Label-free and derivatization-assisted techniques for the analysis of small molecules via mass spectrometry

By

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Label-free and derivatization-assisted techniques for the analysis of small molecules via mass spectrometry

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Abstract

Mass spectrometry is a key technique in the multi-omics field of disease biomarker discovery, enabling scientists to quantitatively analyze all ionizable species in a sample. Small molecules, such as metabolites (<1500 Da), are of particular interest in disease characterization and biomarker discovery, as they can provide useful insights into how larger biological mechanisms function in a diseased state. Metabolites encompass a wide range of biomolecules, including lipids, fatty acids, amino acids, organic acids, nucleotides, and glycans, allowing for a variety of sample handling techniques to effectively separate molecules of interest.

This dissertation focuses on common approaches to small molecule analysis using mass spectrometry: derivatization and label-free analysis. In derivatization methods, MS1-centric isotopic N,N-dimethyl leucine (iDiLeu) has been established as a reliable tool for accurately quantifying amine-containing metabolites, such as amino acids. This is followed by leveraging the change in polarity provided by tagging to improve sample recovery for amine-containing small molecules. In label-free analysis, a traditional dilute-and-shoot (DnS) approach is adapted to characterize changes in metabolite profiles resulting from two different extraction solvents. This work offers affordable solutions to major challenges in small molecule research.

Chapter 1

Introduction and research summary

Introduction

Quantification of signaling molecules such as metabolites and peptides is essential for understanding the downstream effects of diseased conditions and is imperative for discovering novel biomolecules [1]. The most common technique for biomolecule detection and quantification is mass spectrometry (MS) where molecules in a sample are ionized, then separated by their mass-to-charge ratio (*m/z*), and detected -- where the peak height and area are proportional to the concentrations of the molecules of interest (MOI) [2]. Because metabolites have diverse physicochemical properties, not all of these small molecules ionize efficiently in traditional positive mode MS analysis – typically, hydrophobic molecules ionize well, while hydrophilic molecules ionize poorly [3]. Additionally, when paired with reversed-phase liquid chromatography (RPLC), many hydrophilic analytes are not retained long enough to be successfully analyzed in tandem LC-MS, or they are poorly separated, and the molecules that ionize well can overshadow the signal of potential analytes. Sample preparation methods aimed at cleaning up metabolite samples often result in significant sample loss and are therefore frequently avoided. As a result, salts are often left in the sample, which further dampens the signal of metabolites and shortens the lifespan of the instruments used in analysis.

Two approaches to metabolite quantification are commonly used in studies: derivatization and label-free analysis. In derivatization-assisted analysis, samples are chemically tagged prior to analysis and then examined using traditional LC-MS techniques. This process helps homogenize the polarity of the molecules of interest (MOI) and increases their hydrophobicity, thereby improving their ionization efficiency and retention

in RPLC-MS analysis. In label-free analysis, the sample is simply extracted from a biological source and diluted prior to analysis in a workflow often referred to as “dilute-and-shoot” (DnS).

The overall aim of this research is to apply chemical logic to enhance the small molecule profiles of samples using both derivatization and label-free techniques. Herein, we establish the effectiveness of an in-house developed isotopic N,N-dimethyl leucine (iDiLeu) tagging and utilize this tagging to improve the recoveries of select neurotransmitters associated with copper toxicity. We also explore how different extraction techniques can be leveraged to improve label-free analysis of small molecules for profiling the effects of ocean acidification.

Research Summary

Quantification practices developed in neuropeptidomics have been swiftly applied to other omics fields, including metabolomics. In **Chapter 2**, we discuss some of these quantitative techniques, how they have been applied, and introduce the challenges associated with metabolomics workflows.

One quantification technique, in which an internal standard is tagged with labels of different masses and injected at varying concentrations to generate a calibration curve, can be implemented using a variety of tags. It should be noted that the in-house developed iDiLeu tagging method, when used for this purpose, is not only quantitatively accurate, but also more affordable than commercially available tags.

In **Chapter 3**, we demonstrate that when applied to metabolites, these tags retain their quantitative accuracy and also lower the limit of detection for important small molecules related to Alzheimer's disease in human cerebrospinal fluid. **Chapter 4** builds on this by leveraging the change in hydrophobicity introduced by tagging to improve sample recovery of five key metabolites related to copper metabolism, using liquid-liquid extraction (LLE) and solid phase extraction (SPE). We then performed absolute quantification of the molecules of interest (MOI) in crustacean hemolymph with and without copper exposure for 30 minutes, 1 hour, and 2 hours; finding that glutamic acid was downregulated in copper-exposed crabs after 2 hours.

Further probing of the crustacean metabolome was conducted to investigate potential changes due to ocean acidification. Crustaceans are an incredibly relevant biological model, having shown some resilience to ocean acidification, yet they still experience population stresses as a result. In **Chapter 5**, we perform two extraction techniques – one using isopropanol, which has recently been shown to more effectively extract small molecules, and another using acidified methanol, a method commonly employed in peptidomics extractions. These approaches aim to broaden the profile of small peptides and small molecules extracted using dilute-and-shoot (DnS) methodologies. We apply these techniques to samples exposed to CO_2-adjusted pH levels with 1-hour, 2-hour, and 4-hour durations, and compare them to a control to generate a comprehensive profile of how the metabolome changes over time.

Finally, **Chapter 6** outlines future directions based on the findings of this work and related studies. Additional research is presented in Appendix II and Appendix III, which

discuss the physicochemical properties of derivatized small molecules and techniques for performing MALDI on these small molecules, respectively.

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Chapter 2

Developing Mass Spectrometry for the Quantitative Analysis of Neuropeptides

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Structured Abstract:

Introduction:

Neuropeptides are signaling molecules originating in the neuroendocrine system that can act as neurotransmitters and hormones in many biochemical processes. Their exact function is difficult to characterize, however, due to dependence on concentration, posttranslational modifications, and the presence of other comodulating neuropeptides. Mass spectrometry enables sensitive, accurate, and global peptidomic analyses that can profile neuropeptide expression changes to understand their roles in many biological problems, such as neurodegenerative disorders and metabolic function.

Areas Covered:

We provide a brief overview of the fundamentals of neuropeptidomic research, limitations of existing methods, and recent progress in the field. This review is focused on developments in mass spectrometry and encompasses labeling strategies, posttranslational modification analysis, mass spectrometry imaging, and integrated multiomic workflows, with discussion emphasizing quantitative advancements.

Expert Opinion:

Neuropeptidomics is critical for future clinical research with impacts in biomarker discovery, receptor identification, and drug design. While advancements are being made to improve sensitivity and accuracy, there is still room for improvement. Better quantitative strategies are required for clinical analyses, and these methods also need to be amenable

to mass spectrometry imaging, post-translational modification analysis, and multi-omics to facilitate understanding and future treatment of many diseases.

Keywords: Imaging, Label-free, Mass Spectrometry, Multiplexing, Multi-omics, Neuropeptides, Peptidomics, PTMs, Quantitation, Stable Isotope Labeling

Article Highlights:

• Developments in quantitative mass spectrometry have enabled greater sensitivity, higher throughput, and more comprehensive analyses of neuropeptidomics, improving understanding of the signaling pathways involved in many diseases.

• Both isotopic and isobaric labeling strategies have seen increased usage, especially as instrument advancements enable greater multiplexing, and labelfree neuropeptidomics remains common due to reduced sample loss and spectral complexity. Recent incorporation of data-independent acquisition strategy has benefits for both labeling and label-free methods.

• Post-translational modification analysis remains challenging, but is in greater demand, especially with the discovery of glycosylated neuropeptides. These analyses have benefited from adapting glycoproteomics methods and improvements in instrumentation, such as the availability of ETD and EThcD for fragmentation.

• Recent advances in normalization methods, matrix development, data analysis, etc., have enabled mass spectrometry imaging to not only be useful for localization, but also for quantitation of neuropeptides.

• Neuropeptides play roles in diverse signaling pathways that involved a suite of co-modulating neuropeptides, proteins, neurotransmitters, and metabolites, highlighting the need for multi-omic workflows. These methods have seen increased use in recent years, facilitated by developments in analyte extraction and separations, differential labeling, and instrumentation.

1. Introduction:

Regulation of the nervous system is a strictly controlled process influenced by a plethora of signaling peptides, neurotransmitters, hormones, and other modulating molecules. [1,2] These neuromodulators are critical to behavior, [3–5] stress responses, [6–11] maintaining homeostasis, [12,13] and many other biological processes. [14–16] Neuropeptides, signaling peptides originating in the neuroendocrine system, are of particular interest as they have highly diverse function and structure, dynamic expression, and many sites of action. [1] Dysregulation of neuropeptides has been implicated in many diseases and biological states, including heavy metal toxicity, [8] hypoxia, [7] Alzheimer's disease, [17] depression, [18] and others. As a result, comprehensive characterization of neuropeptides could have many benefits such as discovering biomarkers or elucidating important biological pathways involved. However, such global analysis is challenging not only due to many neuropeptides having low in vivo abundance and high structural diversity, but also because neuropeptide function is influenced by many factors, such as location, post-translational modifications, and the presence of other co-modulating neuropeptides. [1]

This complexity inherent to all neuropeptidomic studies is exacerbated by the difficulties in determining possible neuropeptide sequences. Neuropeptides are produced by the select processing of precursor proteins (i.e., preprohormone) encoded within the genome. [19] These preprohormones contain a signaling sequence and the remaining prohormone. After cleavage of the signaling sequence, the prohormone is selectively and specifically cleaved by endopeptidases, such as various prohormone convertases, to produce several peptide sequences from a single precursor protein. [20] The peptides are then processed further and post-translationally modified to produce the bioactive neuropeptides. [20] The intricate pathways from genome to active neuropeptide, splice variants, and diversity of post-translational modifications lead to many possible peptide forms that are difficult to predict from genomics or even transcriptomics alone. This compounded with the fact that many model organisms do not have a fully sequenced genome to use as a reliable starting point for predicting a full neuropeptide database makes neuropeptide studies even more challenging.

Mass spectrometry (MS)-based approaches have led to enhanced workflows for detecting, characterizing, and quantifying neuropeptides in various samples. MS can provide detailed, high-accuracy information about the intact mass, sequence, modifications, and expression levels of a detected neuropeptide. [1] Furthermore, MS analyses can profile many analytes (neuropeptides) from a sample in a single experiment without requiring extensive a priori knowledge. This unbiased, untargetedmethod facilitates discovery-based neuropeptidomics. As neuropeptide function is influenced by the other neuropeptides present, methods to detect the suite

of neuropeptides expressed in a sample are critical to understanding the underlying signaling mechanisms of neuropeptides.

Using various labeling and label-free strategies, MS can further provide useful quantitative information that can be used to determine neuropeptide expression differences between samples. [21] Alternative MS analysis methods, such as dataindependent acquisition (DIA) can enable greater depth of coverage for the neuropeptidome. [22] These quantitative strategies can be applied not only to the detection and identification of neuropeptides, but also to identifying different forms of neuropeptides with post-translational modifications. [23] Additionally, there have been many developments in the field of MS imaging (MSI) to enable the detection of neuropeptides in specific locations of a tissue or tissue section. [13,24,25] This spatial distribution provides an additional level of information for quantitative neuropeptidomics. Furthermore, MS has applications in numerous -omics (e.g., proteomics, metabolomics), that can offer complementary information to the quantitative neuropeptidomic workflows. By combining the structure elucidation, quantitative information, spatial distribution, and analysis of correlated biomolecules, analysis by MS has greatly improved our understanding of neuropeptides and their roles in many biological processes. This review aims to highlight recent developments in the broader field of neuropeptidomics with an emphasis on quantitative strategies.

2. Quantitative Strategies:

As neuropeptide function has some dependence on concentration, [26] being able to reliably identify and quantify neuropeptides is necessary for understanding their function, especially as it relates to different biological states. A recent review has been published detailing mass spectrometry strategies applied to functional neuropeptidomics. [27] These functional studies are challenging, however, due to the low concentrations (as low as femtomolar) in vivo [26] of neuropeptides, highlighting the continual need for improved quantitative methods. Although MS is not inherently quantitative, its widespread application and growth in the -omic fields has led to the development of various strategies for accurate and sensitive quantitation. The quantification of neuropeptides has been achieved with label-free methods that allow analysis of the neuropeptides without modification and minimal sample loss; [28] a variety of labeling strategies that facilitate more reliable quantitation and greater throughput analyses; [29] and more recently, DIA workflows to increase reproducibility and enable detection of more low abundance analytes and thus deeper profiling of the neuropeptidome. [22] These methods are of course not the only quantitative methods for neuropeptidomics and others have been reported and summarized in other reviews. [30,31]

2.1 Label-free Quantitation:

In MS workflows, certain steps, such as desalting, are crucial, but researchers will often forgo extraneous steps to reduce sample loss for low abundance species like manyneuropeptides. Label-free quantitation (LFQ) strategies may suffer from reduced throughput, but they often provide the least amount of sample processing (and fewer losses from it). In LFQ methods, samples are analyzed independently of each other,

commonly using LC-MS or LC-MS/MS approaches. [21,32] Quantitation is typically performed by comparing signal intensities (e.g., chromatographic peak area). By examining the extracted ion chromatogram (XIC) of analytes, quantitation is achieved by comparing peak areas between sample runs. Controlling for run-to-run variability is critical for reliable quantitation as samples are run separately. [28] Software is typically used to align the XICs by retention time and filter data, such as ensuring the precursor/fragment ions and the charge states match between aligned peaks. [33,34] Additionally, normalization between analyses needs to be considered to facilitate more accurate comparisons between samples, as variations can arise from instrument calibration, sample preparation, and ambient temperature during analysis. Various methods and software packages for normalization exist and are systematically evaluated in a recent article by Välikangas et al. [35] Ye et al. demonstrated the efficacy of LFQ to quantify neuropeptide expression changes as a result of feeding in a rat model. [36] Anapindi, et al. similarly used XIC analysis to examine neuropeptide expression, but on a much larger scale; over 200 LC-MS runs were performed to identify and quantify over 1500 neuropeptides to examine their effect on chronic migraine and opioid-induced hyperalgesia. [4] Targeted approaches have also proven useful; Salem et al. performed relative quantitation of surrogate neuropeptides and their fragments to characterize the processing of pro-neuropeptides to mature neuropeptides. [37] Frequently, targeted approaches will employ parallel reaction monitoring (PRM) in which a targeted precursor ion and subsequent fragment ion are analyzed. Similarly, multiple reaction monitoring (MRM) analyses a single precursor ion and multiple fragment ions from that precursor ion. As these approaches minimize interference

from other matrix components, sensitivity and throughput are greatly enhanced. [38] Although often used for measuring surrogate peptides from a targeted protein, MRM and PRM have seen use in peptidomics workflows, such as the analysis of orexin in mice cerebrospinal fluid [39] and the identification of endogenous signaling peptides in insects. [40] While MRM and PRM have high sensitivity, the simplicity of using XICs for quantitation allows it to be easily applied to novel and/or less developed MS workflows. Bianco et al., for example, used XICs to characterize differences between arginine and lysine vasopressin after analysis by Fourier transform ion cyclotron resonance (FTICR) MS with multiphoton dissociation. [41]

LFQ workflows can also employ spectral counting for quantitation. Spectral counting assesses protein abundance by correlating concentration to the number of times a constituent peptide is identified, with the idea being that more abundant proteins will be identified more frequently in a single run. [33] The protein abundance index (PAI) is calculated from the normalized level of observed peptides per protein and its exponential modification (emPAI) is used to estimate protein concentrations. [42] In neuropeptidomic experiments, however, identifications are not made based on constituent peptides, as done in bottom-up proteomics, because the endogenous neuropeptide is generally not digested prior to LC-MS analysis. This greatly reduces the applicability of spectral counting in neuropeptidomic experiments, but it still has gained some use, typically with XIC information used for improved confidence. Southey et al. has shown that spectral counting and spectral indexing (based upon the cumulative intensity of product ions) provide more informative characterization of neuropeptides in the rat suprachiasmatic nucleus over XIC.

[28] Other groups have published quantitative methods that utilize both XIC and spectral counting for characterizing endogenous peptides outside of the central nervous system. By comparing the peptidomes of patients with systemic juvenile idiopathic arthritis (SJIA) to healthy patients, LFQ methods enabled the detection of 17 potential biomarkers for SJIA in urine. [43] Similarly, Labas et al. used LFQ methods to characterize peptides in chicken semen to identify key peptides for phenotyping. [44] Although these applications do not address neuropeptides specifically, the peptidomics workflow is translatable and provides future directions for neuropeptide analyses. Even as other quantitative methods are developed, the scalability and ease of using LFQ will likely ensure its continued use in the future.

2.2 Label-based Strategies:

Although label-free quantitation is adaptable, requires fewer sample processing steps, and is amenable to many samples, it often requires a greater number of LC-MS runs and can suffer from run-to-run variability. Conversely, many labeling strategies exist that allow samples to be run simultaneously, enabling accurate multiplex quantitation with fewer control samples required, reduced instrument time, and decreased effects due to instrument variation. [21] For neuropeptide applications, most labeling reactions occur post-extraction and create a mass difference between channels via stable isotope incorporation to differentially label samples. Reductive dimethylation is frequently used to label neuropeptides as it targets primary amines (N-termini and lysine residues), common to most neuropeptides. Additionally, this reaction is low cost and easily accessible, requiring only isotopic formaldehyde and a reducing agent such as borane pyridine or

cyanoborohydride. [45] Isotopic dimethylation has been used extensively in the Li lab to study neuropeptidomic changes in crustacean models. [7,10,11,46,47] Moreover, the group has shown the method is compatible with both electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) methods to provide complementary coverage of neuropeptides in a single sample. [7] Wilson et al. has developed an online dimethyl labeling system in which neuropeptides are derivatized on-column with either light or heavy reagents. [48] The Fricker lab has also published several articles using isotopic labeling for quantitative peptidomics. They have expanded the reductive dimethyl labeling scheme to an impressive five channels while improving accuracy with isotopic correction calculations. [45] Similarly, the lab has expanded the use of trimethylammoniumbutyryl (TMAB) chemical tags to a 4-plex. [49,50] TMAB tags add a permanent positive charge via a quaternary amine to N-termini and lysine residues of peptides using an amine-reactive NHS ester. These tags have been widely used in peptidomics to study numerous biological processes, including peptide degradation, [51] prohormone processing, [52] narcotic effects, [53] and can be transferred to neuropeptidomic applications. These tags are effective, easily synthesized, and increase signal intensity by incorporating a permanent positive charge. This permanent charge, coming from a charged quaternary amine, does make the peptide more prone to dissociation, thus increasing instability and limiting application.

One of the major limitations with isotopic labeling, as evidenced by Buchberger et al.[7] and others, [45] is the added spectral complexity with higher multiplexing. This can lead to difficulties in accurately calculating relative abundances, even with isotopic corrections.

Additionally, given the wide dynamic range of neuropeptides, it is possible (and perhaps likely) that peaks corresponding to downregulated neuropeptides could be too low in intensity to be quantified or even be detected. Even analytes of high enough intensity might still not be selected for fragmentation and tandem MS analysis by commonly used data-dependent acquisition (DDA) settings, simply due to the greater number of precursor peaks that come with an isotopic labeling workflow. Isobaric tagging–where neuropeptides are labeled with isotopically encoded tags that add the same mass but produce unique reporter ions upon tandem MS analysis–can offer advantages over conventional isotopic labeling. **Figure 1** depicts the differences between isotopic (1A) and isobaric tagging (1B). These methods are capable of significantly higher order multiplexing, such as 8-plex with iTRAQ, [54] 16-plex with TMTpro, [55] or 21-plex with DiLeu, [56] because only the MS2 spectra have added complexity due to the unique reporter ions. Moreover, the tags are strategically designed to create reporter ions in regions of the spectrum that do not contain other useful peaks. Isobaric tags have been used often for proteomics applications, but have had fewer applications with peptides, especially neuropeptides. Dimethylated leucine (DiLeu), for example, has been used to quantify neuropeptides in lobster brains as a function of growth cycle, [15] and also as part of a multi-omic profiling of the mouse hypothalamus, [57] but the low abundance of many neuropeptides still presents challenges. As quantitation occurs at the MS2 level, isobaric tagging requires analytes be selected for fragmentation and subsequent tandem MS analysis. Low abundance analytes, like many neuropeptides, are often omitted from tandem MS analysis using conventional DDA strategies. Recently, Sauer and Li have shown that optimization of the DDA

parameters can facilitate a greater depth of coverage in the crustacean neuropeptidome. This enabled the characterization of many neuropeptides dysregulated in response to copper toxicity. [8] Similar optimization strategies have been used to improve proteomic coverage, [58,59] highlighting a problem not unique to the field of neuropeptidomics. As instrumentation and analytical capabilities improve, it is likely that we will see an increased prevalence of isobaric and isotopic labeling methods reported for neuropeptides and other low abundance analytes.

2.3 Data-Independent Acquisition:

Conventional identification and quantitation of peptides and proteins by mass spectrometry typically involves DDA to trigger and obtain MS2 fragmentation spectra. Prioritizing fragmentation of the higher signal intensity ions, this acquisition method is biased towards higher abundance and more easily ionizable species. Fragmentation spectra are crucial to obtain confident identification of biomolecules. Neuropeptides are often of low abundance compared to other biological and neurological matrix components; [60] their analysis can benefit greatly by using a less biased fragmentation scheme. DIA methods were developed to overcome the limitations of DDA, by fragmenting all molecules within a desired m/z window, not only the highest signal intensity ions, [61] demonstrated in **Figure 2**. During DIA MS, all molecules within a m/z isolation window of user-defined width are fragmented, without a precursor selection to trigger fragmentation. Fragmentation ion spectra are collected from different isolation windows in a cyclic manner, from the beginning of the defined m/z survey range to the end before repeating. MS1 spectra are collected every cycle, or however often is desired. The use of DIA enables

the identification, and therefore potential quantitation, of more molecules compared to DDA, [61–64] including lower abundance molecules such as neuropeptides. [22] As a newer method, DIA MS does not have the breadth of application that DDA does and is not yet commonly applied to neuropeptide analyses. However, the field of neurobiology has seen great benefit from the adoption of quantitative DIA MS, as discussed in a recent review on its application to quantitatively analyze the brain proteome. [65] Although not all quantitative, a few MS analyses using DIA have recently been performed to better improve the characterization of neuropeptides. DIA MS was used in the untargeted quantification study of neuropeptide expression changes in response to feeding activity in crustacea. 66 The authors were able to detect and quantify 137 peptides directly from microdialysate with minimal sample preparation; no extraction or precipitation steps were required, demonstrating the utility of DIA for the quantification of limited samples. 66 While DIA MS is a powerful technique for untargeted quantification of neuropeptides, Saidi et al. demonstrated its limitations and higher variances during targeted quantification studies of neuropeptides found in animal spinal cord tissues. [38] Delaney and Li also evaluated the utility of DIA MS for the quantification of neuropeptides from crustacean neural tissue. While improving the technical and biological reproducibility of analysis and number of overall neuropeptides identified compared to DDA, the DIA method showed poor quantitative accuracy using a label free approach. [22] To improve LFQ of peptide hormones using DIA MS, a method was developed using an internal standard peptide, enabling accurate quantitation. [67] As the field of DIA MS quantification further develops,

new strategies to improve its abilities to characterize neuropeptides will need to be developed.

The utility of DIA searches using a spectral library-free approach and database searches generated from FASTA sequences has been demonstrated for analysis and quantitation. Model spectra generated from these methods are not always as reliable for neuropeptides as the algorithms are often developed for tryptic peptides. PTMs and structural diversity of endogenous peptides complicate these matters, making it difficult to obtain good results without high-quality spectral libraries. Although identification through spectral library searches have been shown to increase identification and quantitation reproducibility, [68] spectral library generation, requiring high amounts of starting material, may not always be feasible due to the limited sample concentration of neuropeptides. Database searching and spectral-free methods [69–73] will likely be more beneficial to the future study of neuropeptides by DIA MS.

Overall, DIA has been shown to improve sensitivity of analysis for both identification and LFQ, [74] enabling consistent detection with up to a 10-fold increase in sensitivity compared to DDA, and improving the quantitative dynamic range of analysis. [61,75] Notably, the reproducibility and quantitative performance of DIA MS methods was evaluated by 11 sites worldwide, and reproducible quantitation of proteins was observed. [75] Reproducibility is very important for the quantitation of samples; the same analytes must consistently be identified across all conditions for comparison. A large limitation to DIA analysis is the duty cycle for each scan and total time it takes to cycle through the entire desired m/z range. Careful consideration is required when choosing parameters

such as maximum ion injection time, automatic gain control target, isolation window width, and full m/z range, among others, as this may cause analytes to not be fragmented during the appropriate window before fully eluting during the LC gradient. [76] This issue is further exacerbated during DIA MS for quantitation as accurate quantitation requires data to be collected at multiple points across a peak profile. Further complicating quantitation by DIA MS, multiplexed label-based methods increase spectral complexity, although such methods have recently been utilized in a few proteomics experiments. [77–80] Additional information about the utility and considerations of DIA MS for neuropeptide analysis and quantitation, including software resources can be found in a recent comprehensive review detailing advances in the MS analysis of neuropeptides [manuscript under review]. More general reviews for proteomics also exist. [63,71,81–83]

DIA can also be beneficial for the analysis of heavily modified analytes where MS signal intensity is distributed across multiple proteoforms, as this increases the number of precursors needed to be selected for fragmentation using DDA. A limitation to this method is the deconvolution of data; multiple precursor ions are co-fragmented in MS2 spectra and PTMs can further complicate fragmentation spectra. Although it has been shown to benefit the analysis of glycosylated proteins, [84–86] this analysis has not yet been applied to the field of neuropeptidomics. Even with the limitations of DIA, it has been shown to successfully identify more peptides and neuropeptides, with improved reproducibly. With a limit of detection (LOD) in the amol range [61] and improved quantitation capabilities, [75] the field of neuropeptidomics would benefit greatly from the adoption of quantitative DIA MS workflows.

3. Post-Translational Modifications:

Neuropeptides and other bioactive peptides are formed after enzymatic cleavage of larger precursors by peptidases. Additional enzymes can alter these peptides with PTMs, altering their structure, function, and stability, among other effects, contributing to the vast diversity of neuropeptides. Neuropeptide modifications can include amidation, phosphorylation, acetylation, glycosylation, and sulfation, to name a few. [87] Identifying and differentiating between these forms is crucial to understand molecular mechanisms in neurobiology, and thus these modified neuropeptides are investigated using MS by a variety of labs, [10,88–90] thoroughly summarized in a few recent reviews. [91] The quantification of post-translationally modified neuropeptides faces additional challenges. Labeling approaches often target specific residues and moieties, so post-translational modification of these residues often inhibits quantitation via labeling. For example, many tags target primary amines and are therefore ineffective or less effective for peptides with acetylated N-termini. Further challenging analysis of modified peptides is the decreased ion signal intensity from distribution of already low abundance neuropeptides across the differentially modified forms. This leads to the need for targeted analyses and enrichment strategies to detect and quantify peptides with PTMs, [92–94] especially for those modified by highly dynamic glycosylation. [94–97] As MS considerations are more prominent for peptides modified by glycosylation, [93,98,99] we will focus on the discussion of glycosylated peptides.

Estimated to modify potentially 33% of all known human peptide hormones, changes in glycosylation have a large impact on the role and efficacy of neuropeptides and other

bioactive peptides. [100–103] It is therefore of interest to improve quantification abilities for these lower abundance peptides with decreased ionization efficiency compared to their non-modified counterpart. 96 During a targeted analysis to characterize insulin and other signaling peptides in pancreatic islets, Yu et al. discovered insulin to be glycosylated and found this form to be differentially regulated in mouse models of diabetes. [104] This demonstrates the need for more attention to be given to the analysis and quantification of modified signaling molecules. To this goal, Hansen et al. investigated the potential presence of glycans on atrial natriuretic peptide (ANP), a peptide hormone with its proteolytic degradation and potency being regulated by glycosylation. They characterized and quantified glycosylated ANPs using a targeted MS approach and demonstrated glycosylation on ANP to impact its stability, circulation time, and receptor activation in rats. [105]

To improve the characterization of glycosylated neuropeptides, sensitive and accurate MS methods must be developed. Several dissociation methods have been investigated to better identify glycopeptides, namely collision-based [106–109] and electronbased [106,108,109] dissociation methods. Hybrid methods have also been developed to improve glycopeptide analysis such as electron-transfer/higher-energy collision dissociation (EThcD), [109] higher energy collision dissociation (HCD) product ion-triggered electron transfer dissociation, [110] and HCD product ion-triggered EThcD. [104] EThcD has also been shown to be valuable for quantitative proteomics of phosphorylated biomolecules as well. [111] The type of fragmentation scheme used is important to consider during glycopeptide analysis. Riley et al. demonstrated the need for optimizing dissociation

methods, finding peptides modified by N-glycans and O-glycans require different dissociation methods for optimal fragmentation. [112] The information-rich fragment ion spectra generated from EThcD, shown in **Figure 3**, is vital for the confident localization of O-glycans. To characterize glycosylated neuropeptides in crustaceans, Cao et al. utilized HCD triggered EThcD. [23] This study demonstrates the method's utility for sensitive glyconeuropeptide analysis. Though not applied to endogenous peptides, Zhu et al. describe a three-part workflow for the in-depth investigation of proteins and glycoproteins in the central nervous system. [113] More broad strategies for the quantification of glycosylated proteins and digested peptides have been discussed thoroughly in a recent review. [114] Although glycoproteomics and its quantification is of great interest to the scientific community, we can see that there has been a disproportional amount of investigation into glycosylated neuropeptides, and even less with quantitative approaches applied. This is an underdeveloped field but as glycoproteomic strategies improve in the future, we expect to see them applied to the field of neurobiology towards quantifying endogenous peptides more readily.

4. **Mass Spectrometry Imaging:**

As various modes of MS are being applied to neuropeptide discovery, there has been an increased emphasis in determining the biological relevance through localizing neuropeptides. MSI has been preferentially implemented in these studies due to the unique advantage of permitting targeted and untargeted detection of analytes within a tissue or cell while still providing spatial information. [115,116] To achieve this, many researchers often use MALDI, where the surface area of a tissue is portioned into

dataattainable units (pixels) and imaged through several laser ablations. Quantitative MSI of neuropeptides can be achieved in different ways including absolute quantification using labels to create a calibration curve and semiquantitative spiking with an internal standard. [115] Here we discuss recent advances in MSI that enable quantitative and semiquantitative analysis of neuropeptides.

Within the field of neuropeptidomics, relative quantitation is widely practiced due to easier sample preparation and reduced costs. Information obtained by these relative quantitation methods can be increased through biological means such as immunohistology assays and Nissl staining. [117,118] These techniques are applied to tissue after MSI to normalize neuropeptide quantities with respect to number of nerves and free amines present. The relative abundance and spatial information from MSI is valuable, but still comes with challenges such as localized ion suppression, availability of software, duration of data collection, and having a sufficient MSI analyzer. [119–122] These issues are exacerbated by the low concentration of neuropeptides in tissue. There have, however, been advances in desorption electrospray ionization (DESI) and liquid extraction surface analysis (LESA) MSI in lipidomics that may help mitigate these issues. [123–125] Zemaitis et al. demonstrates an increased signal intensity in DESI-MSI compared to MALDI-MSI, leading to higher resolving power for lipids. [123] This can likely be attributed to a lack of matrix clusters, which in MALDI, contribute to a pattern of low ionization efficiency. [123,124] Some imaging techniques seek to circumnavigate these issues by performing DESI and MALDI on the same tissue, permitting both proteomic/peptidomic and metabolomic analyses. [125] The use of LESA shows increased signal intensity due to larger sampling size, making this

method faster and more sensitive at the sacrifice of spatial resolution. [125] Both LESA and DESI come at the expense of spatial resolution; where MALDI can often distinguish sub-micron distances, DESI is limited to 50-100 micron and LESA images often have pixel sizes of 1000 microns. These techniques can be coupled with subsequent LC-MS/MS to verify trends of relative abundances seen in imaging experiments or XIC for LFQ. [116,126] Practices are being explored in drug MSI for better absolute quantitation using more sensitive mass analyzers, such as FTICR, coupled with an increased number of internal standards as well as adjusting calibration curves to pixel deviation. [122,127] Techniques under development work to decrease the LOD such as those discussed in metabolomics works implementing surface-assisted laser desorption/ionization (SALDI) [128] and other matrix-free MSI methods where nanoparticles are used to coat the tissue to enhance ionization efficiency as well as spatial resolutions (**Figure 4**). [129,130] These methods incorporate metals and have been found to retain spatial resolution of MALDI (~2-5 microns) while increasing ionization efficiency for small molecules. They are, however, presently limited to small molecules like drugs and metabolites. Such relative abundance techniques, combined with LFQ via LC-MS/MS back-correlation and software development, could greatly improve the accuracy and performance of quantitative MSI studies. The many forms of MSI complement the diversity of biological problems being studied, and advancements in MSI of neuropeptides will provide insights into the anatomy and physiology of neurological function and regulation. This in turn will help clinicians and other researchers identify and treat various health conditions.

5. **Multi-omics:**

The exact roles of neuropeptides are often difficult to discern when strictly observing only neuropeptides. Their receptors are typically proteins, such as G protein-coupled receptors (GPCRs); [131] they regulate biochemical pathways with downstream metabolite products; [132] and are often co-expressed and co-released with small molecule neurotransmitters. [133] Multi-omic workflows have emerged in which elements of proteomic, peptidomic, metabolomic, etc. experiments are integrated to provide more comprehensive information. [134–136] As a result, multi-omic experiments have seen increased popularity in neuropeptide experiments in recent years.

5.1 Small Molecule Studies:

Characterizing neuropeptides and relevant small molecules in a single experiment is difficult due to stark differences in extraction efficiencies, solubilities, ionization efficiencies, fragmentation patterns, and subsequent data analysis. Specific applications have further constraints, such as matrix compatibilities for MSI. Nevertheless, researchers have made recent advances in combining neuropeptidomics with small molecule analyses (e.g., lipidomics and metabolomics), typically by processing the two separately, then co-analyzing the data. Keller et al. addressed the extraction issues using both acidified methanol and methanol/water/chloroform extractions to efficiently recover proteins, peptides, and metabolites. Two molecular weight cut-off (MWCO) filters were used to separate metabolites (30 kDa). [136] Although not specifically studying neuropeptides, the reported extraction methods (i.e., acidified methanol), have been used previously for neuropeptides. [7,8,22,46] Gutierrez et al. reported a similar strategy in which an acetone/chloroform precipitation was used to separate proteins and metabolites. [135]

This method, dubbed SPOT (sample preparation for multi-omic technologies), is not described for the study of endogenous peptides, but could be adapted by adding a MWCO step similar to Keller et al. For some approaches, neuropeptides do not necessarily need to be isolated from metabolites prior to LC-MS analysis. Chen et al. used atmospheric pressure (AP) MALDI to study neuropeptides, lipids, and other biomolecules from the same tissue section, but incorporated different ionization methods. [137] This niche strategy eliminates issues with extraction and ionization efficiency differences by collecting data directly from the tissue section. The lack of tandem MS data, however, does require researchers to rely on accurate mass matching, possibly losing confidence in data interpretation.

Often co-expressed and released with neuropeptides, neurotransmitters are another target for multi-omics. Wojnicz et al. report a method that combines short neuropeptides (4 residues) and metabolite analyses to study bovine cells without the need for separate extractions. Using synthetic standards, they created a calibration curve that allowed absolute quantitation of both neuropeptides and neurotransmitters. [132] These methods would likely have limited applicability to larger neuropeptides but are important in establishing multi-omic strategies. Similarly, zwitterion exchange has been used for online separation prior to LC-MS to quantify neurotransmitters and select neuropeptides (oxytocin and vasopressin) simultaneously from blood. [138] Alternative separation and sampling methods, like microdialysis coupled to LC-MS, have offered sensitive assays for quantifying both neuropeptides and neurotransmitters, summarized in a review by Zestos and Kennedy. [139]

5.2 Proteomics:

Although similar in structure to proteins, neuropeptides require different analytical workflow than proteins largely due to size differences. Most multi-omic workflows that combine proteomics with the study of endogenous peptides like neuropeptides analyze the two separately and interpret the combined data. For example, Liu et al. describes the use of label-free neuropeptidomics with multiplex DiLeu-labeled neuroproteomics, workflow highlighted in **Figure 5**, to profile changes in the mouse hypothalamus resulting from the gut microbiome. [57] This study demonstrated the impact of the gut microbiome on neurochemical processes. [57] Similarly, Chen et al. combined label-free and labeled data to interrogate proteomic, metabolomic, and peptidomic (translatable to neuropeptides) dysregulation in metabolic diseases. [134] Neuroproteomics has also revealed dysregulation of neuropeptide and neurotransmitter release with impacts in neuropsychiatric diseases, such as addiction, using LFQ MS. [140] Conversely, Hook et al. characterizes neuropeptide variants to inform neuroproteomics and precursor protein analysis to better understand proteolytic processing and how it relates to cell-cell signaling. [87] The study of endogenous peptides outside of the neuroendocrine system can also provide translatable methods for multi-omic neuropeptide studies. For example, Labas et al. do not specifically study neuropeptides, but report a LFQ assay to combine proteomic and peptidomic data to phenotype chicken semen in a multi-omic experiment using spectral counting and XICs from LC-MS data. [44] By omitting the digestion step typically used to study proteins (bottom-up proteomics), Li et al., describe the use of top-down MS methods to study microproteins and endogenous peptides in mouse brain tissue

extracts. [141] Top-down MS methods like these are crucial for studying different protein and peptide forms but need adaptation to studying larger proteins and neuropeptides simultaneously.

While several advances have been made to address MS concerns when studying classes of molecules with distinct chemical properties, such as sequential extractions, methods employing simultaneous co-analysis are far from being common. To fully understand the interplay between different types of biomolecules, improvements into interpreting large amounts of data is also required. As investigations into complicated multiinteraction diseases and biological functions are increasingly being pursued by the scientific community, we expect to see more extensive multi-omics experiments adopted into future workflows.

6. Conclusion:

The field of neuropeptidomics is constantly evolving and recent improvements have led to enhanced detection and quantitation of neuropeptides. Their low in vivo abundance, complex functions, and structural diversity make their analysis challenging, but MS analyses have mitigated many of these difficulties. [142] Isotopic labeling strategies, such as dimethyl labeling45 and TMAB labeling, [49] differentiate neuropeptides at the precursor ion level but are only used to compare a few experimental conditions. Enhanced multiplexing can be achieved with the incorporation of isobaric tagging because it does not significantly increase spectral complexity. [8] Labeling strategies do enable higher throughput and typically more reliable quantitation, but additional sample processing

steps often cause sample loss which is detrimental for low abundance analytes like many neuropeptides. As a result LFQ methods are still common. [140] These methods are also more compatible with DIA methods, facilitating greater sensitivity and neuropeptidome coverage. [22] Quantitative strategies are also employed in imaging workflows to provide spatial distribution, utilizing a variety of normalization methods to ensure quantitation is accurate, summarized nicely in a review by Tobias and Hummon. [115] Analysis of PTMs, especially glycosylation, has greatly benefited from improvements in hybrid MS fragmentation method, such as the use of HCD-triggered EThcD to improve fragmentation of glycopeptides (and glyconeuropeptides). [23] As neuropeptidomic workflows become more common and accessible, their findings can be incorporated into larger multi-omic workflows. [57] There is still much room for improvement in terms of technology and method development, but recent advances in neuropeptidomics have provided much insight into the complex signaling pathways involving neuropeptides, with impacts in the fields of biomarker discovery and drug development.

7. Expert Opinion:

The field of neuropeptidomics is instrumental to our understanding of neuromodulation and signaling. This in turn has applications in clinical settings in the areas of biomarker discovery, drug discovery, and drug action. [131,133,143] Neuropeptide dysregulation has been linked to many biological problems and diseases, such as Alzheimer's disease, [144] obesity, [145] cancer, [146] and depression. [147] The advances in neuropeptidomics have enabled researchers to better understand many of these conditions and the related signaling pathways. This can facilitate the discovery of new, more reliable biomarkers.

Additionally, knowledge gained from these signaling pathways may provide new drug action sites or possibly even modifying neuropeptides to act as drugs themselves.

Adoption of neuropeptidomics in a clinical setting does still present challenges, however. As neurochemistry is incredibly complex, most studies are performed in organisms with simpler neuroendocrine systems, including some mammals like mice and rats, [148] and many invertebrates, such as crustaceans [149] and nematodes. [150] Translation from these models to humans is difficult and, at the very least, will require more sophisticated, less invasive sampling methods (e.g., microdialysis). Clinical research involving humans also has the added complexity of genetic diversity. Biomarker discovery and genetic risk score assessments have historically been biased, and more and more research is demonstrating the importance of clinical research that accounts for sex, age, and racial diversity. [151–158] Additionally, MS analyses of neuropeptides, while sensitive, accurate, and quantitative, may not offer high enough throughput for clinical applications. Multiplexing techniques, such as the aforementioned DiLeu tags, [8,56,159] can greatly improve throughput, but are not commercially available. Conversely, the tags that are commercially available (e.g., TMT) [55] are expensive and do not offer as high a degree of multiplexing. Furthermore, clinical applications could necessitate absolute quantitation, often requiring expensive isotopic peptide standards. The Li Lab has developed chemical tags that enable low cost, absolute quantitation. These isotopic DiLeu (iDiLeu) tags are used to create a calibration curve from a synthesized peptide and compare the target peptide to the calibration curve. [160] Methods like this still require synthetic peptides, albeit much cheaper as the isotopes are incorporated via chemical labeling, not during the

synthesis of peptides. When the iDiLeu tags are combined with multiplex isobaric tagging reagents, the throughput of absolute quantification can be greatly enhanced, which could be highly beneficial for absolute quantification and biomarker validation with large cohort of clinical specimens.

Advances in quantitation are often aided by chemical labeling methods, but improvements in sample analysis are other avenues to consider. As mass spectrometers become more sophisticated, neuropeptidomic analyses are greatly enhanced. For example, increased scan times enable greater depth of coverage, especially for low level analytes like neuropeptides. Increased resolving power can help differentiate between neuropeptides with similar masses, and can facilitate greater multiplexing, as seen by the incorporation of mass defects in DiLeu 12- and 21-plex tags. [56,159] Newer instruments are also capable of performing alternative fragmentation methods, such as ETD and EThcD, to provide more detailed MS2 data. This is especially beneficial for analysis of PTMs like glycosylation. [23] DIA experiments have also offered many improvements, especially for low concentration analytes that normally are missed by DDA settings (e.g., neuropeptides) [22], despite being a relatively new method. These benefits are certain to increase as DIA sees more popularity and data analysis/deconvolution software improves. Advances in MALDI-MS have given rise to instruments with high acquisition rates and decreased laser size to generate MSI data with high spatial resolution without requiring extra time. [161] Further improvements in instrumentation will undoubtedly enable more robust neuropeptidomic experiments.

We speculate the field of neuropeptidomics to continue thrive on its current trajectory, but with increased prevalence, informing many biological studies. This is largely due to recent

advances in quantitation and the many possibilities of its application for quantitative neuropeptidomics. As instrumentation enables faster analyses, either through increased scan times, or faster separation modes like capillary electrophoresis, [162] LFQ could see increased use as throughput improves. Conversely, these advances could also lead to an increase in the use of labeling techniques as instruments are able to resolve miniscule mass differences to enable more accurate and sensitive analyses of neuropeptides. Ultimately these methods will both see continued use depending on application, but it will be interesting to see how they are incorporated into larger experiments. As neuropeptides have profound impacts on many biochemical and physiological processes, their study will undoubtedly be important in larger proteomic and metabolomic experiments, and we predict an increase in multi-omic studies, even at the single-cell level with continued improvements in instrumentation and microscale sample preparation. Advances in neuropeptide analyses are also not limited to quantitation. Spatial distribution information gained from MSI can be used to characterize neuropeptide function, receptors, etc.[117] Additionally, ion mobility MS (IMMS) is routinely used to provide structural information that can be used to distinguish isobaric neuropeptides and better understand neuropeptide conformation and possibly functional roles dependent on its tertiary/quaternary structure. [95,163] As IM-MS and MSI methods are further developed, our understanding of neuropeptides will greatly improve. Combining the structural and quantitative aspects of mass spectrometry will provide richer characterization of neuropeptides, and thus has the power to lead to better biomarkers and drug design to help combat neurological disorders, obesity, and other common health concerns.

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All authors have substantially contributed to the writing and design of the review article. The original draft was written by C.S.S., A.P., and O.L.R., with revising and rewriting performed by L.L.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Figures:

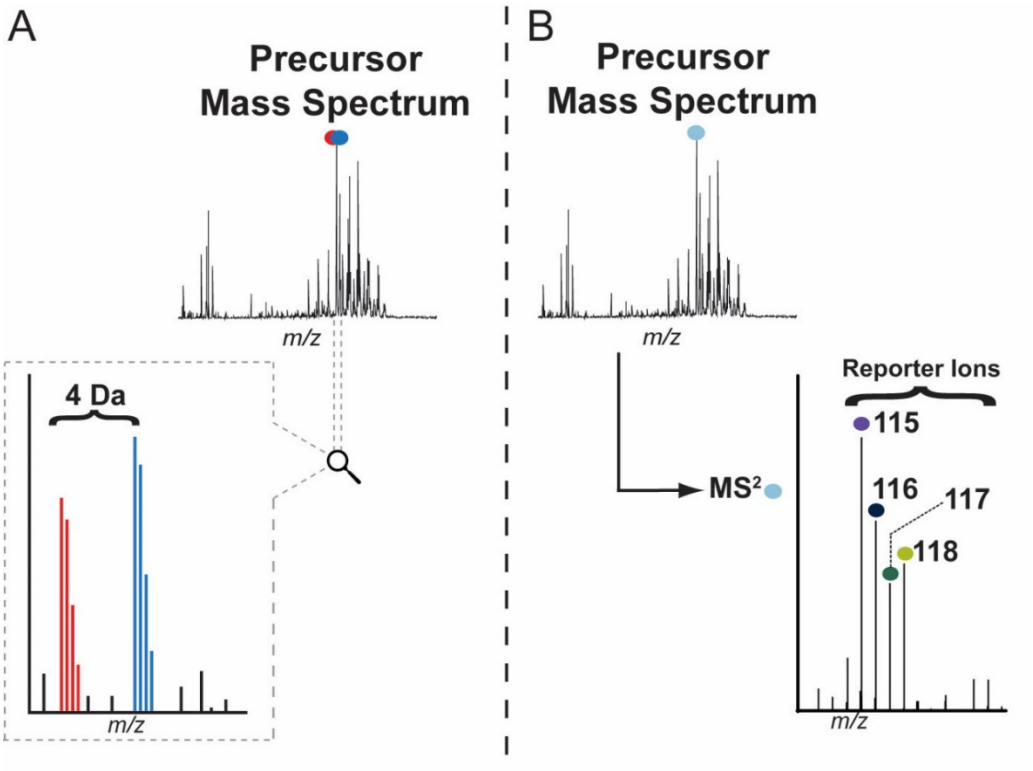

Figure 1: Comparison between isotopic (A) and isobaric (B) labeling. In isotopic labeling strategies, analytes are differentiated at the precursor mass level due to the incorporation of light and heavy tags. Isobaric workflows result in no differentiation at the precursor mass level, but upon fragmentation, unique reporter ions for each channel form, giving rise to quantitation based on relative intensities of reporter ions.

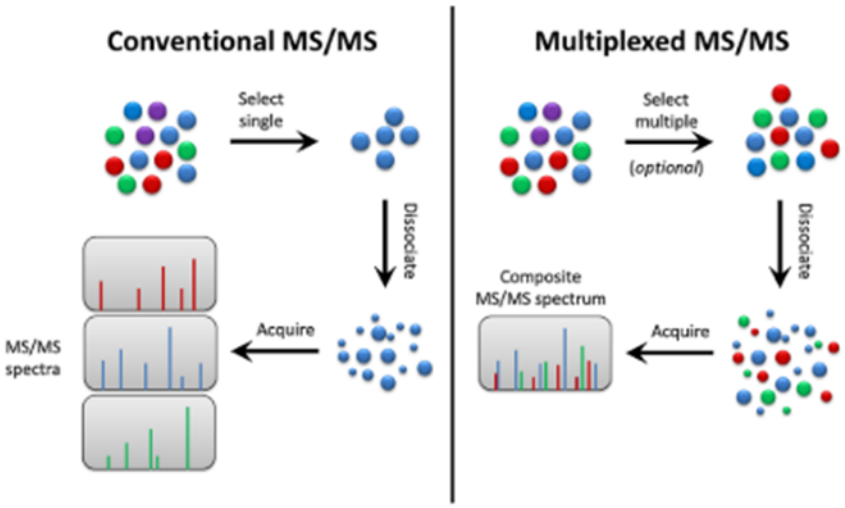

Figure 2: Depiction of differences between DDA and DIA. In conventional MS/MS (DDA), a single analyte is selected at a time for MS/MS. In multiplexed MS/MS (DIA), several analytes are selected for simultaneous MS/MS and the composite mass spectrum is deconvoluted during data analysis to discern constituent analytes. Figure taken with permission from Chapman et al. [58].

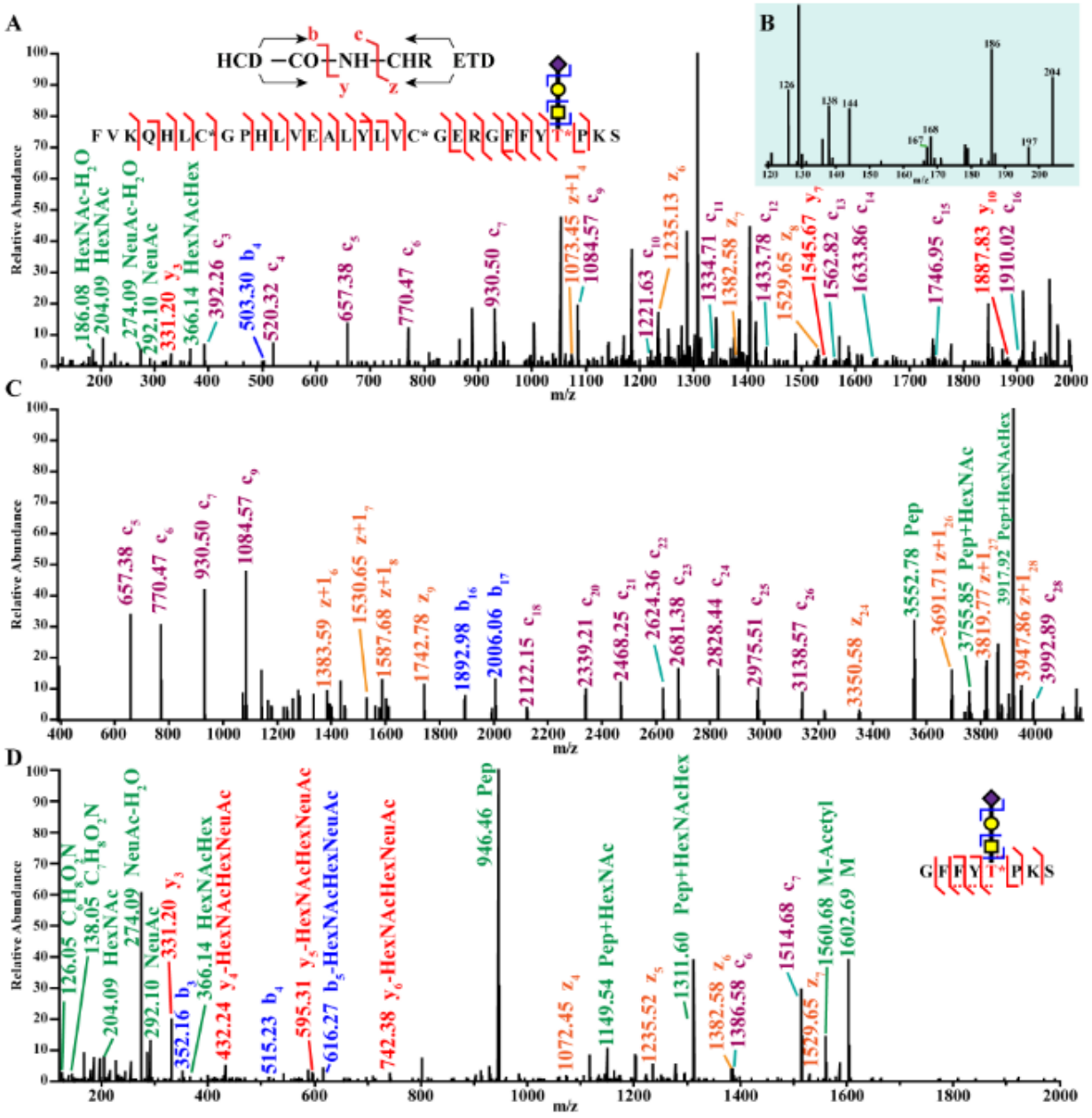

Figure 3: Sample spectra from mouse insulin. Using EThcD (A) and charge deconvolution (C) resulted in high sequence coverage due to production of both b/y-and c/z-ions. The spectrum in D highlights the glycan localization and the low mass region is shown enlarged in (B). Figure taken with permission from Yu, et al. [102].

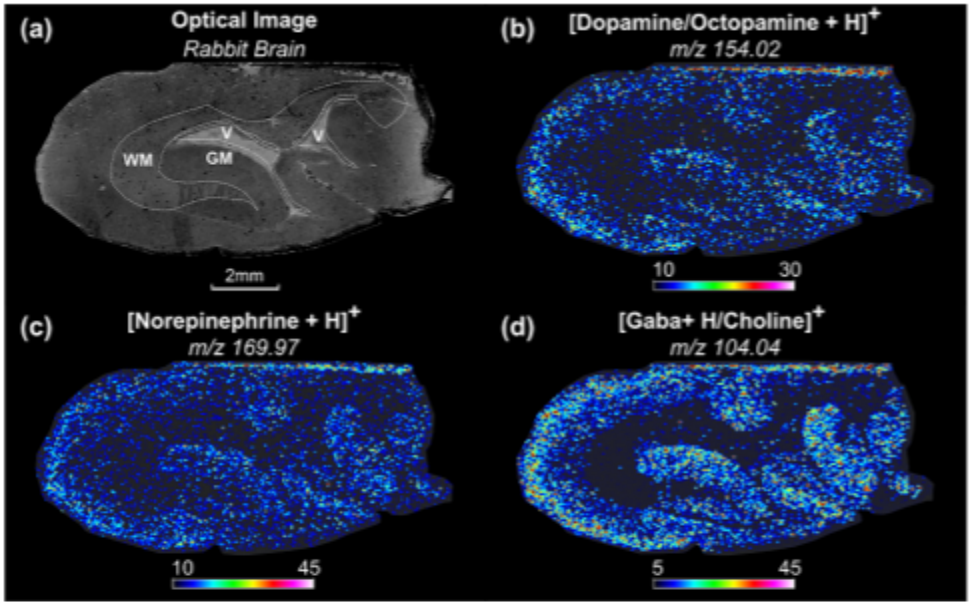

Figure 4: MSI enhancements from application of gold nanoparticles. Comparison of images from extracted neurotransmitters (b-d) to the optical image (a) shows differential expression based on grey/white matter regions of the brain. Image reprinted with permission from McLaughlin et al. [128]

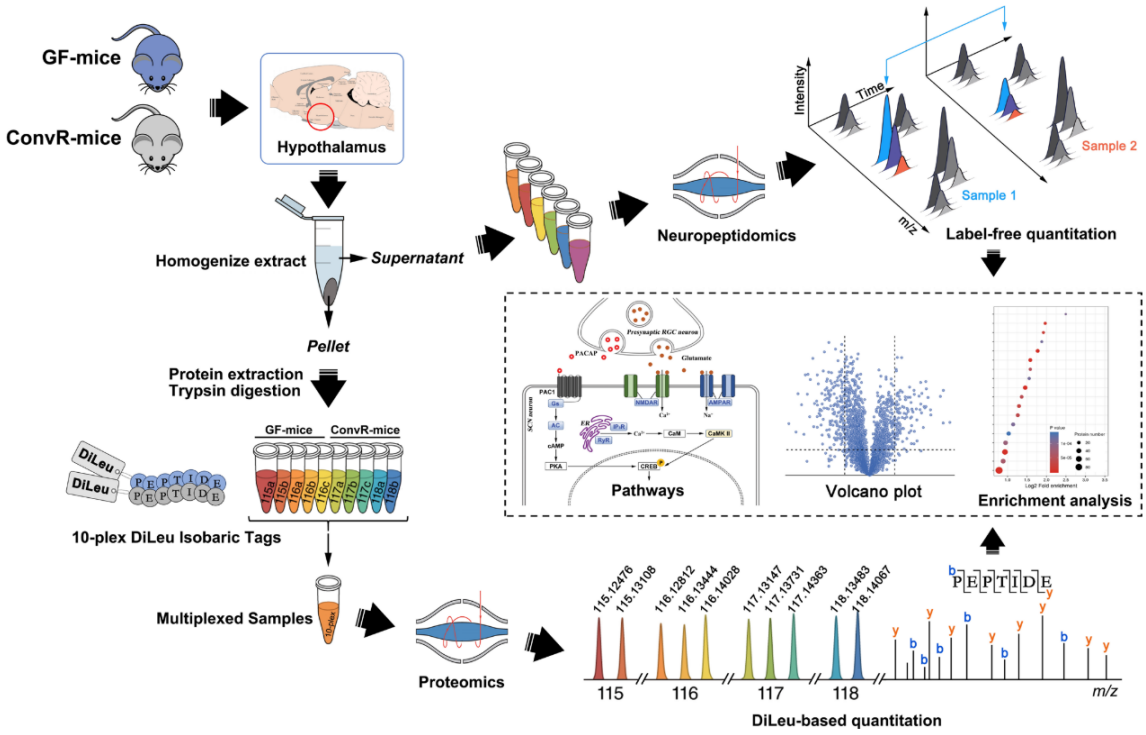

Figure 5: Multi-omic workflow for studying neuropeptides and proteins. Neuropeptides were extracted from the hypothalamus of germ-free (GF) and conventionally raised (ConvR) mice and analyzed using label-free quantification. Proteins were pelleted, digested, and differentially labeled with 10-plex DiLeu isobaric tags. The differentially labeled samples were pooled together and analyzed simultaneously to perform relative quantitation based on reporter ion intensities.

Chapter 3

Absolute Quantification of Amine Metabolites in Human Cerebrospinal Fluid via MS1-centric Isotopic N,N-dimethyl-Leucine (iDiLeu) Labeling

Adapted from: Riusech, O., Hao, L., Li, L. Absolute Quantification of Amine Metabolites in Human Cerebrospinal Fluid via MS1-centric Isotopic N,N-dimethyl-Leucine (iDiLeu) Labeling. Analytical Bioanalytical Chemistry. 2025.

Abstract

Quantitative measurement of metabolites is essential to understand biological and disease processes. Absolute quantification by spiking isotope-labeled internal standards and analyzing on mass spectrometry (MS) platform is a key method to determine the concentration of metabolites in biological samples. However, MS-based absolute quantification is often limited by the commercial availability and high costs of isotope labeled internal standards. Here, we establish an absolute quantification method for amine metabolites utilizing isotopic *N*,*N*-Dimethyl Leucine (iDiLeu) tagging on the LC-MS/MS platform. Absolute quantification of metabolites with excellent accuracy and precision can be achieved with five-plex iDiLeu labeling without the need of expensive isotope labeled internal standards. We demonstrated that iDiLeu labeling improved the separation and detection limits of polar metabolites. Particularly, detection limits for glycine, GABA, and serotonin have been improved by more than 20 folds, and valine by more than 2000 folds. With iDiLeu tagging, 87 amine-containing metabolites were identified and quantified in human cerebrospinal fluid (CSF) samples, revealing potential metabolic changes in Alzheimer's disease patients.

Keywords

iDiLeu, Metabolites, Absolute quantification, Mass Spectrometry, Human Cerebrospinal Fluid, Alzheimer's disease

Introduction

Quantitative analysis of small molecule metabolites is important to understand biological systems and disease processes [1]. Liquid chromatography coupled with mass spectrometry (LC-MS) has become a central technology for metabolite identification and quantification owing to its superior sensitivity, accuracy, reproducibility and dynamic range [2]. MS-based quantification can be categorized into relative and absolute quantifications. Relative quantification through label-free or chemical derivatization compares the relative abundances of metabolites in different biological states, which has been successfully applied to discover candidate metabolic biomarkers of various human diseases [3-5]. Absolute quantification determines the absolute concentration of metabolites, typically achieved with an isotopic analogue of the analyte (e.g., ^{2}H- or ^{13}C-analogues) spiked into samples as an internal standard to construct a calibration curve [6,7]. This technique has been a golden standard for targeted biomarker analysis and is often employed for measuring the concentration of biomarker candidates [4,8].

However, absolute quantification of metabolites is often limited by the commercial availability and high costs of isotopic internal standards. Alternative approaches have been developed in recent years via chemical derivatization, particularly stable isotope labeling. Stable heavy isotopes can be differentially incorporated into analytes to allow simultaneous comparison of multiple samples and the construction of calibration curves without the need of expensive isotopic internal standards. When derivatization is implemented, samples can be multiplexed, allowing the simultaneous analysis of various biological replicates for relative or absolute quantification of biomolecules. Derivatization-

assisted LC-MS analysis of small molecule metabolites has been widely adopted in biomarker discovery due to the saved instrument time and solvent usage [3,5,9,10]. LC-MS analysis is impacted by the incorporation of derivatization, where the retention times in RPLC-MS increase with commercially available tags in addition to the improved ionization of small molecules that has been shown to improve the limit of detection of these analytes. Quantification can be achieved by MS2-centric isobaric labeling or mass difference labeling on the MS1 level [11]. MS1-centric mass difference labeling is often more accurate since it is virtually free from precursor isolation interferences as with isobaric labeling [12]. Yet isobaric labeling provides greater multiplexing capability than mass difference labeling, which is an important feature in terms of constructing multi-point calibration curves.

To develop an MS1-centric mass difference labeling technique with greater multiplexing capability, our research group has designed and synthesized the 5-plex isotopic *N,N*-dimethyl leucine (iDiLeu) reagents for the absolute quantification of peptides/proteins [13]. Each iDiLeu reagent can be synthesized with a simple synthetic route, high yield, and greatly reduced cost compared to commercial mTRAQ labels [14]. The amine reactive group in iDiLeu label can target the N-terminus and lysine side chains of peptides which should also be applicable to amine-containing small molecules. We aim to expand the 5-plex iDiLeu labeling technique to the analysis of amine-containing metabolites in the current study (**Scheme 1**). Proof-of-principle experiments are conducted with metabolite standard mixture on a standard flow LC-MS platform which is routinely used for label-free metabolomics studies. We also applied the iDiLeu labeling

method to the absolute quantification of amine metabolites in human cerebrospinal fluid samples, where two iDiLeu channels can be used to label CSF samples and the other three channels can be used to label metabolite standards. Thus, three-point calibration curve and multiplexed quantification can be efficiently achieved in a single LC-MS run.

Experimental

Preparation of standards

Metabolite standard mixture was prepared using 14 equimolar metabolite standard stock solutions: glycine, alanine, GABA, creatinine, valine, isoleucine, leucine, lysine, methionine, dopamine, phenylalanine, serotonin, tyrosine, and tryptophan. The mixture was then serial diluted to construct label-free standard curves. Aliquots of the metabolite standard mixture were dried down and stored at -20 °C prior to derivatization.

Human cerebrospinal fluid sample preparation

Human CSF samples were collected from consenting human subjects in the Wisconsin Alzheimer's Disease Research Center (ADRC), approved by the University of Wisconsin Institutional Review Board. Each of 16 human subjects, 7 control and 7 stage 3 AD patients (4 males, 3 females in each group), underwent a 12-hour fast, followed by administration of local anesthesia and CSF collection via lumbar puncture. CSF samples were collected and dispensed into individual tubes, gently mixed, and centrifuged at 2000 g for 10 min. Metabolite-containing supernatant was collected, stored at -80 °C, and

thawed on ice before sample preparation. Further matrix simplification was performed using 3 kDa molecular weight cut-off (MWCO) ultracentrifugation (Millipore Amicon Ultra, MA). Forty microliters of each MWCO flowthrough were freeze dried and stored at -80 °C until labeling.

iDiLeu synthesis and labeling reactions

The synthesis procedure of five-plex iDiLeu reagents was performed as previously described [13]. Briefly, one milligram aliquot of each iDiLeu tag was vortexed with activation solution, yielding the tag's triazine ester form [13]. Tagging was then performed using the dried CSF metabolite samples or metabolite standards dissolved in 0.5 M triethylammonium bicarbonate (TEAB) solution and labeled with an excess of activated iDiLeu. Anhydrous DMF was added to reach 70% of organic: aqueous ratio and the labeling reaction was maintained for 2 hours with vortexing at room temperature. The labeling reaction was quenched for 5 minutes with 0.25% hydroxylamine (v/v), and each labeled sample was dried in *vacuo* separately and combined afterwards.

LC-ESI-MS analysis

Ultra-performance LC-MS analysis was conducted using a Dionex UltiMate 3000 LC system coupled with a Q-Exactive™ Orbitrap mass spectrometer (San Jose, CA). A Phenomenex C18 column (2.1 × 100 mm, 1.7 µm, 100 Å) was used for metabolite separation at a column temperature of 30 °C and a flow rate of 0.3 ml/min. Mobile phase A was 0.1% formic acid in optima water and mobile phase B was 0.1% formic acid in optima acetonitrile.

For label-free metabolite samples, a 15 min gradient was set as the following: 0-5 min, 0.5-3% solvent B; 5-10 min, 3-20% B; 10-12 min, 20-80% B; 12-13min, 80% B; 13-15 min, 0.5% B. Full MS was acquired in selected ion mode (SIM) with an inclusion list of fourteen standards at a resolution of 70 K, an automatic gain control (AGC) of 1×10^6 and a maximum injection time (IT) of 100 ms.

For iDiLeu labeled metabolite samples, a 23 min gradient was set up as the following: 0-5 min, 3% solvent B; 5-16 min, 3-30% B; 16-17 min, 30-80% B; 17-19min, 80% B; 19-23 min, 3% B. The first 5 min of LC flow was diverted to waste to remove impurities. Full MS scanned from *m/z* 200-1000 at a resolution of 70 K, an AGC of 1×10^6 and a maximum IT of 100 ms.

Data analysis

Raw data files were acquired by Thermo Scientific Xcalibur software and then uploaded to SIEVE™ software for data processing. Peak alignment and framing algorithm were selected with a frame time width of 2 min and *m/z* width of 5 ppm. The maximum retention time shift was 0.2 min for peak alignment. ICIS algorithm was used for peak detection. A database lookup file (.csv) was created containing the accurate masses and names of 14 metabolite standards. Targeted quantification and identification of metabolites was achieved in SIEVE using the database lookup file with a mass tolerance of 5 ppm. For human CSF samples, the complete component table was downloaded, and the molecular weight of each compound was calculated based on the charge and mass shift caused by iDiLeu labeling in an Excel sheet. MetaboSearch software was then used for

metabolite identification (mass error < 5 ppm) by searching against multiple online databases: Human Metabolome Database (HMDB), Madison Metabolomics Consortium Database (MMCD), Metlin, and LIPID MAPS [15]. Five channels of iDiLeu labeled metabolite samples were separately analyzed by LC-MS to calculate the purity of each iDiLeu label, and purity correction was performed in an Excel sheet following a previously described method [16]. The resulting list of metabolites was then manually examined to only include primary and secondary amine-containing molecules.

Results and discussion

Comparing iDiLeu labeling and label-free metabolite analyses

Five-plex iDiLeu labeling was originally designed for protein/peptide quantification as MS1-centric mass difference reagents [13]. For the first time, we expanded this technique to the analysis of amine-containing metabolites. The general structures of activated iDiLeu reagents are shown in **Scheme 1**. After the labeling reaction, mass shifts of 141.1154, 144.1313, 147.1409, 150.1631, and 153.1644 were introduced into metabolites by the d0, d3, d6, d9, and d12 labels. These mass shifts serve to make metabolites of interest more hydrophobic, increasing their retention time and ionization efficiency [17]. First, a mixture of 14 metabolite standards was used to characterize the performance of iDiLeu labeling. The accurate masses before and after labeling are listed in **Table 1**. Peak areas of extracted ion chromatograms (EIC) of the labeled metabolites were used for quantification. Since metabolomics analysis is typically performed on a standard

flow LC system, here, we aimed to evaluate the performance of iDiLeu labeled metabolites on a standard flow RPLC-MS platform and its comparison with the label-free approach.

Both label-free and d0-labeled metabolite standard mixture were analyzed on a RPLC-MS for comparison. The chromatographic separations are illustrated in **Figure 1**. Free metabolites eluted within the first few minutes. Particularly, small polar metabolites like glycine, alanine, GABA, lysine, and creatinine were barely retained on the column and eluted before 1 min. Free isoleucine and leucine were not completely separated in this label-free platform (**Figure 1A**). Optimization of LC gradient was not helpful to separate these early eluted metabolites. After iDiLeu labeling, polar metabolites can be better retained on the RPLC column and well separated from each other with modified hydrophobicity and increased molecular size (**Figure 1B**). Isomers like leucine and isoleucine were completely separated with retention times of 9.7 min and 10.7 min, respectively.

To compare the detection sensitivity for iDiLeu labeling vs. label-free approach, metabolite samples before and after iDiLeu labeling were serially diluted to construct calibration curves and determine their limits of detection. The results are listed in **Table 2**. Limits of detection and of quantitation (LODs and LOQs) of most metabolite standards were greatly improved after iDiLeu (d0) labeling. Particularly, detection limits for glycine, GABA, and serotonin have been improved by more than 20 folds, and valine by more than 2000 folds. Although the improvements of metabolite detection sensitivity for some metabolites were not as significant as using nanoLC-MS or CE-MS system, the present results demonstrated the utility of iDiLeu labeling on the routine standard flow RPLC-MS

platform with the advantages of better metabolite separation and creating calibration curves in a single run [13,17,18]. More importantly, absolute quantification of metabolites can be achieved by iDiLeu labeling without the need of expensive stable isotope labeled internal standards.

Evaluating the quantification accuracy of iDiLeu labeling

Five-plex iDiLeu labeled metabolites created five distinct peaks, differing from one another by 3 Da, making them easily capable of isolation from other labeled products without isotopic interference. An example iDiLeu labeled serotonin standard was illustrated in **Figure 2**. Deuterium effect was minimized by grouping deuterium atoms around polar amine group of leucine, so that five labeled peaks can elute at roughly the same time [13]. EIC peak areas of five isotopic forms of the same labeled metabolite can be compared for quantification. Quantitative accuracy of iDiLeu labeling was demonstrated by labeling 14 metabolite standards and mixing five channels at theoretical 1:1:1:1:1 and 1:2:5:8:10 ratios. As shown in **Figure 3**, satisfactory accuracies were achieved with an average accuracy < 12% error. Median ratios measured among 14 metabolites (three replicates each) were 1:1.05:0.98:1.02:1.10 and 1:2.06:4.49:7.11:9.64, respectively.

Absolute quantification of a molecule is typically achieved by constructing a calibration curve where quantification accuracy is highly dependent on the curve's linearity (also called correlation coefficient). The linearity of iDiLeu-based quantification was characterized by two approaches. Firstly, iDiLeu labeled metabolite standards were

serially diluted and calibration curves were constructed across multiple LC-MS runs; Alternatively, five channels of iDiLeu labeled metabolite standards were mixed at different ratios across two orders of magnitude, and calibration curves were established in a single LC-MS run. We compared these two methods with the label-free approach and the correlation coefficients of 14 metabolites standards are provided in **Table 2**. The linearity using serially diluted labeled metabolites was comparable with free metabolite standards (R> 0.99) except for creatinine, while the single-run calibration curves demonstrated the best correlation coefficient (R>0.999) because of the ability to generate calibration curves in a single LC-MS injection to avoid run-to-run variations.

Labeling amine metabolites in human CSF samples with iDiLeu reagents

CSF is a valuable biofluid sample circulating within the brain ventricular system. It protects the brain and maintains the metabolic homeostasis of the central nervous system [19,20]. Amino acids in CSF samples serve as the basic building blocks and key regulators in CSF metabolism, which has been found to be involved in various neurological diseases, such as Alzheimer's disease and Parkinson's disease [21,22]. The established absolute quantification method using iDiLeu labeling was applied to determine the concentrations of amine-containing metabolites in CSF samples. By using three channels of iDiLeu to label standards and the remaining channels to label two human CSF samples, we constructed a three-point calibration curve and determine the metabolite concentration in two samples in a single MS scan. Because of the improved chromatographic separation of iDiLeu labeled metabolites, absolute quantifications of many amine metabolites can be achieved in a single LC-MS run. To minimize the influence of variations in the five iDiLeu

tags, we carefully designed the experiment to rotate different iDiLeu channels for the labeling of standards and CSF samples (**Supplemental Table S1**). Eight labeling experiments were conducted for the measurement of 16 human CSF samples.

Using our iDiLeu labeling strategy, a total of 87 amine-containing metabolites were identified from human CSF samples (**Supplemental Table S2**). We then matched the metabolites with the human CSF metabolome database and found that 47 of them are documented in the database and 40 additional amine metabolites were identified in our study [20]. Since iDiLeu tags can only target primary and secondary amine groups, the confidence of metabolite identification was also enhanced by the presence of five isotopic peaks. We then compared the concentration of amino metabolites in CSF samples from Alzheimer's disease patients and control. As shown in **Figure 4**, concentrations of the example measured molecules (lysine, tryptophan, valine, phenylalanine, tyrosine, methionine, creatinine, alanine, isoleucine, and leucine) ranged from 5.16 to 109.09 μM. As expected, Creatinine was found to be the most abundant compound with an average concentration of 109.09 ± 30.77 μM. Example calibration curves were shown in **Supplemental Figure S1**. Additionally, we also revealed potential sex-dependent metabolite regulations in AD patients. For example, leucine was only decreased in female AD patients but not male patients. Tyrosine was only increased in male AD patients but not female patients. Although the connection between branched chain amino acids and AD has been explored, there is limited evidence of how these metabolites behave in CSF. Leucine, isoleucine, and valine treatment has been reported to yield a lower incidence of dementia and AD in mouse models compared to control with a higher concentration of the

three in the blood and a more significant change in the female mouse model [23]. The tyrosine-dopamine metabolism has also been implicated in past studies for AD research, however there is limited evidence that this pathway might be sex-linked [23]. Worth noting that given the small sample cohort, the biological findings need to be validated by future studies with larger sample size.

Conclusions

To summarize, an accurate, cost-effective, and high-throughput absolute quantification strategy was developed for amine-containing metabolites using custom 5-plex iDiLeu labeling. The labeling technique was characterized on a standard flow RPLC-MS platform for the first time and achieved enhanced metabolite separation and detection sensitivity compared with routine label-free approach. Accurate absolute quantification was achieved on the MS1 level with the ability to construct calibration curves in a single LC-MS run. We then successfully applied this method to the identification and quantification of amine metabolites in human CSF samples. We believe that iDiLeu labeling based absolute quantification will be a useful tool to measure the concentration of amine metabolites in biological samples, serving as a promising validation platform for future disease biomarker studies.

ASSOCIATED CONTENT

Supporting Information:

Supplemental Figure S1. Example calibration curves for the absolute quantification of CSF metabolites using iDiLeu labeling.

Supplemental Table S1. Experimental design for the absolute quantification of amine metabolites in 16 human CSF samples using iDiLeu labeling.

Supplemental Table S2. The list of detected amine metabolites in human CSF samples.

DECLARATIONS

Author Contribution: L.L. and L.H. conceptualization and methodology. O.R. and L.H. experiment, investigation, data analysis, and writing. L.L. review, editing, supervision.

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Notes:

The authors declare no conflict of interests.

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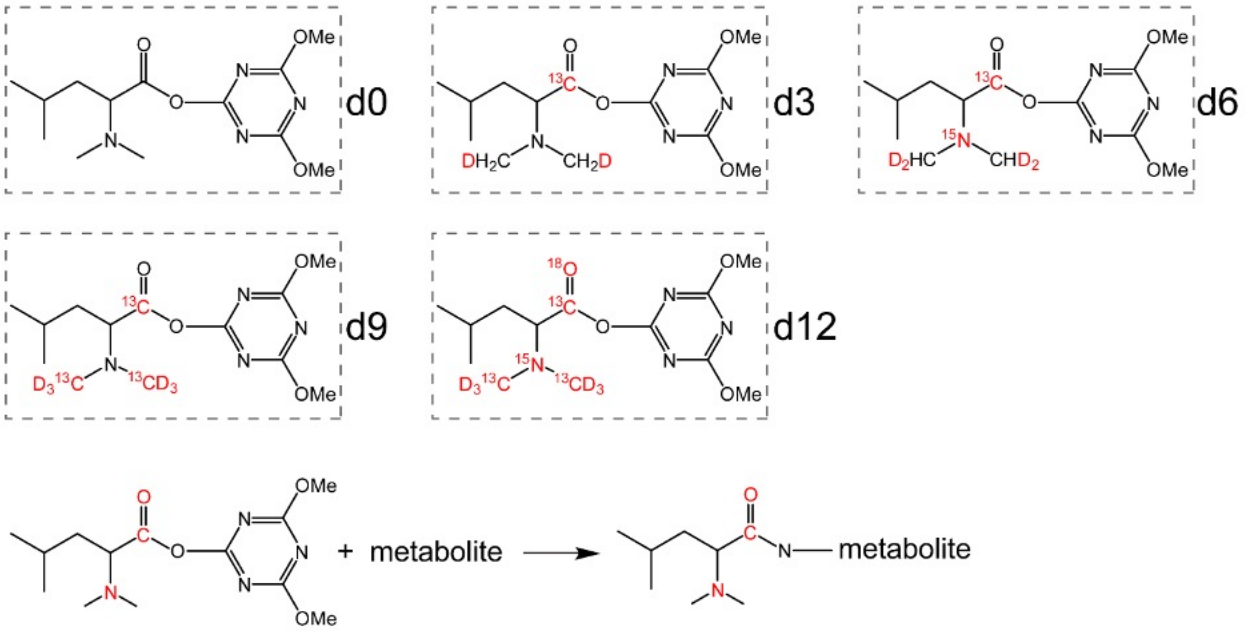
Figures and Tables



Scheme 1 General structures of iDiLeu reagents (d0, d3, d6, d9, and d12) in their activated triazine ester forms, and the reaction to label amine-containing metabolites.

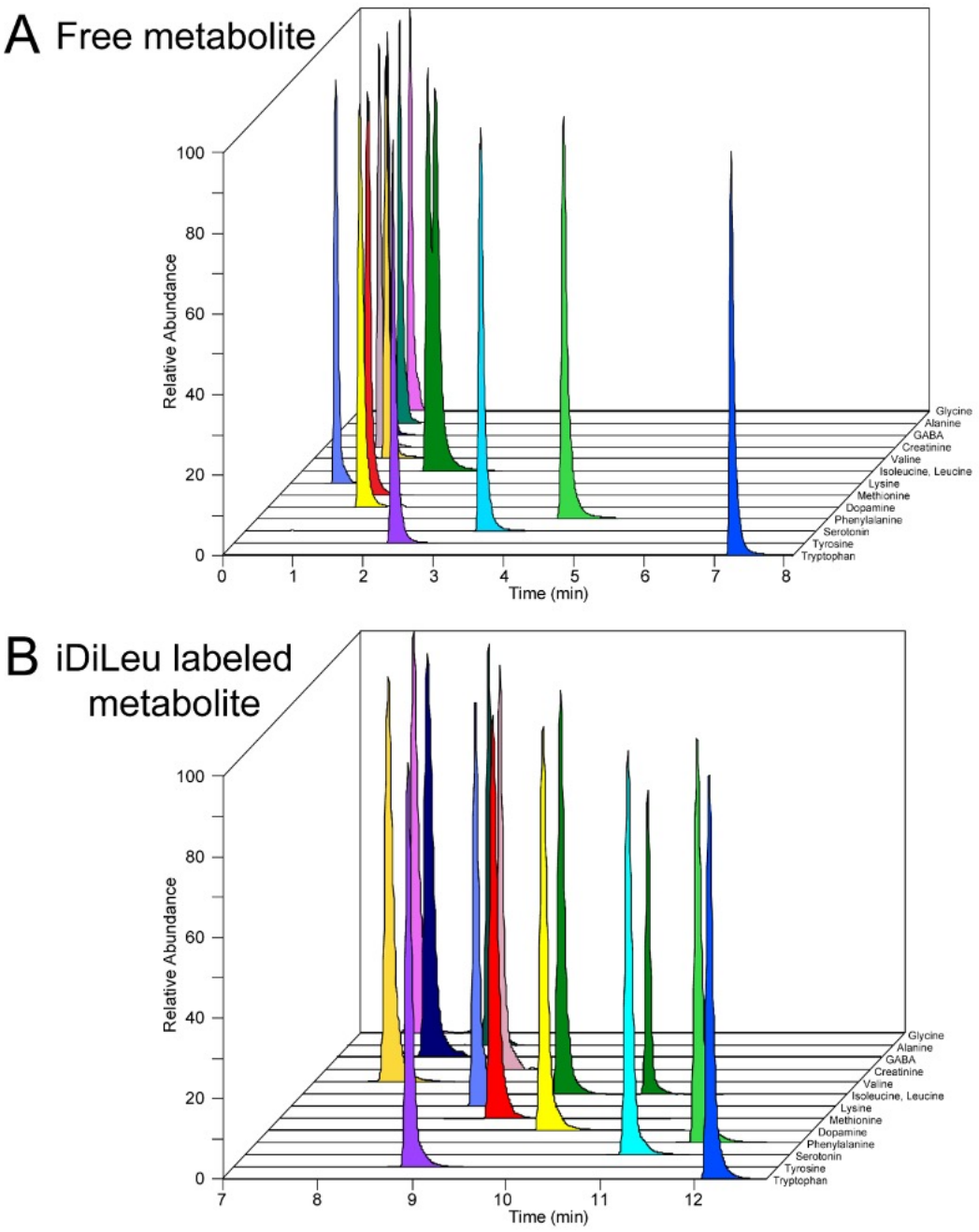


Fig. 1 Chromatographic comparison of label-free metabolites (A) and iDiLeu (d0) labeled metabolite standards (B) in standard flow UPLC-MS platform.

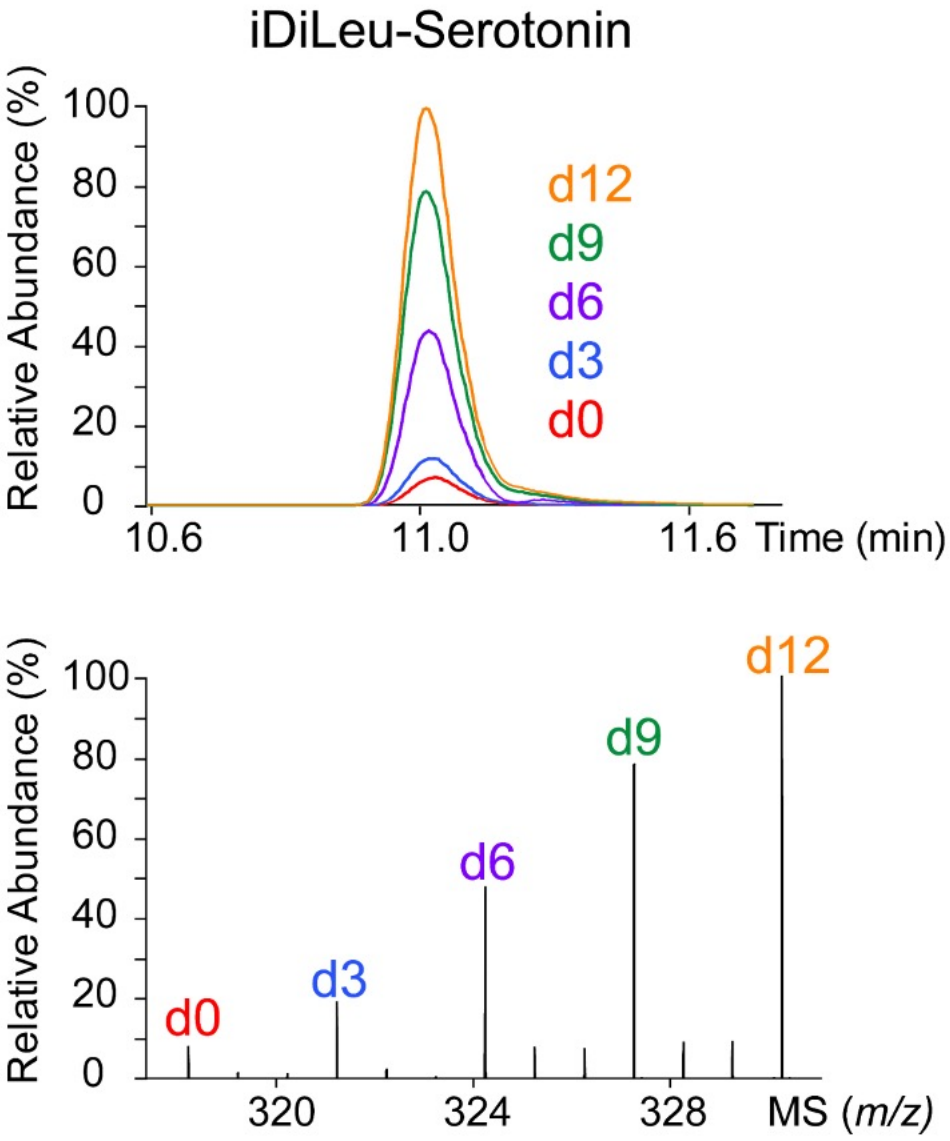


Fig. 2 Example chromatogram and MS spectrum of 5-plex iDiLeu labeled serotonin standard. Five-plex iDiLeu labeled serotonin samples were mixed at 1:2:5:8:10 ratio.

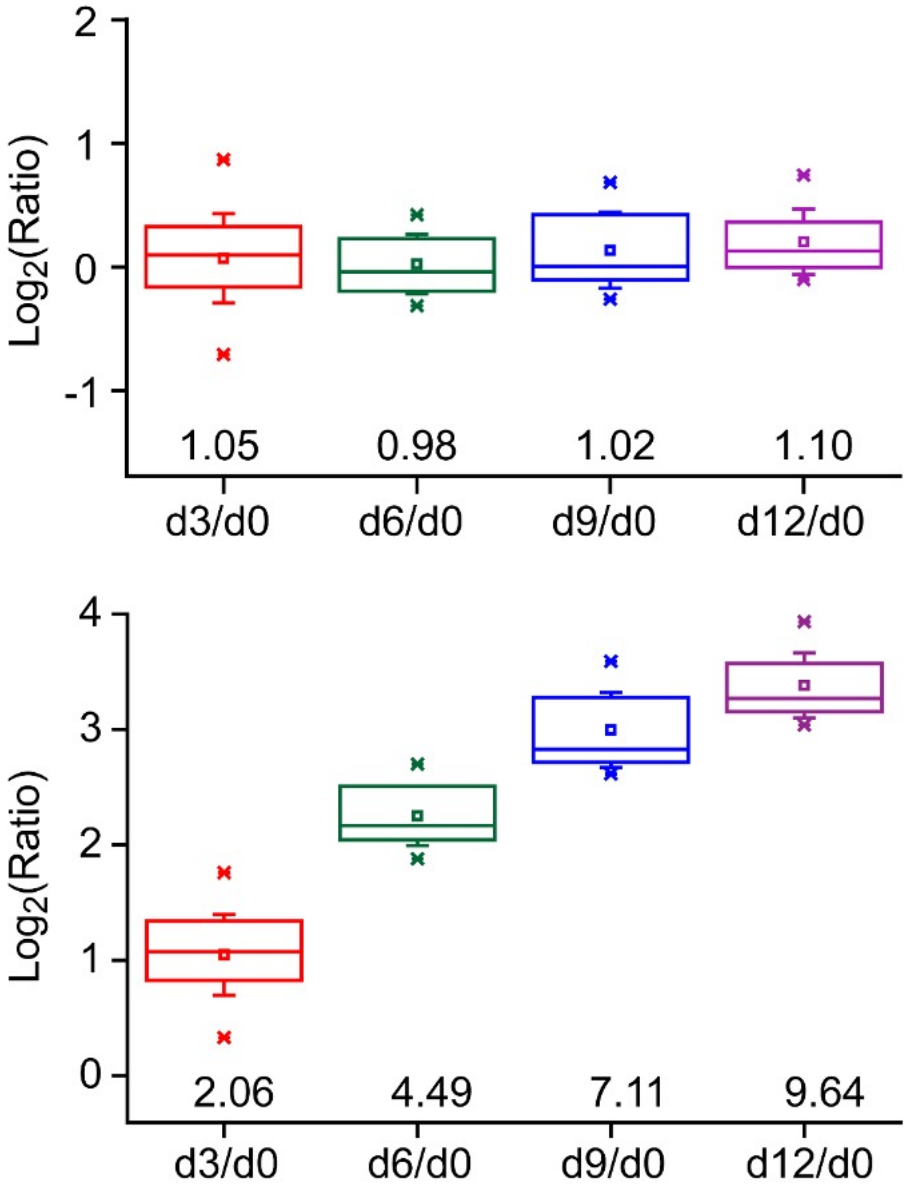


Fig. 3 Quantification accuracy of iDiLeu labeling; each box contains results from 14 metabolite standards labeled by iDiLeu where the box denotes 25th and 75th percentiles, the line within the box denotes 50th percentile, and the whisker denotes standard deviation.

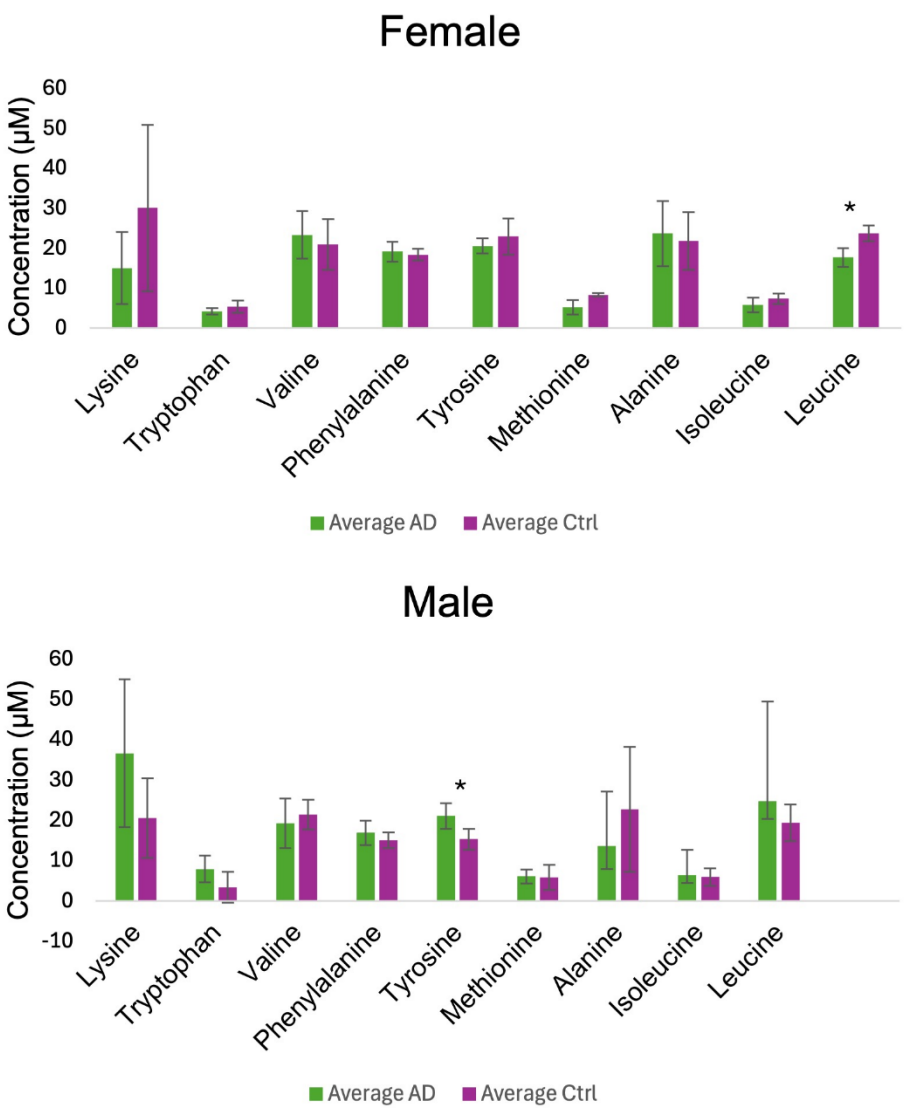


Fig. 4 Example quantification of amino acids in cerebrospinal fluids from female and male AD patients and controls. Three biological replicates were used for female AD and control subjects. Four biological replicates were used for male AD and control subjects. Statistical analysis was conducted by the Student's t-test with * denotes statistically significance ($p<0.05$).

Graphical Abstract

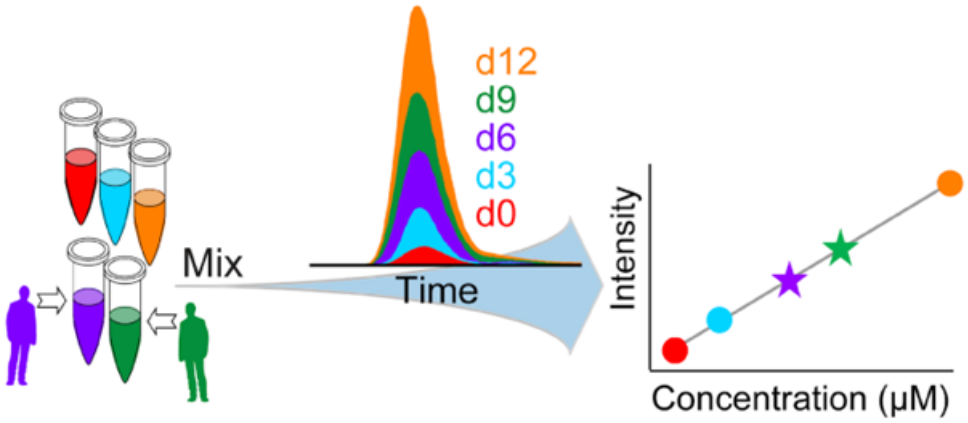


Table 1 Accurate masses of 14 metabolite standards before and after iDiLeu labeling

Metabolite	$[M+H]^+$	d0 labeled	d3 labeled	d6 labeled	d9 labeled	d12 labeled
Glycine	76.03983	217.1552	220.1711	223.1807	226.2029	229.2042
Alanine	90.05553	231.1709	234.1868	237.1964	240.2186	243.2199
GABA	104.0711	245.1865	248.2024	251.212	254.2342	257.2355
Creatinine	114.0667	255.1821	258.198	261.2076	264.2298	267.2311
Valine	118.0868	259.2022	262.2181	265.2277	268.2499	271.2512
Isoleucine	132.1024	273.2178	276.2337	279.2433	282.2655	285.2668
Leucine	132.1024	273.2178	276.2337	279.2433	282.2655	285.2668
Lysine	147.1133	429.3441	435.3759	441.3951	447.4395	453.4421
Methionine	150.0588	291.1742	294.1901	297.1997	300.2219	303.2232
Dopamine	154.0868	295.2022	298.2181	301.2277	304.2499	307.2512
Phenylalanine	166.0868	307.2022	310.2181	313.2277	316.2499	319.2512
Serotonin	177.1028	318.2182	321.2341	324.2437	327.2659	330.2672
Tyrosine	182.0817	323.1971	326.213	329.2226	332.2448	335.2461
Tryptophan	205.0977	346.2131	349.229	352.2386	355.2608	358.2621

Table 2 Retention time, sensitivity, and linearity (calibration curves) of metabolite analysis with iDiLeu labeling and label-free approaches

iDiLeu Labeling						Label-Free			
Metabolite	Time[a]	LOD[b]	LOQ[c]	R (d0)[d]	R (5-plex)[e]	Time	LOD	LOQ	R
Glycine	7.5	4.20E-04	7.00E-04	0.994 8	0.9996	0.72	9.53E-03	1.59E-02	0.999 4
Alanine	8.5	2.17E-03	3.62E-03	0.994 5	0.999	0.7	3.45E-03	5.75E-03	0.998 8
GABA	7.9	5.06E-05	8.43E-05	0.999 1	0.9993	0.71	1.27E-03	2.12E-03	0.999 7
Creatinine	8.8	1.13E-04	1.88E-04	0.975 7	0.9998	0.74	1.20E-04	2.00E-04	0.996 6
Valine	7.7	5.23E-05	8.72E-05	0.996 8	0.9999	1.02	1.09E-01	1.82E-01	0.999 8
Isoleucine	9.7	1.15E-04	1.92E-04	0.980 7	0.9999	1.79	9.63E-04	1.61E-03	0.999 5
Leucine	10.7	6.93E-05	1.16E-04	0.995 6	0.9999	1.82	6.04E-04	1.01E-03	0.999 7
Lysine	8.9	5.06E-05	8.43E-05	0.999 5	0.9998	0.65	4.49E-04	7.48E-04	0.999 8

Methionine	9.2	4.74E-05	7.90E-05	0.9969	0.9998	1.26	1.77E-04	2.95E-04	0.9999
Dopamine	9.9	1.20E-05	2.00E-05	0.9913	0.9991	1.34	4.62E-05	7.70E-05	0.9999
Phenylalanine	11.6	1.43E-03	2.38E-03	0.9985	0.9999	4.31	7.74E-05	1.29E-04	0.9998
Serotonin	11	4.16E-05	6.93E-05	0.9985	0.9991	3.24	9.51E-04	1.59E-03	0.9996
Tyrosine	8.8	4.98E-05	8.30E-05	0.9968	0.9999	2.26	3.33E-04	5.55E-04	0.9999
Tryptophan	12.1	1.18E-04	1.97E-04	0.9887	0.9999	7.34	4.29E-04	7.15E-04	0.9995

[a]Retention Time, min

[b]Limit of detection, μM

[c]Limit of quantification, μM

[d]Linearity of calibration curve generated by serial dilution of iDiLeu d0-labeled metabolite

[e]Linearity of calibration curve generated by five plex iDiLeu in a single LC-MS run

Chapter 4

iDiLeu-Based Mass Spectrometric Quantification of Metabolites Following Copper Exposure

Abstract

Crustaceans are particularly sensitive to copper toxicity, and although the downstream effects of increased copper exposure on the metabolome are often postulated and observed, they are rarely measured. To perform absolute quantification of hydrophilic small-molecule metabolites in the hemolymph of the crustacean Cancer *borealis*, we derivatized targeted metabolites related to copper toxicity using in-house developed isotopic *N*,*N*-dimethyl leucine (iDiLeu) tags. Selected analytes were pooled at previously determined concentrations to serve as internal standards, and a calibration curve was generated. Sample loss was minimized by optimizing derivatization-assisted sample cleanup using dispersive liquid-liquid microextraction (DLLME) and hydrophilic lipophilic balance (HLB). Calibration curves were then used for the absolute quantification of metabolites of interest following 30-minute, 1-hour, and 2-hour exposures to 10 µM $CuCl_2$. We found that glutamic acid and taurine were downregulated after 2 hours of copper exposure, which may disrupt cellular metabolism and increase oxidative stress in crustaceans. These changes could have significant impacts on crustacean populations and the ecosystems they support.

Introduction

Ocean acidification, caused by the absorption of excess atmospheric carbon dioxide (CO_2) by seawater, is a major environmental threat that has been shown to increase the bioavailability of copper (Cu) in marine environments [1]. It is currently predicted that a drop in ocean pH of 0.3 will double the proportion of dissolved copper present as the free metal ion Cu^{2+} -- the most bioavailable form of copper [1]. This copper ion is a potent toxin that can have a range of negative effects on marine organisms. Copper is a micronutrient responsible for coordinating protein structures, facilitating oxygen transport, and maintaining red blood cells, nerve cells, and the immune system [2]. However, copper

homeostasis is delicate; excess copper introduced into the environment through increased ocean acidification, industrial waste, and urban runoff can lead to its accumulation in marine species [3-6].

Dysregulated copper homeostasis has been linked to various neurological conditions, such as Alzheimer's Disease (AD) and Wilson disease [7]. While the role of copper in the pathogenesis of these diseases remains unclear, the ability of copper ions to generate reactive oxygen species (ROS) and hydroxyl radicals is of growing interest. A hallmark of AD is the formation of amyloid-beta plaques, which require robust metal ion interactions. It has been suggested that these copper-containing plaques catalyze the formation of ROS, ultimately leading to oxidative damage [8]. The discovery and quantification of potential biomarkers of copper toxicity are crucial for understanding the links between copper dysregulation and these genetic neurodegenerative conditions.

Small molecule quantification provides distinct insights into cellular and enzymatic activities, making these molecules strong candidate for potential biomarkers. Mass spectrometry (MS) offers an ideal platform for biomarker discovery due to its inherent quantitative capabilities and its ability to analyze multiple molecules of interest within a specific *m/z* range in a single run [9]. Many common small-molecule metabolites such as amino acids, neurotransmitters, di- and tripeptides, and certain lipids fall under the category of small hydrophilic molecules. This class of analytes is challenging to quantify using MS alone due to discrepancies in their ionization efficiencies: more hydrophilic metabolites are known to ionize poorly, while more hydrophobic metabolites ionize more readily. This results in higher limits of detection for hydrophilic metabolites and reduced MS sensitivity [10,11]. In addition to poor ionization, the hydrophilic nature of these metabolites leads to excessive sample loss during sample preparation techniques such as desalting, and to low retention times during reversed-phase liquid chromatographic (LC) analysis [12-14].

To improve retention time and ionization efficiency, chemical derivatization is often employed in small molecule MS analysis [15]. Various derivatization labels exist, including TMT and iTRAQ -- two

commercially available tags with high multiplexing capabilities that enable high-throughput sample analysis while simultaneously amplifying isobaric signals. Alternatively, our in-house-designed 5-plex isotopic *N,N*-dimethyl leucine (iDiLeu) tags offer a more cost-effective and quantitatively accurate method for performing absolute quantification of amine-containing compounds [16,17]. However, these derivatization methods require additional matrix simplification, which may lead to further sample loss.

An approach often reported to enhance small-molecule sample analysis is a hybridized liquid-liquid extraction combined with solid-phase extraction, which serves to remove tagging byproducts, salts, and high-abundance lipids [18,19]. Dispersive liquid-liquid microextraction (DLLME) is a technique in which an aqueous sample is rapidly injected with organic solvents, resulting in a subtle biphasic separation [18,20,21]. It is used to isolate small hydrophilic molecules from complex matrices such as blood or serum. Various combinations of DLLME and derivatization exist: some methods implement tagging prior to DLLME, others tag during/after DLLME, and some omit tagging altogether. A variety of solid-phase extraction methods are also available, including C18, strong cation exchange (SCX), and hydrophilic-lipophilic balance (HLB). Previous studies have utilized C18 and SCX after derivatization to leverage the increased hydrophobicity for small-molecule enrichment; however, the efficiency of this enrichment has not been formally evaluated.

Here, we explore matrix simplification methods for the enrichment of iDiLeu-derivatized hydrophilic small-molecule metabolites. We then apply iDiLeu tagging to crustacean hemolymph from Jonah crab samples for the absolute quantification of copper-related metabolites: glycine, gamma-aminobutyric acid (GABA), glutamic acid, dopamine, and glutathione (GSH) -- five metabolites associated with copper metabolism. Using an optimized workflow, we perform derivatization-assisted matrix simplification. Tagged internal standards were used to implement a 3-point calibration curve, reserving two channels for samples and enabling the simultaneous quantification of copper-exposed and control crab

hemolymph. We also perform label-free analysis to discover novel metabolites related to copper toxicity.

2. Methods

2.1 Materials

Methanol (MeOH), acetonitrile (ACN), glacial acetic acid, and LC-MS solvents were purchased from Fisher Scientific (Pittsburgh, PA). Triethylammonium bicarbonate (TEAB), *N*,*N*-dimethylformamide (DMF), 4-(4,6-dimethoxy- 1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM), chloroform-d, and copper(II) chloride were purchased from Sigma-Aldrich (St. Louis, MO). *N*-methylmorpholine (NMM) was purchased from TCI America, (Tokyo, Japan). C18 and strong cation exchange (SCX) ZipTips were purchased from Millipore (Burlington, MA). Hydrophilic lipophilic balance (HLB) columns (3cc) were purchased from Waters (Milford, MA).

2.2 Derivatization

1 mg aliquots of iDiLeu tags were obtained and dried down during activation solution preparation. Activation solution consisting of 14.08 mg DMTMM BF_4, 495 µL dimethyl formamide, and 4.72 µL N-methyl morpholine was prepared and 50 µL of which was used to resuspend dry aliquots of iDiLeu tags. After vortexing for 1 hour, 35.71 µL of activated iDiLeu tag was added to internal standard or 500 µg hemolymph previously reconstituted in 53.57 µL 0.25 M TEAB in 50% acetonitrile. Samples were tagged via vortex for 1 hour at room temperature at a 1:1 m/m ratio, then quenched with 4.70 µL 5% NH_2OH for 5 minutes while vortexing. Tagged samples were dried down and stored at -80 C.

2.3 Evaluating sample loss in SPE methods

C18 zip tips, SCX zip tips, and OASIS HLB columns were used for iDiLeu tag removal from aliquots of iDiLeu tagged GABA. For C18 matrix simplification, zip tips were wetted using 50% ACN and equilibrated in 0.1% FA before loading iDiLeu tagged GABA in 0.1% FA. Sample was then washed using 0.1% FA and eluted in water, 50% MeOH, and 100% MeOH. Elute fractions were combined. SCX was achieved

similarly; loading samples in 0.1% FA, washing in 0.1% FA, and eluting in 5% NH_3H_2O, 30% MeOH as previously reported. Samples desalted with HLB were resuspended in 1 mL 0.1% formic acid (FA) then pipetted onto columns previously conditioned with 1 mL MeOH and 1 mL water. Samples were subsequently rinsed with 2 mL water and eluted in 1 mL MeOH, then dried down and stored at -80 C until LC MS/MS analysis. The flowthrough from load, wash, and elute steps from all three methods were collected and analyzed via direct infusion.

2.4 Optimizing DLLME workflow

9 aliquots of 500 µg hemolymph were used to determine if derivatization coupled with optimized desalting method would yield the highest signal in the five metabolites of interest or if DLLME could be performed before or after derivatization to enrich these small molecule metabolites.

DLLME protocol was completed as described: samples were resuspended in 200 µL optima grade water and rapidly injected with 200 µL 1:10 chloroform/acetonitrile via syringe. Samples were then vortexed for 2 minutes and centrifuged for 5 minutes at 4.5 x 1000 rcf, producing two clear layers. Supernatant was pipetted off to waste and the sediment layer was dried down and stored at -80 C.

2.5 Copper exposure experiments

Male Jonah crabs, *Cancer borealis*, were purchased from local store (Global market, Madison, WI) and equilibrated in tanks for one week prior to experiments. Crabs were exposed to copper (10 µM $CuCl_2$) or control conditions for 30 minutes, 1 hour, and 2 hours. After incubating on ice for 30 minutes, 400 µL hemolymph was collected using a syringe and directly deposited into 400 µL 90:9:1 MeOH/water/acetic acid (AcMeOH). Samples were then vortexed and centrifuged for 15 minutes at 18000 rcf for protein precipitation. The supernatant was collected into weighed microcentrifuge tubes, dried down, and stored at -80 C prior to sample preparation. For label-free analysis, sample was aliquoted and resuspended at 1 µg/µL in 0.1% FA for LC MS/MS analysis.

500 µg hemolymph was derivatized using either d0 or d3 channels for targeted analysis of metabolites of interest. For consistent sample loss, samples were pooled with 10 µM, 1 µM, and 0.5 µM of d6, d9, and d12 channels respectively. Pooled samples were then extracted using DLLME and desalted using HLB. Samples were then resuspended in 100 µL 0.1% FA for LC MS/MS analysis.

2.6 LC MS/MS

An Ultra-High-Performance LC (UHPLC) Dionex UlitiMate 3000 system equipped with a Kinetex 2.6 µm Polar C18 LC Column (100 Å, 100 x 4.6 mm) was coupled with the Q-Exactive Orbitrap mass analyzer for LC MS/MS analysis of both derivatized and label free metabolites. All experiments utilized the same 15-minute gradient with 0.1% FA as solvent A and acetonitrile with 0.1% FA as solvent B; 5-15% B from 0-7 minutes, 15%-90% B from 7.1-10.5 minutes, 90% B from 10.5-12 minutes, 90-5% B from 12-12.5 minutes, and 5% B from 12.5-15 minutes.

MS was performed using positive ion mode with a range of m/z 200-500 for derivatized metabolites and a range of m/z 70-1000 for label-free metabolites, both at a resolution of 70 K with an AGC of 10^6 and a maximum IT of 100 ms. An inclusion list was used for all channels of iDiLeu tagged glycine, GABA, glutamic acid, dopamine, and GSH.

2.7 Data analysis

Data was processed using Xcalibur 4.4 and Compound Discoverer 3.3 (Thermo Fisher Scientific, Inc.). Label-free and derivatized samples were analyzed separately. For tagged samples, extracted ion chromatograms (EICs) were exported to excel from Xcalibur where calibration curves generated from channels d6, d9, and d12 were used for the absolute quantification of samples from channels d0 and d3. Label-free data was analyzed using Compound Discoverer where positive ID's were defined as those assigned by mzcloud.

3. Results and discussion

3.1 SPE sample loss analysis

While SPE is often avoided in “dilute-and-shoot” approaches, it is highly recommended in derivatization workflows. iDiLeu-derivatized GABA was selected to evaluate sample loss across the three selected SPE methods. We found that C18 and SCX cleanup steps resulted in nearly 80% sample loss during the loading of samples onto the ZipTip, whereas HLB showed less than 3% loss. Sample loss during the wash step was comparable across all SPE methods, at approximately 10%, bringing the total sample loss to over 90% for C18 and SCX cleanup of derivatized GABA, as shown in **Figure 1**. For this reason, HLB was selected for sample desalting and tag removal.

3.2 DLLME

DLLME is an extraction method developed as a “greener” chemistry technique, requiring lower volumes of organic solvent. The results in **Figure 2** compare the standard protocol (derivatization followed by desalting) with DLLME performed before derivatization and DLLME performed after derivatization. While glycine is neither enriched nor depleted by the addition of DLLME to the protocol, glutamic acid and dopamine show statistically significant enrichment when DLLME is incorporated after derivatization. The enrichment of GABA and dopamine using DLLME after derivatization exceeded that of DLLME before derivatization, leading us to adopt the post-derivatization DLLME method in subsequent experiments.

3.3 Targeted quantification results

Analytes were successfully detected and quantified down to concentrations as low as 10 nM (dopamine), as shown in **Figure 3**. No statistically significant changes were observed in copper-exposed carbs after 30-minute and 1-hour exposure times. In the 2-hour exposure experiments, control crabs exhibited a significant downregulation of GABA, consistent with stress conditions previously reported in PTSD studies [22].

Glutamic acid has been reported as a potential antioxidant treatment for copper toxicity and is an essential component in the synthesis of the tri-peptide glutathione (GSH) [23]. GSH protects the cells

from copper-induced cytotoxicity by binding to copper and maintaining it in a reduced, non-toxic state [24]. Glutamic acid was found to be downregulated in crabs exposed to copper for 2 hours, with concentrations averaging 11.8 µM. Glutathione plays a primary role in copper transport [2]. The downregulation of glutamic acid suggests that glutathione is being produced at a rate that exceeds the synthesis of glutamic acid, potentially leading to decreased future glutathione concentrations in the hemolymph. This finding aligns with other research suggesting that oxidative stress induced by copper may be primarily driven by the depletion of glutathione [24].

2. Label-free results

Volcano plots generated by Compound Discoverer revealed the upregulation and downregulation of several hundred compounds. Only eight compounds were identified with positive MS/MS matches supported by mzCloud, all of which were downregulated under copper-toxic conditions. Among these downregulated compounds with confirmed identities are nortriptyline, 2-(cyclohexylmethylidene)-1,2,3,4-tetrahydronaphthalen-1-one, taurine, (2E)-3-[(1R,4S,7R,7aR)-1-hydroxy-3,7-dimethyl-2,4,5,6,7,7a-hexahydro-1H-inden-4-yl]-2-methylprop-2-enoic acid, prolylleucine, leucylproline, 5-methoxyindole, and amobarbital.

Taurine is a sulfonated amino acid hypothesized to influence drug and metal absorption through indirect antioxidant activity [25-28]. Previous studies suggest that dietary taurine intake can enhance copper excretion, providing a protective effect on mouse kidneys in one study [26,27]. Specifically in hemolymph, using a mussel model, taurine downregulation has been reported as a possible trigger for glutamic acid downregulation [26]. In contrast, few reports link the other downregulated metabolites to copper toxicity. However, nortriptyline -- a tricyclic antidepressant -- has been reported to exhibit neuroprotective properties by preventing Ca^{2+} overload [29,30]. This behavior is similar to that of mitochondrial taurine, which is known to help maintain Ca^{2+} homeostasis.

Conclusions

Metabolomics profiling of hemolymph provides valuable insight into cellular changes, with mass spectrometric quantification enabling the simultaneous measurement of multiple metabolites wthin a sample. Using both iDiLeu tagging and label-free methodologies, we identified and quantified metabolites associated with copper toxicity. While no statistically significant changes in the metabolome were observed in 30-minute and 1-hour copper exposures, glutamic acid concentrations were found to decrease in crustacean hemolymph, a change that may be linked to taurine downregulation. Future studies exploring longer exposure durations may reveal further dysregulation of the metabolites of interest.

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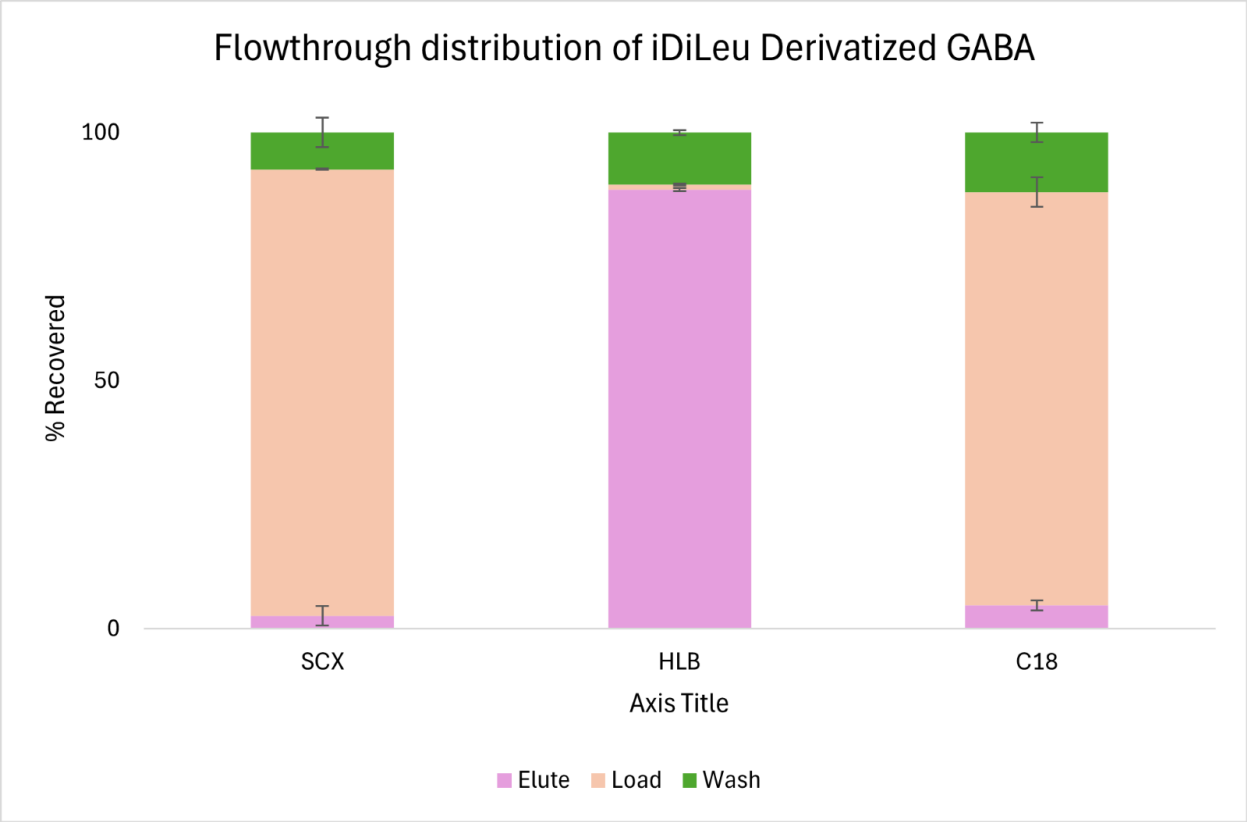

Figure 1. Flow through from elution, loading, and washing fractions of strong cation exchange (SCX), C18, and hydrophilic lipophilic balance (HLB) solid phase extraction (SPE) techniques.

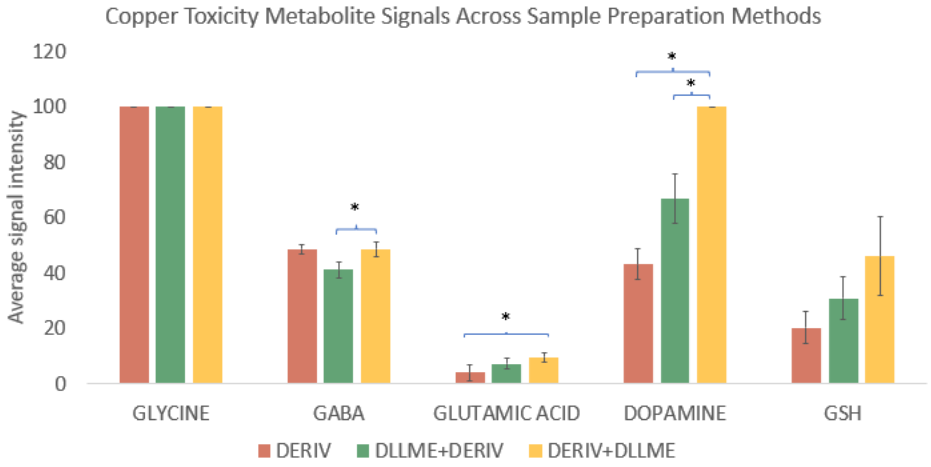

Figure 2. Signal intensities of the five metabolites of interest -- glycine, gamma aminobutyric acid (GABA), glutamic acid, dopamine and glutathione (GSH).

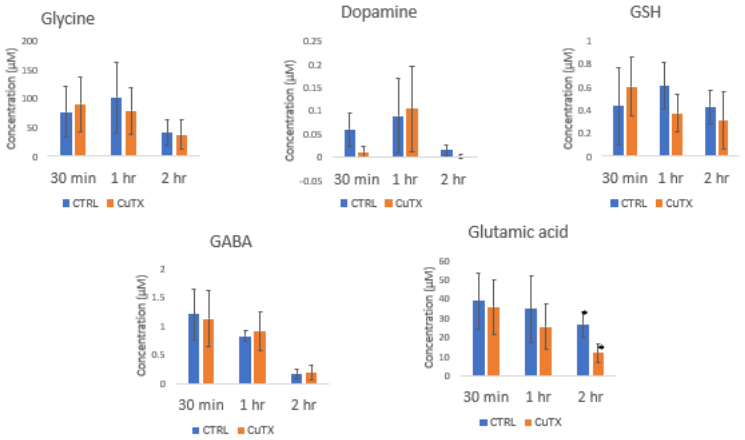

Figure 3. Absolute concentrations of glycine, dopamine, glutathione (GSH), gamma aminobutyric acid (GABA), and glutamic acid are reported. GABA concentration is shown to steadily decrease over time, while glutamic acid is downregulated at the 2 hour mark in copper-exposed crabs.

Chapter 5

Untargeted Mass Spectrometric Approach to Investigating Ocean Acidification in *Cancer Borealis*

Abstract:

Metabolite profiling of jonah crabs can help us understand the stress response produced by marine invertebrates faced with extreme environmental conditions. To successfully profile the crustacean metabolome, many different approaches can be taken, however first employing a dilute-and-shoot (DnS) approach can ensure that a complete picture of the metabolome is captured during preliminary analysis, allowing for further interrogation of molecules of interest via absolute quantification techniques such as derivatization in future studies. Two extraction techniques, isopropanol and acidified methanol protein precipitations, were applied to jonah crab hemolymph for metabolite and small peptide profiling. Comparing 4 hour control experiments of isopropanol and acidified methanol extractions, it was found that 365 unique molecules could be identified using MS-Dial to analyze isopropanol extractions (n=5) compared to the 322 molecules identified using acidified methanol extractions (n=5). This

Introduction:

Ocean acidification (OA) is the systematic lowering of ocean pH caused by the adsorption of CO_2 in the water from the atmosphere; a non homogeneous process that is exacerbated in regions with dense industrial runoff [1-5]. While the ocean's surface pH of ~8.2 is widely accepted, it is projected that the pH will drop 0.3-0.4 pH units by the year 2100, resulting in the thinning and acid erosion of calcium carbonate shells as evidenced in studies using *Limacina helicina* pteropods as potential OA indicators [6-8]. In addition to the obvious pH changes that come with OA, salinity, temperature, and nutrient availability are expected to change over the course of the 21st century, impacting the health of marine species everywhere [9,10].

Many marine species have been studied for their abilities to withstand OA effects, including the jonah crab *Cancer borealis* which is well known for its ability to withstand temperature and pH changes [11,12]. Understanding how marine invertebrates can withstand

OA has been of interest to many researchers trying to understand and predict metabolic changes to help envision the potential biodiversity and economic challenges that might be incurred as a result of global climate change. To achieve this, mass spectrometry (MS) is the ideal tool for completing a metabolic profile as it can be used for quantification of all ionizable molecules in a sample of a selected mass-to-charge ratio (m/z) range.

Small molecule metabolite (<1500 Da) identification and quantification using mass spectrometry can be difficult as they are incredibly diverse and therefore require different sample preparation to isolate molecules of different chemical properties. A common sample preparation approach to getting a wide range of metabolites is often referred to as dilute-and-shoot (DnS) where a sample is extracted using a protein precipitation (ppt) method prior to liquid chromatography (LC) MS/MS analysis [13]. Many different ppt methods exist for analysing metabolites in positive and negative mode LC-MS/MS analysis, each giving a different profile.

Here we explore two different ppt techniques and compare their extraction profiles for small molecule metabolites as well as their ability to extract small peptides. We further dissect the completeness of the metabolome profile provided by the two individual extraction methods.

Methods:

Materials

Optima grade solvents Methanol (MeOH), glacial acetic acid, acetonitrile (ACN), isopropanol (IPA) and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acidified methanol (AcMeOH) was prepared by combining MeOH, water, and glacial acetic acid at a 90:9:1 ratio. AcMeOH and IPA ppt solutions were stored on ice at least 30 minutes prior to ppt extraction experiments.

Ocean Acidification Experiments

Male *Cancer borealis* jonah crabs were purchased from local store (Global market, Madison, WI) and equilibrated in an Aquaneering (California, USA) circulating water system

maintained with 30 ppm salts and 12 hours light, 12 hours dark cycled light for one week prior to experiments. Crabs were then exposed to either control conditions (pH 8.2) or ocean acidified conditions (adjusted to pH 7.8 via CO_2 sparging) for 1 hour, 2 hours, or 4 hours; then were incubated on ice for ~30 minutes prior to hemolymph extraction.

200 uL hemolymph was extracted and immediately combined with 200 uL ppt solution (IPA or AcMeOH). Samples were then sonicated at 4 C and incubated overnight at -20 C before centrifuging for 15 minutes at 16000 rcf, 4 C. The supernatant was then collected into weighed centrifuge tubes, dried down, and stored at -20 C prior to sample preparation. Samples were resuspended in 300 uL 50/50 optima grade ACN/water and centrifuged again at 17000 rcf at 4 C for 5 minutes to remove any insoluble debris before LC-MS/MS analysis.

LC-MS/MS Analysis

An Agilent 1290 Infinity II HPLC system was equipped with a Kinetex 2.6 um hydrophilic interaction liquid chromatography (HILIC) column (100 A, 150 x 4.6 mm) and coupled with an Agilent 6560 QTOF (Agilent Technologies, USA), ranging 50-1200 m/z. 25 mM ammonium hydroxide (NH4OH) and 25 mM ammonium acetate (NH4OAc) in water was selected as mobile phase A and ACN was selected as mobile phase B. A flow rate of 0.45 mL/min was used and the following gradient was implemented as previously described: 0-1 min 95% B, 1-14 min 95-65% B, 14-16 min 65-40% B, 16-18 min 40% B, 18-18.1 min 40-95% B, 18.1-23 min 95% B. After data collection, data was processed using MS-Dial (version 4.9) using less than 20 ppm mass error and a signal-to-noise greater than 5.

Results:

IPA v AcMeOH Metabolite ID's

Acidified methanol has been an important extraction solvent in multi-omics for the simultaneous extraction of proteins, peptides, and metabolites [14]. Isopropanol has also been useful for metabolomics studies as it is capable of extracting a wide range of metabolites [15]. In the interest of characterizing the Jonah crab metabolome for future ocean acidification

experiments, it would be prudent to compare the two extraction methods for a more complete profile.

Control experiment hemolymph was compared at the 4 hour time point for metabolites extracted by isopropanol and acidified methanol. **Figure 1** shows the difference in MS-Dial positive ID's between IPA and AcMeOH. Overall, IPA extractions are more robust, elucidating twice as many lipids, steroids, and peptides as AcMeOH. Between all 5 trials, AcMeOH averaged 112 ID's while IPA averaged 129 ID's with 322 and 365 unique ID's respectively and 153 common ID's between the two. **Table 1** lists the amine-containing metabolites and peptides that can be further quantified using DiLeu tagging.

Conclusions:

Jonah crab hemolymph is a valuable and complex sample good for examining a variety of environmental stressors. Using IPA and AcMeOH extractions, a snapshot of the metabolome can be easily obtained and analyzed using mass spectrometry. Upon comparison of the two extraction methods, it is evident that IPA provides a slightly more complete and more consistent extraction than AcMeOH. It would be interesting to see how the two compare for multi-omics workflows with tandem peptide and protein analysis.

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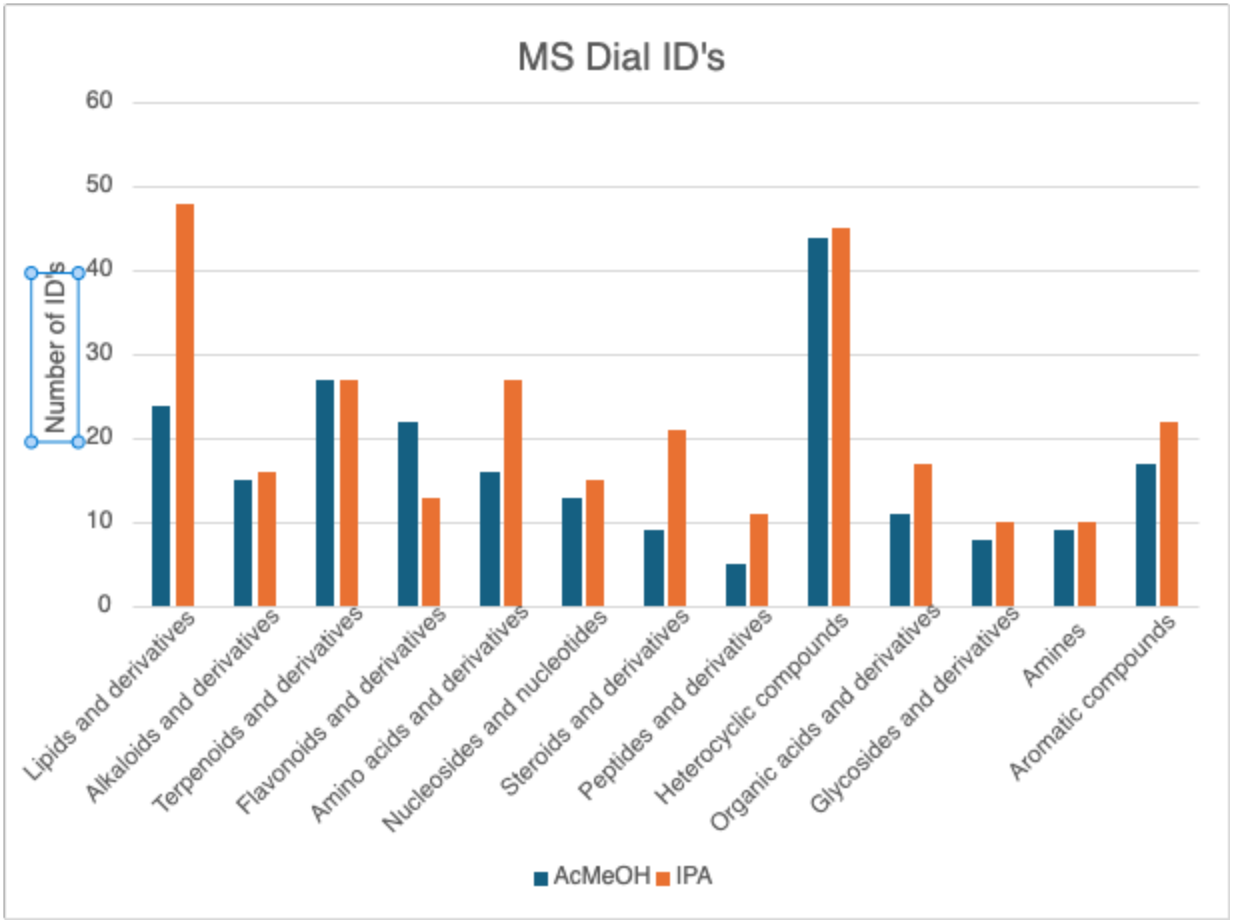

Figure 1. Distribution of ID's between AcMeOH (n=5) and IPA (n=5) for major metabolite groups.

Amine-containing molecule	IPA/AcMeOH/ Both
Acetochlor-OXA	AcMeOH
L(+/-)-Alliin	AcMeOH
Pentose + Proline; PlaSMA ID-1252	AcMeOH

NCGC00384639-01_C14H16N2O3_Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-[(4-hydroxyphenyl)methyl]-	AcMeOH
Dimethachlor OA	AcMeOH
N-Fructosyl alliin - H2O; PlaSMA ID-1715	AcMeOH
Glu-Glu	AcMeOH
6-Aminopenicillanic acid	AcMeOH
Betaine	BOTH
Phosphoarginine	BOTH
C14-homoserine lactone	BOTH
N-Fructosyl isoleucine; PlaSMA ID-1287	BOTH
Dimethachlor-OXA	BOTH
Yersiniabactin	BOTH
3-hydroxy-C14 homoserine lactone	BOTH
Lauramidopropyl betaine	BOTH
H-gamma-glutamyl-glutamine	BOTH
(NAG)(NAM)-AqKA[3-NH2-GGSGG]	BOTH
Alachlor-OXA	IPA
Myristamidopropyl betaine	IPA
2-amino-3-prop-2-enylsulfinylpropanoic acid	IPA
N-Acetylarginine; CE30; SNEIUMQYRCDYCH-LURJTMIESA-N	IPA

(E)-5-hydroxy-3-isobutyl-6-(3-methylbenzylidene)-1,6-dihydropyrazin-2(3H)-one	IPA
CGA62826 (2-[2,6-dimethylphenyl)-methoxyacetylamino]propionic acid	IPA
N-(1-(2-amino-2-oxoethyl)piperidin-4-yl)-3-(4-hydroxy-6-methyl-2-oxo-2H-pyran-3-yl)-3-(2,3,4-trimethoxyphenyl)propanamide	IPA
Glu-Gln	IPA
Neotame	IPA
(R)-2-((S)-2-((S)-2-(2-hydroxy-4-oxoquinazolin-3(4H)-yl)-3-(1H-indol-3-yl)propanamido)-3-methylbutanamido)propanoic acid	IPA
Microcolin E	IPA

Table 1. Amine-containing metabolites and peptides found in AcMeOH and IPA extractions.

Chapter 6

Conclusions & Future Directions

Conclusions

Metabolites are widely considered the link between genotype, phenotype, and environment, making these small biomolecules excellent potential biomarkers for both disease and environmental conditions [1]. Their structural diversity results in uneven distribution within an ESI droplet, leading to variable ionization efficiencies across functional groups and challenges in separating certain molecules from salts [2,3]. This dissertation focuses on method development to improve the mass spectrometry (MS) detection of small molecules using both derivatization and label-free techniques. A general overview of the techniques and associated challenges is provided in **Chapter 2** and further addressed throughout the remainder of this thesis.

One factor that impacts the profile of small molecules detected in MS analysis is the hydrophobicity bias, where more hydrophobic molecules ionize more readily than hydrophilic ones [2]. Derivatizing small molecules allows more hydrophilic compounds to co-ionize more competitively, bringing them into the frame for MS analysis and enabling sample multiplexing. In **Chapter 3**, a 5-plex isotopic *N*,*N*-dimethyl leucine (iDiLeu) tagging method was developed to label amine-containing metabolites. When applied, this tagging method resulted in a lower limits of detection (LOD) and quantification (LOQ) for most of the metabolites of interest (MOI) explored in the subsequent Alzheimer's disease study, along with increased liquid chromatography (LC) retention times. In the established method, the first three minutes of each run were diverted to waste to remove salts and tagging byproducts – a process that limits the automation potential of standard LC-MS

workflows. Nevertheless, two potential biomarkers were successfully identified: – tyrosine in male AD patients and Leucine in female AD patients.

In lieu of physically diverting the sample to waste and potentially missing some early-eluting analytes, **Chapter 4** explores sample preparation methods that can be paired with derivatization to allow uninterrupted automated LC-MS analysis. It was found that while tagging increases the hydrophobicity of some analytes, the change is not sufficient to make neurotransmitters and amino acids compatible with traditional desalting methods, such as C18 Ziptip desalting, as detailed in previous metabolomics and neuropeptide studies [4]. Additionally, the green technique dispersive liquid-liquid microextraction (DLLME) is applied to five MOI to demonstrate its ability to further enhance the signal of derivatized metabolites. [5] The five MOI -- dopamine, glutathione, glutamic acid, gamma-aminobutyric acid (GABA), and glycine -- were all successfully quantified in hemolymph at three different time points (30 minutes, 1 hour, and 2 hours), with and without copper exposure. This analysis established glutamic acid as a potential biomarker for copper toxicity.

To improve label-free analysis, the acidified methanol extraction profile is compared with the isopropanol extraction profile in the context of ocean acidification in **Chapter 5**. A dilute-and-shoot (DnS) approach is employed, and it is found that the more recently established chilled isopropanol method can recover a diverse range of peptides as well as a broad spectrum of amino acid metabolites [3].

Future Directions

While this thesis proposes some affordable workarounds for metabolomics, there are still additional improvements that can be made. Enhancing metabolomics workflows can take many forms, including advancements in data search algorithms, green chemistry practices, and integration with multi-omics approaches. Striving for financially sustainable solutions in small molecule metabolomics not only advances the field but also enables broader adoption by laboratories that may lack access to the latest instrumentation. With more researchers contributing to the characterization of drug, disease, and environmental metabolomes, our fundamental understanding of the biological systems at play can continue to evolve rapidly.

Workflows adapted from **Chapters 3 and 4** can be readily applied in other laboratories; however, it should be noted that changes in hydrophobicity are not consistent across all derivatization methods. Therefore, recovery using smaller tags may not be comparable to that achieved with larger tags, such as the isotopic *N*,*N*-dimethyl leucine used in this study. Additionally, in lieu of dispersive liquid-liquid microextraction, other microextraction techniques -- potentially greener alternatives – could be explored, such as salting-out assisted liquid-liquid microextraction (SALLME), stirbar microextractions, or ionic liquid microextractions [6,7,8]. Although all of these techniques have been used to improve small molecule extraction profiles, none have yet been applied in tandem with derivatization. Thus, there is a possibility that they may also be compatible.

Label-free workflows, such as those demonstrated in **Chapter 5**, were performed using strictly data-dependent acquisition (DDA), which only interrogates high-abundance MS1 peaks with MS2. This approach may limit the number of possible identifications for small molecules, as it overlooks low-abundance signals. Data-independent acquisition (DIA), described in **Chapter 2**, may ultimately benefit small molecule and peptide analysis by capturing low-abundance peaks in the spectrum for MS2 analysis. DIA has already been applied in both peptidomics and metabolomics studies to enhance molecular profiling and may help identify more hydrophilic analytes in small molecule mass spectrometry.

Matrix-assisted laser desorption/ionization (MALDI) workflows, such as those described and explored in Appendix III, can be easily applied to mass spectrometry imaging (MSI) experiments. However, it may be beneficial to seek methods for consolidating the peaks under a single adduct. Salt doping has been successfully used to improve the ionization of neutral molecules, such as certain neutral lipid species in MSI experiments. The application of salts does decrease the ionization efficiencies of many molecules, so the salt dosage must be carefully selected. When combined with the TiO_2 matrix, the expected result would be a spectrum with low-abundance polyethylene glycol background peaks and high-intensity peaks for small-molecule metabolites, with a mass shift corresponding to the salt that best ionizes the sample.

Many MSI experiments involve tissue imaging, which relies on sacrifice for metabolomic, peptidomic, lipidomic, or proteomic profiling. It would be prudent to begin profiling highly abundant biofluids, such as sweat, for mass spectrometric analysis in order to explore noninvasive biomarkers that may be more comfortable to collect from patients.

One such application that has been realized is fingerprint MSI for drug analysis, where fingers serve as a popular source of sweat. With improved ionization efficiencies, desorption electrospray ionization (DESI) might be a viable alternative to MALDI MSI. DESI can also be coupled with TiO_2 nanoparticle matrices to further enhance the ionization efficiencies of small molecules [9].

To conclude, metabolomics presents some compelling challenges at first glance that, with careful consideration of the physical properties of the sample, can be overcome. Whether with or without derivatization, sample recovery can always be improved to expand coverage of the metabolome. The methods described can be applied to many other sample fluids, to multi-omics workflows, and to expanding the peptidome. Overall, this dissertation provides foundational work for the sensitive analysis of metabolites in future metabolomics and multi-omics research.

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Appendix 1

List of Publications, Presentations, and Grants

Publications

1. Riusech, O., Yao, Y., Xu, S., Appell, J., Li, L. In preparation.
2. Riusech, O., Li, L. Mass spectrometric absolute quantification of metabolites after copper exposure using iDiLeu tagging. In preparation
3. Riusech, O., Hao, L., Li, L. Absolute quantification of amine metabolites in human cerebrospinal fluid via MS1-centric isotopic N,N-dimethyl leucine (iDiLeu) labeling. Analytical and Bioanalytical Chemistry, January 24, 2025.
4. Sauer, C., Phetsanthad, A., Riusech, O., Li, L. Developing mass spectrometry for the quantitative analysis of neuropeptides. Expert Review of Proteomics, 1-15, August 26, 2021.
5. Borchers, J.; **Riusech, O**.; Rasmussen, E.; Anand, R. Visual Voltammogram at an Array of Closed Bipolar Electrodes in a Ladder Configuration, Journal of Analysis and Testing, 3, 150-159, March 30, 2019.

Presentations

- **Riusech, O.**; Li, L. Enhancing Hydrophilic Metabolite Detection and Quantifiability via Derivatization-Assisted Sample Preparation and Dispersive Liquid-Liquid Microextraction (DLLME) Techniques, Oral. ASMS National Meeting Jun 2023
- **Riusech, O.**; Li, L. Amine-containing metabolite quantification via 5-plex isotopic N,N-dimethyl leucine (iDiLeu) tagging: Examining the effect of cleanup methods to reduce sample loss, Poster. ASMS National Meeting, June 2022

- **Riusech, O.**; Sauer, C.; Li, L. Metabolite Quantitation using Multiplex Isotopic N,N-Dimethylated Leucine (iDiLeu) Tags, Poster. ASMS National Meeting, October 2021
- **Riusech, O.**; Sagan, C.; Garand, E. Determining the Splitting and Order of the Singlet & Triplet Excited States of Cycl[3.3.3]azine, Poster. ACS National Meeting, August 2020
- **Riusech, O.**; Garcia, A.; Forsyth, E.; Smidzic, Q. The Effect of Physical Activity vs. Cognitive Activity in Reaction Time, Poster. Undergraduate Research Symposium, Iowa State University, April 2015

Grants

1. Student Research Grants Competition (WISH) | 2023
2. Advanced Opportunity Fellowship (AOF) | 2023
3. ACS Bridge Fellowship | 2019
4. Plagen's Research Scholarship | 2018

Appendix II

Physicochemical investigation of the impact of common derivatization techniques on hydrophilic small molecule ionization in mass spectrometry

Abstract

Small hydrophilic molecules such as amino acids make valuable biomarkers for biological studies, however are difficult to ionize and therefore difficult to analyze using mass spectrometry (MS). To solve this problem, derivatization is often implemented to make hydrophilic molecules ionize more readily, however not all molecules have the same response to derivatization. A better understanding of how derivatization impacts the ionization efficiencies of small molecules can be obtained by looking at how the proton affinities and hydrophobicities are affected. In comparing three different derivatization methods - dansylation, N,N-dimethyl leucine (DiLeu) tagging, and isobaric tags for relative and absolute quantification (iTRAQ) - we find that dansylation and DiLeu produce a greater change in hydrophobicity than iTRAQ does, leading us to conclude that the two methods might be more effective at ionizing amino acids. Dansyl chloride also showed the most consistent increases in proton affinities compared to both label-free and other derivatization methods.

Background

Mass spectrometry (MS) is a key tool for investigating potential biomarkers as it allows for the simultaneous quantification of all ionizable molecules in a sample within a given range of mass-to-charge ratios (m/z). [1,2] It works by ionizing analytes, often achieved using electrospray ionization (ESI), and subsequently separating ionized molecules under vacuum by their m/z via electric field migration and subsequently detecting the current produced by these ions. Ionization can be performed in positive and negative mode, where positive often implies the protonation of a molecule and negative incorporates a negative charge on a sample. For biomolecule discovery purposes, positive mode mass spectrometry is most popular as it permits the

quantification of peptides, proteins, and many metabolites. The ability for a molecule to adhere to a proton is inherently linked to the ionization efficiency, affecting how much signal is produced by an analyte and whether or not it can be detected in a given sample.

Small polar molecules, such as amino acids, neurotransmitters, di- and tri-peptides, and some lipids often suffer poor ionization efficiencies, leading to decreased sensitivity in MS analysis. [3] Chemical derivatization or “tagging” is often used in MS analysis of hydrophilic small molecule analysis to improve ionization efficiency, prolong liquid chromatographic retention time, and increase analysis throughput. [4] Many different derivatization techniques exist, both commercially available and not, where the mass addition makes these hydrophilic compounds more hydrophobic, often associated with a higher ionization efficiency. Some common derivatization techniques include isobaric tags for relative and absolute quantification (iTRAQ), dansyl chloride, and our lab’s in-house synthesized N,N-dimethyl leucine (DiLeu) tags. [5,6]

Three key components to ionization efficiency are proton affinity, hydrophobicity, and surface activity of a molecule of interest. [7] While the change in hydrophobicity caused by derivatization is experimentally apparent due to the changes in retention time during reversed phase liquid chromatography, changes in other physicochemical properties related to ionization are largely unknown. [8] Experimental determination of proton affinity and hydrophobicity are complex and require expensive equipment, however computational evaluations of each of these properties can be performed using simple, cost-effective calculations.

Here we explore the physicochemical properties of amino acids and the different chemical structures resulting from different chemical derivatization techniques. Using MedChem+, we calculate theoretical hydrophobicity utilizing the free logD value predictor feature. We also calculate the gas phase basicity using geometry optimizations of TMT-tagged, DiLeu tagged, and dansylated amino acids to explore how different derivatization techniques impact the ionization properties of each of the hydrophilic molecules of interest.

2.0 Methods

2.1 Proton affinity and gas phase basicity calculations

Gaussian 16 was used to perform geometry optimizations and vibrational frequency calculations at the B3LYP level theory with 6-31G(d) basis sets. To calculate the proton affinity, the enthalpies of each protonated amino acid were subtracted from the enthalpies of their respective neutral molecules. [9]

$-\Delta H = -(E(MH+) - E(M))$

Traditionally recorded as a positive value although the enthalpy is itself negative. To calculate the enthalpies of the protonated amino acid, a proton was placed 1.200 Angstroms away from the amine of each amino acid both before and after derivatization.

2.2 Log D calculations

LogP is defined as a partition coefficient of an unionized molecule that can reside in either octanol or water layers of a traditional organic biphasic separation.

$log([M]octanol/[M]water) = logKow = logP$

This property has been criticized as an incomplete representation of the polarity of a molecule as the partition coefficient can drastically change at different pH values. For this reason it has become commonplace to describe a molecule's polarity using it's calculated logD value or distribution coefficient of logP values.

Structures of amino acids and derivatized amino acids were drawn using simulations plus software Medchem designer 5.5. ADMET calculations of Log D were performed using the free software package and recorded on excel.

3.0 Results and discussion

3.1 Proton affinities of amino acids compared to derivatized amino acids

Amino acids, generally accepted to have low ionization efficiencies, can be compared to their derivatized counterparts using proton affinity calculations. When calculated at the same level of theory and compared with the three derivatization methods of interest, no trends are apparent in improved proton affinity (**Table 1**). In fact, label-free amino acids don't prove to have the lowest proton affinities in any of the amino acids calculated except for Valene (VAL). Though there are no trends, dansyl chloride has the highest number of high proton affinities when compared to label free, DiLeu labeled, and TMT labeled amino acids. This could indicate that dansyl chloride would ionize most favorably, but experimental data would need to be collected to confirm this.

3.2 Log D values of derivatized amino acids

All amino acids were correctly calculated to be more hydrophilic than their derivatized counterparts as shown in **Figure 1**. Dansyl chloride resulted in the largest change in hydrophobicity overall, however DiLeu tagging was competitive in changing

the hydrophobicities of these molecules. TMT, the most expensive of these derivatization techniques, showed consistently lower changes in hydrophobicity than the other two methods, indicating that TMT may not facilitate ionization as well as the other methods of interest.

Conclusions

Computational experiments can provide cost effective insights into the reactions of interest and the physicochemical properties of products and reactants. In general, the data suggests that hydrophobicity may be more of a driving force in the ionization efficiencies of these small molecules than proton affinity, as it has previously been experimentally demonstrated that each of these derivatization techniques produce larger signal-to-noise ratios in mass spectrometric analysis. It may also be true that the level of theory selected for modeling proton affinity was not sensitive enough to capture these slight differences in proton affinity. Further insight into how derivatization facilitates ionization could be an important step to selecting derivatization techniques specifically for molecules of interest without having to spend money on trial-and-error experiments.

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Amino Acid	Label free	DiLeu	iTRAQ	Dansylated
GLY	-0.346436	-0.327463	-0.322112	-0.347366
ALA	-0.333502	-0.338358	-0.322996	-0.323407
CYS	-0.343629	-0.330865	-0.33253	-0.34589
SER	-0.348921	-0.306662	-0.326016	-0.35251
VAL	-0.318356	-0.342473	-0.344356	-0.340002
THR	-0.341645	-0.323108	-0.33282	--
LEU	-0.354266	-0.374619	-0.325686	-0.353823
ILE	-0.353455	-0.347784	-0.380645	-0.347031

Table 1. Calculated proton affinities (Hartrees) of selected amino acids where the highest proton affinities are highlighted in blue and the lowest proton affinities are highlighted in gray.

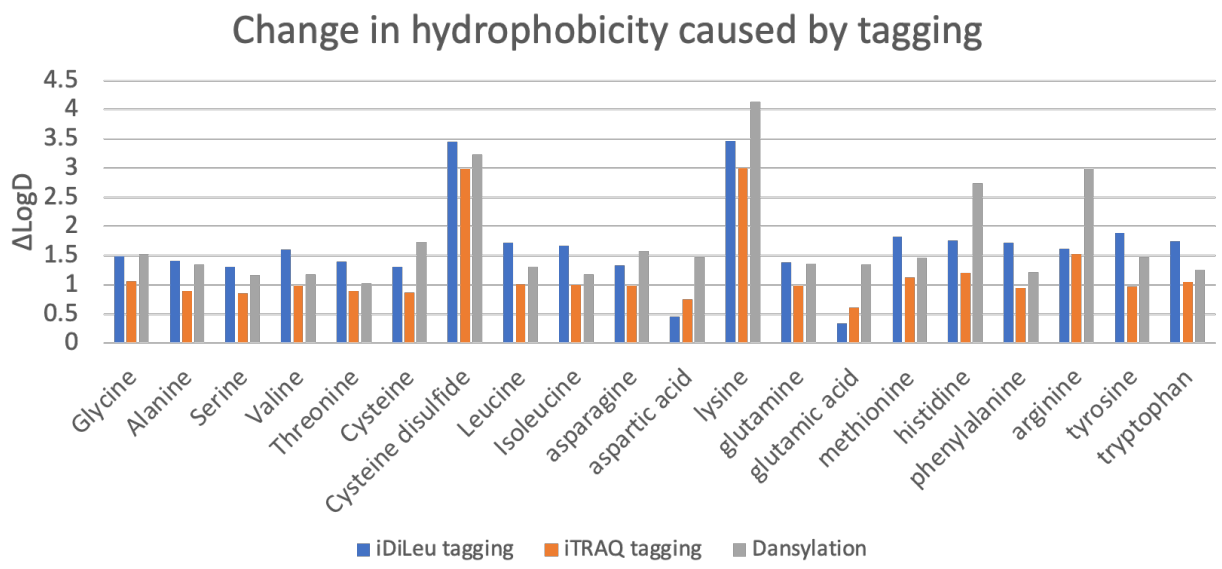

Figure 1. Changes in hydrophobicity predicted by MedChem LogD analysis across three derivatization methods.

Appendix III

MALDI Mass Spectrometry of GABA using combined tagging and SALDI

Abstract

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) is a highly accelerated field for biomolecule localization within tissue samples, originally applied to proteins and other large molecules. Surface-assisted laser desorption/ionization (SALDI) has been developed as a MSI technique that can be made more compatible with small molecules, as the matrix peaks are less prominent in low m/z areas. Small amine-containing metabolites such as GABA also suffer from poor ionization efficiencies; an issue better addressed by applying derivatization to improve the limit of detection. This work analyzes how nanosecond photochemical reaction (nsPCR) tagging might be combined with a TiO_2 SALDI matrix to both improve the ionization efficiency and remove matrix. I found that although the TiO_2 matrix works well for GABA imaging, salts in the TiO_2 storage solution contribute adduct signals at a high percentage. This problem is exacerbated when using nsPCR tagging, where tagging is not very successful in traditional organic matrix CHCA nor in TiO_2 and creates a complicated spectrum full of salt adducts on both tagged and untagged GABA.

1. Introduction

Matrix-assisted laser desorption/ionization (MALDI) is commonly used for mass spectrometric imaging (MSI) to observe the spatial distribution of a molecule or set of molecules of interest. MALDI, traditionally partnered with a time-of-flight (TOF) mass analyzer, involves the ionization of an organic matrix-embedded sample by laser ablation as opposed to the traditional electrospray ionization method [1,2]. The organic matrix used to embed these samples

facilitates ionization such that the molecules within a sample can maintain charge without falling apart [3]. This soft ionization technique is commonly performed on tissues such as brain, where an image of the sample can be pixelated, each pixel containing a mass spectrum reporting the approximate quantities of each molecule in the spectra in the form of a heat map.

Performing MSI to localize small molecule metabolites (<1500 Da) is important to elucidating biological functions, however small molecules face issues in traditional MALDI analysis that make MSI sensitivity suffer [4]. For example, many MALDI matrixes are organic acids, which themselves are small molecules and are usually sprayed onto tissue samples at a high concentration to facilitate ionization. The high concentration increases matrix effects where the signals produced by low-abundance molecules are effectively “drowned-out” by the large signal produced by these organic matrixes.

One important metabolite in neurological studies is GABA, where it’s localization can be used to track GABA receptor activity in the central nervous system. Many different techniques have been used to enrich neurotransmitters and amino acids to give them higher signals, the most common of these techniques being on-tissue derivatization where a small molecule is tagged with another molecule, giving it a unique m/z and sometimes a higher ionization efficiency. One recently developed ionization technique, nanosecond photochemical reaction (nsPCR), developed by the Li-lab requires minimal incubation and tags analytes during the laser ablation process of MALDI sample analysis [5].

Another method of metabolite enrichment is using a nanoparticle matrix instead of a traditional organic matrix; generally referred to as surface assisted laser desorption/ionization (SALDI) [6]. Many different SALDI matrixes exist, ranging from gold nanoparticles to graphene oxide, where many people experiment with mixing multiple matrixes together to expand the metabolite profile. TiO_2 is an inexpensive nanoparticle matrix which has been used to successfully image GABA in the past [7]. Here, I characterize and compare nsPCR tagging in

traditional CHCA MALDI matrix and TiO_2 nanoparticle SALDI matrix. I then perform MSI on crab brain tissues comparing CHCA with TiO_2.

2. Methods

2.1 Materials

Matrixes used include α-cyano-4-hydroxycinnamic acid (CHCA) and TiO_2 nanoparticles (Sigma-Aldrich) resuspended in optima grade acetonitrile (ACN) and optima grade water. To characterize nsPCR tagging, 2-hydroxy-5-nitrobenzaldehyde (2-NBA) was purchased from Thermo Fisher (Waltham, MA). Spotting experiments were conducted using gamma-aminobutyric acid (GABA) standards purchased from Sigma-Aldrich (St. Louis, MO), 2-nitrobenzaldehyde (2-NBA), and 5-hydroxy-2-nitrobenzaldehyde (5-OH-2-NBA)

2.2 Spotting experiments

CHCA and TiO_2 matrixes were each resuspended in 50/50 ACN/water at 10 mg/mL and 25 mg/mL respectively. Matrixes were spotted independently to characterize background noise, followed by the spotting with 500 uM GABA. Each nsPCR reagent, 2-NBA and 5-OH-2-NBA, was then spotted with GABA and each of the two matrixes. Analysis performed with a Bruker Rapiflex MALDI-TOF/TOF instrument (Bruker Daltonik, Bremen, Germany) used a laser power of 40% for CHCA analysis and a laser power of 60% for TiO_2 spots.

2.3 Tissue collection and sectioning

Female blue crabs were purchased from Global Market and Food Hall (Madison, WI) and equilibrated for a week, maintained in an Aquaneering tank system circulating ~30 ppt salinity with a 12 h/12 h light/dark cycle [8]. Prior to dissections, crabs were incubated on ice for ~20 minutes to anesthetize them, then brains were extracted and embedded in 100 mg/mL gelatin in water as established in previous protocols [9]. Brain tissues were then stored in -80 C

freezers until cryostat sectioning (Thermo Scientific Microm HM 525) 16 um thick slices at -20 C and thaw-mounting slices on ITO slides as previously described [10].

2.4 MSI

Brain tissue slices were dried in a dessicator for ~30 minutes prior to matrix application via handheld sprayer (Beauty Airbrush System, Rhinowisdom). Samples were manually sprayed with 20 passes of CHCA (mg/mL) or 20 passes TiO_2 (mg/mL) at a distance of roughly 13 cm, with 30 seconds inbetween each pass. Samples were then dried in a dessicator for 30 more minutes prior to imaging experiments. Imaging experiments were conducted on a Bruker Rapiflex at 90% laser power with a frequency of 200 Hz, laser 355 nm, calibrating the low mass range using red phosphorus.

3. Results and Discussion

3.1 Spotting experiments

Background spots revealed 17 prominent peaks with CHCA with a laser power of 40 while TiO_2 had 27 prominent peaks and required a higher laser power of 60. Still, once GABA was introduced, many of the prominent peaks from the TiO_2 matrix were less than 20 percent of the signal intensity, including background peaks ~44 Da apart which are presumably from polyethylene glycol used in TiO_2 synthesis (**Figure 1**).

In CHCA, label-free GABA produced a signal intensity of 7% with no visible salt adducts where the highest abundant peaks were all matrix peaks. When 5-OH-2-NBA was added, label-free GABA was still visible at m/z of 104, with the tagged GABA visible at m/z 253, 275, and 291 as protonated, sodiated, and potassiated species. The tagged, potassiated species was seen at the highest abundance with a mass addition of 149 from the tag and 39 from the potassium. Upon addition of 2-NBA, the mass addition of 133 which chronicles successful tagging was less than 10 percent of the total signal while label-free GABA was 50% of the signal intensity and the highest intensity was still reserved for matrix peaks. While this indicates greater success tagging

with 5-OH-2-NBA, the concentration spread across three different adducts makes even the most abundant signal relatively low, and the salt presence impedes proper ionization. Even at a high concentration, the matrix peaks overwhelm the detector.

In the TiO_2 matrix, GABA appeared protonated, sodiated, and potassiated where the highest peak was the sodiated GABA with a mass addition of 23, m/z 126 (Figure 1). With 5-OH-2-NBA, the tagged species appeared more than 50% abundant signal split across the protonated, sodiated, and potassiated while with 2-NBA only about 25% of GABA signal was from successfully tagged GABA, sodiated and potassiated only.

3.2 Mass Spec imaging experiments

CHCA deposition in excess with handheld sprayer was proven unsuccessful upon reviewing imaging experiment where large portions of the crab brain tissue were left bare, as seen in **Figure 2**. TiO_2 imaging on the other hand revealed many details in the low mass region with a laser diameter of 10 um, showing that the cheap nanospray setup combined with affordable nanoparticles can be used to image in the low m/z range. Images at m/z 104 reveal discernable structures within the slice of the crab brain with TiO_2 imaging, though MS/MS imaging would be needed to distinguish this peak as either GABA or Choline.

4. Conclusions and Future Directions:

Surface-assisted laser desorption/ionization (SALDI) provides a promising alternative to traditional matrix-assisted laser desorption/ionization when it comes to small molecule ionization, as its low m/z range peaks are of low abundance. TiO_2 provides a kind of salt-doping effect that might be helpful for separating GABA from Choline in mass spec imaging if combined with the proper salt. Further salt doping might help consolidate GABA signal into one peak, however further experimentation is needed to confirm. TiO_2 hand-sprayed onto tissue appears to have promising S/N at a reasonably high resolution without mandating expensive equipment and can be an affordable solution to small molecule mass spectrometry imaging.

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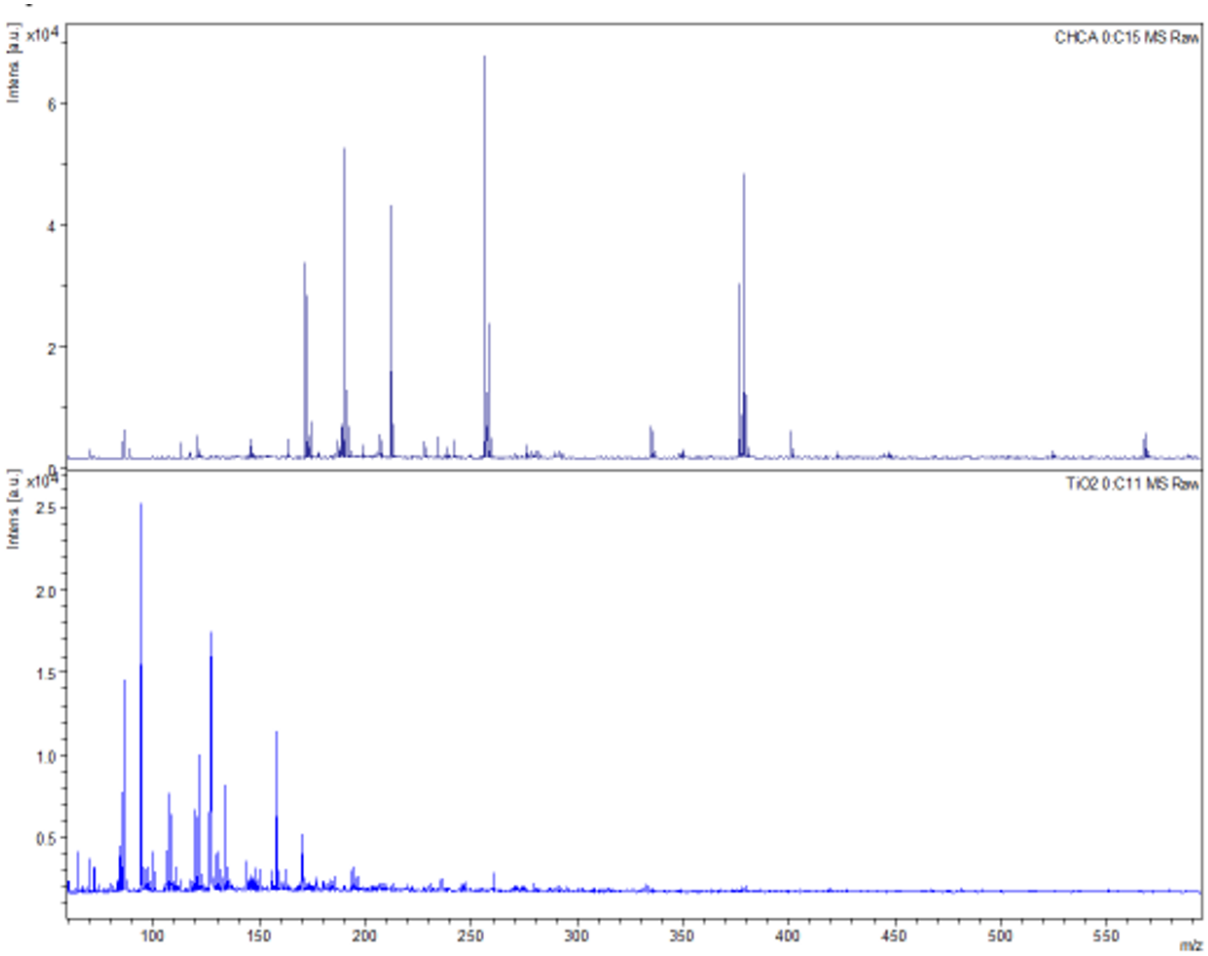

Figure 1. Background peaks for CHCA (top) versus TiO_2 (bottom) where TiO_2 peaks are 1/4th of the intensity of CHCA with a faint presence of PEG.

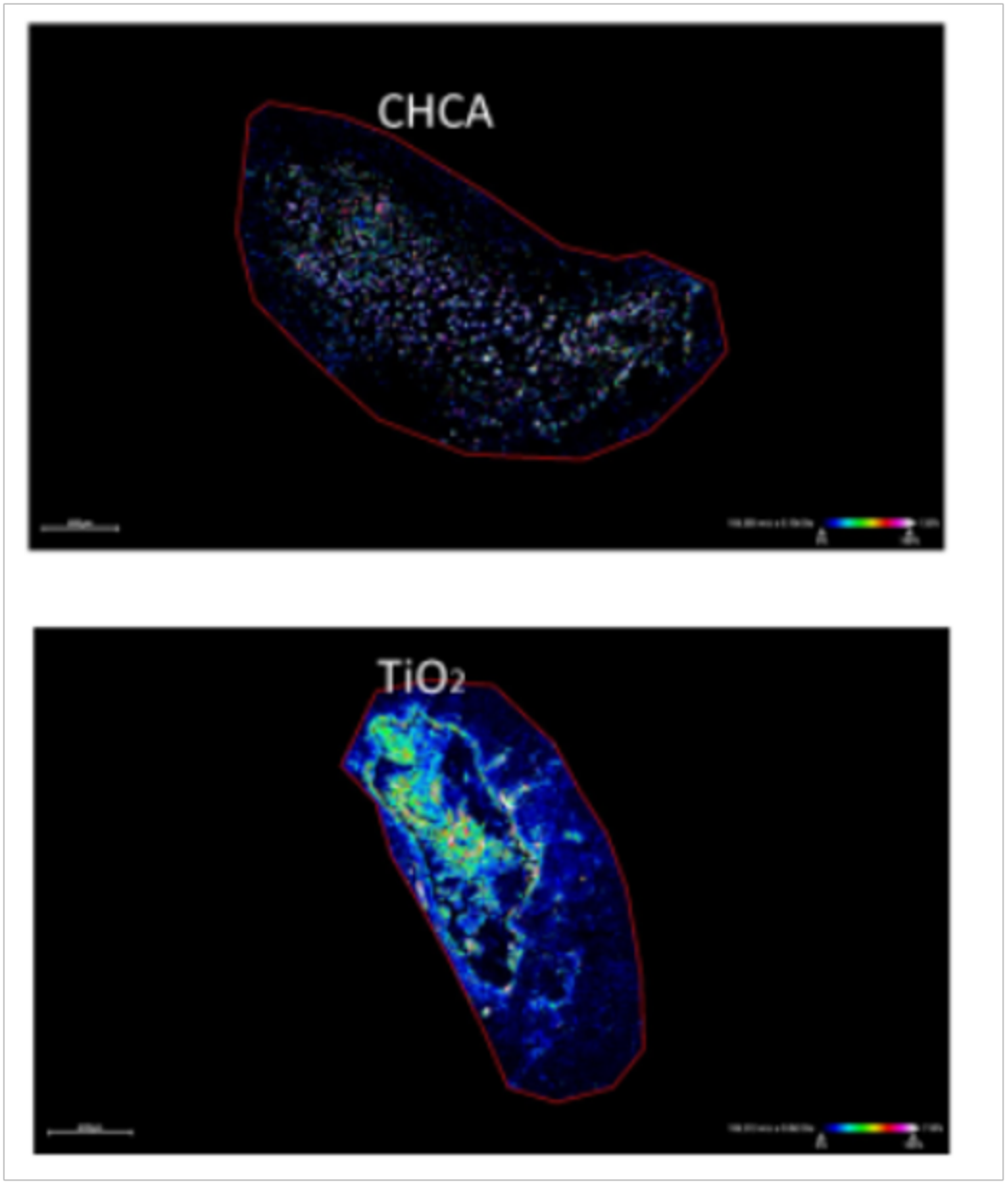

Figure 2. Comparison of CHCA imaging with TiO_2 imaging.