products [45]. Tertiary amine, however, is used in our SUGAR tags as reporter structures to produce high-intensity fragment ions. N-oxide side products were observed when reacting labeled FAs with *m*-CPBA and whose reporter ion yields are low. Therefore, it is essential to perform epoxidation prior to SUGAR tag labeling in the experimental workflow. The structure and isotopic configuration of 4-plex SUGAR tags are shown in **Fig. 1B**. Since the hydrazide group does not react with carboxylic acids directly, carboxylic acids were first activated by EDCI and HOBt which are commonly used reagents to conjugate biomolecules [46,47].

To achieve maximum labeling efficiency, reaction conditions were examined by using different solvents, additives, and temperatures (**Fig. 3**). Since the carboxylic acid groups of FAs will be converted to amide groups that are analyzed in positive ion mode, while negative ion mode is more suitable for carboxylate molecules, FA 19:0 was added as an internal standard before and after the labeling to estimate the amount of remaining FA in different conditions by using the negative ion mode. In our initial tests, room temperature only reached around 50% of the reaction yield, so a higher temperature at 50°C was used in the following experiments. Since the absence of NMM gave inconsistent results among FA species, 3% of NMM in solvents was used in the reaction. It was also found that a higher concentration of NMM made no difference in production yield but increased impurities. With the optimized temperature and additives, DMF was able to reach a high labeling efficiency of 95%, and ACN and DCM solvents could even achieve nearly complete labeling without any hydrolysis of epoxides observed.

After *m*-CPBA epoxidation and SUGAR labeling, a substantial amount of side-product, SUGAR-labeled chlorobenzoic acid, would exist in the mixture crudes. To remove this side

product, we found that 0.1% formic acid in water was able to wash out SUGAR-labeled chlorobenzoic acid using Sep-Pak C18 cartridges (**Fig. S1**).

MS and chromatographic properties of SUGAR labeled FAs

In general, FAs are analyzed in the negative ion mode due to carboxylate groups and generate few informative fragment ions in MS2 for structural elucidation. In our method, the characteristics of FA derivatives have been changed in many aspects. First, with the conjugation of SUGAR tag, the tertiary amine protonated under acidic conditions enables analyses of FA in the positive ion mode with strong signal intensities. Upon HCD fragmentation, FA derivatives would not only generate reporter ions with high yields for relative quantification but also diagnostic ion pairs for C=C localization. As an example, FA 18:1 had a precursor ion at *m/z* of 515 in the full MS scan (**Fig. 4A**). In its MS2 spectrum, reporter ions from the SUGAR tags were observed in the low mass range. A pair of diagnostic peaks at m/z of 373.30 and 389.30 generated from the epoxide rings indicated a double bond between the 9^{th} and 10^{th} carbons. Additionally, the peaks located at m/z 203.16 and 235.20 were also generated from the SUGAR tag, and a neutral loss of 18 Da from the precursor ion stood for a water loss of FA derivatives. For polyunsaturated FAs, since the generation of diagnostic ions was less efficient, not all diagnostic ions would be detected, where unsaturated FA database was used as comparison. Informative MS^2 spectra of FA 18:2 and FA 20:4 were shown in **Fig. S2.**

In terms of LC, LC separation prior to MS analysis was essential to distinguish unsaturated FA positional isomers that have the same masses. Therefore, LC methods were carefully optimized in this study. FAs with longer aliphatic chains tended to retain for longer time on a C18 column. In addition, unsaturated FAs tended to elute earlier than saturated FAs with the same aliphatic

chains. However, the conversion of C=C bonds to epoxide rings would significantly increase the hydrophilicity of FAs, which led to a shorter retention time (**Fig. S3**). Thus, a lower percentage of organic solvent was used in LC methods. In order to identify FAs with high confidence, all patterns in MS2 spectra and LC properties were considered together to validate the identification of FAs along with accurate mass matching.

Evaluation of quantification performance of 4-plex SUAGR labeling

Quantitative performance was assessed using 8 FA standard mixtures (**Table S1**). Four aliquots of FA mixture were epoxidized, labeled with 4-plex SUGAR reagents respectively, and combined at molar ratios of 1:1:1:1 and 1:2:4:8 prior to clean-up and LC-MS/MS analysis. A representative MS2 spectrum is shown in **Fig. 4B**, where reporter ions had a relative abundance 1:1:1:1. The retention time extracted for 4 channels showed no obvious retention time shift between each other despite a slight amount of skew of the peak caused by the use of deuterium in the tag design (**Fig. 4C**). The ratios of all quantified FAs were plotted against each other in a boxplot (**Fig. 5**). Mean error less than 5% was observed for both theoretical ratios of 1:1:1:1 and 1:2:4:8, suggesting that our approach offered a great quantitative accuracy.

FFAs profiling in human serum samples

To further demonstrate the applicability of our method to complex biological samples, FFA extracts from healthy human serum were used for 4-plex quantitative analysis. 4 aliquots of FFA extracts were subjected to epoxidation and SUGAR labeling and pooled before LC-MS/MS analysis. A relatively short dynamic exclusion time and the TopN 15 were employed in data-dependent acquisition (DDA) mode to avoid exclusion of low-abundance isomers from

fragmentation. FAs were identified by accurate mass matching and verified using MS2 fragments and retention time. In total, 43 FA species were successfully identified in human serum samples, including 13 saturated FAs and 31 unsaturated FA isomers. In biological triplicate, 35, 37, and 39 FAs were identified (**Table S2**). 86% of FAs were found in at least two replicates (**Fig. 6A**), demonstrating a high reproductivity. The quantitative performance also showed accurate results of 1:1:1:1 among unsaturated FAs (**Fig. 6B**), which means that the current method is highly applicable to complex biological systems. Next, FA isomers with different C=C bond positions were examined. The identification of isomers was based on the diagnostic ion pairs in MS2 spectra and compared with unsaturated FA database [48]. In traditional analysis where double bond positions were not characterized, the presence of isomers might be covered up by higher signal peaks or counted as the same FA species, which hinders the detection of the low abundant isomers. By contrast, our method was able to simultaneously detect and quantify FA isomers with different C=C bond positions. In an example of an extracted ion chromatograph of FA 16:1 (**Fig. 7**), 3 peaks appearing on LC chromatogram were clearly assigned to 3 different isomers FA16:1 $\triangle 7$, $\triangle 9$, and $\triangle 11$ according to their unique diagnostic ion pairs. Furthermore, FA 18:1 was found to be a mixture of $\triangle 9$, $\triangle 11$, $\triangle 12$ C=C location isomers. C18:3 was a mixture of FA 18:3 (Δ9, 12, 15) and (Δ6, 9, 12). All identified FA isomers were summarized in **Table S2**. More FA isomers were expected to be present in human serum samples since co-isolation of different FA isomers for fragmentation were observed due to the limited LC separation. Those co-isolation spectra were not included in **Table S2.**

Quantification of FFAs in human serum from healthy people and AD patient serum

FAs and their metabolic products play important roles in brain structure and functions, such as cell membrane, inflammation, and neurotransmission. Both brain and plasma FA levels have been reported to be associated with Alzheimer's disease. In a recent systematic review [7], the authors collected hundreds of articles studying FAs in plasma, serum, or the cerebrospinal fluid and summarized all FA levels that were commonly studied. In plasma, most of the unsaturated FAs did not show significant changes between groups, while the percentages of total FAs for docosahexaenoic acid, linoleic acid, and palmitoleic acid were significantly downregulated in AD patients, and mead acid and vaccenic acid were elevated significantly in AD. This suggested that the quantification of FAs in AD plasma might facilitate biomarker discovery and study of FA functions. However, the discussion on FA isomers present in low abundance was limited in the previous studies. Here, we adopted our method to perform quantitative analysis on 6 plasma samples, 3 from healthy people and 3 from AD patients (**Table S3**). The quantitative results of selected FAs were summarized in **Fig. 8.** In our study, docosahexaenoic acid and linoleic acid showed down-regulated in AD. In the meantime, mead acid and vaccenic acid were up-regulated in AD, which was consistent with the previous studies. Interestingly, we found that same as mead acid (FA 20:3 (Δ11, 14, 17)), the unsaturated FA positional isomers, FA 20:3 (Δ5, 8, 11) also trended upwards in AD, while FA 20:3 (Δ8, 11, 14) kept similar levels between healthy people and AD patients. As a proof of concept, this study revealed the distinct trends among FA positional isomers at different disease states and future investigations are needed to further explore the role of FA isomers during disease progression.

Conclusions

In this study, we introduced a novel strategy coupled with LC-MS/MS to simultaneously identify and quantify unsaturated FA isomers with different C=C bond positions. SUGAR isobaric mass tags that were developed for glycomic analysis were applied to FA quantitative analysis successfully. After careful optimization, both epoxidation and SUGAR tag labeling efficiencies were able to achieve nearly quantitative yields without observing hydrolyzed FA derivatives. With SUGAR labeling, FA derivatives could be analyzed in the positive ion mode with high sensitivity. With adjusted LC settings, FAs with the same mass were well-separated before MS analysis. Upon HCD fragmentation, reporter ions for multiplexed quantification were generated at high yields, and diagnostic ions for C=C localization enabled quantitation of individual FA isomers. In addition, all the sample preparation steps could be done in a day. With 4-plex SUGAR tags, rapid, accurate and high-throughput FA lipidomic quantification was achieved. Overall, this strategy features high labeling efficiency, quantitative performance, compatibility of complex samples, and the capability in multiplexed quantification and double bond localization. Future application of this novel strategy to different biological systems or clinical samples are expected to provide deeper insights into the roles of FAs in physiological possesses or disease progression.

Methods

Chemicals and reagents

All FA standards, mCPBA, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI), hydroxybenzotriazole (HOBt), N-methylmorpholine (NMM), ACS grade 2,2,4-Trimethylpentane (iso-octane), ACS grade dichloromethane (DCM), ACS grade acetone, dimethyl sulfoxide (DMSO), N,N-dimethylformamide (DMF), hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St. Louis, MO). Optima UPLC grade ACN, Optima UPLC grade water, and Optima LC/MS grade formic acid were purchased from Fisher Scientific (Pittsburgh, PA). Phosphate buffered saline (PBS) was purchased from Crystalgen (Commack, NY). Sep-Pak C18 1 cc Vac Cartridges were purchased from Waters Corporation (Milford, MA). SUGAR tags were synthesized in-house as previously described [29].

FFAs extraction from humane serum

FFAs were extracted from human serum according to Fatty Acid Mass Spectrometry Protocol from LIPID MAPS with slight modification [44]. A 50 µL serum sample was added to 75 µL dPBS and 350 µL methanol. Then the mixture was acidified with HCl to a final concentration of 25 mM and vortexed for 20 min, followed by the addition of 500 µL iso-octane. The mixture was vortexed for 20 min and centrifuged at 5000 rpm for 5 minutes to separate layers. The top organic layer was transferred to a new centrifuge tube. The iso-octane extraction was conducted twice. The collected organic layers were dried *in vacuo* and stored at -20^{o}C until use.

FAs epoxidation and SUGAR tag labeling

10 μg FA standards or FFA extracts were mixed with 10 μL of m-CPBA (10 μg/μL in DCM). The reaction was incubated at 35 °C for 1 h, followed by quenching with acetone. The mixture was dried under a gentle nitrogen stream. The epoxidized FA sample was then dissolved in 50μL DCM and mixed with 1 mg SUGAR tag. 50μL solution containing 50 mM EDCI and 10 mM HoBt was added to the sample to activate the labeling reaction. The reaction mixture was incubated at 50 °C for 1 hour and dried *in vacuo*. For multiplexed analysis, the mixtures were pooled before dried down. The sample was cleaned via Sep-Pak C18 Cartridges. The C18 cartridge was conditioned with 1 mL ACN with 0.1% formic acid and 2 mL 0.1% formic acid in water. The reaction crude was resuspended in 50 μL ACN and loaded onto the cartridge that was filled with 1 mL 0.1% formic acid in water. The cartridge was then washed with 0.1% formic acid in water, and 2 mL of ACN/H2O/formic acid (95/5/0.1) was used to elute labeled FAs. The eluate was dried *in vacuo* and kept at −20 °C until further processing.

Matrix-Assisted Laser Desorption/Ionization (MALDI) analysis for epoxidation and labeling efficiency

Samples were mixed with the matrix and spotted onto the MALDI target plate. For the negative ion mode, 1,5-diaminonaphthalene (10 mg/mL in acetonitrile/water (v/v, 70/30)) was used as a matrix, and 2,5-dihydroxybenzoic acid (25 mg/mL in acetonitrile/water (v/v, 70/30)) was used for the positive ion mode. Samples were analyzed using a Bruker RapifleX MALDI TOF mass spectrometer (Bruker Scientific, LLC, Bremen, Germany). The Smartbeam laser was set to 20% and 70% power for the negative and positive ion modes, respectively. Spectra were acquired with a mass range of 100-1000 Da.

LC-MS/MS analysis

A binary nanoAcquity UPLC system (Waters, Milford, MA) coupled with an Orbitrap Elite mass spectrometer (Thermo Scientific, San Jose, CA) was used to monitor the progress of labeled FAs. Labeled FAs were dissolved in 20% ACN in water. The samples were loaded onto a self-fabricated microcapillary column packed with C18 beads (Waters Bridged Ethylene Hybrid, 1.7 μm, 130 Å, 75 μm x 15 cm), and for mobile phases, phase A was composed of water with 5% DMSO, and 0.1% formic acid, and phase B was composed of ACN, 5% DMSO, and 0.1% formic acid. The gradient started with 20% B (0 min), 50% B (65 min), 99% B (85 min), 99% B (95 min), 20% B (100 min), 20% B (110 min) at a flow rate of 300 nL/min. For precursor MS scans, 350 to 800 m/z were collected at a resolving power of 60k (at 400 m/z) with automatic gain control (AGC) target of 1 x 10^6 and a maximum injection time of 100 ms. Tandem MS spectra were performed using HCD with 30% normalized collision energy at a resolving power of 30k, and the top 15 precursors were selected for HCD analysis. The AGC, maximum injection time, resolution (at m/z 400), and lower mass limit for tandem mass scan were 1 × 10^5, 110 ms, and 30k, 100 m/z respectively. Precursors were subjected to dynamic exclusion for 5 s. FA identification was performed by accurate mass matching with a tolerance of 5 ppm and diagnostic ions in MS2 spectra. The FA database was obtained from LIPID MAPS. Quantitative results were normalized with total ion intensities.

Supplementary table

Supplementary tables to this article can be found online at https://doi.org/10.1016/j.aca.2022.340215.

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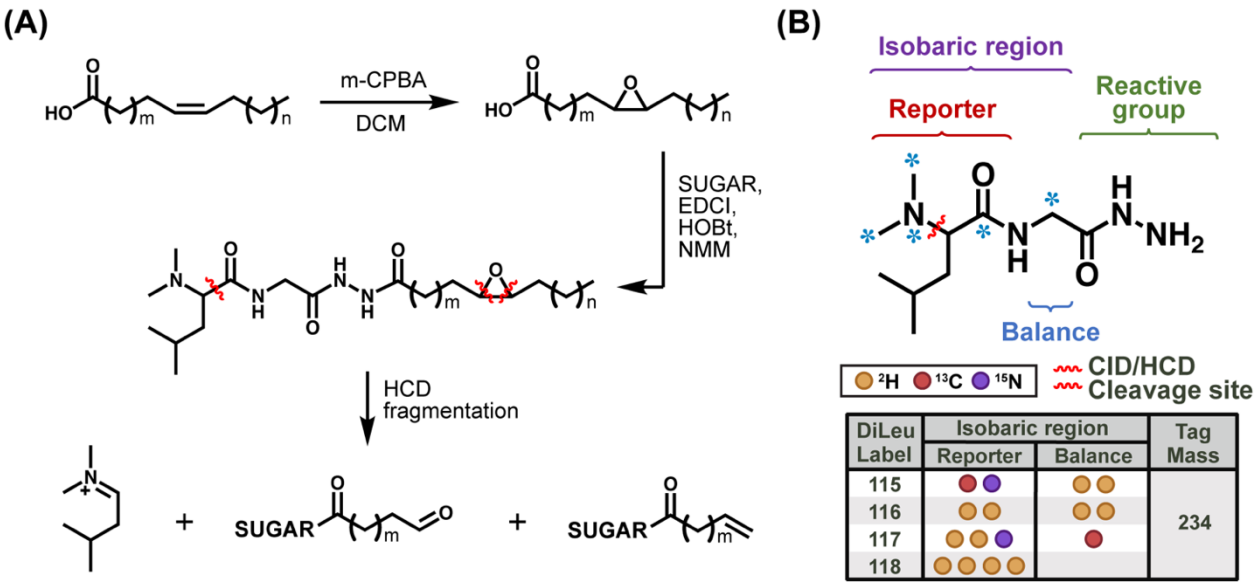

Fig. 1. Structure and mechanism of SUGAR tags. (A) isotope configurations of 4-plex SUGAR mass tags. (B) C=C epoxidation followed by SUGAR tag labeling and the fragmentation pattern of FA derivatives.

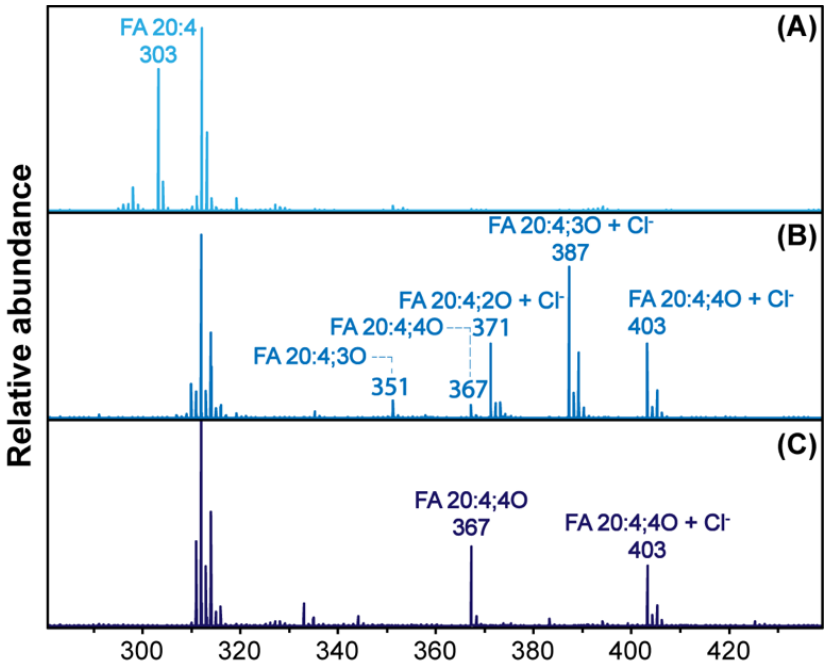

Fig. 2. MALDI spectra of (A) unreacted FA 20:4, (B) epoxidation of FA 20:4 at room temperature, and (C) epoxidation of FA 20:4 at 35 °C.

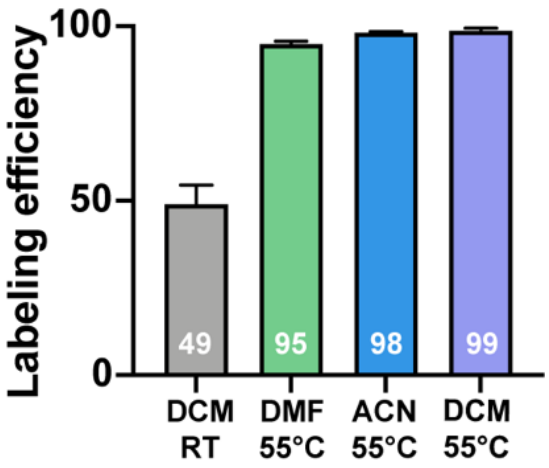

Fig. 3. Labeling efficiencies of SUGAR tags using 4 conditions, DCM at room temperature, DMF at 50 °C, ACN at 50 °C and DCM at 50 °C.

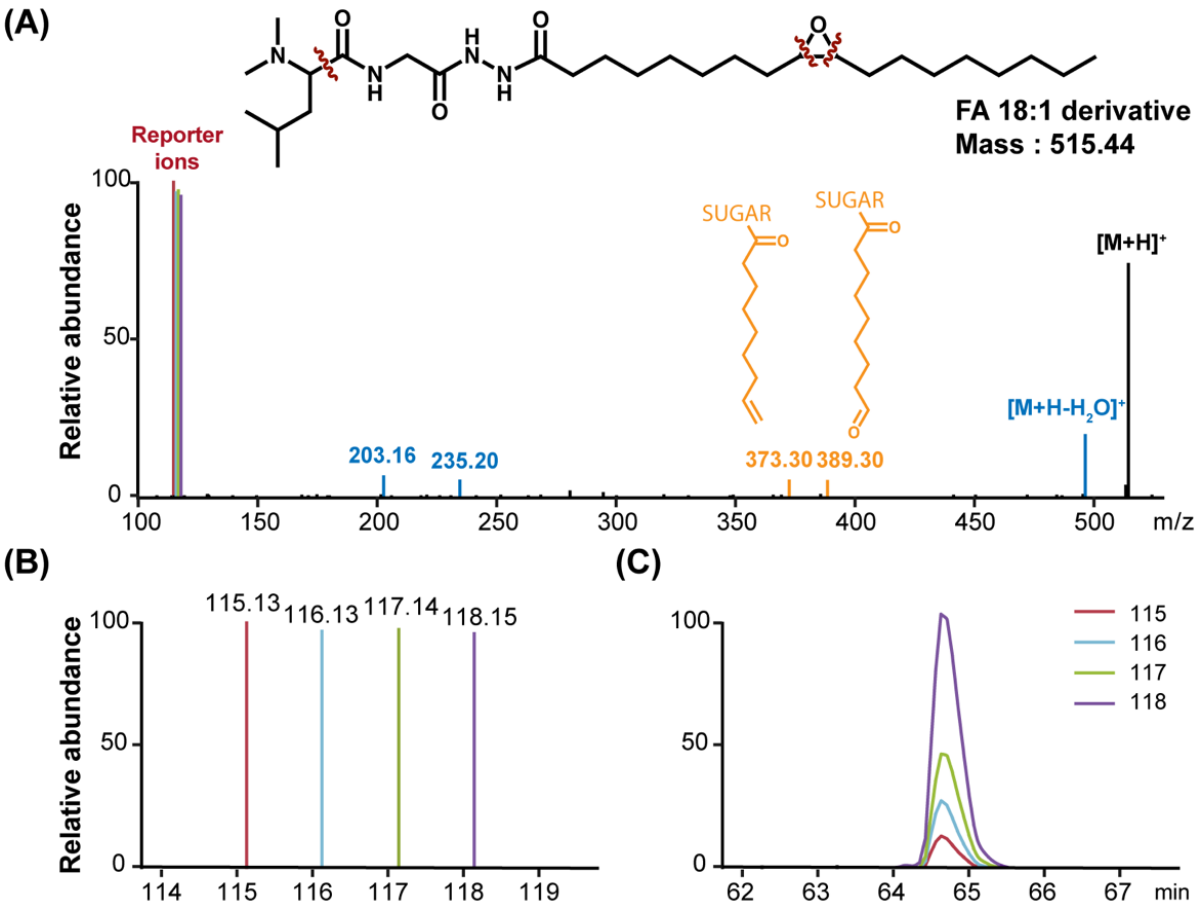

Fig. 4. Characteristics of 4-plex SUGAR labeled FAs. (A) MS2 spectrum of FA 18:1 derivative. (B) 4-plex reporter ions in low mass range. (C) Retention time of 4-plex SUGAR-labeled FAs.

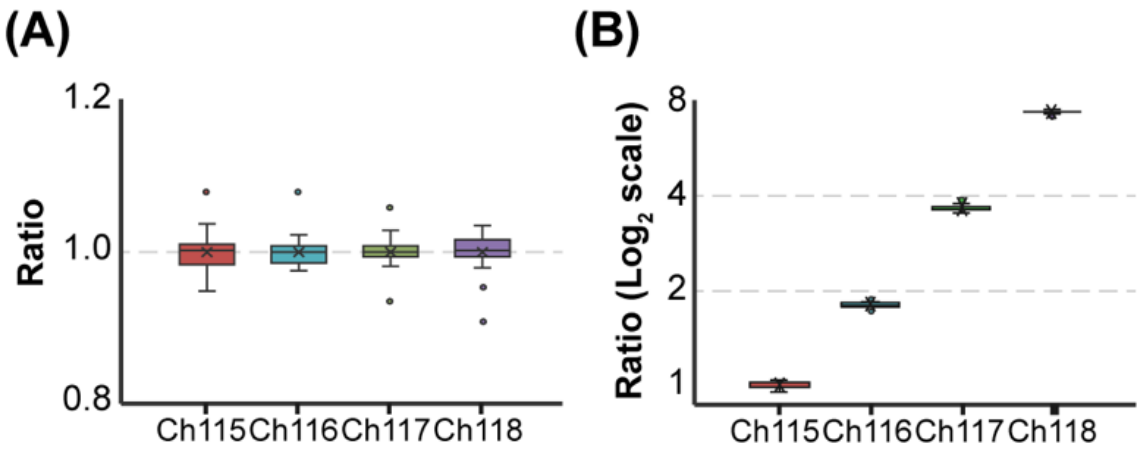

Fig. 5. Quantitative performance of 4-plex SUGAR-labeled FA standards. (A) relative ratio for 1:1:1:1 mixture. (B) relative ratio for 1:2:4:8 mixture. Box plots demarcate the median (line), the 25th and 75th percentile (box), and the 5th and 95th percentile (whiskers).

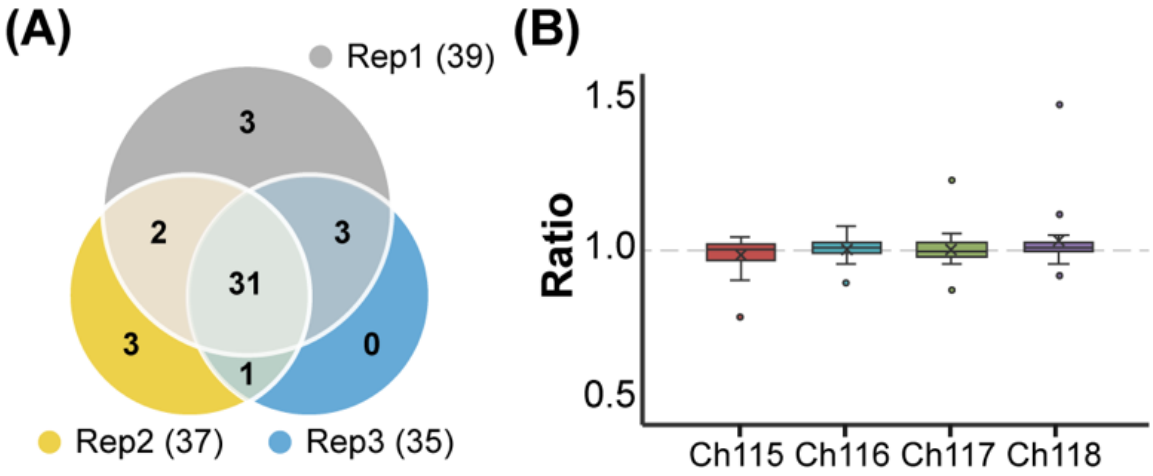

Fig. 6. Application of 4-plex SUGAR tags to human serum. (A) Venn diagram showing the overlap of identified FA isomers from triplicates. (B) Quantitative performance of 4-plex SUGAR-labeled FFA.

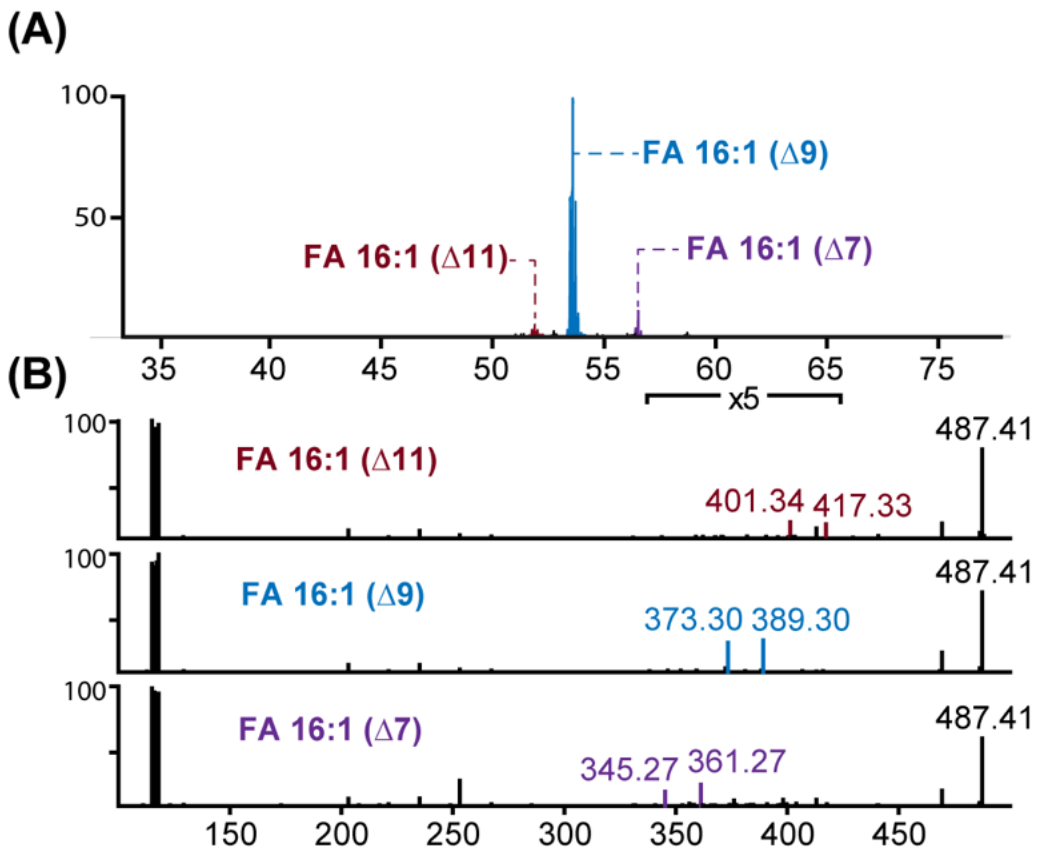

Fig. 7. LC-MS/MS analysis of FA 16:1 derivatives. (A) Extracted ion chromatogram of FA 16:1 derivatives. (B) MS^2 spectrum of FA 16:1 (Δ11), FA 16:1 (Δ9), FA 16:1 (Δ7) respectively.

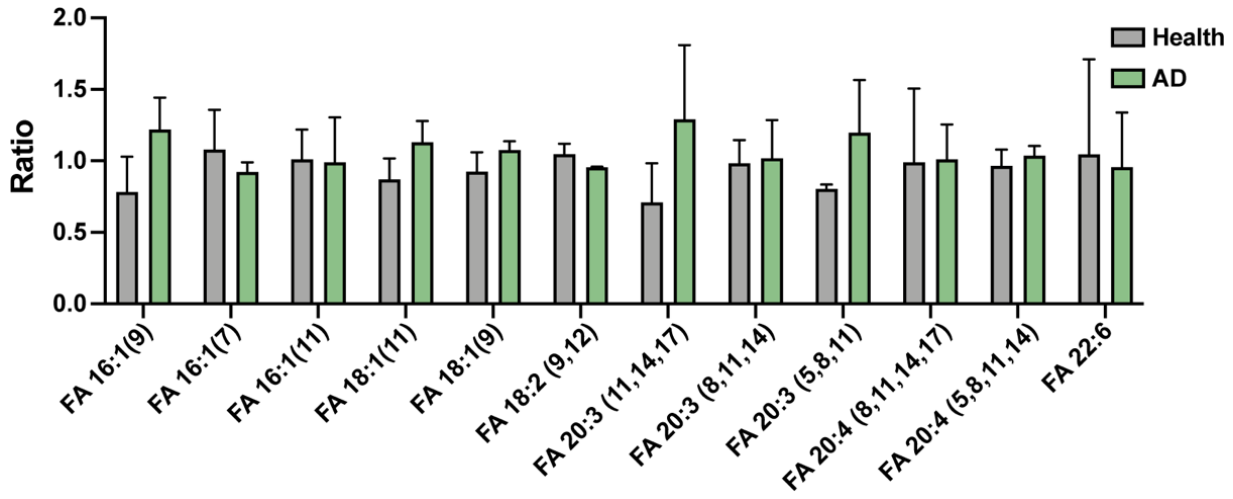

Fig. 8. Selected FA isomer relative quantification of human serum from healthy people and AD patients. Ratios represent intensities of reporter ions for FA derivatives. Error bars represent the standard deviation of three biological replicates.

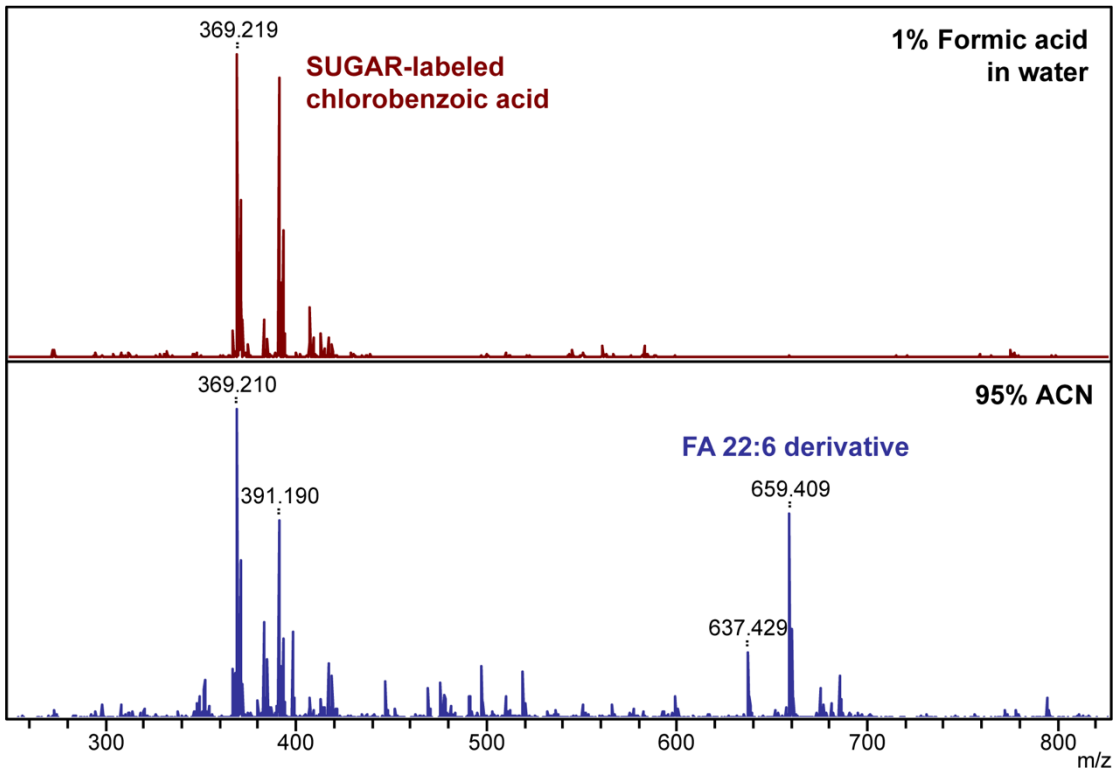

Fig. S1. MALDI spectra of washing fraction (top) and eluate (bottom) of SUGAR-labeled mixture using Sep-pak C18 cartridge.

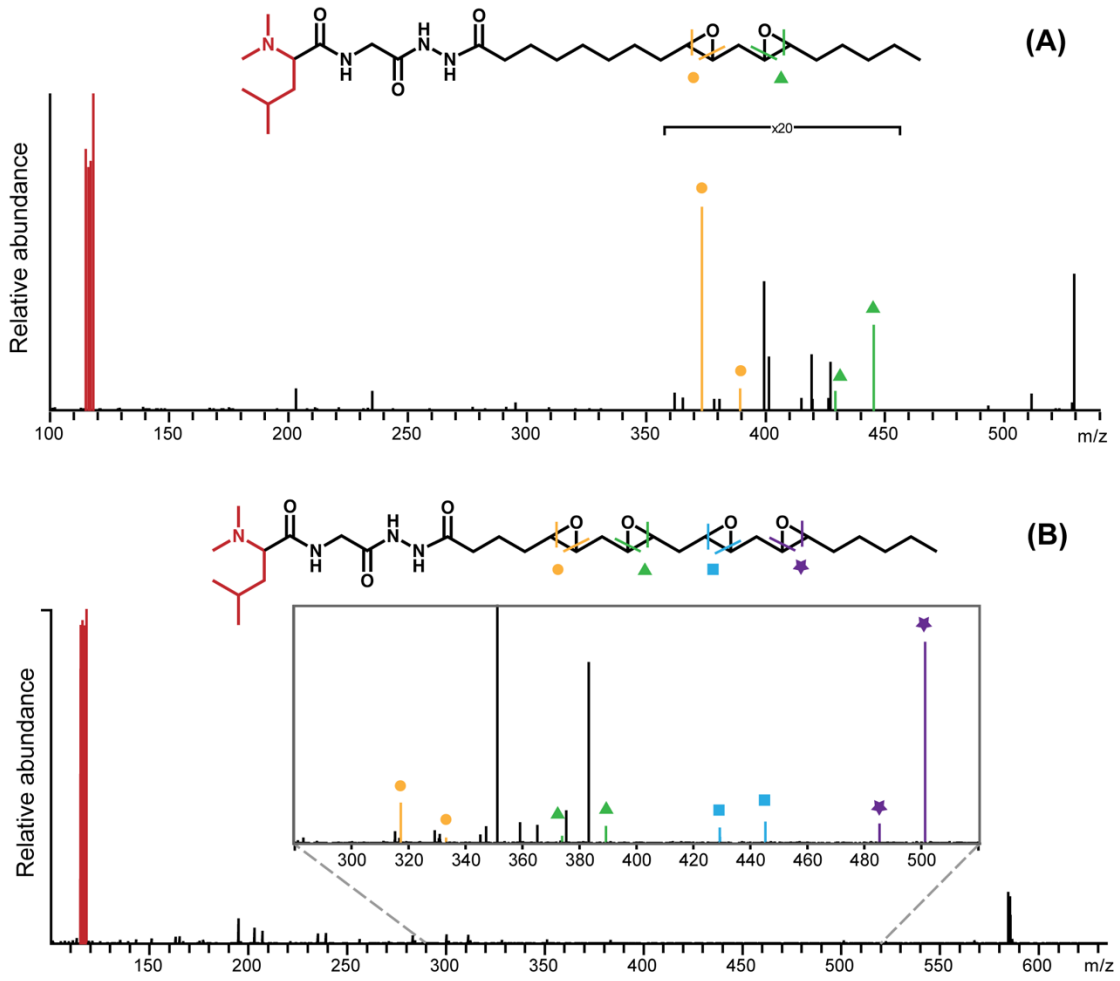

Fig. S2. MS^2 spectrum of FA 18:2 (Δ9, 12) (A) and FA 20:4 (Δ5, 8, 11, 14) (B).

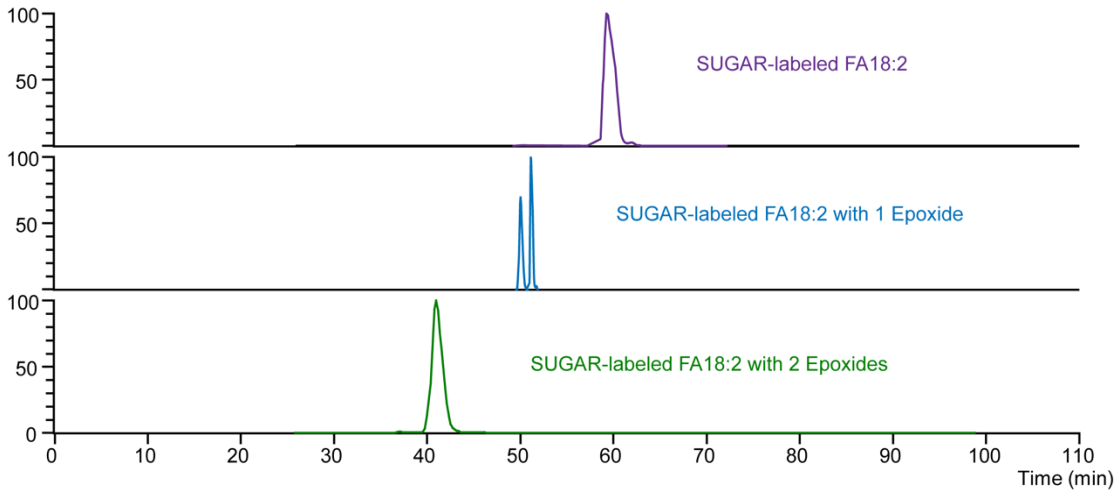

Fig. S3. Extracted ion chromatograms of SUGAR-labeled FA 18:2 (top), SUGAR-labeled FA 18:2 with only one epoxide (middle), SUGAR-labeled FA 18:2 with two epoxides (bottom).

Chapter 5

A complementary ion-based strategy enables multiplexed quantification in ETD analysis using DiLeu isobaric tags

Adapted from: **Gu, T. J.,** Lui, P.K., Wang, D., Wang, Z., Frost, D. C., and L. Li. A complementary ion-based strategy enables multiplexed quantification in ETD analysis using DiLeu isobaric tags. *In preparation.*

Abstract

LC–MS/MS is a popular technique to perform protein post-translational modification (PTM) analysis, where collisional dissociation approaches are frequently used to induce MS/MS fragmentation. Unfortunately, many PTMs are labile under collisional dissociation leading to the loss of PTM localization. Alternatively, electron transfer dissociation (ETD) is a method that could localize PTM on peptides. ETD approach induces dissociation at the N-Cα bonds by transferring electrons, which generates fragments of c and z-type ions while keeping PTMs intact. To expand ETD technology to high-throughput quantitative PTM proteomics, we seek to combine isobaric labeling with ETD. However, traditional isobaric tags fail to generate consistent reporter ions for quantification. Here, we present a novel 3-plex DiLeu tagging method for ETD-based analysis employing a complementary ion-based strategy, which enables the consistent generation of complementary ions across diverse peptide sequences. This strategy overcomes the limitations of existing isobaric mass tags in ETD fragmentation and demonstrates promising quantitative performance and PTM localization. The method has potential to facilitate the multiplexed quantitative proteomics with PTM modification sites.

Introduction

Post-translational modifications (PTMs) refer to numerous types of chemical groups that covalently attach to certain amino acids at different sites of peptides or proteins. These modifications occur on a majority of proteins leading to changes in protein states or characteristics to govern protein–protein interactions, protein folding, and protein localization [1]. PTMs play a significant role in many biological processes including cell signaling, gene expression regulation, and immune response[2]. Mass spectrometry (MS)-based PTM analysis opened up a new field to qualitatively and quantitatively analyze thousands of protein modification across biological states, which facilitate the identification of PTM, monitor abundance changes, and determine modification sites[3]. Alterations in various modifications have been extensively reported in disease progression to provide deeper insight in PTM functions and pathogenesis[4].

In MS-based PTM analysis, proteins or peptides are usually subjected to MS fragmentation to generate peptide backbone and PTM-related fragment ions for peptide sequence and PTM elucidation[5]. Several fragmentation methods such as collision-induced dissociation (CID)[6], higher energy collisional dissociation (HCD)[7], electron capture dissociation (ECD)[8], and electron transfer dissociation (ETD) have been applied to generate informative fragment ions for different research purposes[9]. In general, collision-induced dissociation-based methods are the most prominent fragmentation techniques due to their high accessibility and fragmentation efficiency. However, the bonds between peptides and several important PTMs including phosphorylation, sulfonation, and glycosylation which are most common PTMs in organism are labile under CID/HCD mechanism causing the losses of PTMs on peptide fragment ions[10].

ETD has been used as a complementary tool to CID/HCD methods. Dissociation mechanism of ETD started with transferring an electron from a radical anion to a protonated peptide. An unstable aminoketyl radical was first generated followed by the cleavage at the N-Cα bond, resulting c and z-type peptide ions series. ETD has been recognized as an efficient tool for the analysis of highly charged peptides and top-down proteomics to achieve better sequence coverage. One of the major advantages of ETD is the ability to preserve vibration activation labile PTM bonds probably due to the cleavage of peptide backbones faster than that of PTM bonds[11]. In such cases, labile PTM can be accurately localized on the target peptides.

Aberrant PTM expression levels have been linked to many diseases, such as Alzheimer's diseases, cancers, and diabetes[12–14]. Either up- or down regulations of them could be pathogenic factors or protective response during disease progression[15]. Thorough investigation is needed to fully understand disease mechanism and discover therapeutical targets. Besides PTM expression levels, individual PTM sites determine protein behaviors in terms of their physical and chemical properties[16], functions [17], protein crosstalk[18], and protein-protein interactions[19], which is another important factor to elucidate the intricate relationship between protein PTM and physiological states. Therefore, a method to accurately quantify the PTM expression levels and localize PTM sites simultaneously is highly desirable.

To quantify proteins in biological samples, MS-based approaches, label-free and stable isotopic labeling methods, are common relative quantitative strategies[20]. Label-free quantification that is performed by comparing chromatographic peaks of peptides is widely used because it does not require stable isotopes, which is cost-effective and less laborious. However, since samples are prepared and analyzed individually, label-free quantification strategy suffers from long instrument time and variations arising from separate sample injections and changes during the long running

time, leading to inaccurate quantification. An alternative approach, stable isotope labeling, incorporates light and heavy isotopic reagents into peptides. Although labeled peptides from separate samples can be distinguished in the MS1 spectra, the increased spectral complexity negatively affects the spectral interpretation. Thus, due to the above factors, these two approaches may not provide accurate results and achieve high-throughput quantification.

Isobaric labeling is a powerful tool to achieve multiplexed quantification without increasing spectral complexity[21,22]. It has become popular for quantitative proteomics and glycomics, featuring high sample throughput, improved accuracy and reproducibility. Isobaric mass tags consist of a reporter group, a neutral balance group, and a reactive group. All mass tags have identical chemical structures but with different isotopic configurations across all tag variants. In such cases, samples are introduced with the same mass increment by each tag variant and exhibit as single precursor ions in full MS scan. Upon MS fragmentation, the distinct isotopic configurations present between reporter and balancer regions result in the generation of reporter ions with unique masses in the low mass range representing the relative abundance of peptides in multiple samples. Thus, a high-throughput quantitative analysis can be conducted within one experiment.

An expanded strategy utilizing the design of isobaric mass tags is complement reporter ion-based multiplexed quantification[23]. Complementary ions were peptide-coupled fragment ions which contain the remaining parts of isobaric mass tags after generating reporter ions. Since complementary ions are unique to peptides, this strategy was developed to circumvent the issue of reporter ion distortion that commonly occurs with traditional isobaric tags.

Here, we presented a novel method to achieve multiplexed quantification for ETD-based analysis using 3-plex DiLeu tags enabling simultaneous quantification and PTM localization in a

high-throughput manner. ETD is an excellent tool to elucidate peptide sequence while providing accurate modification sites of PTM which might not be accessible using CID/HCD methods. However, currently, no existing isobaric mass tags can generate reporter ions in the low *m/z* range effectively under ETD fragmentation, where inconsistent reporter fragment ions and low reporter ion yields have been reported[24]. In contrast, we found that complementary ions with high signal intensity were generated in DiLeu-labeled peptides in the previous study[25]. Therefore, we aimed to explore the possibility of using complementary ions to achieve multiplexed quantification for ETD analysis. The quantitative performance including complementary ion yields, peptide identification rates, quantification accuracy, and dynamic range has been evaluated, showing promising results.

Result and discussion

The design of isobaric labeling for ETD-based analysis

The traditional isobaric labeling methods where reporter ions are generated in the low mass ranges is a well-established strategy for multiplexed quantification. Several search engines have incorporated this analysis scheme as one of the features allowing users to perform protein identification and multiplexed quantification at the same time. In our initial method development, we attempted to design a novel isobaric tag that could generate reporter ions in the low mass ranges under ETD fragmentation for easy and straightforward data processing. Unfortunately, although we have designed several isobaric mass tag structures based on ETD cleavage mechanism, none of the structures were capable of generating desired fragment ions (Data not shown). Upon ETD fragmentation, several reporter ion-related ions were generated with insufficient signal intensities,

and some of them failed to be cleaved between reporter and balancer regions, unable to provide any quantitative information. The generation of these candidate ions was also inconsistent across different peptide sequences. However, during the initial tests, we observed complementary ions, neutral loss peaks of the proposed reporter ions, were generated in high fragment ion yields, which was also found in our previous work using DiLeu isobaric tags. This observation indicated that the bond between reporter and balancer groups can be fragmented readily, while the resulting reporter ions might not preserve a positive charge efficiently. Therefore, as opposed to using reporter ions, we aimed to achieve multiplexed quantification using complementary ion-based strategy.

Acquisition mode and tag design

Compared to traditional isobaric labeling, the development of complementary ion-based strategy, despite lower ratio distortion, was limited due to the complicated data processing and the lack of software that can quantify peptides based on peptide-coupled ions. To quantify peptides based on complementary ions, two acquisition modes regarding isolation windows have been developed[26], a wide isolation window that can isolate the entire envelope and a narrow isolation window to isolate only one single peak from the isotopic clusters.

Natural isotopic peaks are present in all peptides usually contributed by Carbon-13. For tryptic peptides whose molecular weights are between 500 – 5000 Da, the abundance of M + 1 or M + 2 peaks might be as high as monoisotopic peak (M). In such cases, when using isobaric mass tags with 1 Da apart from one another, desired complementary ion peaks will be complicated by peptides' natural isotopic peaks which also have a 1 Da difference. Therefore, by using the wide isolation windows to isolate the entire isotopic envelope, extensive deconvolution is required to obtain quantitative results. On the other hand, narrow isolation windows avoid the inferences from

other isotopic peaks and generate clean complementary ion clusters representing the abundance of peptides across all samples.

Narrow isolation window methods have been reported to provide more accurate quantification. In order to develop a simple and accurate method, we aimed to utilize narrow isolation window methods for our study. However, unlike the previous studies where HCD fragmentation is used, ETD analysis has some inherent limitations[27]. First, ETD reaction rates and efficiency are dependent on the peptide charge states. Doubly charged peptides tend to have poor fragmentation regardless of long reaction times used. Second, ETD suffer from lower fragmentation efficiency in general compared to CID/HCD and can result in several nondissociated but charge reduced precursor products. Therefore, to avoid the long duty cycles of doubly charged peptides which result in low-quality MS2 spectra and low identification rates, only 3+ charge or above peptides were chosen for MS2 analysis in our study.

Fig. 1 describes the generation of complementary ion clusters from charged 2 and 3 peptides with different MS acquisition settings. Charged 3 and 4 peptides have mass increments of ~0.33 and 0.25 *m/z* between each isotopic peak respectively which are smaller than charged 2 peptides (0.5 *m/z* mass increment). According to the previous reports[26], 0.4 Th, the lowest isolation window the current instrument can reach, is sufficient to generate clean complementary ion clusters. Although the instrument vendor stated that the isolation windows is centered at the target peaks with a total width of the assigned Th, the present instruments are unable to attain a completely rectangular shape for an isolation window and a non-zero efficiency beyond its boundaries[28]. When analyzing charged 3 peptides, some studies applied an asymmetric isolation window around M peaks using offset settings to avoid the isolation of M + 1 peaks[29]. With isolation window offsets, we observed a clean complementary ion cluster from a labeled peptide using 4-

plex DiLeu tags with a 1 Da mass difference (**Fig. 2**). Unfortunately, we found that narrow isolation windows have a serious impact on MS2 quality due to the lower fragmentation efficiency of ETD. **Fig. 3A** compared MS2 scores derived using Sequest HT algorithm from different isolation window settings. The MS2 scores were normalized with the highest scores of each labeled peptide derived from all isolation windows. We found that the scores decreased and the deviations of the scores increased as the isolation windows become narrower, resulting in low identification rates for complex samples. This tendency can be explained by the mechanism that not all of the ions in a mass spectral peak and in neighboring isotopic peaks are isolated when using a narrow isolation window, where lower abundances of precursor ions were collected for fragmentation. Although a decreased transmission was not observed in the previous HCD methods[29], ETD analysis required certain abundance of precursor ions to compensate its inherent limitations.

In order to utilize the concept of narrow isolation windows while keeping decent MS2 quality for peptide identification, we designed a set of isobaric mass tags with 2 Da mass increments within the reporter and balancer regions (**Fig. 4**). We hypothesize that by employing this 2 Da mass difference, we can apply a wider isolation window (1 *m/z*) to accommodate M and M + 1 peaks for the generation of peptide fragment ions, and meanwhile generate clean complementary clusters for accurate quantification. Considering the fact that M and M + 1 peaks are usually the most abundant peaks in tryptic peptides, the isolation of M and M + 1 peaks was presumed to lead to a great improvement in MS2 spectral quality. In the analysis of cell lysate samples, the identification numbers increased from 1685 to 3050 using the isolation windows of 0.4 Th and 1 Th, which represented an 80% of increase (**Fig. 3B**). In terms of the complementary ion clusters, six complementary ion-related peaks appeared upon ETD fragmentation. Based on our 2 Da mass difference design (**Fig. 5**), the first, the third, and the fifth peaks would be chosen

and extracted for relative quantitative purposes. By combining a greater mass difference in the isobaric mass tags and a wider isolation window, we successfully achieved multiplexed quantification and decent identification rates for ETD analysis without requiring complicated data processing.

3-plex DiLeu complementary ion-based quantification

Method performance was carefully assessed from several aspects including quantitative accuracy, quantifiable peptides, and applicability to PTM analysis. Since DiLeu isobaric tags were developed in our group and have been well-optimized, the labeling efficiency was not evaluated in our study due to the same tag structures used with different isotopic configurations. The evaluations were conducted using bovine serum albumin (BSA) and alpha S1 casein protein digests and peptide aliquots extracted from HTB-26 cell line using 3-plex DiLeu complementary ion-based strategy (**Fig. 6**). Digested peptide aliquots were labeled with 3-plex DiLeu tags respectively and pooled with the ratios of 1:1:1 and 1:3:6 to examine different dynamic ranges prior to further enrichment and purification. In order to explore the applicability to modified peptides, we conducted experiments of phosphoproteomics using IMAC enrichment which is well-established enrichment methods for phosphopeptides with high specificity[30]. For MS acquisitions, we focus on the analysis of charged 3 and 4 peptides using ETD fragmentation technique ensuring a decent identification rate. Data was processed by Proteome Discoverer for peptide identification. The MS2 spectra from the identified peptides were further processed to extract the correspondent complementary ion clusters for relative quantification.

In two analyses, 85% of identified peptides were able to generate complementary ion clusters with sufficient ion yields for quantification, demonstrating excellent reproducibility for

generating quantitative fragment ions under ETD fragmentation. Compared to the previous analysis where only 38% of identified peptides were quantifiable[24], the complementary ion-based strategy greatly enhanced the quantification capability of ETD-based analysis. All quantified peptides were plotted against each other (**Fig. 7**). Averages of the median coefficient of variation (CV) of 16% and 19.95% for the theoretical ratios 1:1:1 and 1:3:6, demonstrating satisfactory quantitative accuracy. It is worth noting that despite a relatively higher CV compared to the traditional isobaric labeling strategy, complementary ion-based strategy eliminates the interferences from co-isolation avoiding the ratio distortion effect.

Additionally, the performance on phosphopeptides was examined (**Fig. 8**). In the comparison between ETD and HCD spectra on the same DiLeu-labeled phosphopeptide, the ETD fragmentation technique can generate desired complementary ion clusters and numerous peptide backbone ions with the phosphate groups attached, while HCD produced few phosphate-attached fragment ions. Several search engines used neutral losses peaks to predict the phosphorylation sites[31]. Yet, since several neutral loss combinations result in the same mass losses, leading to mis-identifications. For example, the elimination of H_3PO_4 can be the direct loss of H_3PO_4 or the loss of HPO_3 and a water molecule, which make analysis challenging to accurately determine the locations of the phosphate group for polyphosphopeptides or peptides with multiple potential modification sites. In the representative DiLeu-labeled phosphopeptides, three amino acids might be potential modification sites. In HCD spectrum, three peptide backbone ions (b_8, b_9, and b_{11}) with phosphate attached were not sufficient to indicate the modification sites on this peptide. In contrast, clear differences of whether phosphate attached between c_5 and c_6 ions as well as z_{10} and z_{11} ions in the ETD spectrum enabled confident assignment of the phosphorylation site to the sixth amino acid, the serine residue. Overall, our method provided a multiplexed quantification for ETD-

based analysis with promising performance of consistent generation of quantifiable complementary ions, reasonable quantitative accuracy, and applicability to PTM analysis.

Conclusion

ETD has been widely used as a fragmentation technique for protein analyses including top-down proteomics, labile PTM analysis, and non-tryptic peptide characterization. Recently, using ETD to avoid hydrogen/deuterium scrambling occurring under CID was also reported in hydrogen/deuterium exchange monitored by mass spectrometry. However, the isobaric labeling strategy for ETD-based analysis is limited due to the inefficient generation of reporter ions. Current isobaric labeling studies which attempted to exploit the advantages of the ETD technique mainly conduct additional MSn fragmentation or supplementary energy to acquire reporter ion information. In such cases, duty cycles and sensitivity of reporter ions and PTM localization might be compromised, thus limiting the overall performance of the isobaric labeling strategy in ETD-based analysis. Therefore, reliable ETD-based isobaric labeling is desirable in order to further expand the utility of ETD in various research areas. In our study, we presented a novel strategy that combined complementary ions and isobaric mass tags with a 2 Da mass increment to circumvent multiple challenges arising from the inherent limitation of the ETD technique. This method has been carefully evaluated and demonstrates promising performance including high labeling efficiency, consistency of quantitative fragment ions, quantitative accuracy, and compatibility with PTM analysis. We found that complementary ions can be generated by all dialkylated amino acid-based isobaric mass tags, and thus this set of 3-plex isobaric mass tags can be adapted to the design of larger tag structures that can accommodate more isotopic combinations

allowing higher multiplexing capacity. As proof-of-principle, we demonstrated the possibility of ETD-based multiplexed quantification and anticipated that this 3-plex DiLeu complementary ion-based strategy can contribute to various fields using ETD fragmentation.

Methods

Chemicals and reagents

All isotopic reagents for the synthesis of tags were purchased from Isotec (Miamisburg, OH). Optima LC/MS grade acetonitrile (ACN), formic acid (FA), and water were purchased from Fisher Scientific (Pittsburgh, PA). Mass spec grade trypsin and dithiothreitol (DTT) were purchased from Promega (Madison, WI). ACS grade *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM), formaldehyde (CH_2O), hydrogen chloride gas (HCl), l-Leucine, iodoacetamide (IAA), N-methylmorpholine (NMM), sodium cyanoborohydride (NaBH3CN), triethylamine (NEt3), triethylammonium bicarbonate (TEAB), trifluoroacetic acid (TFA), and tris hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Hydroxylamine solution was purchased from Alfa Aesar (Ward Hill, MA). All reagents were used without additional purification.

Cell Sample Preparation

HTB-26 cells were pelleted and lysed by sonication in 100 mM TEAB with 1% (v/v) protease inhibitor and phosphatase inhibitor. Proteins were precipitated by the precipitation buffer (50% acetone in ethanol) at -20°C overnight. The mixture was centrifuged at 18,000 *g* at 4 °C for

10 min, and the pellet was washed by the precipitation buffer. The precipitate was dissolved in 8M urea and 50 mM Tris-HCl (pH = 8) and reduced in a solution of 10 mM DTT for 1 h followed by alkylation of free thiols with 20 mM iodoacetamide and incubation in the dark for 30 min. Sample was diluted to 1 M urea with 50 mM Tris-HCl buffer. Proteins were proteolytically digested by addition of trypsin at a 1:50 enzyme-to-protein ratio and incubation at 37 °C for 16 h. The digestion was quenched with 10% TFA to a final concentration of 0.3%, followed by desalting with Sep-Pak C18 cartridges (Waters Corporation, Milford, MA).

3-plex DiLeu Synthesis

L-Leucine (300mg, 2.28mmol) and sodium cyanoborohydride (369mg, 5.87mmol) were suspended in MeOH (15mL) and the mixture was cooled in an ice-water bath. Formaldehyde (803 μL, 4.46mmol, 37% w/w) was added dropwise in an ice-water bath, and the mixture was stirred at room temperature for 4 hours. The target product was purified by flash column chromatography (MeOH/DCM) and dried in vacuo to obtain white solid *N*,*N*-dimethyl leucine (DiLeu). The 3-plex DiLeu reagents was synthesized following the same procedures using commercially available isotopic reagents (**Fig. 4**). For 48-channel DiLeu reagent, isotopic leucine was dissolved in the solution of 1 N HCl $H_2{}^{18}O$ solution (pH = 1) and stirred on a hot plate at 65 °C for 4 hours. Following evaporation of HCl from the solution in vacuo, trace amounts of acid were removed with StratoSpheres PL-HCO_3 MP resin (Agilent Technologies) to obtain ^{18}O L-leucine in a free base form.

DiLeu Activation and Labeling Procedure.

DMTMM and NMM at 0.7× molar ratios were added to a solution of DiLeu reagent in anhydrous DMF. The mixture was vortexed at room temperature for 45 minutes. Then, the mixture was centrifuged at 14,000 xg for 1 min, and the resulting supernatant was transferred immediately for peptide labeling. Peptides were resuspended in 0.5 M TEAB buffer and labeled by adding the activated DiLeu at a tags-to-peptides ratio of 20:1 (w/w). The reaction was vortexed for 2 hours and quenched by adding hydroxylamine to a final concentration of 0.25%. The excess amount of DiLeu tags were removed using SCX SpinTips (PolyLC, Columbia, MD). The labeled sample was cleaned via Sep-Pak C18 Vac cartridge for non-phosphopeptide or IMAC enrichment for phosphopeptide prior to LC-MS analysis.

IMAC Enrichment

Titanium (IV) magnetic immobilized metal affinity chromatography (IMAC) beads were home-made and adapted to the work of Zhou's group for phosphopeptide enrichment.[30] Briefly, the Ti^{4+}-IMAC beads were equilibrated by washing three times with loading buffer (80% acetonitrile, 6% TFA). The beads were incubated with the peptides in loading buffer with a beads-to-protein ratio of 20:1 (w/w). The mixture was vortexed for 30 min at room temperature. Then, the beads were washed once with washing buffer 1 (50% ACN, 6% TFA, 200mM NaCl), and twice with washing buffer 2 (30% ACN, 0.1% TFA) to remove non-specific adsorbed peptides. Finally, phosphopeptides were eluted with 150 μL elution buffer (10% NH3•H2O) and lyophilized for LC-MS analysis.

LC-MS/MS analysis.

Sample was analyzed using a Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, San Jose, CA) coupled with a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific, San Jose, CA). Samples were reconstituted in 0.1% FA and injected onto a self-fabricated microcapillary column packed with C18 beads (Waters Bridged Ethylene Hybrid, 1.7 μm, 130 Å, 101.3 μm × 15 cm) in 100% mobile phase A (0.1% FA in water) at a flow rate of 0.3 μL/min. Samples were separated using a linear gradient elution of 0-35% mobile phase B (0.1% FA in ACN) over 90 min. For precursor MS scans, 320–1600 *m/z* were collected at a resolving power of 60k with automatic gain control (AGC) target of 4×10^5 and a maximum injection time of 100 ms. Precursors were subjected to dynamic exclusion for 15 s. Tandem MS spectra of ETD fragmentation were acquired at a resolving power of 60k, AGC target of 1×10^5, and maximum injection time of 118 ms. MS/MS spectra were collected via a top 10 data-dependent acquisition method with a charge state between 3-4. Precursor ions were isolated with an asymmetric 0.9-Th window (offset –0.04 Th) for charge state 3 and with an asymmetric 0.7-Th window (offset –0.06 Th) for charge state 4. ETD experiments were carried out with optimized reaction time according to their charge state of precursor ions.

Data Analysis.

Mass spectra were processed using Proteome Discoverer (version 1.4.0.288, Thermo Scientific) to identify peptides. Raw files were searched in Proteome Discover against UniProt human protein database using Sequest HT. Searches were performed with a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.02 Da. Static modifications were specified as carbamidomethylation (+57.02146 Da) on cysteine residues and 3-plex DiLeu (+145.12801 Da)

on peptide N-terminus and lysine residues. Dynamic modifications consisted of the oxidation of acetylation (+42.01057 Da) of protein N-terminus, deamidation (+0.984016 Da) of asparagine and glutamine residues, and oxidation of methionine (+15.99492 Da). Peptide spectral matches (PSMs) were validated on the basis of q-values to 1% FDR using percolator. The peaks of complementary ions were assigned and extracted the signals through an in-house R script. Quantification of complementary ions in MS2 spectra was performed in a R script using an integration tolerance of 10 ppm. Only the PSMs that contained 3 complementary ions were considered, and protein quantitative ratios were determined using a minimum of one quantified peptide. Complementary ion ratio values for protein groups were exported to Excel workbook format.

Acknowledgements:

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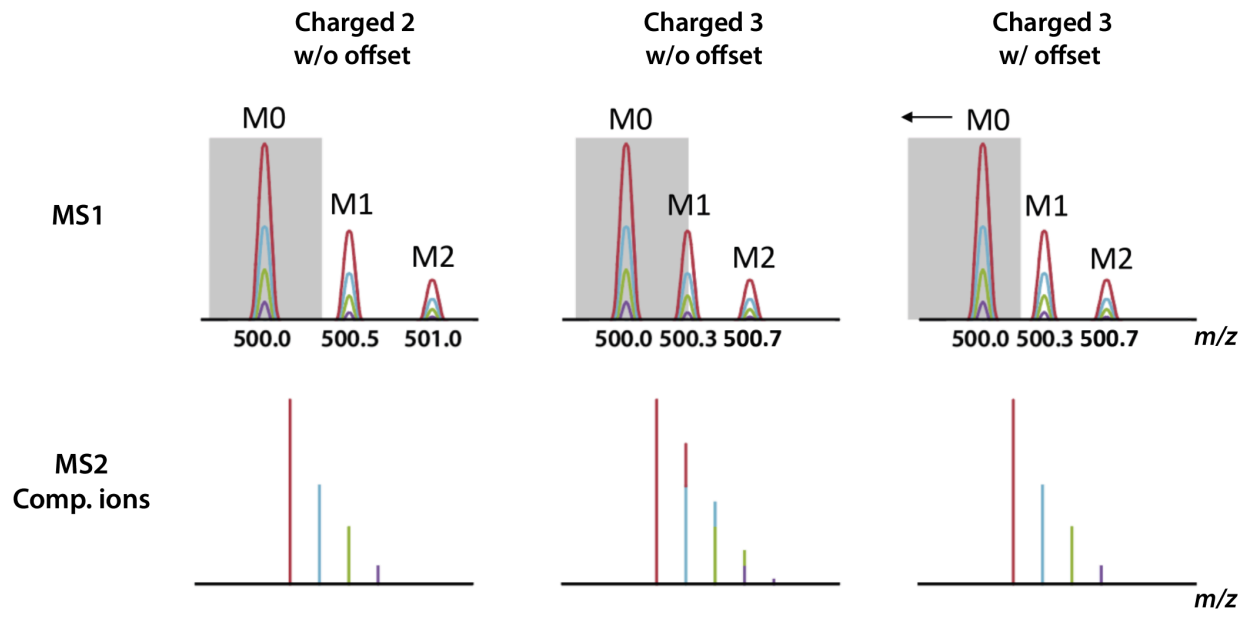


Fig. 1. The generation of complementary ion clusters on charged 2 and 3 peptides using different MS acquisition settings. Isolation windows affect the numbers of isotopic peaks that will be co-isolated. With the same isolation window, charged 2 peptides will generate clean complementary ion clusters, while charged 3 peptides will have complementary ion clusters that are interfered by other natural isotopic peaks. An asymmetric isolation window that suppresses the signal from adjacent isotope peaks and enabled interference-free complementary clusters.

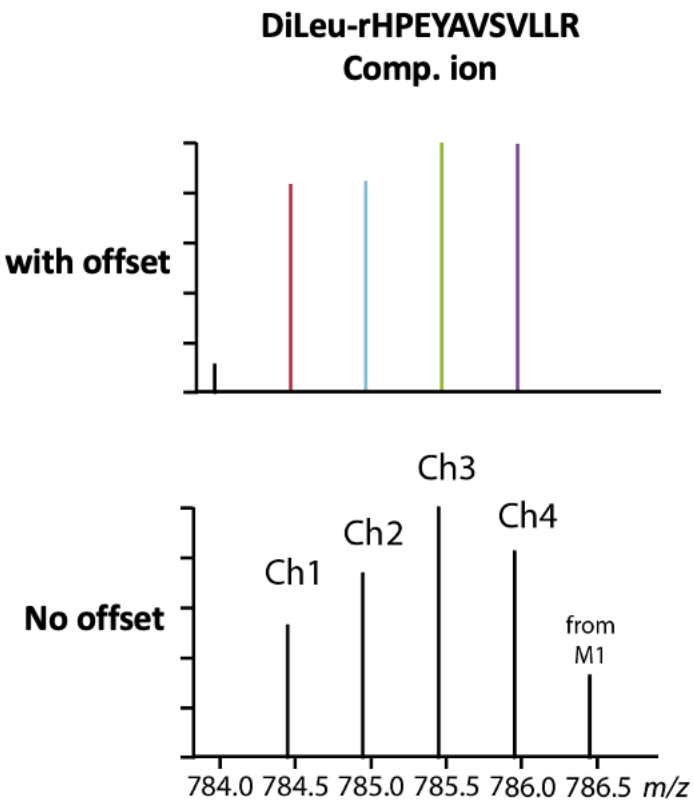


Fig. 2. Complementary ion clusters generated with or without isolation window offsets. The complementary ions from the same peptide, 4-plex DiLeu-labeled RHPEYAVSVLLR, with or without isolation window offset settings. With offsets, the expected ratio can be observed in the complementary ion cluster, while a skewed ratio was obtained if no offset used.

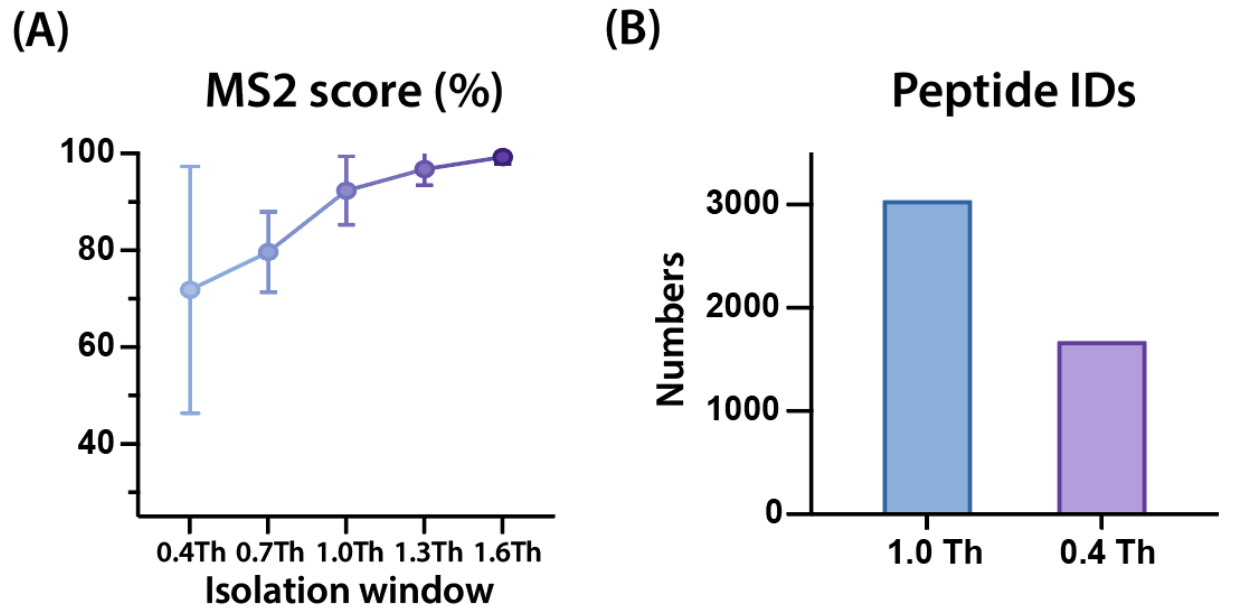
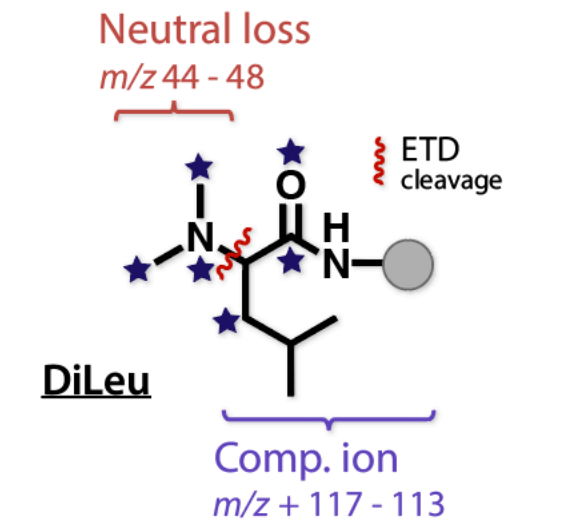


Fig. 3. The negative impacts from narrow isolation windows for ETD-based analysis. (A) The MS2 scores of the cross correlation with the theoretical spectra were compared for different isolation window settings. For each DiLeu-labeled peptide, the highest score across all isolation windows was set as a value of 100%, and other scores were calculated against the highest score. (B) The number of peptides that was identified from HTB-26 cell line digests using 1.0 Th and 0.4 Th isolation windows.



DiLeu Label	Isobaric region		Comp. ion	Tag Mass
	Reporter	Balance		
44		● ● ●	+117	146.15
46	● ●	●	+115	
48	● ● ● ●		+113	

● ^{2}H ● ^{13}C ● ^{18}O

Fig. 4. The 3-plex DiLeu general structure and isotopic configurations. The reporter ion part will not be detected under ETD fragmentation. The complementary ions which are precursor ions with neutral losses will be generated and detected.

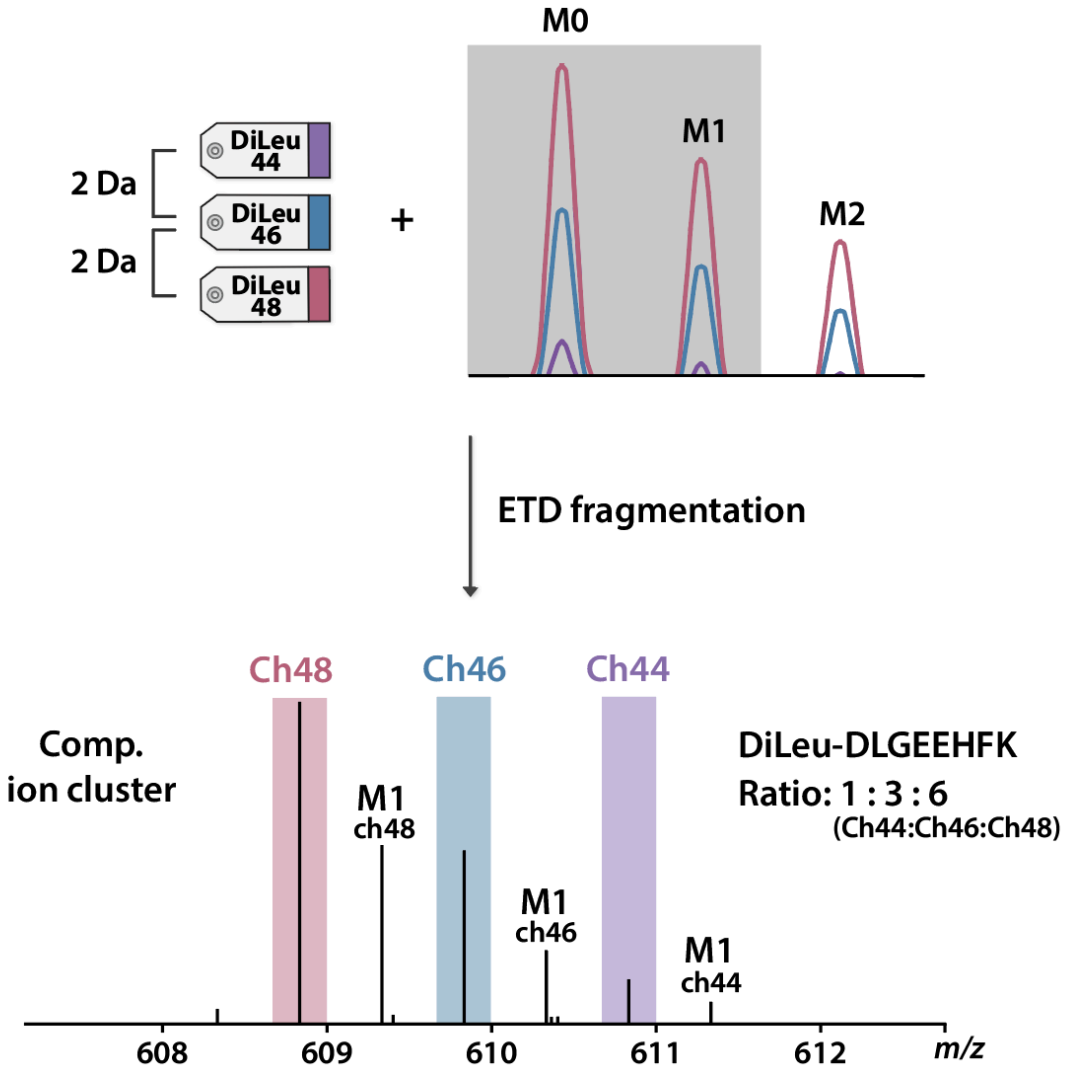


Fig. 5. The new strategy and the resulting complementary ions. Isobaric tags with a 2 Da mass difference and 0.9 Th isolation window were used for the ETD-based analysis. The first, the third, and the fifth peaks were extracted for quantification to avoid interference.

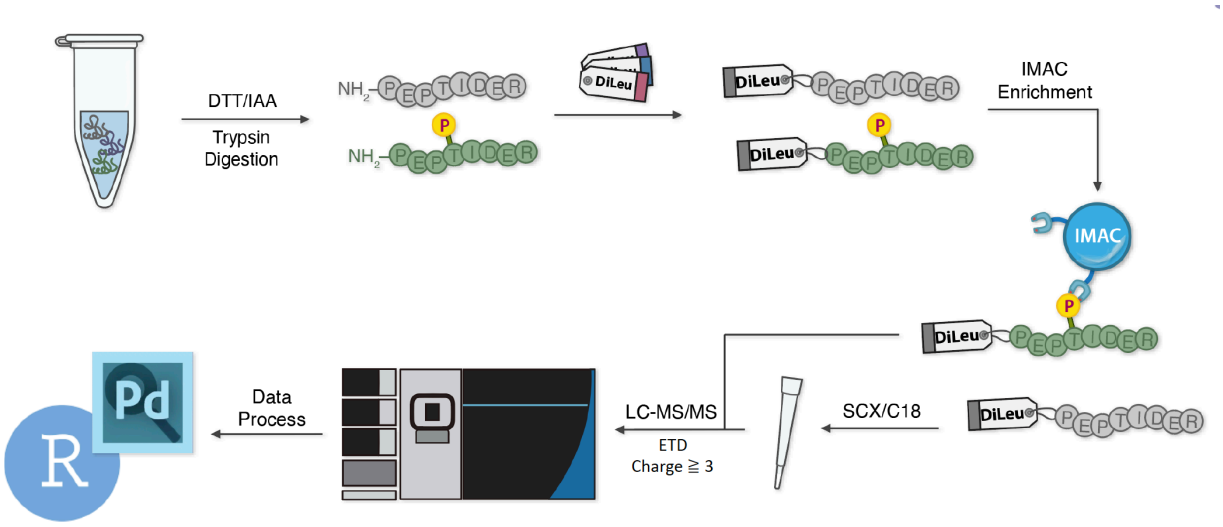


Fig. 6. The workflow of 3-plex DiLeu complementary ion-based quantification for the ETD-based analysis. Protein samples were digested by trypsin followed by 3-plex DiLeu labeling respectively. Samples were pooled prior to cleanup or enrichment step. IMAC enrichment was conducted for enriching phosphopeptides. LC-MS-ETD analysis was performed after sample cleanup and desalting.

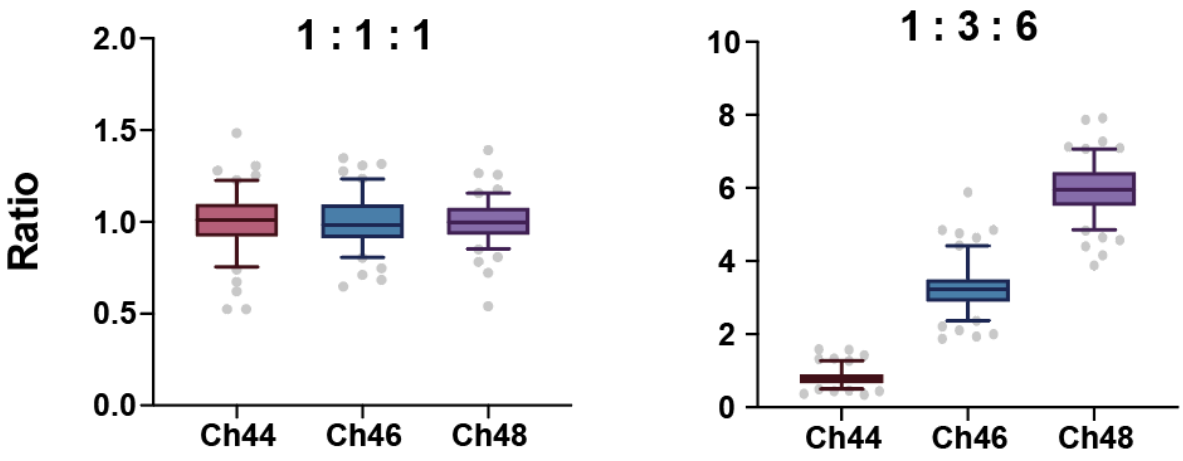


Fig. 7. Quantitative performance of 3-plex DiLeu labeling using complementary ion-based strategy. Two ratios, 1:1:1 and 1:3:6 were examined to represent different dynamic ranges.

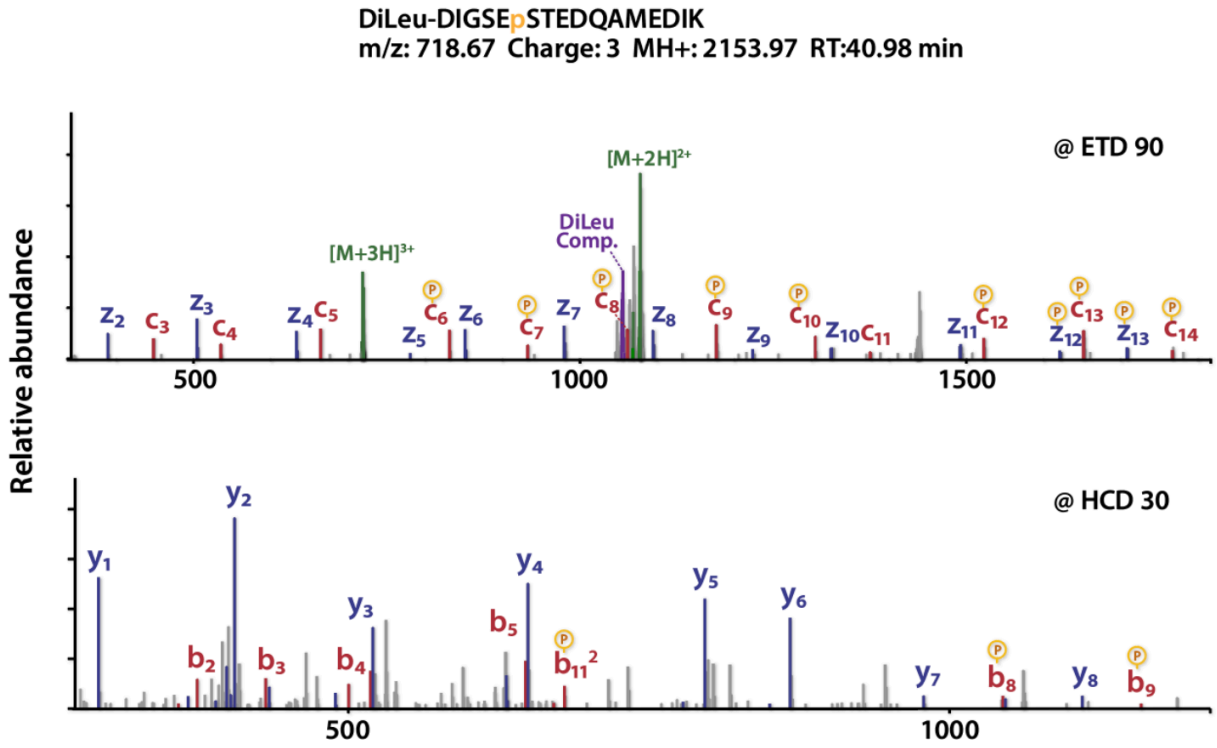


Fig. 8. ETD and HCD MS2 fragmentation spectra of DiLeu-labeled phosphopeptides. In ETD spectrum, c and z ions were generated with phosphate group attached to all peptide fragment ions which contained phosphorylation sites. In contrast, HCD spectrum shows only a few peptide backbone ions with phosphate group attached.

Chapter 6

DiLeuPMP: a multiplexed isobaric labeling method for quantitative analysis of O-glycans

Adapted from: Li, M.[#], **Gu, T. J.[#],** Lin, X., & Li, L. (2021). DiLeuPMP: a multiplexed isobaric labeling method for quantitative analysis of O-glycans. *Analytical chemistry*, *93*(28), 9845-9852. (# co-first author).

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Contribution: The study was designed by Li, M., **Gu, T. J.,** and L. Li.; experiment and data analysis were performed by **Gu, T. J.** and Li, M.; manuscript was written by Li, M. and edited by **Gu, T. J.** and L. Li.

Abstract

As one of the most important post-translational modifications, glycosylation plays a pivotal role in many essential physiological functions, including cell recognition, signaling, and immune response. Thus, various qualitative and quantitative analytical strategies for glycomic profiling have been developed in recent decades. However, while extensive efforts have been devoted to the analysis of N-glycans, high throughput quantitative analysis of O-glycans is often overlooked and underexplored. This is partially due to the lack of universal enzyme for the release of O-glycans from protein backbone. Furthermore, the traditional chemical releasing method suffers from severe side reactions and involves tedious sample preparation procedures. Here, a multiplexed isobaric labeling method enabled by *N*, *N*-dimethyl leucine containing pyrazolone analog (DiLeuPMP) is introduced. This method combines the release and labeling of O-glycans in a one-pot reaction and achieves accurate MS^2-based relative quantification with the ability to process four samples at a time. The method has been applied to core-1 O-glycan standard and three glycoproteins first and the results demonstrated its validity. Following this proof-of-principle demonstration, we analyzed more complex biological specimen using human serum samples. Overall, this method provides an effective and reliable approach for the profiling and high-throughput quantitative analysis of O-glycans in complex samples.

Introduction

Protein glycosylation is a pivotal post-translational modification (PTM) and plays essential roles in many biological processes such as immune response, receptor recognition, cellular communication, and embryonic development[1–3]. The important biological functions and ubiquitous existence of glycosylated proteins in nature have made pressing needs for developing simple and sensitive platforms capable of analyzing glycans. Typically, glycosylation is classified into two types, N-linked and O-linked glycosylation, based on the attachment between oligosaccharides and amino acid residues. N-glycans conjugate to asparagine (N) residues in the consensus peptide motif of Asn-X-Ser/Thr (where X is any amino acid except proline) with a core structure (GlcNAc2Man3), while O-glycans are conjugated to serine (S) or threonine (T) without a clear consensus motif and consistent core structure.

For a typical glycomics analysis, cleavage of glycans from the peptide backbone is commonly the first step. For N-glycans, they can be readily released by enzymes such as PNGase F and PNGase A[4]. In contrast, the release and recovery of O-glycans remain to be a very challenging problem. Due to lack of a common core structure, O-glycans are typically categorized into eight subclasses (core-1 to core-8)[5]. Although endo-α-N-acetylgalactosaminidase (O-glycanase) is reported to be able to cleave the core-1 type O-glycans (Gal-GalNAc), there is no universal O-glycosidase that enables removal of all O-glycans. Thus, the chemical method so-called β-elimination, is often employed for releasing O-glycans in practice. β-elimination is performed under alkali conditions, and the strong base removes the β-proton on amino acid residue where an elimination reaction is triggered making for an unsaturated amino acid (from serine or threonine residues) together with the released O-glycans. However, this process can proceed

stepwise and remove one saccharide residue at a time from the polysaccharide backbone, which results in the degradation of released glycans (**Fig. S1**). This side reaction is known as 'peeling'[6–8]. Previously, scientists found that adding blocking reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) during β-elimination can suppress side reactions since PMP derivatization of the released O-glycans proceeds faster than peeling degradation and thus is able to competitively inhibit peeling process[9–11]. Mass difference-based isotopic labeling strategy using light and heavy isotopic PMP reagent was also reported to achieve quantitative glycomics analysis[11, 12]. However, the multiplexing capacity was limited to dual-plex due to the increase in spectral complexity and limited commercially available isotopic PMP reagents.

Mass spectrometry (MS) has emerged as a prominent analytical tool for the O-glycomics analysis[13, 14]. Currently, developed methods have largely improved the recovery and facilitated the profiling of O-glycans, but most of them suffer from low throughput and limited capacity for quantitative analyses. Furthermore, the procedures are usually time-consuming and tedious. Isobaric labeling is a popular approach in MS to achieve high throughput analysis based on MS^2 fragmentation and reporter ion quantification. Our laboratory has previously developed a set of custom isobaric labeling reagents, *N*, *N*-dimethyl leucine (DiLeu), for large-scale quantitative proteomics and peptidomics[15]. Very recently, the general architecture of DiLeu was borrowed and extended for the development of a novel set of isobaric multiplex reagent for carbonyl-containing compound (SUGAR) tags capable of quantitative N-glycomics[4]. The SUGAR tags employed reductive amination chemistry on the reducing end of released N-glycans, thus are not applicable for direct analysis of O-glycans. Herein, we combined the isobaric labeling strategy employed by DiLeu with PMP-aided β-elimination to develop a novel chemical tool, 4-plex *N*, *N*-dimethyl

leucine containing pyrazolone analog (DiLeuPMP), which provides the first isobaric tag aiming at high throughput quantitative O-glycan analysis and facilitates the application of O-glycomics.

Results and discussion

Numerous challenges exist for O-glycan analysis. In contrast to N-glycosylation, O-glycosylation lacks a known amino acid consensus sequence and Mucin-type O-glycosylation is usually highly heterogeneous. However, among the various challenges, the major difficulty lies in the lack of universal enzymatic tools for release of O-glycans from proteins. Therefore, the chemical method is currently the most effective and reliable way of acquiring released O-glycans, where β-elimination is the most common one. However, β-elimination demands alkaline conditions and usually results in degradation which was referred as "peeling". Previously, researchers found that adding PMP can efficiently block the reducing end of released glycans and prevent them from sequential peeling degradation. The derivatization reaction itself is a Michael addition involving a two-step labeling process in which Michael donor molecules of the labeling reagent are formed and consecutively added to the reducing end of the glycan with a stoichiometry of two label molecules per glycan[19]. The use of PMP and related tags was restricted in fluorescence detection initially[20] and was recently employed in the field of mass spectrometry[10, 21, 22].

O-glycans are known to be associated with many critical biological functions. Abnormal O-glycosylation has been implicated in a variety of diseases, including familial tumoral calcinosis[23], Tn syndrome[24], and IgA nephropathy[25]. Thus, global profiling and quantitative analysis of O-glycans is crucial to understand the structures and functions of O-glycosylation in the development of these diseases. The current analytical tool suffers from low throughput and

tedious sample preparation procedures. Mass spectrometry (MS)-based, isobaric labeling strategy for relative quantification allows for parallel multiplexing of experiments, which provides an opportunity to address these limitations for O-glycan analysis. Generally, multiple samples are chemically labeled with isobaric chemical tag variants and each variant has the same chemical structure and nominal mass which is indistinguishable in full MS spectrum. However, each variant is fragmented to produce a unique "reporter ion" during tandem MS and the signal intensity can be used for relative quantification. Commercially available reagents such as isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tags (TMT) are widely used in quantitative proteomics[26]. In our lab, we previously developed DiLeu isobaric tags for cost-effective proteomics[27] and isobaric multiplex reagents for carbonyl-containing compound (SUGAR) tags for N-glycan analysis[4, 28].

By combining the PMP labeling and DiLeu isobaric labeling strategies together, we designed and synthesized a set of 4-plex DiLeuPMP tags to enable high-throughput quantitative O-glycomics (**Fig. 1**). The reactive site of Michael donor PMP is on the five-member ring. Hence, we incorporated DiLeu reporter ion onto the benzene moiety. DiLeuPMP was created in a two-step synthesis with an overall yield of 70%. The 4-plex isobaric version of DiLeuPMP tags were synthesized using heavy isotope-coded starting materials accordingly (**Fig. S2**). The isotope purities of 4-plex DiLeuPMP were over 98% (**Fig. S3**). As two label molecules were added per glycan, the chromatographic properties of the derivatized oligosaccharides were largely affected by the tags, thus we avoided incorporating deuterium atom into 4-plex DiLeuPMP structures, using only ^{13}C, ^{15}N, and ^{18}O, to prevent possible retention time shift.

Releasing and labeling of O-glycans was performed in a one-pot manner. The general workflow is illustrated in **Fig. 2.** After *in situ* releasing and labeling of O-glycans during β-

elimination, the pH of the system was adjusted to neutral, and chloroform was used for liquid-liquid extraction. The organic layer was discarded to get rid of excess tags and the remaining aqueous phase was desalted using Sep-Pak C18 cartridges. The elute was dried and reconstituted for MALDI-MS or LC-MS/MS analysis. Compared to developed methods[29, 30], we further lowered the alkalinity of the system, choosing 5% NH_4OH instead of 100 mM NaOH or 50% hydrazine and found that in 5% NH_4OH, the releasing of O-glycans was still efficient and the peeling degradation can be effectively suppressed.

Commercially available core-1 O-glycan standard was used for method validation. As shown in **Fig. 3,** after labeling, the signal corresponding to bis-DiLeuPMP labeled glycan (*m/z* 1026.5495) was abundant, while mono-DiLeuPMP labeled (*m/z* 696.3434) and peeling product (*m/z* 864.4965) were also observed. However, they were less than 5% of overall signal intensity, which indicated satisfactory labeling efficiency of DiLeuPMP and its efficient peeling suppression. It is worth mentioning that because two tags are added per glycan and it is the tag that commonly carries charges, on ESI-MS spectrum, the dominant peak corresponding to glycan conjugates carries +2 charges (*m/z* 513.7766 $[M+2H]^{2+}$, *m/z* 524.7674 $[M+H+Na]^{2+}$) while +1 charged signals are also noticeable (*m/z* 1026.5466 $[M+H]^{+}$, *m/z*1048.5283 $[M+Na]^{+}$). The elevated charge states facilitate the fragmentation process and produce abundant fragment ions for structural elucidation.

Various model glycoproteins were used to further evaluate the feasibility of the described method for O-glycan analysis on glycoproteins. Bovine fetuin, bovine submaxillary mucin (BSM) and porcine stomach mucin (PSM) were chosen as model glycoproteins as the O-glycans on these glycoproteins are well studied[4, 28]. Bovine fetuin, which contains both N- and O-linked glycans, has a relatively simple O-glycosylation pattern. BSM belongs to the class of salivary glycoproteins,

and it consists of a long protein chain with numerous disaccharide and oligosaccharide side chains, some of which are known to be sialylated. PSM is primarily composed of carbohydrate units with protein contributing to only 20% of the molecular weight. Following the one- pot procedure described above, the O-glycans were released from these glycoproteins and labeled by DiLeuPMP. We set a control group using PMP and compared our results with previously established methods. As shown in **Fig. 4,** for MALDI-MS profiling, 3 O-glycans were found on bovine fetuin, 14 O-glycans were identified on BSM and 26 O-glycans were identified on PSM using DiLeuPMP labeling. Our results showed great overlap between PMP control as expected and the identified O-glycans were similar to those previously reported[31, 32] (**Fig. S6, Table S1**). After coupling with LC separation, a higher number of identifications and coverage were achieved in LC-MS/MS results (**Table S2**). The distinct profiling, high coverage, and good reproducibility of identified glycans between PMP and DiLeuPMP demonstrated the consistency and robustness of our new method compared with existing methods.

The quantification performance of the 4-plex DiLeuPMP tags was evaluated next by labeling O-glycans on PSM at known ratios. Denatured PSM was aliquoted into four equal portions in triplicate and labeled with 4-plex DiLeuPMP tags respectively, and then they were mixed with known ratios at 1:1:5:10 before performing liquid extraction and subsequent desalting. The combined desalted samples were then vacuum dried and reconstituted before performing LC-MS/MS analysis. The intensities of reporter ions in MS/MS spectra for each conjugate were used to calculate the experimental ratios. In **Fig. 5A**, experimental ratios of 4-plex DiLeuPMP-labeled O-glycans are plotted against theoretical ratios 1:1:5:10. Representative MS^2 spectra of low mass range containing reporter ions are shown in **Fig. 5B**. For all three known ratios, less than 15% relative errors were observed with standard deviations of 0.15, 0.19, and 0.23, demonstrating that

the DiLeuPMP quantification approach offers an accurate tool for quantitative analysis of O-glycans in a high-throughput manner. Moreover, no retention time shift was observed for 4-plex DiLeuPMP tag labeled O-glycans on the C18 column, as shown in **Fig. 5C**.

Lastly, we applied our DiLeuPMP labeling strategy to the O-glycan analysis of healthy human serum for exploration in real biological samples. Since numerous clinically relevant analyses utilize human blood serum or plasma for both routine clinical measurements and potential disease biomarker discovery, the ability to analyze real biological specimen will surely expand the scope of usage of DiLeuPMP tags. The healthy human serum was collected at a FDA-approved collection center. The sample preparation was followed by a previously reported method with moderate modifications[33]. Briefly, the proteins were extracted and denatured firstly, and N-glycans were removed prior to O-glycoprotein/peptides enrichment. The enriched samples were then aliquoted into four equal portions before being labeled by 4-plex DiLeuPMP. After the derivatization procedure, four samples were equally mixed followed by cleanup procedure prior to LC-MS analysis. The data analysis revealed that 12 O-glycans could be reliably recorded in a profile with a quantitative ratio error of less than 15% (**Table S3**). We anticipate that these are among the most abundant O-glycans in human serum, and it is consistent with previous studies[33, 34]. Furthermore, it is noted that the fragment ions from the precursors are abundant, which aids manual confirmation and structural elucidation of O-glycans (**Fig. 5D and Fig. S7**). In summary, our results shed light on the potential application of the DiLeuPMP labeling strategy for high-throughput quantitative O-glycomic analysis of clinical samples.

Conclusion

In summary, 4-plex DiLeuPMP isobaric tags were developed in this study for quantitative O-glycomics. This is the first isobaric tag designed and developed for high-throughput quantitative analysis of O-glycans. By combining traditional PMP-aided β-elimination method and DiLeu enabled isobaric labeling strategy together, DiLeuPMP achieved releasing and labeling of O-glycans simultaneously as well as enabling high throughput MS^2-based quantitative analysis. The labeling efficiency of DiLeuPMP is high and the peeling degradation is suppressed in a decreased alkali condition. The labeling pattern of two tags per glycan elevated the charges of labeled glycan conjugates and thus facilitated the fragmentation during MS/MS and produced abundant fragment ions for structural elucidation and reporter ions for quantification. We also demonstrated accurate relative quantification across an order of magnitude dynamic range using standard glycoproteins with these novel isobaric tags and applied the method to the analysis of human serum sample, suggesting the potential applications for large-scale analyses of biological and clinical specimens. Overall, the successful development of DiLeuPMP tags offers a powerful chemical tool for glycomics study in many biological and clinical applications and makes parallel profiling and quantitative analysis of O-glycans readily implemented. In conclusion, we anticipate that the novel DiLeuPMP labeling approach can be widely applied in a variety of biomedical research areas.

Methods

Materials and reagents

Acetic acid (AA), acetonitrile (ACN), *N*, *N*-dimethylformamide (DMF), tetrahydrofuran (THF), carbonyldiimidazole (CDI), tris-(2-carboxyethyl)phosphine (TCEP), formic acid (FA), methanol (MeOH), chloroform ($CHCl_3$), 20% ammonium hydroxide (NH_4OH) and water (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). 2-(4-aminophenyl)-5-methyl-2,4-dihydro-3H-pyrazole-3-one was purchased from Matrix Scientific (Columbia, SC). Core-1 O-glycan standard, bovine fetuin, porcine stomach mucin (PSM) and bovine submaxillary mucin (BSM) were purchased from Sigma-Aldrich (St. Louis, MO). Single donor healthy human serum was purchased from Innovative Research Inc. (Novi, MI). PNGase F was purchased from Promega (Madison, WI). Microcon-10kDa centrifugal filters (10K MWCO) were purchased from Merck Millipore Ltd. (Darmstadt, Germany). Sep-Pak C18 Cartridges were purchased from Waters Corporation (Milford, MA). Pierce™- C18 Tips, 10 ul bed were purchased from Thermo Fisher Scientific (Waltham, MA). Ethylene Bridged Hybrid C18 packing material (1.7 μm) was purchased from PolyLC Inc. (Columbia, MD). Fused silica capillary tubing (inner diameter 75 μm, outer diameter 375 μm) was purchased from Polymicro Technologies (Phoenix, AZ). All reagents were used without additional purification.

DiLeuPMP labeling on standard glycoproteins and human serum

The release of O-glycans from glycoproteins was performed according to the published procedure with moderate modifications[16]. Briefly, 100 μg of standard glycoproteins were dissolved in 10% NH_4OH/DMF (50/50 v/v), and DiLeuPMP was added to make the final

concentration at 0.5 M. After incubating at 70°C for 24h, the pH of the solution was adjusted to neutral by acetic acids followed by liquid extraction using chloroform for three times. Organic phase was discarded and remaining aqueous phase was desalted using Sep-Pak C18 Cartridge. The eluted fractions were dried in vacuo and reconstituted in 20 μL of 5% ACN, analyzed by MALDI-MS or LC-MS/MS. For 4-plex DiLeuPMP labeled samples, they were combined firstly before extraction.

In terms of human serum sample preparation, we followed FASP protocol with minor modifications.[17] In brief, 100 μL healthy human serum was mixed with 300 μL 0.1% SDS and 100 mM TCEP in 10 mM sodium phosphate (pH 7.5) at 60 °C for 1 h. After the sample was cooled down, 10 kDa MWCO was used for buffer exchange. 400 μL of 10 mM sodium phosphate was used to rinse the filter three times, following additional three times of 0.5 M TEAB buffer exchange. 8U of PNGase F in 100 μL TEAB buffer was then added onto the filter and the sample was incubated at 37°C for 18 h to release and remove N-glycans. 10 kDa MWCO filters were used to separate proteins and glycans. After the proteins were recollected, ZIC-HILIC were applied for O-glycoprotein/peptide enrichment[18]. HILIC beads were first activated with 200 μL of elution buffer (0.1% TFA, 99.9% H_2O) for 30 min and then washed with binding buffer (0.1% TFA, 19.9% H_2O, 80% ACN) twice. Proteins were dissolved in 300 μL of binding buffer and mixed with 7 mg activated ZIC-HILIC resin at a 1:50 peptide-to-material mass ratio in a microcentrifuge tube. The tube was shaken over a vortex mixer for 1 h and the supernatant was removed by centrifugation. The beads were washed with 70 μL binding buffer three times and glycoprotein/peptides were eluted with 70 μL elution buffer. Same labeling procedure with DiLeuPMP was conducted subsequently as mentioned above.

LC-MS/MS analysis

A self-fabricated nano-C18 column (15 cm, 75 μm i.d., 1.7 μm Ethylene Bridged Hybrid C18 packing material) was used for glycan separation. A Dionex Ultimate 3000 nanoLC system was coupled to a Q Exactive HF Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Scientific, Bremen, Germany) for all LC-MS/MS analyses. Mobile phase A was water with 5% DMSO, and mobile phase B was ACN with 5% DMSO. The flow rate was set at 0.3 μL/min, and the injection volume was 2 μL. The following gradient was used (time, % mobile phase B) unless otherwise specified: (0 min, 5%), (28 min, 5%), (38 min, 9%), (128 min, 37%), (133 min, 95%), (143 min, 95%), (148 min, 5%), (158 min, 5%).

The following mass spectrometer parameters were used for all data acquisition. Samples were ionized in positive ion mode with a spray voltage of 3 kV. S-lens radio frequency (RF) level was set to be 30, and capillary temperature was set to be 300 °C. Full MS scans were acquired at *m/z* 350–2000 with resolving power of 120 K. Maximum injection time of 100 ms, automatic gain control (AGC) target value of 5×10^5, and 1 microscan were used for full MS scans. Top 15 data-dependent MS^2analysis was performed at a resolving power of 15 K with higher-energy collisional dissociation (HCD) operating with normalized collision energy (NCE) of 25. The first fixed mass sets to 100 *m/z* in order to obtain reporter ions. The isolation window was set as 2.5 *m/z*and the dynamic exclusion of acquired precursors was set to 15 sec with a ± 20 ppm tolerance.

O-glycan data analysis

The raw data was compared against an in-house database including the most possible combinations of O-glycan units (Hexose (H), HexNAc (N), Fucose (F), and NeuAc (S)).

DiLeuPMP-labeled O-glycans were identified by accurate mass matching in full MS with a mass tolerance of 10 ppm and fragmentation matching in MS/MS spectra assisted by GlycoWorkbench. Peak areas of reporter ions for DiLeuPMP-labeled glycans were used for relative quantification. Microsoft Excel and Origin were used for calculations and statistical analyses.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website: https://pubs.acs.org/doi/10.1021/acs.analchem.1c01433.

- β-elimination causing "peeling" degradation (**Fig. S1**); synthetic route and starting materials for 4-plex DiLeuPMP tags (**Fig. S2**); ^{1}H NMR of DiLeuPMP tag (**Fig. S3**); isotope purities of 4-plex DiLeuPMP (**Fig. S4**); HPLC chromatogram of DiLeuPMP (**Fig. S5**); MALDI-MS profiling of PMP-labeled *O*-glycans released from standard glycoproteins (**Fig. S6**); representative MS/MS of *O*-glycans quantified in PSM (**Fig. 7**); observed *O*-glycans in MALDI-MS from glycoproteins using the two methods, DiLeuPMP and PMP (**Table S1**) (PDF)
- Full list of quantified *O*-glycans labeled with DiLeuPMP from PSM (**Table S2**); Full list of identified *O*-glycans labeled with DiLeuPMP from healthy human serum (**Table S3**) (XLSX)

Acknowledgement

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Figures

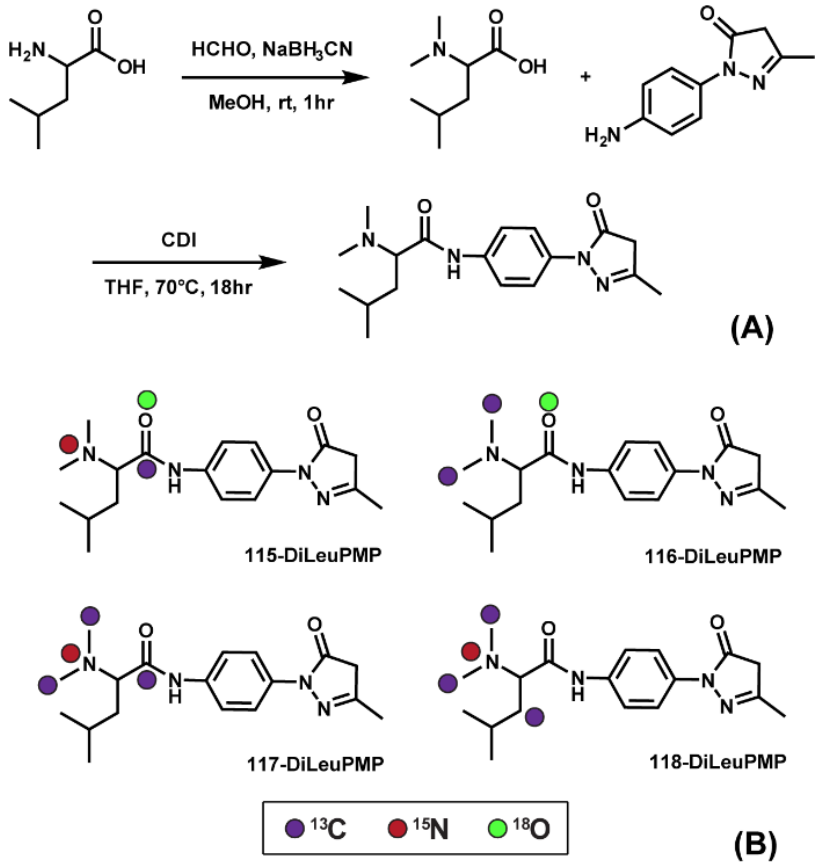

Fig. 1. **4-plex DiLeuPMP tag structure and reaction scheme**. (A) Structure and synthetic routes of DiLeuPMP tag; (B) Isotopic configurations of 4-plex DiLeuPMP tags.

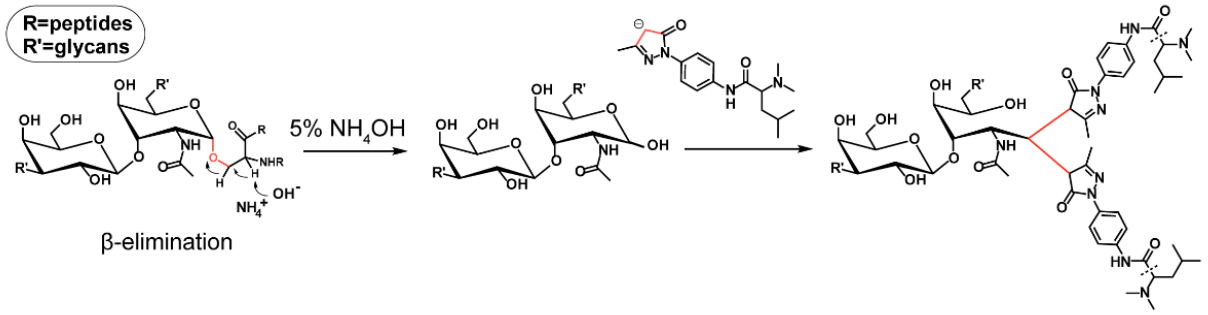

Fig. 2. Mechanism of releasing and labeling O-glycan by DiLeuPMP. Two units of DiLeuPMP will be added to one O-glycan. The reaction site is highlighted in red.

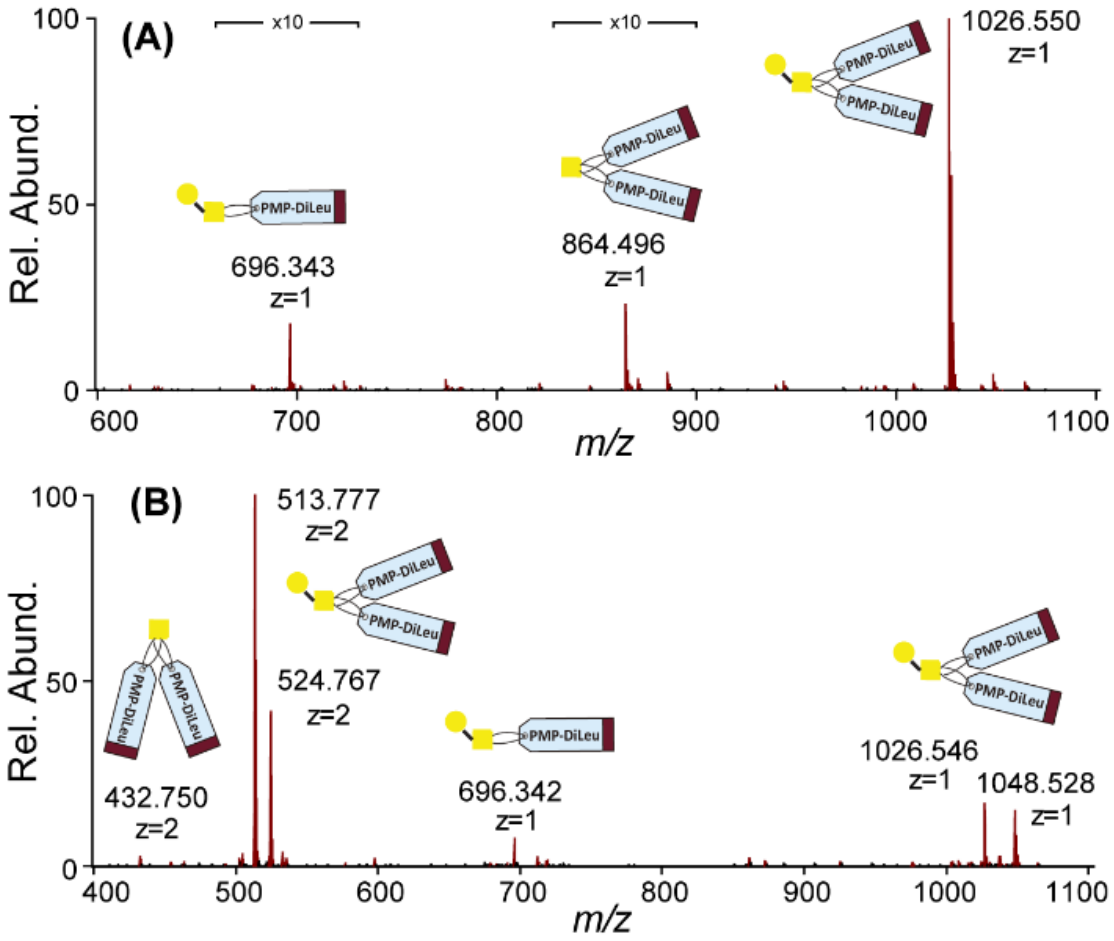

Fig. 3. MS analysis of DiLeuPMP labeled core-1 O-glycan standard. (A). In MALDI spectrum, charged 1 species were detected. (B) ESI-MS spectrum, the analytes tended to exhibit in charged 2 forms.

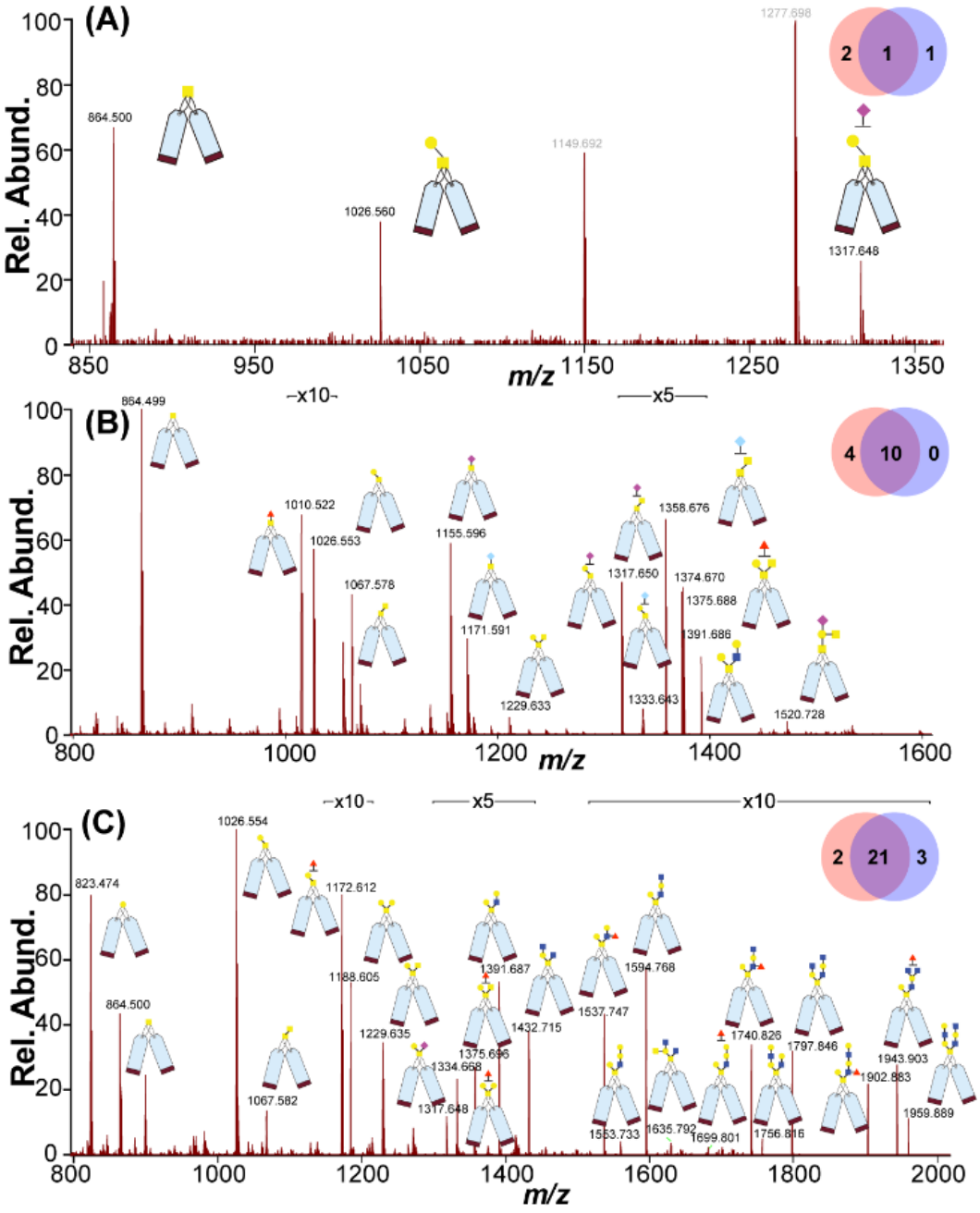

Fig. 4. **MALDI-MS profiling of DiLeuPMP labeled O-glycans released from standard glycoproteins**. (A) Bovine fetuin; (B) Bovine Submaxillary Mucin (BSM); (C) Porcine Stomach Mucin (PSM). The inset Venn diagrams above each spectrum show the numbers of identified glycans from PMP control (blue purple, right) and DiLeuPMP (orange red, left).

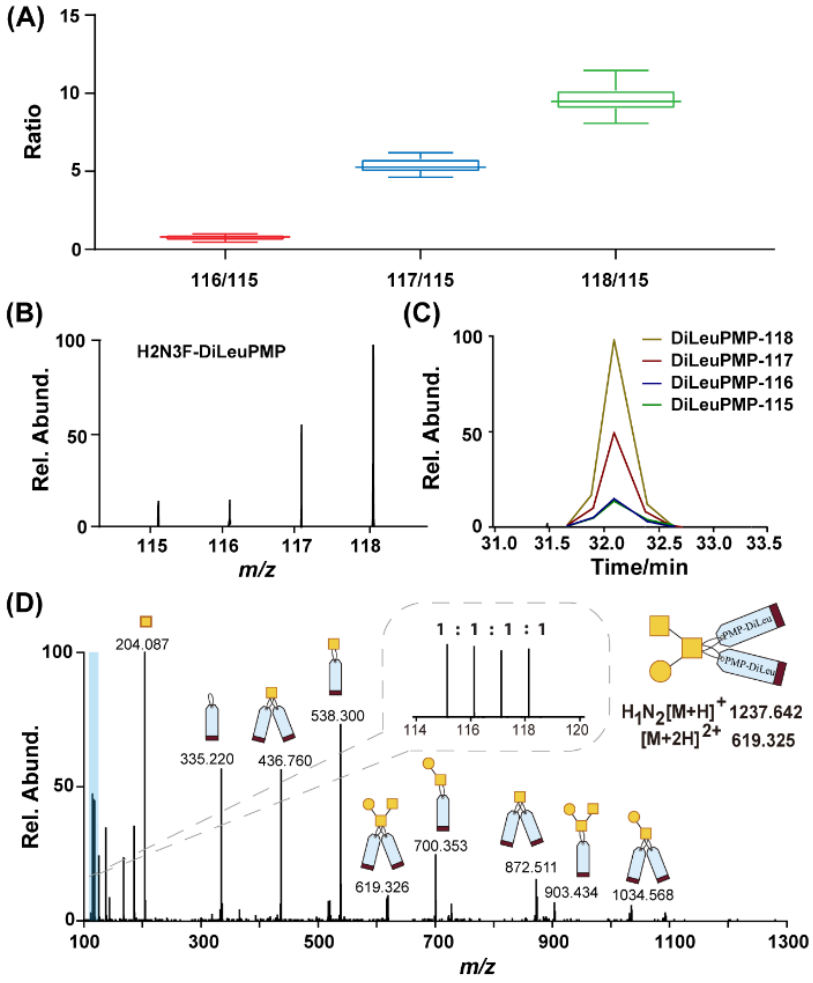

Fig. 5. **Relative quantification performance of 4-plex DiLeuPMP labeled O-glycans released from glycoprotein PSM.** (A) Labeled O-glycans were mixed at ratios of 1:1:5:10 and analyzed in triplicates. Box plots show the median (line), the 25th and 75th percentile (box), and the 5th and 95th percentile (whiskers); (B) Representative MS spectrum of reporter ion region for 4-plex DiLeuPMP labeled H_2N_3F; (C) LC retention times of reporter ions generated from 4-plex DiLeuPMP labeled H_2N_3F; (D) ESI-MS/MS fragmentation of DiLeuPMP labeled H_1N_2 from healthy human serum sample showing efficient fragmentation and abundant reporter ions in the zoomed inset allowing quantification. Note: H-hexose, N-N-acetylhexoamine, F-fucose.

Chapter 7

Conclusions and Future directions

In this dissertation, several isobaric labeling strategies have been described to analyze various biomolecules such as phospholipids, fatty acids, peptides, and glycans. These cutting-edge methodologies included new linker reagents, novel coupling methods, advanced fragmentation techniques in MS, enhanced quantification approaches, and novel isobaric mass tags. These developments were applied to human cells, plasma, as well as mouse tissues and utilized to investigate intricate biological systems and disease models, improving current analytical technology for biomolecule quantification and characterization, and showcasing their potential applications in pharmaceutical sciences and clinical research.

In **Chapter 2**, we provide an overview of several isobaric tags and recent advancements, including improvements in sensitive quantitation, multiplexing capabilities, and targeted analysis strategies, while discussing their limitations and potential solutions. Isobaric tags have various applications, such as biomarker discovery, structural investigations, single-cell analysis, top-down proteomics, and analysis of different molecules, including neuropeptides, glycans, metabolites, and lipids. The number of applications of isobaric labeling in MS-based studies has increased exponentially, along with the development of new labeling reagents and strategies. We expect the isobaric labeling strategy will continue to grow to achieve increased multiplexing capabilities and expand to various fields for quantitative purposes.

In **Chapter 3**, we present a diazobutanone-assisted isobaric labeling strategy that enables rapid and robust multiplexed quantitative lipidomics for various lipid classes, including phospholipids and glycolipids. A 6-plex quantification analysis of lipid extracts from lean and obese mouse livers was performed, and 245 phospholipids from central phospholipid classes were identified and quantified to reveal lipidomic changes in mouse obesity. In the future, deeper and more comprehensive studies will be conducted to profile the changes in all phosphate and sulfate

group-containing lipids, such as lysolipid, sphingomyelin, and sulfated glycolipids, which have not yet been explored in the biological samples in our study to investigate lipidomic changes from different diseases models.

We coupled SUGAR isobaric mass tags and *m*-CPBA epoxidation strategy to enable simultaneous multiplexed quantifying fatty acids and pinpointing C=C positions in **Chapter 4**. In this study, several fatty acid positional isomers were discovered in human plasma samples using their unique diagnostic ion pairs. From the quantitative analysis of plasma from healthy individuals and AD patients, although similar trends of fatty acid changes have been observed, no significant difference was found in this study probably due to the small sample size. To confidently discover AD biomarkers, a larger sample size is needed. Currently, SUGAR mass tags are available in up to 12-plex in our lab. We expect that this 4-plex quantitative strategy can be easily scaled up to 12-plex by employing a new set of 12-plex SUGAR isobaric tags to enable analysis with larger sample sets.

The isobaric labeling strategy has been extensively utilized in quantitative proteomics and PTM analysis, while the methods using other MS2 fragmentation techniques are still limited. In **Chapter 5**, we explored the application of isobaric labeling to ETD-based analysis, favorable to labile PTM proteomic analysis. By designing the DiLeu mass tags with a 2 Da mass difference and MS acquisition settings, multiplexed quantification was achieved for ETD-based analysis using complementary ions. We plan to apply this method to mouse brain tissues from healthy mice and AD mice to investigate the differences in phosphorylation sites on proteins.

In **Chapter 6**, a novel set of 4-plex DiLeuPMP isobaric mass tags has been developed to quantify O-glycosylation on proteins. This method allows one-pot releasing and isobarically labeling O-glycans from the peptide backbone to achieve multiplexed quantification. So far, the

application to human plasma has been conducted, while limited numbers of O-glycans were identified. Further optimization might be needed to increase the overall coverage of O-glycosylation in the biological samples.

Appendix I

Publications and Presentations

Publications

1. **Gu, T. J.[#],** Lui, P.K.[#], Wang, Y. W., Flowers, M. T., Xu, S., Lui, Y., Davis, D. B., & Li, L. Diazobutanone-assisted isobaric labeling of phospholipids and sulfated glycolipids enables multiplexed quantitative lipidomics using LC-MS/MS. *Under revision*. (#co-first authors)

2. **Gu, T. J.,** Lui, P.K., Wang, D., Wang, Z., Frost, D. C., and L. Li. A complementary ion-based strategy enables multiplexed quantification in ETD analysis using DiLeu isobaric tags. *In preparation.*

3. **Gu, T. J.,** Feng, Y., Wang, D., & Li, L. (2022). Simultaneous multiplexed quantification and C= C localization of fatty acids with LC-MS/MS using isobaric multiplex reagents for carbonyl-containing compound (SUGAR) tags and C= C epoxidation. *Analytica Chimica Acta*, *1225*, 340215.

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Conference Presentations

1. **Gu, T. J.**, Liu, P.K., Wang, Y. W., Flowers, M. T., Xu, S., Lui, Y., Davis, D. B., & Li, L. Diazobutanone-assisted isobaric labeling of phospholipids and sulfated glycolipids enables multiplexed quantitative lipidomics using LC-MS/MS. 70th ASMS Conference, Minneapolis, MN, June 2022, Poster.

2. **Gu, T. J.**, Feng, Y., Wang, D., & Li, L. Simultaneous multiplexed quantification and C= C localization of fatty acids with LC-MS/MS using isobaric multiplex reagents for carbonyl-containing compound (SUGAR) tags and C= C epoxidation. 69th ASMS Conference, Philadelphia, PA, Nov 2021, Oral presentation.

3. **Gu, T. J.**, Li, M., Lin, X., & Li, L. DiLeuPMP: a multiplexed isobaric labeling method for quantitative analysis of O-glycans. 68th ASMS Conference, Virtual, June 2020, Poster.

4. Frost D, **Gu, T. J.,** Li M, Feng Y, Li L. Increased Multiplexing of DiLeu Isobaric Tags with Enhanced Linker Using Mass Defect Isotope Encoding, *67th ASMS Conference*, Atlanta, GA, June 2019, Poster.