Parallel Metabolome-Wide Quantification of Metabolites with ^{15}N-Labeled Mouse Specimens through Mass Spectral Accuracy

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Introduction

The parallel quantification of a great number of metabolites in tissues and biofluids is currently not possible. Instead, individual stable isotope-labeled reference material for each metabolite of interest needs to be obtained and spiked into the sample. The goal of our project is to develop a more robust and cost-effective biomolecular quantification method that allows for the parallel quantification of large numbers of metabolites in human tissues and biofluids for pharmaceutical, medical, and research applications.

Similar to the approach reported for proteins (Ref 1), we developed a new method for the global quantification of nitrogen-containing metabolites in human and mouse tissues and plasma. The method leverages *in vivo* metabolic stable isotope labeling strategies of live rodents combined with quantitative mass spectrometry-based metabolomics data acquisition and bioinformatic analysis.

Method

Sample Preparation, LC/MS Data Acquisition and Post-Acquisition Software Analysis

The livers of C57BL/6 mice, ^{15}N-labeled at approximately 93%, and unlabeled C57BL/6 mice were subjected to extraction using 40:40:20 methanol:acetonitrile:H2O, 0.5% formic acid. After protein precipitation the metabolite extracts were analyzed by LC/MS using a Thermo Exploris 480 Orbitrap mass spectrometer coupled with a Vanquish UPLC under both 240K and 480K resolving power via two injections of the same sample in ESI+ and ESI- modes. Spectral accuracy (SA, Ref 2) calibration was performed with MassWorks software using the sCLIPS (self-Calibrating Lineshape Isotope Profile Search) feature for a metabolite and its various ^{15}N-labeled versions with both SA and relative fractional concentrations reported. The incomplete ^{15}N-labeling of the samples resulted in complex and overlapping isotopologue patterns for nitrogen-containing metabolite features and were accounted for and quantitated by a multivariate regression approach (Ref 3).

To establish the feasibility of this new approach, from the hundreds or even thousands of potential metabolites, a few with good abundances were selected for evaluation. These metabolites contained different numbers of N atoms and were analyzed in both ESI+ and ESI- mode.

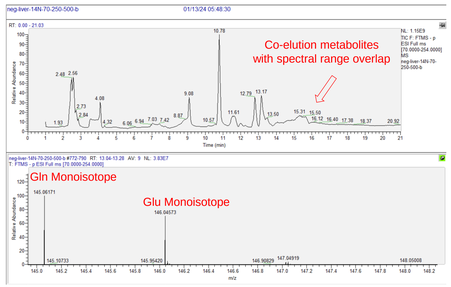
Results and Discussion

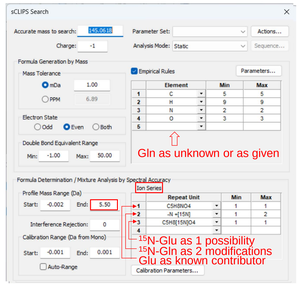
Earlier published work (Ref 4-6) has studied the key factors affecting the achievable SA on the Orbitrap LC/MS systems. To assess the SA for this work, the unlabeled metabolites were first evaluated to help establish the level of SA expected (shown in table below).

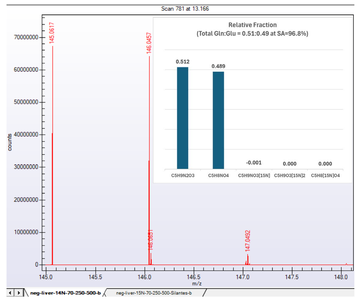
Metabolite	Ionization	Ion Formula	Exact Mass (Da)	RT Range (Min)	Resolving Power	Accurate Mass (Da)	SA (%)
Glutamine (Gln)	ESI-	$C_5H_9N_2O_3^-$	145.0619	13.04-13.28	240K	145.0617	96.8
Glutamic Acid (Glu)	ESI-	$C_5H_8NO_4^-$	146.0448	13.04-13.28	240K	146.0457	96.8
Adenosine Triphosphate (ATP)	ESI+	$C_{10}H_{17}N_5O_{13}P_3^+$	508.0030	13.86-14.18	480K	508.0027	96.3
Nicotinamide Adenine Dinucleotide (NAD)	ESI+	$C_{21}H_{28}N_7O_{14}P_2^+$	664.1164	13.52-13.89	480K	664.1157	95.7

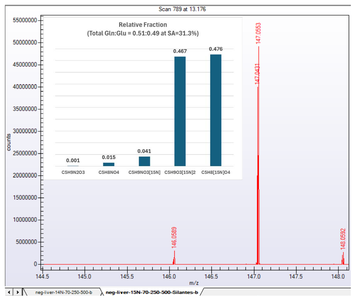
As shown in the next graph, Gln and Glu happen to co-elute with mutual spectral range overlap due to the ~1 Da difference in their monoisotopic masses, very similar to the mixture of unlabeled and labeled ions if the labeling is incomplete. For metabolites with multiple *m* nitrogen atoms, there may be a mixture of all possible labels with *0-m* ^{15}N incorporation. The sCLIPS accounts for these additional ion species in the form of spectral accuracy mixture analysis with ion series (shown below).

The sCLIPS mixture analysis is able to analyze multiple co-eluting labeled or unlabeled metabolites in the same spectral window. For unlabeled Gln and Glu (below 2nd right-most graph), the labeled species automatically came back with essentially zero concentrations. For labeled sample (below far right), the total Gln:Glu ratio compared very well with that from the unlabeled sample by summing variously labeled and unlabeled species, lending confidence to the approach employed.

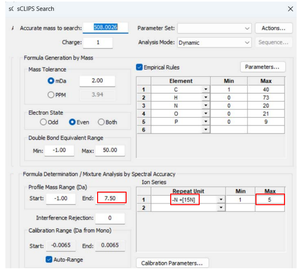


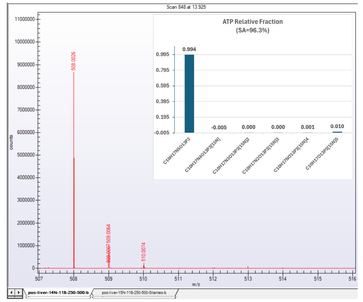


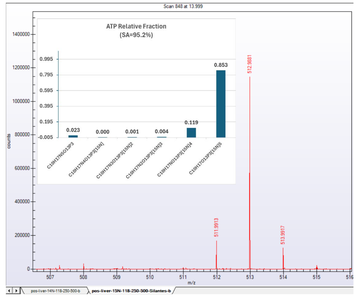




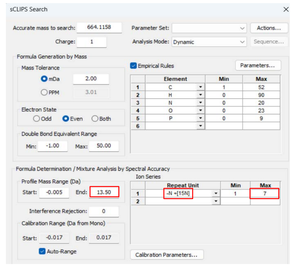
Due to the 5 N atoms in ATP, a wider profile mass spectral range was used along with 0-5 ^{15}N incorporations were provided by the sCLIPS in order to handle a mixture of potentially up to 6 different ion species. When applied to the unlabeled sample, the estimated fractional concentrations for all labeled ion species came back with essentially zero, again validating the approach employed. In the labeled sample, ATP was found to be composed of 2.3% unlabeled, 11.9% with four labels and 85.3% with all five labels, indicating reasonably good incorporation.

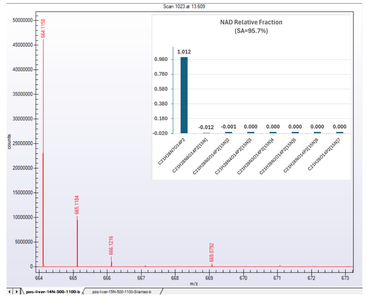


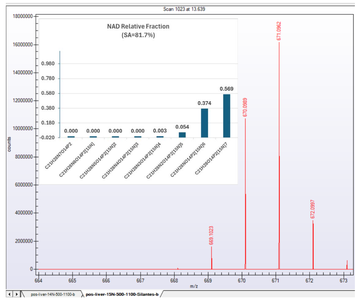




With an even greater number of N atoms, the analysis of NAD required an even wider profile mass spectral range to potentially handle a mixture containing 8 possible ion species. Similarly good confirmation results were obtained for the unlabeled sample. In addition, the ^{15}N incorporation was found to be quite high as well, with 56.9% for full incorporation, 37.4% for six ^{15}N incorporation, and 5.4% for five ^{15}N incorporation.
These incorporation levels and their distribution will not only provide good feedback and confirmation about the labeling process and quality control, but could also be utilized for better and more accurate quantitation of real samples containing many different metabolites across the metabolome.







Conclusions

- Initial results were obtained to validate this new approach for metabolome-wide labeling and quantitation.
- More work is needed to better understand and optimize the spectral accuracy of labeled mixtures, e.g., by exploring other resolving power levels less than 480K/240K, including possibly on a triple quad MS.
- Further confirmation and validation of this approach includes the analysis of mixtures of unlabeled and labeled compounds containing a single N atom.
- For the eventual implementation of this approach, fully automated software tools and solutions will be needed to search for possible metabolites and quantify them in a single process.

References

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Conflict of Interest (COI)

The authors declare no competing financial interest.