Journal of proteome research

Letter

pubs.acs.org/jpr

Combining Mass Spectrometry and NMR Improves Metabolite Detection and Annotation

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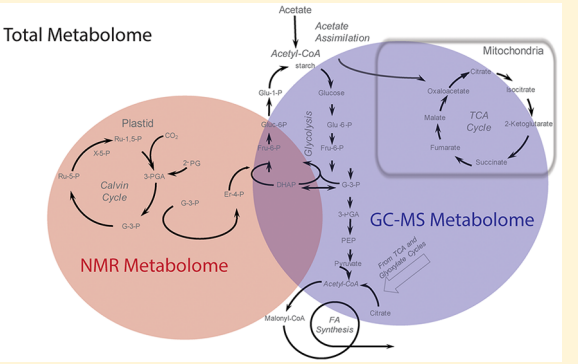
Supporting Information

ABSTRACT: Despite inherent complementarity, nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) are routinely separately employed to characterize metabolomics samples. More troubling is the erroneous view that metabolomics is better served by exclusively utilizing MS. Instead, we demonstrate the importance of combining NMR and MS for metabolomics by using small chemical compound treatments of *Chlamydomonas reinhardtii* as an illustrative example. A total of 102 metabolites were detected (82 by gas chromatography−MS, 20 by NMR, and 22 by both techniques). Out of these, 47 metabolites of interest were identified: 14 metabolites were uniquely identified by NMR, and 16 metabolites were uniquely identified by GC−MS. A total of 17 metabolites were identified by both NMR and GC−MS. In general, metabolites identified by both techniques exhibited similar changes upon compound treatment. In effect, NMR identified key metabolites that were missed by MS and enhanced the overall coverage of the oxidative pentose phosphate pathway, Calvin cycle, tricarboxylic acid cycle, and amino acid biosynthetic pathways that informed on pathway activity in central carbon metabolism, leading to fatty-acid and complex-lipid synthesis. Our study emphasizes a prime advantage of combining multiple analytical techniques: the improved detection and annotation of metabolites.



Metabolomics is experiencing exponential growth[1] and has made substantial contributions to various research areas, such as nutrition, plant physiology, cellular metabolism, disease diagnosis and biomarker detection, and drug discovery and development.[2−4,5,6] To date, metabolomics has primarily relied on the separate application of mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR), but there are also notable examples of the application of surface enhanced Raman spectroscopy and Fourier transform infrared spectroscopy (FTIR).[7] Nevertheless, the vast majority of recently published metabolomics studies are only making use of GC−MS or liquid chromatography (LC)−MS despite prior contributions from NMR and other analytical techniques.[8] In 2017, only 5% of metabolomics manuscripts published in PubMed described any form of a combined NMR and GC−MS approach to metabolomics (Figure 1). This may be explained, in part, by an erroneous belief that mass spectrometry is the optimal analytical technique for metabolomics. Unfortunately, this false perspective has begun to negatively impact the field and will likely limit the coverage of the metabolome, potentially diminish the quality of research, and hamper progress. Instead, metabolomics should seek to maximize (not limit) the number of analytical techniques used to characterize the entirety of the metabolome. Moreover, the confidence and accuracy of metabolite identification and quantification is improved by the application of multiple analytical techniques. Thus, the goal of the field should be to accurately address scientific questions by striving for the broadest coverage of the metabolome, not by focusing on the type of instrumentation used.

NMR and MS are inherently complementary due to their distinct strengths and weaknesses. This, in turn, leads to different sets of metabolites that are uniquely detected by NMR and MS. Accordingly, combining both NMR and MS will result in a greater coverage of the metabolome. Simplistically, NMR detects the most-abundant metabolites, and MS detects the metabolites that are readily ionizable. This arises from fundamental differences between NMR and MS. For example, NMR requires minimal sample handling, but chromatography is a necessary component of MS metabolomics because of the relatively narrow molecular-weight distribution of the metabolome.[9] Chromatography methods are plagued by non-uniform metabolite derivatization, incomplete column recovery, decomposition during derivatization, ion-suppression due to the coeluent matrix, and misaligned retention times, to name a few reasons.[10−14] Similarly, small molecules exhibit variable thermal stability that

Received: July 24, 2018
Published: October 10, 2018

DOI: 10.1021/acs.jproteome.8b00567
J. Proteome Res. XXXX, XXX, XXX−XXX

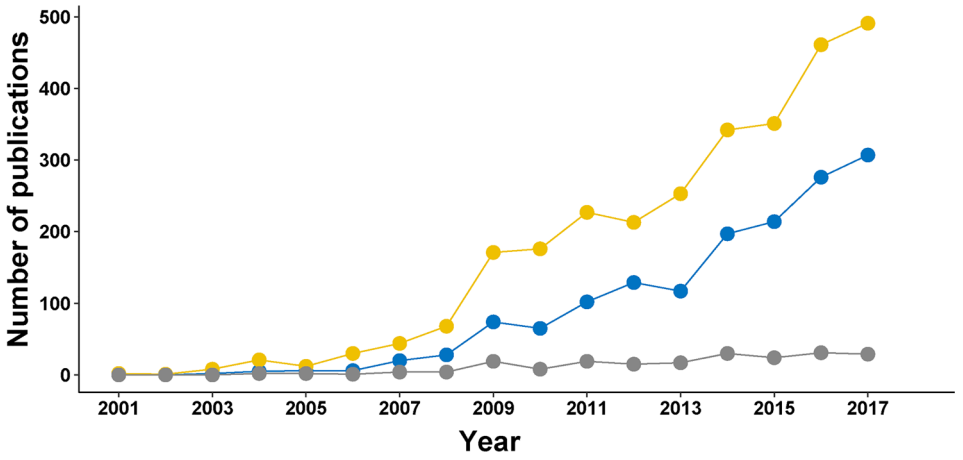


Figure 1. Summary of metabolomics publications in PubMed that refer only to NMR (yellow), only to GC−MS (blue), or to both GC−MS and NMR (gray).

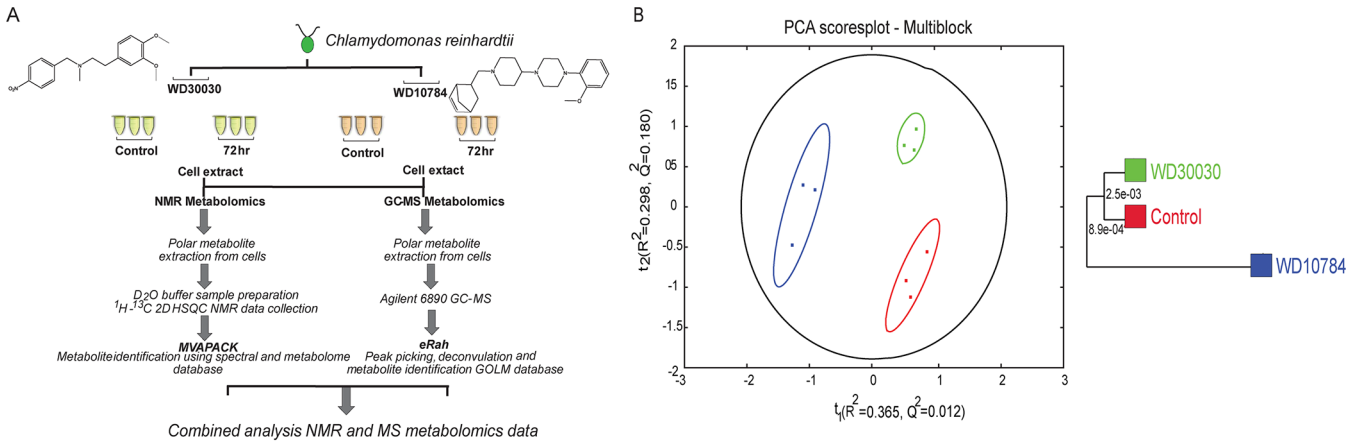


Figure 2. (A) Workflow schematic showing the key steps in the combined NMR and GC−MS analysis of the *C. reinhardtii* metabolome. Three biological replicates were prepared for each group consisting of the untreated controls, WD30030-treated cells, and WD10784-treated cells. A GC−MS spectrum and a 2D $^{1}H-^{13}C$ HSQC NMR spectrum were collected for each biological replicate. (B) Multiblock PCA scores plot generated from the combined GC-MS and 2D $^{1}H-^{13}C$ HSQC NMR data sets illustrating a distinct clustering for untreated controls (red squares) and the WD30030- (green squares) and WD10784- (blue squares) treated cells. A total of three biological replicates are displayed per group, and each data point represents the combined GC−MS and 2D $^{1}H-^{13}C$ HSQC NMR data sets plotted in the PC space. The ellipses represent a 95% confidence limit of the normal distribution of each cluster. The associated dendrogram was derived from the PCA scores plot, and each node is annotated with a Mahalanobis distance-based *p* value. The separation between untreated controls and WD30030 (p value of 2.5×10^{-3}) and WD10784 (p value of 8.9×10^{-4}), respectfully, is considered statistically significant ($p < 0.05$). The color scheme for the dendrogram is the same as the scores plot.

may lead to the loss of metabolites and the erroneous accumulation of degradation products at temperatures routinely used for gas chromatography (GC).[15] Conversely, NMR lacks the sensitivity to detect metabolites in the submicromolar range (≥1 μM) and has limited spectral resolution that often results in peak overlap.[16] MS also has a higher resolution (~10^3 to 10^4) and dynamic range (~10^3 to 10^4) relative to NMR.

Ambiguous peak assignments are a common problem encountered by both NMR and MS. This issue is attributed to limitations in the availability of reference spectra, insufficient software and databases, and our incomplete knowledge of the metabolome. It is believed that nearly all metabolomics investigations have at least one misidentified or unidentified metabolite.[17] Natural product chemistry has routinely employed protocols involving both NMR and MS data to identify novel compounds, but the application of this combinatorial approach has seen limited usage in metabolomics.[18] Nevertheless, a few methods have recently been described that combine NMR and MS to assign metabolites and identify unknowns.[19−21] Notably, the community has recognized that metabolomics needs to continue to move in this direction.[8,21−26] There have also been a few recent examples that highlight the utility and complementarity of combining 1D ^{1}H NMR with direct injection or LC− and GC−MS experiments for metabolomics.[27,28] Most of these examples are methodology-driven; are focused on improving statistical tools and modeling; or performed parallel, but separate, sample analysis.[29−31] In this regards, NMR is routinely only used as a supplement to MS or in a secondary confirmatory role. Accordingly, the full impact of using NMR to characterize a metabolomics sample is missed.

Current estimates suggest the size of the human metabolome is approximately 150 000 metabolites, but only ~~those~~ upward of a few hundred metabolites are typically identified in a given metabolomics study.[32] Combining MS with NMR and other analytical techniques is necessary to move beyond this self-imposed limit.

DOI: 10.1021/acs.jproteome.8b00567
J. Proteome Res. XXXX, XXX, XXX−XXX

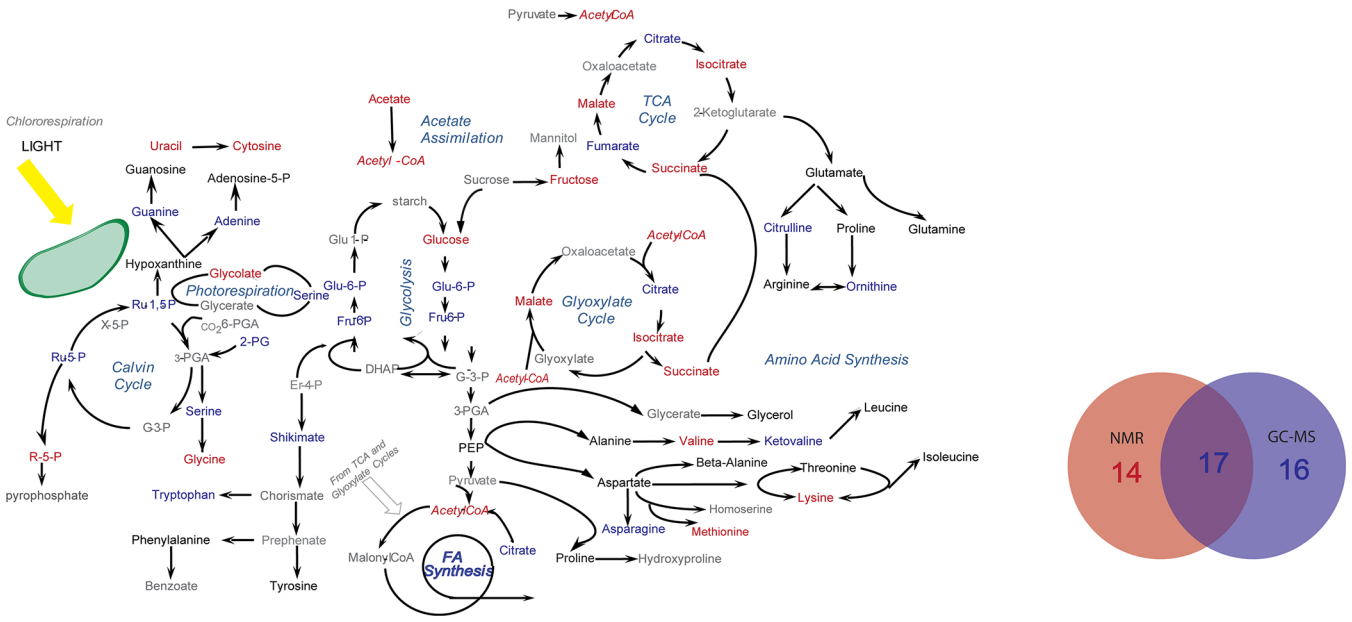


Figure 3. Metabolic pathway summarizing the coverage of the *C. reinhardtii* metabolome (metabolites of interest) from the combined application of NMR and GC−MS. Metabolites that were only identified by NMR are colored blue. Metabolites that were only identified by GC−MS are colored red. Metabolites identified by both methods are colored black, and metabolites that are not identified are colored gray. The embedded Venn diagram identifies the total number of metabolites of interest within these metabolic pathways that were identified either by NMR, by GC−MS, or by both techniques.

To address this need, a global metabolomics study was performed in a platform-unbiased fashion to highlight the intrinsic benefits of combining NMR and MS. In this regard, NMR and MS data were collected on a similar set of samples without complicating existing workflows or requiring major protocol modifications. Accordingly, there were no serious experimental barriers encountered that would prevent the metabolomics community from adapting a combined NMR and MS approach as a standard for the field. As an illustrated example, the metabolome of *Chlamydomonas reinhardtii* grown in tris-acetate phosphate (TAP) media ($^{13}C_2$-acetate for NMR) was characterized by NMR and GC−MS. The cells were also treated with two lipid accumulation modulators (WD30030 and WD10784) as described by Wase et al.[33] The aqueous-extracted metabolomes from treated and untreated cells were then compared to identify metabolic variations due to the compound treatments. The eRah package was used to perform peak picking, retention-time alignment, and metabolite library search for the GC−MS data set.[33,34] Similarly, NMRpipe[35] and NMRviewJ[36] were used for processing and peak picking the NMR data set and metabolite assignments were performed using spectral databases.[37] A schematic overview of the workflow is shown in Figure 2A. Details of data handling, processing and analyses are available as Supporting Information.

The complete 2D $^{1}H-^{13}C$ HSQC NMR spectra obtained from *C. reinhardtii* metabolome extracts were used for unsupervised multivariate analyses to generate a principal component analyses (PCA) scores plot with an associated dendrogram (Figure S-1A). Statistical models were generated after the data was processed as a matrix to be standard normal variate (SNV) normalized and unit variance scaled. The WD30030- and WD10784-treated cells formed distinct clusters separate from the untreated control. The dendrogram generated from the Mahalanobis distances between each point in the PCA scores plot and the resulting p value between each node indicates a statistically significant ($p < 0.05$) separation between each group. Similarly, metabolite assignments from the GC−MS spectral data set were obtained from the eRah package and identified using the GOLM database.[38] The assigned metabolite peak areas were then imported as a matrix into MVAPACK to obtain a comparable PCA scores plot and dendrogram as described above (Figure S-1B).[39] A similar statistically significant group separation between the WD30030- and WD10784-treated cells and the untreated controls was obtained. Importantly, the NMR and GC−MS data sets were successfully combined to generate a comparable multiblock (MB)-principal component analysis (PCA) model with a corresponding dendrogram (Figure 2B).[30] The MB-PCA model provides a single statistical model for both data sets. In this manner, key metabolite differences between the treated and untreated controls can be identified irrespective of the analytical method.

Overall, 82 compounds were identified by GC−MS alone and 20 by NMR alone, and 22 were common to both methods (Tables S-1−S-3). Of these 102 detected metabolites, a total of 47 metabolites of interest were perturbed upon compound treatment (Table S-4). Thus, a greater coverage of compound-induced changes in the *C. reinhardtii* metabolome was obtained by combining the metabolite assignments from the NMR and GC−-MS data sets. Specifically, 14 unique metabolites were identified from the NMR analysis of $^{13}C_2$-acetate labeled *C. reinhardtii* cells that were significantly perturbed upon treatment with either WD30030 or WD10784. Metabolites were assigned using the Biological Magnetic Resonance Bank (BMRB) metabolomics database.[40] Similarly, 16 unique metabolites were identified from the GC−MS spectra using the GOLM database. Furthermore, an additional 17 metabolites were identified by both NMR and GC−MS. In total, the metabolites comprise the following metabolic pathways: the oxidative pentose phosphate pathway, the Calvin cycle, the tricarboxylic acid cycle, and the amino acid biosynthetic

DOI: 10.1021/acs.jproteome.8b00567
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pathways. A summary of the *C. reinhardtii* metabolic changes of interest resulting from treatment with WD30030 and WD10784 is shown in Figure 3.

NMR and GC−MS identified nine glycolytic intermediates in which fructose, glycerol, and pyruvate were uniquely identified by NMR, and fructose-6-phosphate was unique to GC−MS. All 20 amino acids were detected from the combined data sets, but asparagine, cysteine, histidine, serine, and tryptophan were only observed with GC−MS. Consequently, glycine, lysine, methionine, and valine were unique to NMR. Tricarboxylic acid cycle and Calvin cycle metabolites exhibited the most variation. Acetate, isocitrate, ketoglutarate, malate, and succinate were identified by NMR, but fumarate was limited to GC−MS. Ribulose and its phosphate derivatives were exclusively assigned through GC−MS. Nucleotide and nucleoside analogs were the metabolite group consistently observed by both techniques. A total of 7 out of the 10 metabolites (2-deoxy adenosine, adenosine, guanosine, hypoxanthine, inosine, thymine, and xanthosine) were observed by both NMR and GC−MS. Cytosine and uridine were uniquely identified by NMR, whereas uracil was only observed by GC−MS. A complete list of metabolites identified by NMR and GC are provided in Tables S-1−S-4).

The complete set of 22 metabolites identified by both NMR and GC−MS, including the 17 metabolites of interest depicted in Figure 3, were further evaluated for overall consistency between the two methods. A correlation between the 22 common metabolites was evaluated using Pearson correlation within the R environment (http://www.r-project.org), and the resulting comparison is plotted in Figure 4. While there is significant scatter, the overall trend is quite similar. It is important to note that only relative changes in metabolite concentrations were compared. Furthermore, the GC−MS metabolomics analysis was untargeted and lacked any metabolite-specific calibration. Conversely, the absolute quantitation of metabolite concentration changes is an inherent strength of NMR. However, NMR was only used to monitor the relative changes in metabolites derived from $^{13}C_2$-acetate, whereas GC−MS captured total metabolite changes. Differences in the number of sample processing steps may also impart unintended variations. Metabolite derivatization has been identified as a major source of sample variation.[10,12,14] Similarly, variable metabolite stability during GC−MS data acquisition is another potential source of error.[15] Finally, a limited number of biological replicates will also contribute to a larger variance. We want to emphasize that, given these unavoidable discrepancies and the limited number of sample replicates, the observed correlation between the relative changes in metabolite concentration is quite notable. Importantly, the overall trend (or direction) in metabolite concentration change is preserved for the majority of metabolites despite the scatter in the magnitude of these changes. Furthermore, a simple comparison of metabolite trends is probably the limit of the data given the distinct and numerous sources of variance.

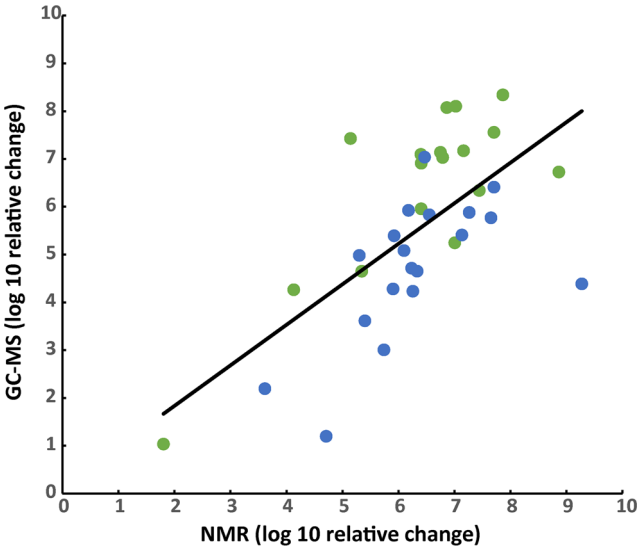

Figure 4. Comparison of the 22 relative metabolite concentration changes detected by NMR and GC−MS. Metabolite changes resulting from treatment with WD30030 and WD10784 are colored green or blue, respectively. The regression line fitted to the data exhibited a correlation coefficient of R^2 0.55 and a confidence interval with a p value of <0.001.

A pair-wise comparison between the 22 individual metabolites identified by both NMR and GC−MS are plotted as line curves in Figure S2. Again, an acceptable level of consistency is achieved in the pair-wise comparisons. A general agreement was also observed in the relative changes between both compound treatments. Any observed discrepancies between metabolite trends may be explained by the fact that GC−MS is capturing the total metabolite change, while NMR is only capturing the changes in metabolites derived from $^{13}C_2$-acetate. In this regard, both measurements are likely correct but are simply observing different aspects of the metabolome. Again, this highlights the inherent strength of combining both NMR and MS. Conversely, if GC−MS observes a significantly lower metabolite concentration relative to NMR, this is a likely an error in the GC−MS data due to a limited thermal stability of the metabolite, variations in derivatization efficiency, and the multipeak phenomena.[12−15] Additionally, given the fact that NMR routinely provides highly accurate sample quantitation relative to MS, NMR is likely to provide the correct metabolite change when the methods disagree (Figure S3).[41]

Extensive (nearly complete) coverage of key metabolic pathways associated with lipid accumulation was only achieved by combining NMR and GC−MS data. In effect, the NMR data filled-in the metabolites that were missed by GC−MS. Importantly, the broader coverage of the *C. reinhardtii* metabolome was able to provide a comprehensive view of the algae's response to a compound treatment. This level of detail is essential to further our understanding of the mechanism of action of drug leads, of drug resistance, and of disease development and progression, among numerous other potential utilities. Achieving this level of coverage of the metabolome requires employing multiple analytical techniques. This viewpoint is consistent with some prior observations.[8,21−26] For example, Chen et al. noted an improvement in biomarker identification by combining 1D ^{1}H NMR and GC−MS for the analysis of urine from patients with bipolar disorder.[42] Another recent example highlighted the use of 1D ^{1}H NMR and GC−MS for the analysis of bronchial-wash fluid to investigate responsiveness to air pollution.[43] Barding et al. have highlighted similar improvements in coverage of the metabolome in molecular response of rice to stress.[44] These studies were able to combine multiple data sets to obtain a robust set of biomarkers, which further emphasizes the benefit

DOI: 10.1021/acs.jproteome.8b00567
J. Proteome Res. XXXX, XXX, XXX−XXX

of combining multiple analytical platforms for metabolomics. These are other recent examples in which both NMR and GC−MS metabolomics data sets have been integrated for applications in biomarker identification, food chemistry, and plant physiology.[45−48]

To date, the majority of metabolomics studies have been self-limited to a single analytical platform (Figure 1). This is despite the fact that NMR and MS (and other analytical techniques) are highly complementary. Furthermore, existing workflows (Figure 2A) can easily accommodate the inclusion of both techniques. Consequently, there is little to no barrier to the broad adoption by the scientific community of a multianalytical approach to metabolomics. Importantly, and as clearly demonstrated herein, combining NMR and MS improves the coverage of the metabolome, increases the accuracy of metabolite assignments,[19−21] and provides redundant validation of metabolite changes. In fact, our results demonstrate a limited overlap in the metabolites identified by both NMR and GC−MS. However, most metabolites in common did exhibit consistent trends in relative concentration changes, showcasing the robustness of the combined approach. Our results provide clear evidence that both NMR and MS are equally valuable and necessary for metabolomics studies and that combining multiple analytical sources is essential to the future of metabolomics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00567.

Additional experimental methods; figures showing PCA scores plots, individual line plots, and a comparison of metabolite changes; tables showing lists of metabolites uniquely identified with analysis methods and a comparison of metabolites of interest (PDF)

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Author Contributions

F.B. and N.W. performed the experiments; R.P. and C.D. designed the experiments; F.B., N.W., C.D., and R.P. analyzed the data and wrote the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Martha Morton, the Director of the Research Instrumentation Facility in the Department of Chemistry at the University of Nebraska-Lincoln, for her assistance with the NMR experiments. This material is based on work supported by the National Science Foundation under grant no. 1660921. This work was supported in part by funding from the Redox Biology Center (P30 GM103335, NIGMS) and the Nebraska Center for Integrated Biomolecular Communication (P20 GM113126, NIGMS). The research was performed in facilities renovated with support from the National Institutes of Health (RR015468-01). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

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DOI: 10.1021/acs.jproteome.8b00567
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