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Evaluating Protocols for Reproducible Targeted Metabolomics by NMR

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Metabolomics aims to study the downstream effects of variables like diet, environment, or disease on a given biological system. However, inconsistencies in sample preparation, data acquisition/processing protocols lead to reproducibility and accuracy concerns. A systematic study was conducted to assess how sample preparation methods and data analysis platforms affect metabolite susceptibility. A targeted panel of 25 metabolites was evaluated in 69 clinical metabolomics samples prepared following three different protocols: intact, ultrafiltration, and protein precipitation. The resulting metabolic profiles were characterized by 1D ^{1}H nuclear magnetic resonance (NMR) spectroscopy and analyzed with Chenomx v8.3 and SMolESY software packages. Greater than 90% of the metabolites were extracted more efficiently using protein precipitation than filtration, which aligns with previously reported results. Additionally, analysis of data processing software suggests that metabolite concentrations were overestimated by Chenomx batch-fitting, which only appears reliable for determining relative fold changes rather than absolute quantification. However, an assisted-fit method provided sufficient guidance to achieve accurate results while avoiding a time-consuming fully manual-fitting approach. By combining our results with previous studies, we can now provide a list of 5 common metabolites [2-hydroxybutyrate (2-HB), choline, dimethylamine (DMA), glutamate, lactate] with a high degree of variability in reported fold changes and standard deviations that need careful consideration before being annotated as potential biomarkers. Our results show that sample preparation and data processing package critically impact clinical metabolomics study success. There is a clear need for an increased degree of standardization and harmonization of methods across the metabolomics community to ensure reliable outcomes.

1 Introduction

Metabolomics is the process of identifying and quantifying the small molecular weight (<1000 Da) metabolites present in a given organism, system, or sample.[1-3] Metabolomics has been applied to clinical, environmental, and agricultural projects to investigate metabolic networks of interest within humans, plants and animals.[4-7] The increased sensitivity of the metabolome to stressors can be leveraged to learn how diet, the environment and diseases affect underlying biochemical pathways.[8-12] This heightened sensitivity of the metabolome relative to the genome or proteome allows researchers to gain deeper insights and to obtain reliable molecular mechanisms of diseases, drugs, and further our understanding of a variety of biological systems. However, a downside of the increased sensitivity of metabolomics are biologically irrelevant changes in response to storage and preparation conditions during analysis.[13-16]

The typical workflow for metabolomics studies involves isolating and extracting fractions of metabolites from the biological samples to be evaluated by an analytical instrumentation before finally assessing the generated data or spectra with univariate or multivariate statistical methods. While the process may seem simple, there are many opportunities for inconsistency when making choices about factors like storage temperature, extraction method, sample preparation and instrumentation type.[17] In this regard, the metabolomics process comprises numerous decision points that may impact the composition of the detectable metabolome and lead to reproducibility and accuracy concerns.[18]

Several studies have shown that metabolites are susceptible to variation due to both pre-analytic and post-analytic

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† Electronic Supplementary Information (ESI) available: Supplementary Fig. S1-S64 showing methods, PCA scores plots and standard deviation scatterplots. Supplementary Tables S1-S7 providing scaled data, p-value calculations and standard deviation analysis as referenced in text. See DOI: 10.1039/x0xx00000x

protocols. For example, Breier et al. 2014 investigated the effects of collection, transport, and sample processing methods on a panel of metabolites and found that amino acids were particularly fragile during simulated transportation.[19] Residual enzymatic activity was also observed to be present even after the sample was frozen, which suggested the potential need for a single freeze-thaw cycle to achieve a stable metabolomics sample.[19] Nevertheless, long term storage even at ultra-low -80°C temperatures resulted in statistically significant (± 15%) changes in 50% of the analyzed metabolites.[14] Want et al. 2009 demonstrated that varying the extraction method and the solvent composition produced unique metabolite panels and yielded distinct extraction efficiencies.[20] A total of 14 different extraction methods were examined and compared in which a 100% methanol extraction of serum was deemed to provide the best comprehensive metabolite profile with a high degree of protein removal.[20] Despite similar on-going efforts by COSMOS: Coordination of Standards in Metabolomics, MSI: Metabolomics Standards Initiative, mQACC: Metabolomics Quality Assurance & Quality Control Consortium, and MANA: Metabolomics Association of North America to establish community standards and best practices, there continues to be a proliferation of metabolomics methodologies populating the scientific literature.[21-27] Simply, the metabolomics community has yet to broadly adopt well-vetted general guidelines for metabolomics studies.[20-22, 28-30]

Choosing the correct sample preparation technique depends on several factors that include both the type and the amount of the biological samples, and the metabolites being investigated. The choice of analytical platform may also be guided by these and other considerations. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are commonly used in metabolomics studies and provide complementary results.[31, 32] NMR detects the most abundant metabolites (> 1 mM) while providing accurate and highly reproducible absolute quantification. Conversely, MS, which is typically combined with liquid- or gas-chromatography, provides a broader coverage of the metabolome with a significantly lower limit of detection and a larger dynamic range. However, one of the important benefits of NMR over MS as an analytical platform for metabolomics is the limited need for sample manipulation, which lowers the variability and inconsistency across and between data sets.[33, 34] Specifically, for liquid biological samples like blood, urine, or other biofluids, an intact sample preparation approach can often be effective in providing reliable and reproducible metabolome concentration changes. An intact sample preparation method requires the simple addition to the sample of a deuterated buffer containing an internal standard. Additional processing, such as the removal of other endogenous biomolecules, is not needed. However, an intact sample approach risks metabolite signal suppression due to metabolite-protein (or other biomolecules) binding, which yields lower resolution and lower intensity peaks with a broadened baseline. The bound metabolites may remain completely undetected in the resulting NMR spectra, which can lead to erroneous conclusions about the perceived changes in the system. While intact sample preparation has advantages, alternative protocols such as solvent-induced protein precipitation or ultrafiltration extract the metabolites of interest while simultaneously removing unwanted biomaterials before analysis.[28, 35-40]

Another potential source of variation or error in a metabolomics study comes from the myriad of data processing tools used to analyze a given spectral data set.[41] For NMR, a few options for metabolomics software toolkits include Chenomx (Chenomx, INC, Alberta Canada), MetaboAnalyst 5.0 (https://www.metaboanalyst.ca), MVAPACK (https://mvapack.unl.edu), and SMolESY (https://github.com/pantakis/SMolESY_platform).[42-44] Each of these software platforms uniquely handles the NMR data by relying on a variety of spectral binning, metabolite identification algorithms, or statistical analysis options. Especially among academia, there is an understandable preference for freely available software like MetaboAnalyst 6.0, MVAPACK, or SMolESY, although commercial software does provide valuable benefits such as customer support and routine updates. The broad range of available algorithms in metabolomics software allows researchers the flexibility to choose and optimize data processing pipelines to meet their individual needs, but difficulties often arise when making inter-laboratory comparisons or assessing data accuracy and reproducibility. Consequently, the harmonization of metabolomics methods, data, and results is an ongoing effort that has only made modest advancements with plenty of opportunities for improvement.[21, 24-26]

Despite the potential influence of other factors on metabolic profiles, such as sample storage, our primary objective was to explore only the effects of sample treatment and the choice of software on the accurate identification and quantification of a common set of plasma metabolites. With our approach, we tried to simulate the typical pipeline followed in conducting an NMR-based metabolomics study, specifically without prior knowledge of the samples content. The reproducibility of metabolome measurements was assessed with a 1D ^{1}H NMR data set of human plasma samples since blood samples are commonly used in clinical metabolomics studies. The data set consists of plasma samples obtained from patients diagnosed with inflammatory bowel disease (IBD) that were age and sex matched to healthy controls. An important feature of our study is that our data was combined with previously published results to achieve a comprehensive view of the degree of variation inherent to individual plasma metabolites. In this regard, the outcomes of our research will contribute to the ongoing metabolomics harmonization efforts by determining how sample preparation methods and NMR acquisition and data processing parameters affect the absolute concentration, statistical significance of between-group and within group metabolite changes, and the reliability and reproducibility of metabolite quantification and annotation.

2 Material and Methods

Details for the study design and cohort demographics, preparation of pooled QC samples, preparation of NMR samples

using ultrafiltration, preparation of NMR samples using methanol precipitation, preparation of NMR samples using intact plasma, NMR data acquisition, ~~data processing~~representative labelled NMR spectra, data processing, —and statistical analyses are included in the Electronic Supplementary Information (ESI†).

3 Results and discussion

3.1 Metabolic Profile is Determined by Protein Removal Protocol and Analysis Method

Principal components analysis (PCA) of the 1D ^{1}H NMR data sets yielded a scores plot containing two distinct clusters comprising either the intact plasma samples or the ultrafiltered and methanol-precipitated plasma samples (see ESI Fig. S1-S2†). Conversely, there was no separation in the PCA scores plot according to clinical group (see ESI Table S1-S2~~2~~†, Fig. S3-S4†). The PCA results for each preparation method indicate that the pooled samples are located in the center of the PCA scores plot of the first two components exhibiting low variability. Additionally, t~~T~~he lack of separation based on pathology increases the level of confidence that the observed group differences were truly derived from only the sample preparation method. ~~Additionally~~Finally, it is encouraging to note that these trends persist across all data irrespective of whether the PCA was ~~modeled~~modelled with binned raw spectral data (see ESI Fig. S3~~2~~a, S4~~3~~a†), Chenomx data (see ESI Fig. S3~~2~~b, S4~~3~~b), or SMolESY data (see ESI Fig. S3~~2~~c, S4~~3~~c).

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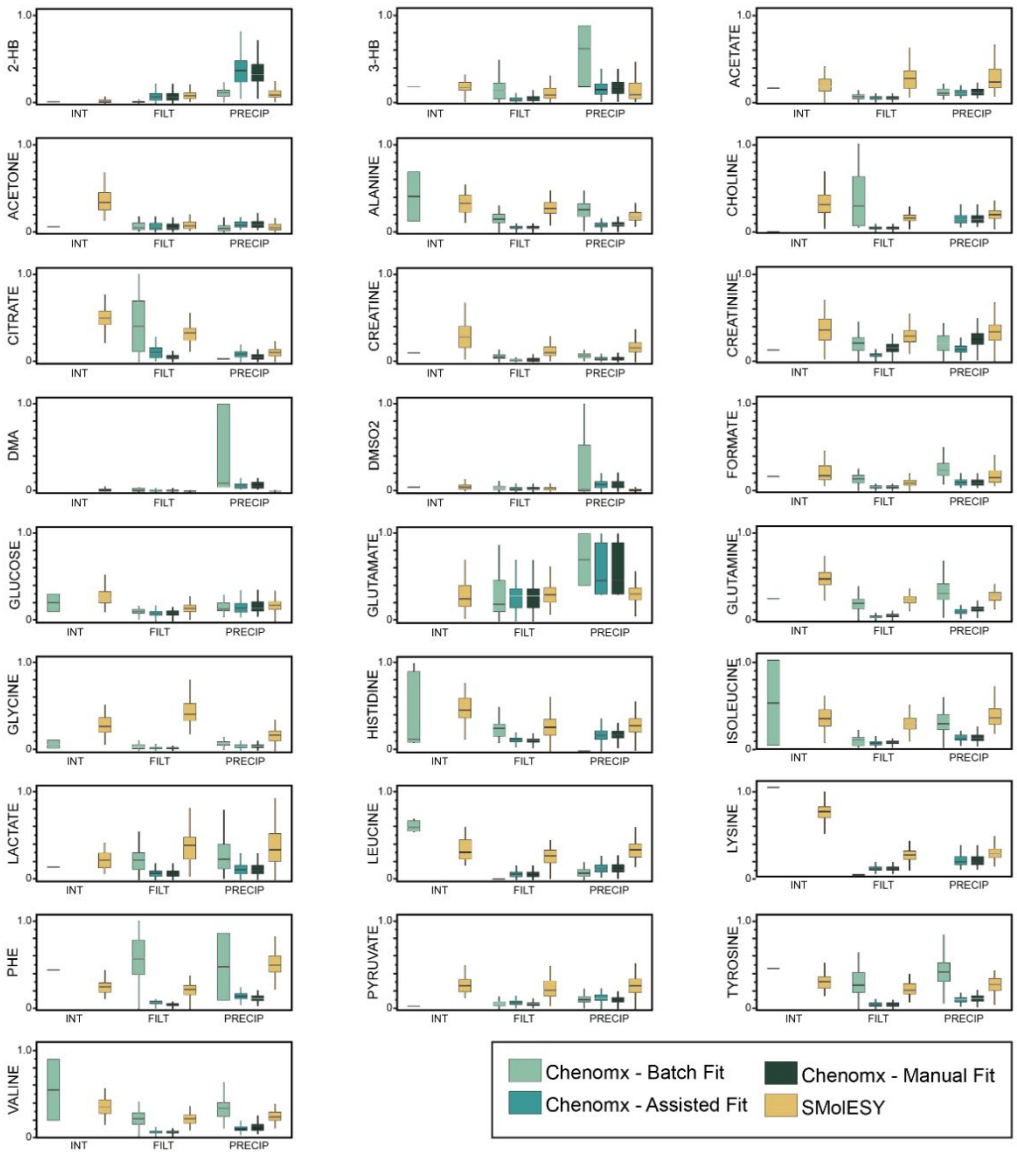


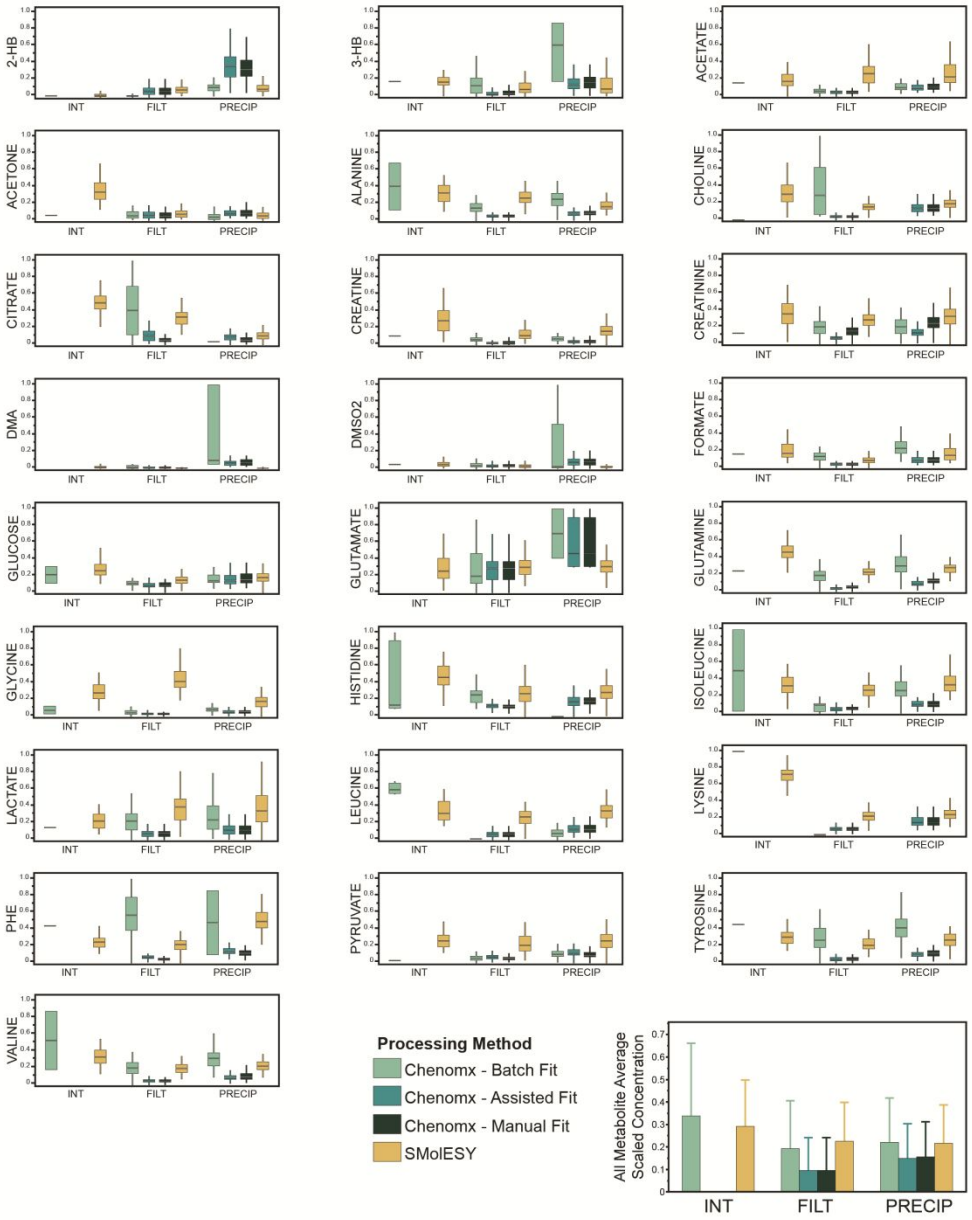


Fig 1 Scaled Metabolite Concentrations Organized by Fit Method and Preparation. Box and whisker plots of range scaled (0-1) data for each of the 25 metabolites. Plots are placed in alphabetical order except for the final panel. ~~Panel 26 is a combined bar chart plotting the average of the 25 previous panels.~~ Each individual box and whisker plot is organized left-to-right by preparation method from intact (INT), to ultrafiltered (FILT), to methanol-induced protein precipitated (PRECIP). The color of each box corresponds to the processing method used to generate the data in the following order: Chenomx – batch-fit (light blue), Chenomx- assisted-fit (teal), Chenomx – manual-fit (dark green), SMolESY (gold). Abbreviations: 2-HB: 2-hydroxybutyrate, 3-HB: 3-hydroxybuytrate, DMSO2: dimethyl sulfone, DMA: dimethylamine.

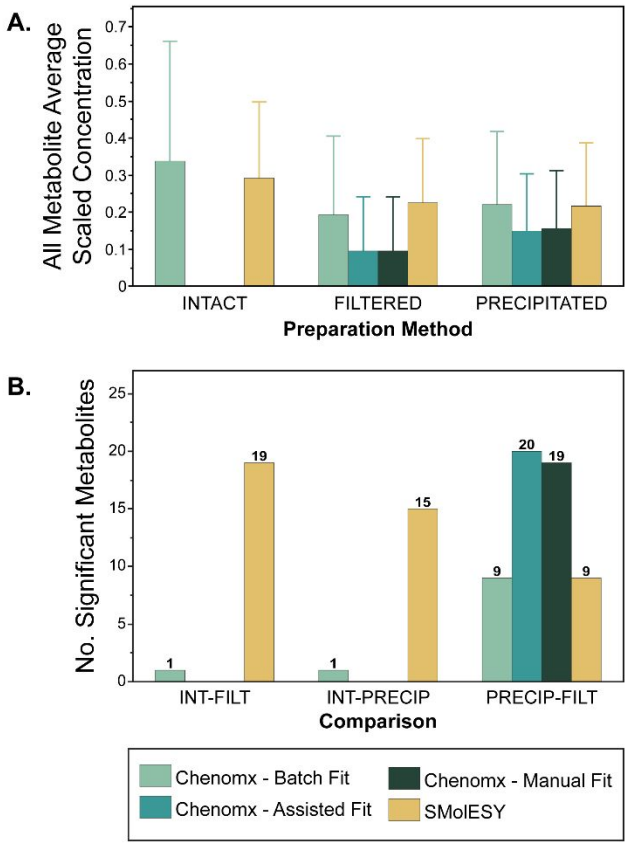
3.2 Methanol Protein Precipitation Leads to Higher Overall Metabolite Concentrations

Fig. 1 and Table S3† summarize the scaled measured concentrations for the 25 targeted metabolites grouped by sample preparation protocol and data processing method. Table S3† also lists the coefficient of variation (CV) and percent differences in the measured metabolite concentrations, which are calculated relative to the manual analysis of protein precipitation plasma samples. Fig. 2 summarizes~~highlights~~ the metabolites that were significantly altered (p < 0.05) in a pairwise comparison of sample preparation protocols and according to processing method. A more detailed overview of the comparisons can be seen in Fig. S5 and ESI Table S4. In nearly all cases, almost all the metabolites in the panel were detected. The Chenomx batch-fit approach missed the greatest number of metabolites, omitting choline, histidine, and lysine when analyzing the plasma NMR samples following methanol-induced protein precipitation. In general, the assisted-fit Chenomx analysis of the 1D ^{1}H NMR spectra acquired for the methanol-induced protein precipitation plasma samples had the most accurate (lowest % difference from Chenomx manual-fit data) metabolite concentrations where the average percent

difference was 7% (for the 19 metabolite concentrations lower in manual-fit) or -16% (5 higher in assisted-fit) (see ESI Table S3†). The same Chenomx analysis of the plasma NMR samples prepared by ultrafiltration yielded a nearly uniform decrease in the measured metabolite concentrations corresponding to an average decrease of 57% for manual-fit and a decrease of 67% for assisted-fit. The observed decrease in ultrafiltered plasma samples aligns well with previous results.[35] Citrate, an endogenous metabolite, was the only notable exception. Overall, 96% (24) of the 25 targeted metabolites were determined to have at a higher concentration in the methanol-precipitated plasma samples with 92% (23) of these concentration differences being statistically significant (Fig. 2, Fig S5, see ESI Table S4†).

3.3 SMolESY and Chenomx Batch-Fit Tend to Overestimate Metabolite Concentrations

The batch-fit Chenomx analysis and SMoIESY processing protocols of both the methanol-precipitated and ultrafiltered NMR data sets yielded similar, but mixed results relative to the manual analysis of the methanol-precipitated data set. In general, metabolite concentrations tended to increase with batch-fit and SMoIESY. For example, batch-fit analysis produced 15 metabolites with an average increase in concentration of 98% (see ESI Table S3†). This trend is clearly apparent for 3-hydroxybutyrate (3-HB), alanine, creatinine, dimethyl sulfone (DMSO2), DMA, formate, glutamate, glutamine, glycine, isoleucine, lactate, phenylalanine, tyrosine, and valine (Fig. 1).


A.
All Metabolite Average Scaled Concentration
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
INTACT
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Preparation Method
B.
No. Significant Metabolites
25
20
15
10
5
0
1
19
1
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20
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INT-FILT
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Comparison
Chenomx - Batch Fit
Chenomx - Manual Fit
Chenomx - Assisted Fit
SMolESY

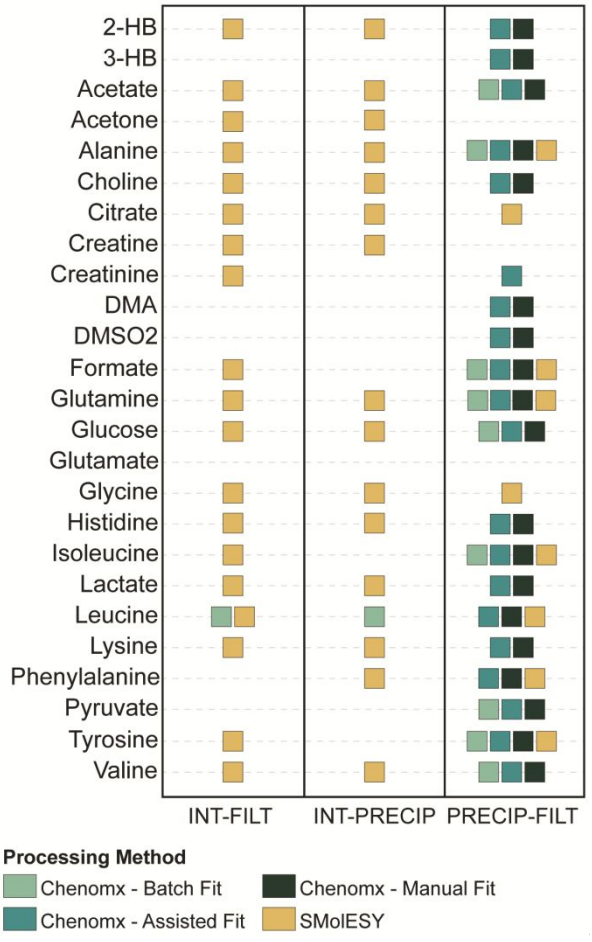
2-HB
3-HB
Acetate
Acetone
Alanine
Choline
Citrate
Creatine
Creatinine
DMA
DMSO2
Formate
Glutamine
Glucose
Glutamate
Glycine
Histidine
Isoleucine
Lactate
Leucine
Lysine
Phenylalanine
Pyruvate
Tyrosine
Valine
INT-FILT
INT-PRECIP
PRECIP-FILT
Processing Method
Chenomx - Batch Fit
Chenomx - Manual Fit
Chenomx - Assisted Fit
SMolESY

Fig. 2 Summary bar charts showing **a.** general trends in metabolite concentration and **b.** the total number of metabolites that had statistically significant concentration variation between sample preparation type. Color-coded table lists metabolites in alphabetical order from top to bottom with statistical significance noted by presence or absence of colored square in the corresponding row. Presence of a square in a metabolite row denotes that it was found to be statistically different ($p < 0.05$) when comparing intact (INT) samples to ultrafiltered (FILT) samples (INT-FILT, left), intact samples to methanol-induced protein precipitated (PRECIP) samples (INT-PRECIP, middle), or methanol-induced protein precipitated to ultrafiltered samples (PRECIP-FILT, right). The color of the square denotes the processing method the metabolite was identified as being statistically different: Chenomx–Batch fit (light blue), Chenomx–assisted fit (teal), Chenomx–Manual fit (dark green), SMolESY (gold). Abbreviations: 2-HB: 2-hydroxybutyrate, 3-HB: 3-hydroxybuytrate, DMSO2: dimethyl sulfone, DMA: dimethylamine.

Alternatively, the batch-fit Chenomx analysis measured 7 metabolites with a 29% decrease in concentration for the methanol-precipitated NMR data set. SMolESY analysis yielded nearly identical results to Chenomx corresponding to 19 metabolites with an average increase in concentration of 98% and 6 metabolites with a 48% decrease in concentration. Similarly, the batch-fit Chenomx analysis of the ultrafiltered NMR data set resulted in 12 metabolites with a 99% average increase in concentration compared to 13 metabolites with a 52% decrease in concentration. Again, SMolESY processing yielded comparable results with 15 metabolites exhibiting a 121% average increase in concentration compared to 10 metabolites with a 38% decrease in concentration.

The metabolite concentrations measured by the batch-fit Chenomx analysis varied significantly ($p < 0.05$) from the values determined by the assisted-fit or manual-fit methods in 52% of the ultrafiltered plasma samples and in 41% of the methanol-precipitated plasma samples (Fig. 2, see ESI Table S4†). Overall, Chenomx determined 80% of the metabolites to be significantly different between the methanol-precipitated plasma samples and the ultrafiltered plasma samples, but SMolESY only found a statistical difference in 36% of the metabolites.

3.4 Biological Variance is Primary Factor Affecting Precision in Measured Metabolite Concentrations

Unsurprisingly for biological samples, the overall variability in the measured metabolite concentrations were high as assessed by CVs (see ESI Table S3, S5†). The Chenomx analysis of the 69 methanol-precipitated plasma samples yielded average CVs ranging from 97 to 102%. SMolESY processing yielded a slightly improved but not statistically significant ($p > 0.4$) average CV of 86%. Given the overall lower metabolite concentrations measured for the ultrafiltration samples, the resulting CVs were equally reduced and ranged from an average of 57 to 82% for Chenomx and a comparable average CV of 75% for SMolESY. Notably, only the manual-fit and assisted-fit Chenomx analysis led to average CV values that were significantly different ($p < 0.0001$) between the methanol-precipitated plasma samples and ultrafiltered plasma samples. The overall average 25% decrease in CV values is partly attributed to the average 55% decrease in metabolite concentrations. Overall, there is no clear evidence to suggest that sample preparation protocol or data processing method produced an inherently better precision. Instead, the natural biological variation appears to be the dominating factor.

3.5 Analysis of Intact Plasma Samples Required Specialized Protocols and Algorithms

The analysis of the intact plasma sample was completed using both the Chenomx and SMolESY software platforms. SMolESY was used to analyze the NOESY and CPMG NMR datasets and Chenomx was only used to analyze the CPMG NMR spectra. Chenomx is not able to account for the large protein background signal present in the NOESY NMR spectra. Conversely, SMolESY was specifically designed to evaluate intact plasma samples acquired with a NOESY pulse sequence. SMolESY mimics the effects of the CPMG NMR experiment and computationally removes the macromolecule baseline from the NMR spectra before evaluating peak areas. In this regard, SMolESY was able to successfully detect 100% of the metabolite panel in 100% of the 69 NOESY and CPMG spectra acquired for the intact plasma samples. Encouragingly, the measured metabolites using both the NOESY and CPMG spectra yielded similar results from the SMolESY platform. The average concentration difference ranged from 17% to 19% with only 6

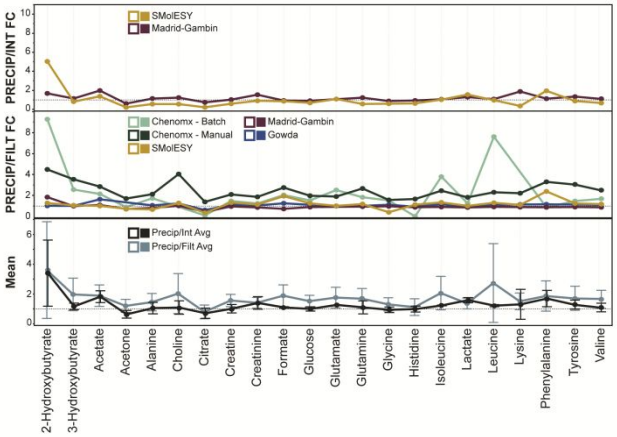

Fig. 3 Comparative Analysis of Results. Line graph plotting the fold change of each metabolite as reported by previously published studies and present results. Fold changes (FC) are organized by group with top panel containing precipitated sample/intact sample fold changes from SMolESY data (gold) and Madrid-Gambin et al. (2023) data (maroon). The middle panel contains methanol-induced protein precipitated (PRECIP) and ultrafiltered (FILT) sample fold changes from Chenomx – batch-fit results, Chenomx – manual-fit results, SMolESY results, Madrid-Gambin et al. (2023) results, and Gowda et al. (2014) results. The bottom panel contains line plots of calculated averages from the top two panels (Chenomx results were excluded from the Precip/Filt Avg line since they were identified as unreliable). The color of the line is correlated to the method or previous publication.[26, 50] A dashed line is present at FC = 1 for reference. All fold changes were calculated from the data generated by each processing method or publication. For example, the gold SMolESY lines are generated from SMolESY data. No cross-comparative fold changes were calculated or plotted.

statistically significant differences ($p < 0.05$). In total, eleven metabolites were measured at a higher concentration using the CPMG NMR spectra with only the differences in 2-HB and formate being statistically significant. A total of 14 metabolites resulted in a lower concentration with the CPMG spectra but only the measured concentration differences for 3-HB, acetone, glutamate, and lysine were statistically significant. Again, the

overall variability in these measured metabolite concentrations were high as assessed by CVs and ranged from 45 to 322%. Overall, the variability in the measured metabolite concentrations appear to be determined by a combination of inherent biological variations and the absolute metabolite concentration.

The SMolESY processing of the intact plasma samples resulted in statistically significant ($p < 0.05$) differences in the measured metabolite concentrations relative to manual-fit Chenomx analysis of the methanol-precipitated plasma samples (see ESI Tables S3, S5†). Specifically, the SMolESY processing of the NOESY data set resulted in an average increase in measured metabolite concentrations by 174% for 20 of the 25 metabolites. Five metabolites were measured with an average 57% decrease in concentration. 16 of these metabolite concentration differences were statistically significant ($p < 0.05$).

Although the CPMG pulse sequence is not recommended for quantitative analyses due to its interference with signal intensities, we also analyzed CPMG profiles since they are more similar to the profiles of the extracted samples (i.e., without the broad macromolecular signals). The intact plasma samples acquired with the CPMG pulse sequence exhibited the poorest Chenomx metabolic profiling across several metrics. Metabolites were only detected in 3% of the intact samples and required extensive manual analysis to retrieve any semblance of usable data. The very sparse results that were produced by this analysis were also clearly incorrect. Chenomx batch-fit results of the CPMG experiments suggested that in the few samples metabolites were detected in, that they were found to be at a much higher or much lower concentration than the NOESY experiments. For example, when measured with the CPMG pulse sequence, glutamine was found to be at 1003.8 mM on average but only at 164 mM in the NOESY experiments and DMA was detected at 0.6 mM in CPMG experiments but 4.85 mM in NOESY experiments.

3.6 Reproducibility of Metabolomics Sample Preparation Protocols

Gowda et al. (2014) previously reported an increase in metabolite concentrations in serum and plasma samples prepared by protein precipitation methods compared to either an ultrafiltration protocol or to an intact sample.[35] Additionally, they determined that intact sample preparation was not appropriate for metabolite quantitation. Thus, the protein precipitation approach was identified as a robust and best-choice method.[35] The higher percentage of metabolites recovered through precipitation is probably a contributing factor to its relative popularity as a metabolite extraction method compared to other options.[45] Precipitating proteins from a biofluid sample removes 98% of the proteins and the addition of organic solvent to a biofluid sample will disrupt any metabolite-protein binding.[45] However, the results from Tiziani et al. (2008) directly contradicts these observations.[35, 46] Specifically, 3-HB, alanine, creatine, citrate, isobutyrate, succinate, threonine, glucose, glutamate, and ethanol were all found by Tiziani et al. (2008) to be significantly increased in filtered samples compared to precipitated samples.46 A recently published study by Madrid-Gambin et al. (2023) found an increase in concentration for 63% of the metabolites measured from protein-filtered samples compared to an increase in concentrations for 37% of the metabolites measured from the protein-precipitated samples.[47] The methanol-induced protein precipitation protocol was observed to produce a non-ideal baseline in the 1D ^{1}H NMR spectra. As a result, glycerophospholipid solid-phase extraction (g-SPE) was reccomended as the prefered sample preparation method. These divergent results and a corresponding lack in reproducibility show a clear gap in our fundamental knowledge of metabolomics and the effects of sample preparation method.

A direct comparison of these published outcomes with our results can be challenging due to missing or variations in experiemental parameters and because of fundamental differences in the scale of reported metabolite concenrations. Fortunately, Gowda et al. (2014) and Madrid-Gambin et al. (2023) provided sufficient information to enable proper scaling of study data for a direct comparison with our results (see ESI Table S6†). Fig. 3 (top) plots the fold change (FC) in metabolite concentrations derived by comparing protein precipitation samples (PRECIP) to intact plasma samples (INT) for the commonly reported metabolites. Similarly, FCs were also calculated between PRECIP and ultrafiltration (FILT) samples (Fig. 3, middle). An average of these previously published FCs was then compared to our results (Fig. 3, bottom). Since Gowda et al. (2014) determined an intact plasma sample was not appropriate for metabolite quantitation, this data was excluded from Fig. 3.[35] The Chenomx analysis of the intact plasma sample was similarly determined to be unreliable and was not included in the fold change comparison presented in Fig. 3. Encouragingly, a qualitative examination of all the FCs suggest similar overall trends that are independent of study. The overall consistency in the FC calculated from the three independent studies and the sample preparation methods is readily apparent in the overlay of the average FC values (Fig. 3, bottom). Please note the overlap of the average metabolite concentrations with the error bars and the close clustering around or slightly above an FC of 1 in most cases. This suggests the relative FCs likely negates sources of error and/or variability readily apparent in the individual measurements of absolute metabolite concentrations.

For the PRECIP/INTACT FC comparison from (Fig. 3, top), it is particularly encouraging to note that our SMolESY derived FC values closely aligns with the results from Madrid-Gambin et al. (2023) with only a few notable deviations. Specifically, SMolESY measured a significantly higher FC for 2-HB. The FC values for 3-HB, alanine, choline, creatine, creatinine, glutamine, leucine, lysine, tyrosine and valine were opposite in the relative directions (i.e., increased or decreased). Madrid-Gambin et al. (2023) did not report a concentration for glutamate. Overall, the Madrid-Gambin et al. (2023) concentrations averaged 39% higher than the SMolESY derived FCs.

In the PRECIP/FILT comparison (Fig. 3, middle), the manually-fit Chenomx FC values were systematically increased relative to SMolESY and the other studies. Chenomx

consistently underestimated metabolite concentrations from ultrafiltered plasma samples and/or overestimated concentrations for the methanol-precipitated plasma samples. The manually-fit Chenomx FC values averaged 2.47 ± 0.81 compared to 1.09 ± 0.18 for Gowda et al. (2014), 0.92 ± 0.25 for Madrid-Gambin et al. (2023), and 1.15 ± 0.44 for SMolESY. A few notable deviations were the relatively large (>200%) FC increases for 2-HB, 3-HB, choline, and citrate. Despite these systemetic offsets, the manual-fit Chenomx metabolite concentrations appears to follow a similar trend as the other studies and SMolESY. For example, the spike in the FC of choline, the relative decrease in the citrate FC, and the relative increase in isoleucine FC were mirrored by SMolESY.

The batch-fit Chenomx analysis provided metabolite concentrations that were also systematically higher than the values determined by other studies and SMolESY with an average FC value of 2.40 ± 2.28. Again, Chenomx consistently underestimated metabolite concentrations from ultrafiltered plasma samples and/or overestimated concentrations for the methanol-precipitated plasma samples. A relatively large FC increase (236% to 589%) was observed for 2-HB, isoleucine, and leucine. Also, the batch-fit Chenomx analysis did not report a concentration for choline, histidine or lysine. It was also notable that the batch-fit Chenomx FC values had the highest variability relative to the other methods and studies. The lower correlation in the Chenomx measured metabolite concentrations from the ultrafiltered and methanol-precipitated plasma samples and the resulting higher FC values were surprising since Chenomx is more compatible with extracted samples. Conversely, the FC values calculated from the Gowda et al. (2014), Madrid-Gambin et al. (2023), and SMolESY analysis yielded average values near one suggesting a good correlation in the measured metabolite concentrations from the ultrafiltered and methanol-precipitated plasma samples. Nevertheless, there are still a few deviations, with a higher FC for 2-HB (Madrid-Gambin et al. (2023)), acetate (Gowda et al. (2014), formate and phenylalanine (SMolESY). A lower FC was observed for acetone (Madrid-Gambin et al. (2023) and SMolESY), citrate (all three), and glycine (SMolESY).

The metabolites most sensitive to sample preparation conditions may be identified by a further comparison of the three independent studies. The average FC and standard deviation values were calculated from the Gowda et al. (2014), Madrid-Gambin et al. (2023), and SMolESY reported metabolite concentrations and plotted in Fig. 3 (bottom) and Table S6†. The Chenomx derived FC values were excluded from the analysis because of the systematically elevated values relative to the other published studies and to the corresponding SMolESY analysis. As evident from the visual evaluation of Fig. 3 (bottom) and confirmed by a pairwise Student t-test ($p > 0.1$, see ESI Table S6†) the results were statistically equivalent with the exception of lactate ($p < 0.02$). As desired, most FC values were approximately one. There were some notable differences in the variability or precision in the measured metabolite concentrations and associated FC values as evident by %CV. The average %CV was 20% ± 14% for the FC values calculated from the comparison of the methanol-induced protein precipitation and ultrafiltration plasma samples. Formate, glycine, and phenylalanine exhibited significantly higher %CV values ranging from 46% to 54%. The average %CV increased to 38% ± 25% for the FC values calculated from the comparison of the methanol-induced protein precipitation and intact plasma samples. 2-HB, acetone, alanine, choline, citrate, glutamine, and lysine exhibited significantly higher %CV values ranging from 48% to 96%. Again, Madrid-Gambin et al. (2023) did not report glutamate.

Taken together, these results clearly demonstrate that fold-change measurements provide an overall higher reproducibility in measured metabolite changes compared to absolute metabolite concentrations. FC values were statistically equivalent regardless of the plasma sample preparation protocol, methanol-induced protein precipitation, protein removal by ultrafiltration, or an intact plasma sample. Presumably, calculating a ratio negates constant sources of errors resulting from the processing, handling, and analysis of the plasma samples. In our study, measurements taken from the methanol-precipitated plasma samples served as the gold standard. However, we posit that employing reference compounds (such as the addition of non-endogenous metabolites to the samples) and/or electronic signals (such as ERETIC[48]) could also effectively mitigate errors stemming from the processing, handling, and analysis of the plasma samples as well as multicentered cohorts. Conversely, Chenomx processing was sensitive to sample preparation protocols and data processing methods, resulting in over a 100% difference in measured FC values. Recall, the manual-fit Chenomx analysis of the plasma samples following methanol-induced protein precipitation produced the highest overall metabolite concentrations, which is likely the primary source of the poor correlation between sample preparation protocols. The results also highlight the metabolites that should be carefully scrutinized to determine if the observed concentration change is truly caused by the condition of interest or is an artifact of sample preparation. For example, metabolites with %CVs and FCs significantly higher than average may be suspect.

3.7 2-HB and Glutamate are sensitive to Sample Preparation Method

To evaluate metabolite stability, we compared average metabolite standard deviations and %CV across each processing method (see ESI Fig. S64, Table S7†). Metabolites with a standard deviation above the group average as determined by Tukey's IQR outlier test were determined to be less stable. Conversely, standard deviations below the group average were determined to be more stable. While %CV is a valuable metric, it is not the most precise measurement of reproducibility since its magnitude varies with absolute concentration. When evaluating each metabolite's %CV, DMA and DMSO2 were the most consistent outliers with %CV's ranging from 158%-519%. However, these two metabolites were also present at the lowest concentration in the plasma samples and their standard deviations were similar to other metabolites. Thus, the lower absolute concentration systematically inflated the %CV to being

a statistical outlier. Therefore, our criteria for determining a metabolite to be significantly more or less stable than average was based exclusively on quantile range outlier testing of the standard deviations.

Lysine and phenylalanine have the highest standard deviation of 0.22 when all the data was analyzed together as one group (see ESI Fig. S64a†). However, lysine and phenylalanine were not statistically different after grouping the data by processing software. The Chenomx data shows an increase in the standard deviation for 2-HB and glutamate (see ESI Fig. S64b†) while the SMolESY analysis resulted in no standard deviation values for the combined dataset being identified as an outlier (see ESI Fig. S64c†). The data from the Chenomx batch-fit analysis was excluded from the pooled Chenomx group since it was previously determined to be an unreliable analysis method, which was further confirmed by the wide spread in standard deviation values as seen in Fig. S64d-e†. However, when examining the Chenomx assisted-fit and manual-fit standard deviations, a clear trend can be seen in both the ultrafiltered and methanol-precipitated plasma samples. 2-HB, DMA and glutamate consistently exhibited standard deviations that were outliers from the rest of the metabolites. Glutamate, in particular, was a clear outlier in all four data groups (see ESI Fig. S64f-i†). Overall, these results suggest that 2-HB, DMA and glutamate are especially sensitive to sample preparation conditions. Additionally, the standard deviaitons from the methanol-precipitated plasma samples have a narrower range (excluding outliers, see ESI Fig. S64g, i†) compared to the ultrafiltered plasma samples (see ESI Fig. S64f, h†). This trend is highlighted by the tight distribution around the dashed average line in the scatter plots shown in Fig. S64d-i†. These results confirm previous studies which concluded that methanol-induced protein precipitation to be the most robust protein removal method for plasma or serum metabolomics.[35,47]

There were no outliers when examining the standard deviation values from the combined SMolESY data set (see ESI Fig. S64c†), but when grouping the data by sample preparation method, 2-HB, choline, and lactate became outliers. Interestingly, 2-HB had a higher standard deviation in the Chenomx analysis of the methanol-precipitated plasma samples, but it had a non-significant standard deviation in the SMolESY analysis of these same samples. A significantly lower standard deviation in the SMolESY analysis was obtained with the intact samples. This wide range of low, normal, and high standard deviations suggests 2-HB may be more sensitive to sample preparation conditions than the other metabolites. For similar reasons, choline and lactate are also suspect.

4 Conclusions

In the present study, we systematically reviewed the effects of sample preparation methods and data analysis platforms on measured metabolite concentrations. The quickly expanding library of metabolomics software provides many alternative options for spectral analysis, but also adds new complications for on-going harmonization efforts by the metabolomics community. We clearly observed that both the choice of sample preparation method and data analysis platform significantly perturbed the resulting metabolic profile, requiring careful consideration of an appropriate metabolomics processing pipeline. Consistent with prior results, we also found that methanol-induced protein precipitation yielded a higher overall concentration of metabolites recovered from plasma samples. Clearly, methanol-induced protein precipitation should be the preferred choice for sample preparation by the scientific community. Importantly, a high level of reproducibility was achieved across our study and the two prior studies by using fold-change measurements relative to absolute concentration changes.[35, 46, 47] Calculating metabolite concentration ratios cancels out sample processing errors, making an additional "normalization step" (e.g., ratio calculation) essential when merging datasets from multicenter cohorts, various studies or laboratories.

Our parallel investigation of Chenomx and SMolESY as analysis platforms offered several valuable insights. Chenomx presents several degrees of automation, batch-fit, assisted-fit, and manual-fit, which can be useful for high-throughput data analysis as the number of samples, or the cohort size increases to comprise hundreds to thousands of NMR spectra. Encouragingly, assisted-fit, and manual-fit Chenomx analysis were found to yield essentially identical results. Assisted-fit Chenomx processing is the preferred choice, especially for a large number of samples, since it drastically reduces the analysis time proportional to the increase in cohort size.

The manual-fit Chenomx processing of the plasma samples prepared by protein-precipitation yielded the highest overall metabolite concentrations relative to SMolESY and the other Chenomx protocols. Accordingly, this increase in measured metabolite concentrations is likely a closer representation of the true metabolite concentrations in the plasma samples. Thus, it is important to recognize that manually adjusting Chenomx profiles will likely lead to a positive increase in metabolite concentrations. However, the comparative analysis of Chenomx to SMolESY and other studies from the scientific literature revealed fold changes that were systematically elevated. This may suggest that Chenomx either overestimates metabolite concentrations from the methanol-precipitated plasma samples or significantly underestimates concentrations from the ultrafiltered plasma samples. Conversely, the overall consistency in the results obtained by Gowda et al. (2014), Madrid-Gambin et al. (2023), and SMolESY is quite encouraging and indicates NMR-based metabolomics data is reproducible. Further, the consistent outcome also suggests that intact plasma samples may be a viable choice.

Finally, while our results align with previously reported data, 2-HB, choline, DMA, lactate, and glutamate were found to deviate from the measurements obtained for other metabolites. 2-HB and glutamate exhibit significant deviations regardless of sample preparation and processing methods. Thus, these metabolites may be specifically sensitive to sample preparation method and may need to be carefully considered before being classified as a biomarker or a group-differentiating metabolite. This work is not the first to investigate the effect of

sample preparation on metabolomics studies and while a handful of other studies have similarly explored sample handling, processing and preparation concerns, the underlying issues to these protocols are complex. Accordingly, a robust and consensus best practice has not yet been reached, but our results reported herein provide an important next step to achieving accurate and reproducible metabolomics data. [29, 35, 45-47, 49-51]

Author contributions

R.P. and P.G.T. conceptualized the study and acquired funding. J.L.A., B.H.M., N.P, J.M., and P.G.T. acquired samples. D.C. completed NMR experiments. D.C. and P.G.T. completed data analysis. D.C., P.G.T., and R.P. wrote the manuscript. All authors read, revised, and approved the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the ESI.†

Acknowledgements

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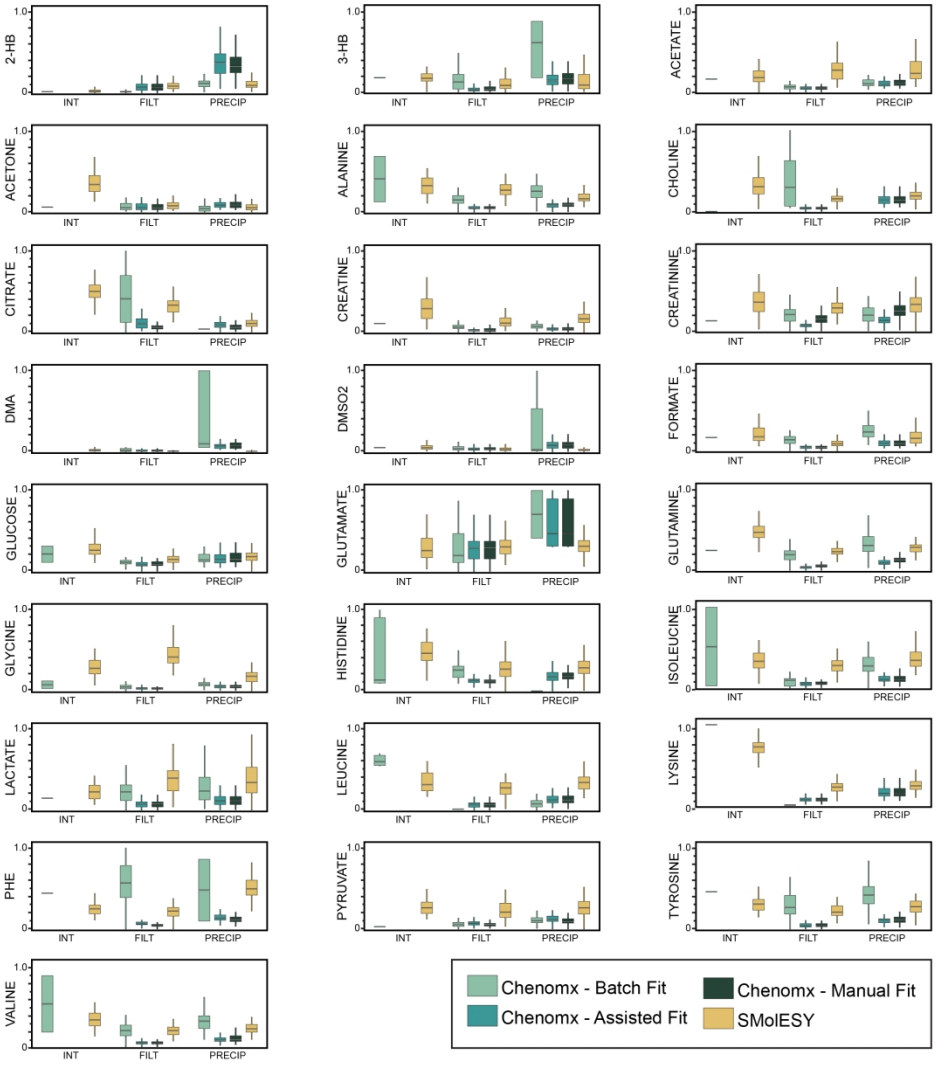
The data supporting this article has been included as part of the Supplementary Information.



Scaled Metabolite Concentrations Organized by Fit and Sample Preparation Method. Box and whisker plots of range scaled (0-1) data for each of the 25 metabolites. Plots are placed in alphabetical order. Each individual box and whisker plot is organized left-to-right by preparation method from intact (INT), to ultrafiltered (FILT), to methanol-induced protein precipitated (PRECIP). The color of each box corresponds to the processing method used to generate the data in the following order: Chenomx – batch-fit (light blue), Chenomx- assisted-fit (teal), Chenomx – manual-fit (dark green), SMolESY (gold). Abbreviations: 2-HB: 2-hydroxybutyrate, 3-HB: 3-hydroxybuytrate, DMSO2: dimethyl sulfone, DMA: dimethylamine.

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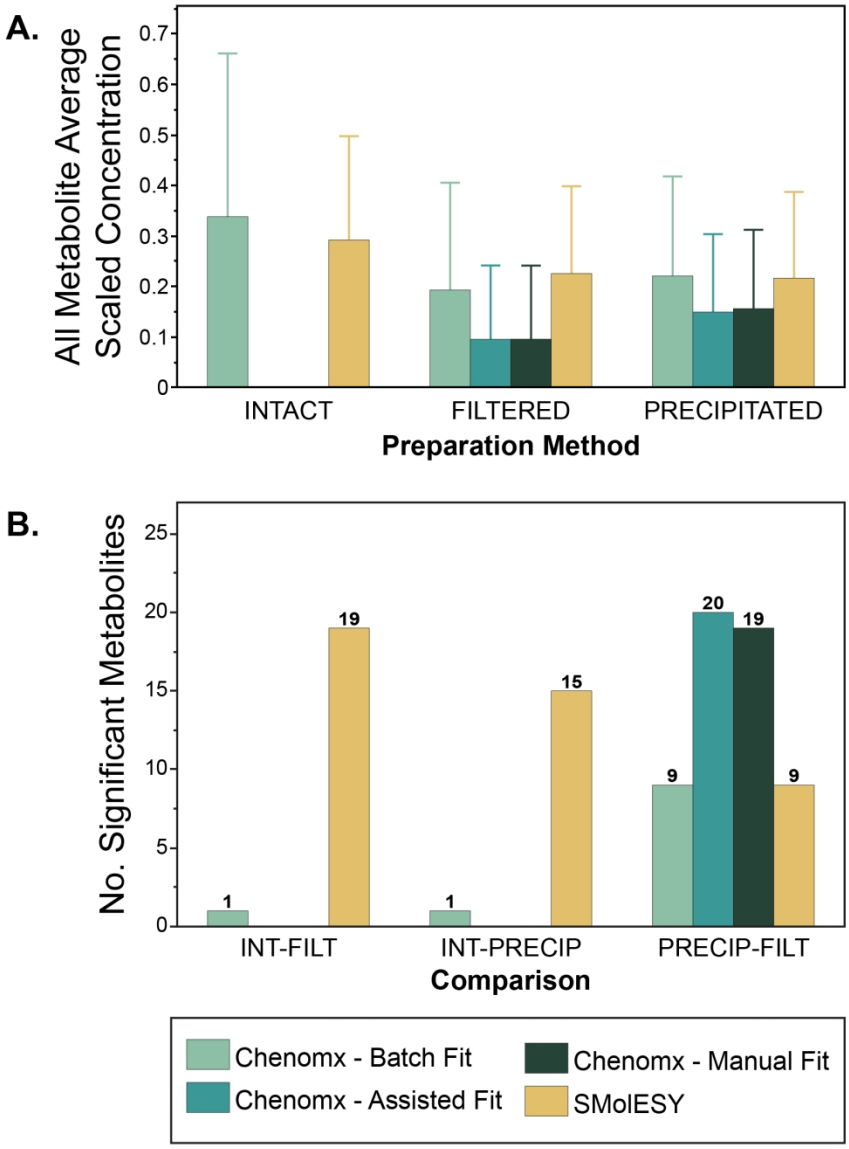

Summary bar charts showing a. general trends in metabolite concentration and b. the total number of metabolites that had statistically significant concentration variation between sample preparation type.

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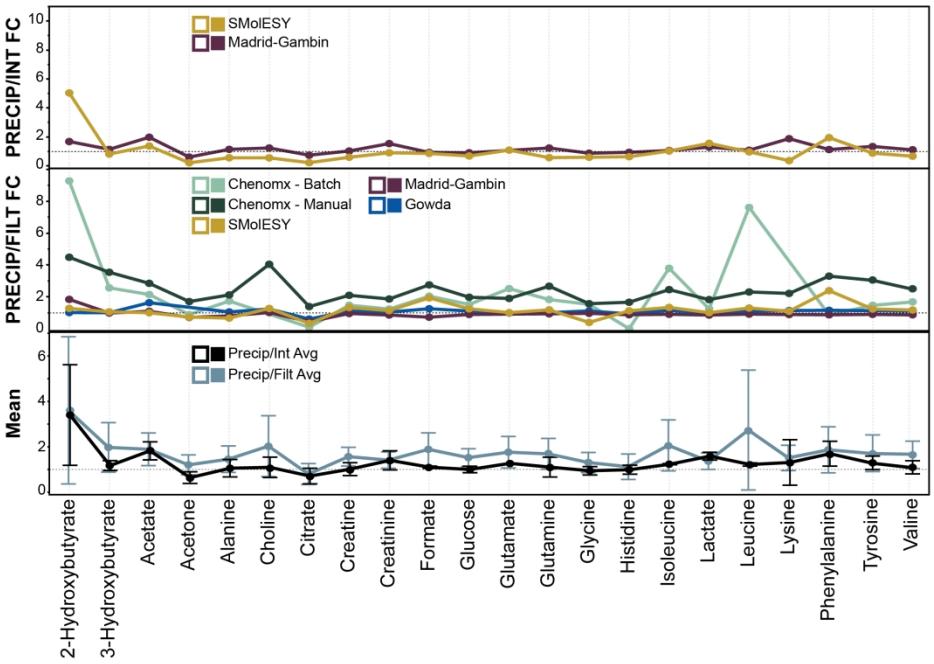

Comparative Analysis of Results. Line graph plotting the fold change of each metabolite as reported by previously published studies and present results. Fold changes (FC) are organized by group with top panel containing precipitated sample/intact sample fold changes from SMolESY data (gold) and Madrid-Gambin et al. (2023) data (maroon). The middle panel contains methanol-induced protein precipitated (PRECIP) and ultrafiltered (FILT) sample fold changes from Chenomx – batch-fit results, Chenomx – manual-fit results, SMolESY results, Madrid-Gambin et al. (2023) results, and Gowda et al. (2014) results. The bottom panel contains line plots of calculated averages from the top two panels (Chenomx results were excluded from the Precip/Filt Avg line since they were identified as unreliable). The color of the line is correlated to the method or previous publication.26, 50 A dashed line is present at FC = 1 for reference. All fold changes were calculated from the data generated by each processing method or publication. For example, the gold SMolESY lines are generated from SMolESY data. No cross-comparative fold changes were calculated or plotted.

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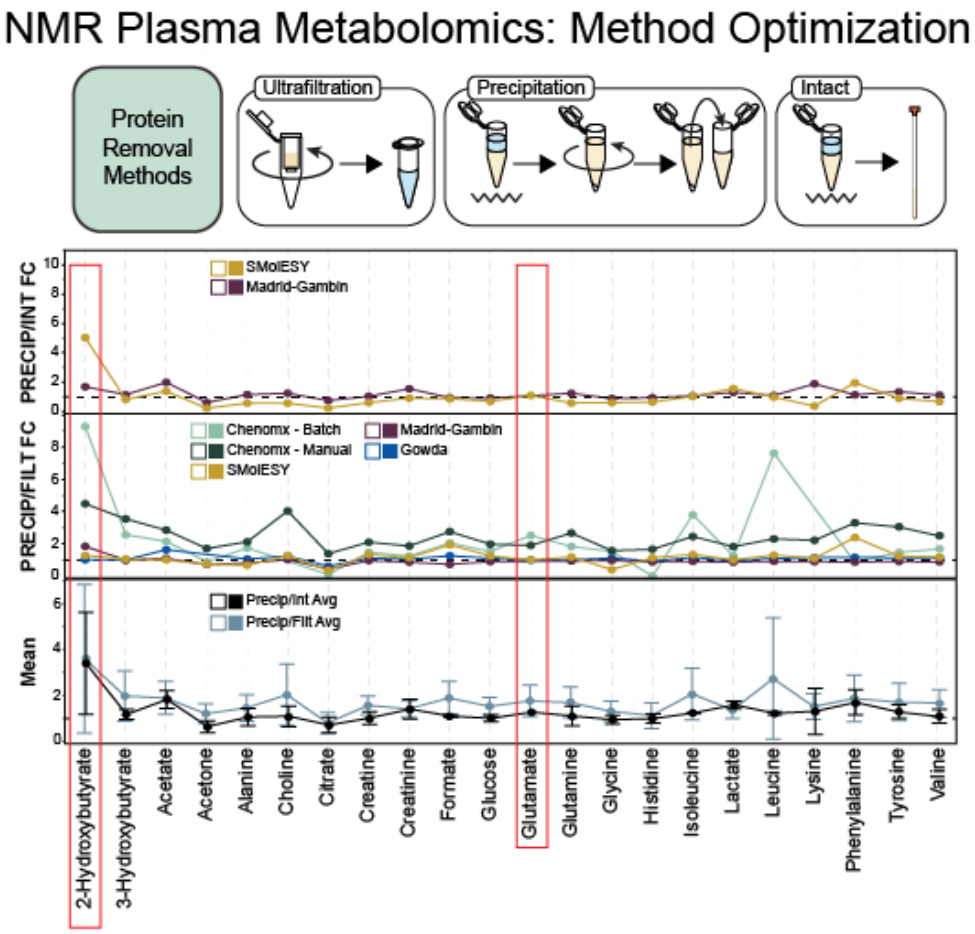

46x44mm (300 x 300 DPI)

Supplementary Information for

Evaluating Protocols for Reproducible Targeted Metabolomics by NMR

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Keywords: nuclear magnetic resonance, harmonization, sample preparation, methods, metabolomics

EXPERIMENTAL DETAILS

Study Design and Cohort Demographics

Plasma samples were acquired from an IBD cohort consisting of 69 patients and healthy volunteers recruited with approval from the research ethics committee (Ref. No. 21/WA/0105). Two identical ~1 mL aliquots of plasma were obtained from each of the 24 healthy age and sex matched controls, 21 patients with Crohn's disease, and 24 patients with ulcerative colitis for a total of 138 plasma samples. Demographic data was gathered at the time of sample collection and is provided in **Table S1**.

To evaluate the effect of sample preparation on metabolite profile detection, three unique protein removal procedures were applied to each individual plasma sample for comparison by 1D ^{1}H NMR. Protein was removed from the plasma samples by either ultrafiltration, methanol precipitation, or the T_2 filtering of intact samples (**Figure S1**). Each sample preparation protocol was used as previously described by Gowda *et al.* (2014) in order to facilitate a direct comparison between these and other previously published results and the data obtained herein.[1-3] The plasma samples were thawed on ice and randomized before the preparation of the 219 NMR samples, consisting of 3 sample preparation protocols for each of the 69 unique plasma samples and the 4 pooled quality control (QC) samples. All sample preparation procedures were completed on the same day to reduce variation and unintended bias. 50 mM phosphate buffer in 100% D_2O at pH 7.2 (uncorrected) with 100 μM of 3-(trimethylsilyl)propanoic-2,2,3,3-D_4 acid (TMSP) was prepared fresh and immediately before NMR sample preparation.[4]

Preparation of Pooled QC Samples

A pooled QC sample was prepared for each of the three clinical groups, Crohn's disease, ulcerative colitis, and healthy controls. 55 µL was removed from each plasma sample from each clinical group and combined into a clean 2 mL centrifuge tube for a total volume ranging between ~1,155 to 1,320 µL for each of the three pooled QC samples. A fourth QC sample was prepared by combining 350 µL aliquots from each of the three individual QC samples. A total of 4 QC samples were then added to the original 69 clinical samples comprised of 21 Crohn's disease, 24 ulcerative colitis, and 24 healthy control samples for a combined total of 73 plasma samples.

Preparation of NMR Samples Using Ultrafiltration

Amicon Ultra centrifugal filters with a 3 kDa cutoff (UFC500396) were purchased from Sigma Aldrich (Saint Louis, MO) and washed 3x with 500 µL Nanopure water (Thermo-Fisher Scientific, Waltham, MA) at 14,000 × *g* for 20 minutes for a total centrifugation time of 60 minutes. 300 µL from each of the 73 plasma samples was transferred to an individual clean filter unit and centrifuged at 14,000 × *g* for 20 minutes and then the filtrate was dried overnight in a SpeedVac. Each dried sample was reconstituted in 300 µL Nanopure water and 300 µL of a fresh 50 mM phosphate buffer pH 7.2 (uncorrected) in 100% D_2O with 100 µM TMSP-D_4 for a final TMSP concentration of 50 µM. Samples were vortexed and then 550 µL was transferred to a 7" Norell Standard Series 5 mm NMR tube (Morganton, NC, USA).

Preparation of NMR Samples Using Methanol Precipitation

A 300 µL aliquot from each of the 73 plasma samples was placed into an individual 2 mL centrifuge tube before adding 600 µL of ice-cold methanol to precipitate the protein. Samples were incubated at -20°C for 20 minutes before centrifugation at 14,000 × *g* for 30 minutes. The supernatant was

transferred to a fresh 2 mL centrifuge tube and then dried overnight in a SpeedVac. Each dried sample was reconstituted in 300 µL Nanopure water and 300 µL of a fresh 50 mM phosphate buffer pH 7.2 (uncorrected) in 100% D_2O with 100 µM TMSP-D_4 for a final TMSP concentration of 50 µM. Samples were vortexed and then 550 µL was transferred to a 7" Norell Standard Series 5 mm NMR tube (Morganton, NC, USA).

Preparation of NMR Sample Using an Intact Plasma Sample

A 300 µL aliquot from each of the 73 plasma samples was placed into an individual 2 mL centrifuge tube. 300 µL of a fresh 50 mM phosphate buffer pH 7.2 (uncorrected) in 100% D_2O with 100 µM TMSP-D_4 for a final TMSP concentration of 50 µM was added and then mixed by vortexing. 550 µL was transferred to a 7" Norell Standard Series 5 mm NMR tube (Morganton, NC, USA).

NMR Data Acquisition

All NMR experiments were performed at 298K on a Bruker Neo 600 MHz NMR spectrometer (Bruker BioSpin, Billerica, MA) equipped with a 5 mm TCI-F cryoprobe, temperature controlled SampleCase automated sample changer, ICON NMR and an automatic tune and match (ATM). All 1D ^{1}H NMR experiments were collected with 1D ^{1}H nuclear Overhauser effect spectroscopy (NOESY, noesygppr1d)[5] or a Carr-Purcell-Meiboom-Gill (CPMG, cpmgpr1d)[6, 7] pulse sequence, 65K data points, a spectral width of 17857 Hz, 64 scans, 16 dummy scans, and a 4s relaxation delay.

Data Processing

The 1D ^{1}H NMR spectra were zero-filled to 132K data points and Fourier transformed following an exponential apodization function of 1 Hz. Baseline and phase corrections were done via the Bruker automation software IconNMR 5.2.3.1 for TopSpin 4.1.3. When needed, a manual zero-order and first-order phase correction were applied, followed by baseline correction via fitting a third-degree polynomial function to regions of the spectrum lacking peaks. Both manual phase and baseline corrections were performed via TopSpin "apk" and “abs” functions, respectively.

Processed spectra were imported into Chenomx NMR Suite Professional Software Package (version 8.3; Chenomx Inc., Edmonton, Alberta, Canada) for metabolite quantification. Chenomx allows users to upload and process raw or pre-processed spectra, and then complete metabolite identification and quantification by comparing NMR peaks in the sample to their proprietary reference libraries as well as the libraries that have been developed in a collaborative effort with HMDB. Chenomx v8.3 offers three methods of metabolite identification that vary in degree of required manual human intervention. A fully automated, completely hands-free method of metabolite detection is possible by using the “batch-fit” method and choosing a library or metabolite list that Chenomx subsequently attempts to fit to the acquired NMR spectra of interest. A second Chenomx process is the “assisted-fit” method which allows users to upload a reference spectrum with metabolites of interest already fit to it that Chenomx then uses as a template for fitting the same metabolite profiles to other 1D ^{1}H NMR spectra in the data set. In this manner, the assisted-fit method allows for a user-directed semi-automated analysis. Finally, the third Chenomx method involves a fully manual approach to confirm the proper fitting of each reference 1D ^{1}H NMR spectra to each experimental NMR spectrum. Manual fitting was completed in combination with either the batch-fit or assisted-fit method used as a first step followed by manual adjustments as needed. A fully manual-fitting approach is highly time-consuming and is not always feasible

when analyzing large-scale studies consisting of hundreds or thousands of samples. Each of these three methods, batch-fit, assisted-fit, and manual-fit was applied to the dataset to evaluate their similarities and differences. The 27 metabolites selected for comparison and assessment are described in **Table S2**, which was based upon the panel of abundant metabolites previously described in the literature.[8] Glycerol and methanol were excluded from all further analysis since the compounds may be contaminants introduced by the sample preparation methodology leaving a total of 25 metabolites for comparison.

First, a blind batch-fit of the entire 600 MHz Chenomx library was applied to the complete dataset. A metabolite was excluded if it was not detected in 80% or more of the samples comprising a clinical group. An assisted-fit was conducted by manually fitting the clusters correlated to the selected set of 25 metabolites for a single reference 1D ^{1}H NMR spectra for each clinical group. This manually fitted spectrum was then uploaded as a template starting point for Chenomx to complete the transformations and assignments automatically. A full manual-fitting of the reference 1D ^{1}H NMR spectra for a select set of 25 metabolites to each of the experimental 1D ^{1}H NMR spectra for each clinical group was completed for spectra acquired with the NOESY pulse sequence. The fit of each metabolite reference 1D ^{1}H NMR spectra was individually evaluated and the Chenomx fit was manually adjusted to properly match chemical shifts and peak intensities.

The data analysis was also completed in parallel using the SMolESY platform with a semi-automated configuration.[9] Briefly, as previously shown[9] and validated in more than 8000 blood NMR spectra, the integral of a SMolESY signal of at least one ^{1}H spin system from the 23 metabolites can be automatically assigned and integrated to provide the relative concentration of each plasma/serum metabolite. Consequently, we employed the same strategy via SMolESY-platform which obtains the assignment and quantification of the metabolites in a semi-automated

manner (for more details see User's guide semi-automated peak picking function of the software: https://github.com/pantakis/SMolESY_platform).

Statistical Analysis

Prior to statistical analysis, all data was range scaled to make a comparative analysis possible between Chenomx and SMolESY. The Chenomx manual-fit analysis of the 1D ^{1}H NMR spectra acquired from the methanol-induced protein precipitation plasma samples was used as the standard data set for relative comparisons. Microsoft Excel and JMP 17.2.0 were used for data analysis. Group differences were statistically evaluated with one-way ANOVA testing followed by Tukey's posthoc test or FDR correction via the Benjamini-Hochberg method. Statistical significance was set at $p < 0.05$. Outlier testing was performed using Tukey's interquartile range (IQR) method and using 1.5 as the Q value for determining the range's upper and lower bounds.

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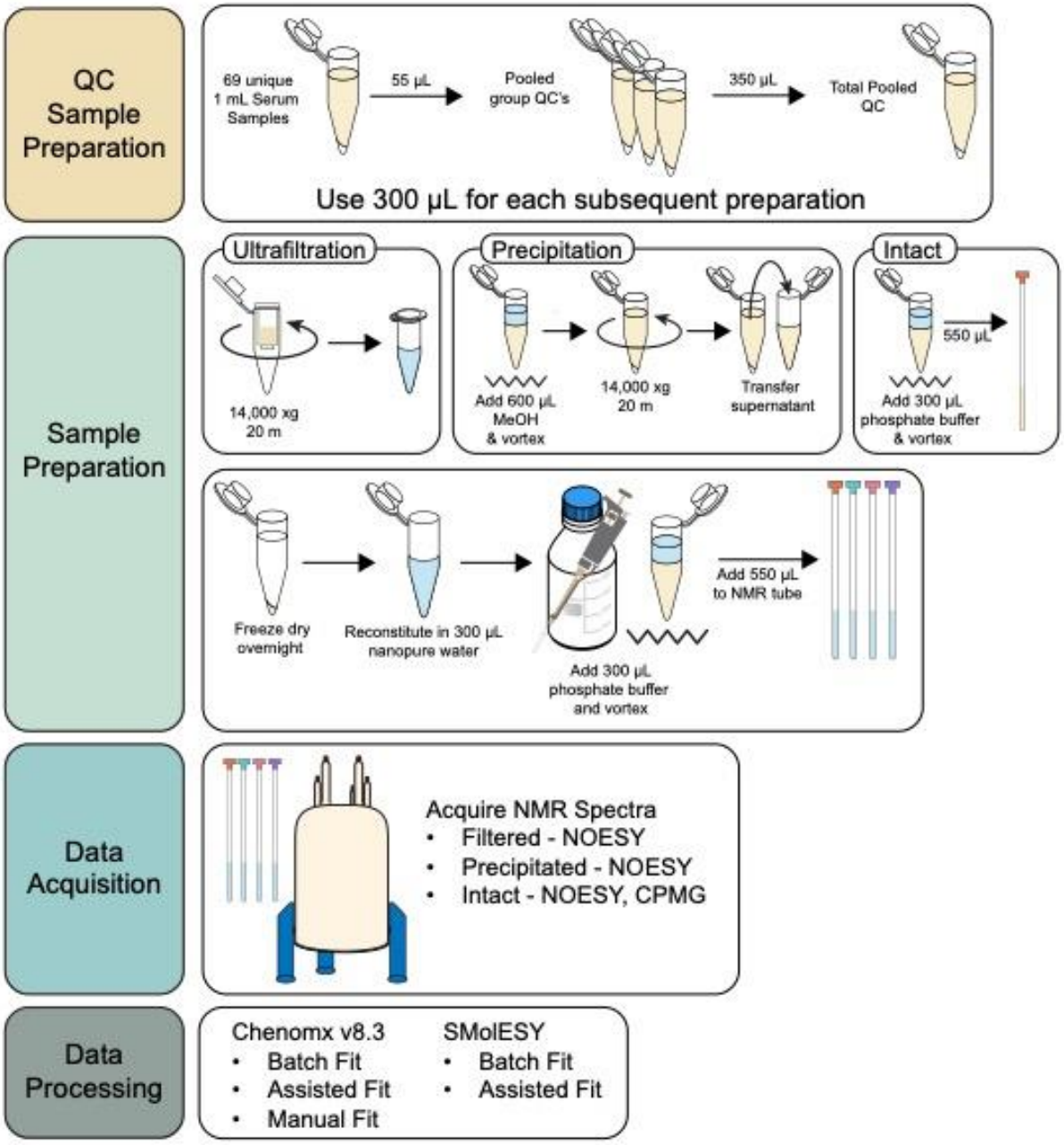

Figure S1. Methodological Workflow. Top to bottom stepwise diagram depicting the experimental workflow. 69 clinical samples and 4 QC samples were prepared with 3 unique sample preparation methods and analyzed by 2 different NMR experiments.

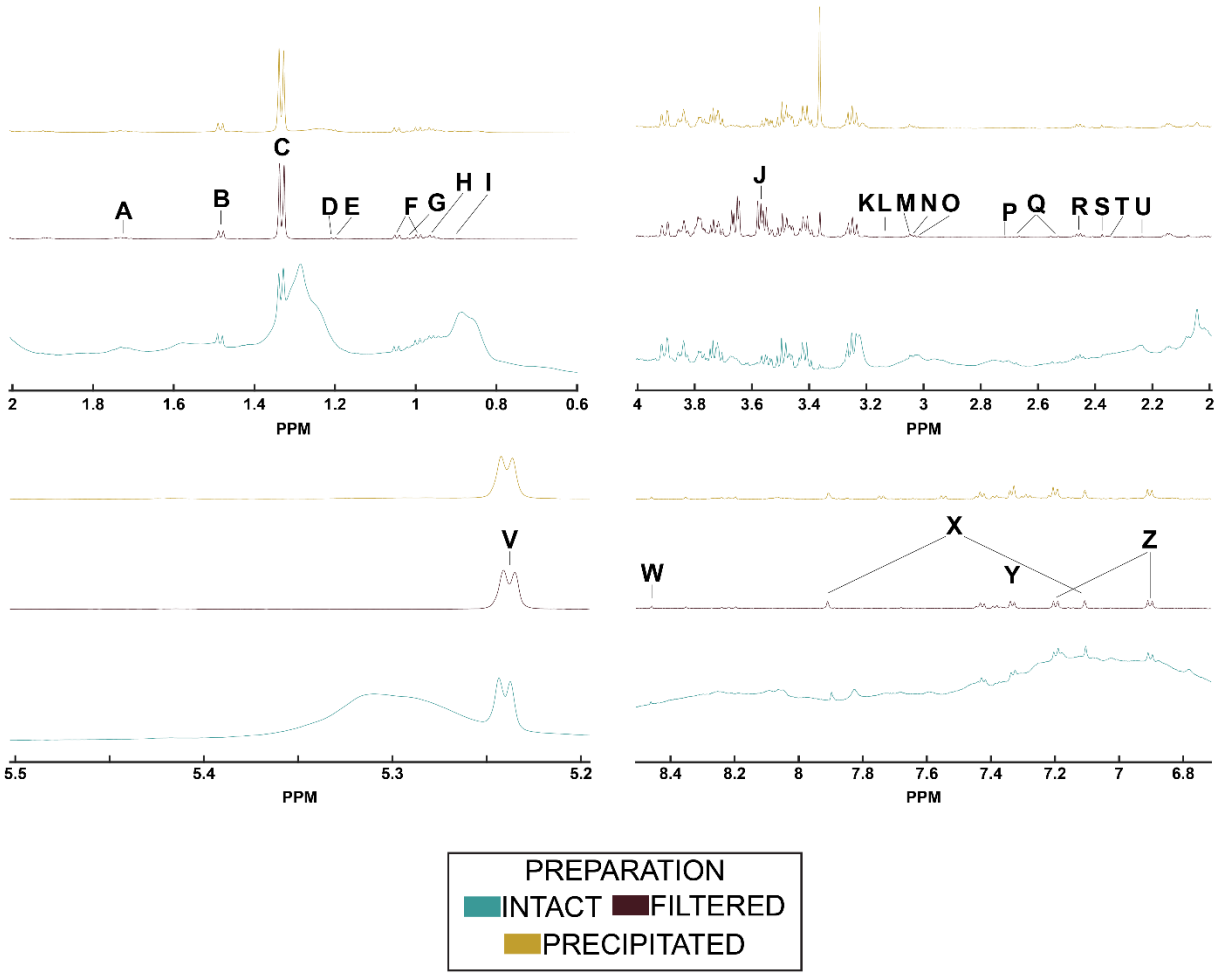

Figure S2. Representative labeled1D ^{1}H NMR Spectra

1D ^{1}H NMR spectra with labelled peaks corresponding to each metabolite of interest.

A – Acetate, B – Alanine, C – Lactate, D – 3-Hydroxybutyrate, E – Ethanol, F – Valine, G – Isoleucine, H – Leucine, I – 2-Hydroxybutyrate, G – Glycine, K – Choline, L – Dimethyl Sulfone, M – Creatinine, N – Creatine, O – Lysine, P – Dimethylamine, Q – Citrate, R – Glutamine, S – Pyruvate, T – Glutamate, U – Acetone, V – Glucose, W – Formate, X – Histidine, Y – Phenylalanine, Z - Tyrosine

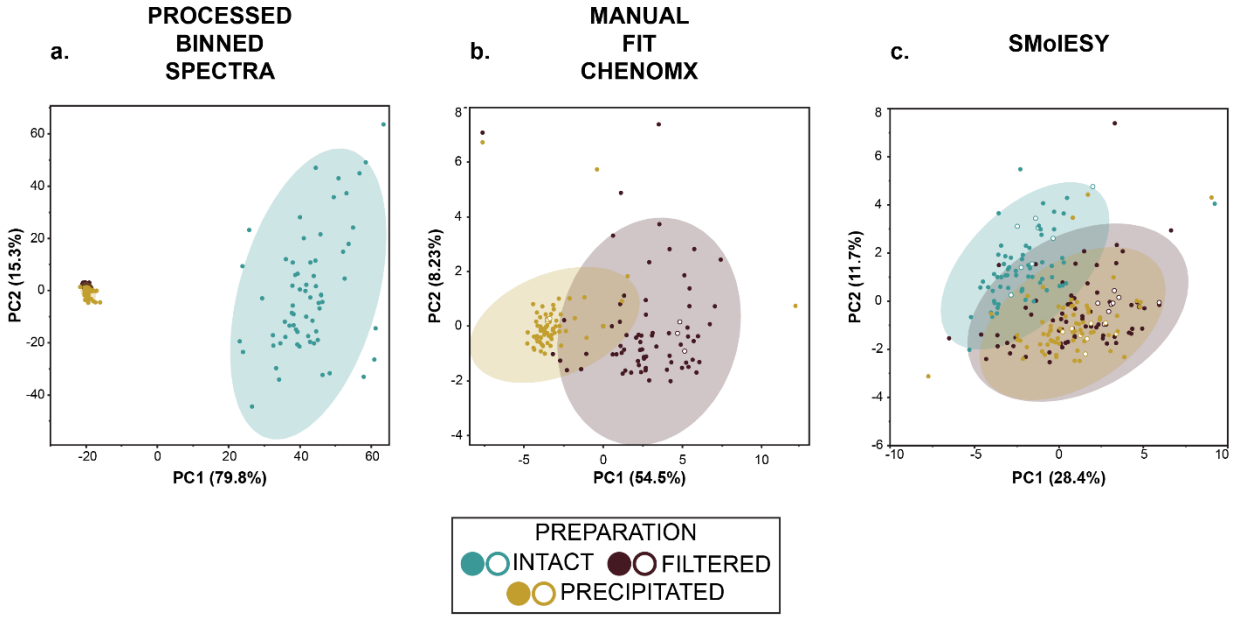

Figure S3. PCA Group Separation.

PCA scores plots demonstrating intact (teal) sample preparation group separation from ultrafiltered (burgundy) and methanol-induced protein precipitated (gold) groups using three data types **a.** binned spectral data, **b.** exported metabolite concentrations derived from manual Chenomx analysis and **c.** peak areas acquired from SMolESY analysis. Pooled quality control samples are open circles, clinical samples are filled circles. No QC samples are shown in panel **a** since the clusters are too compact.

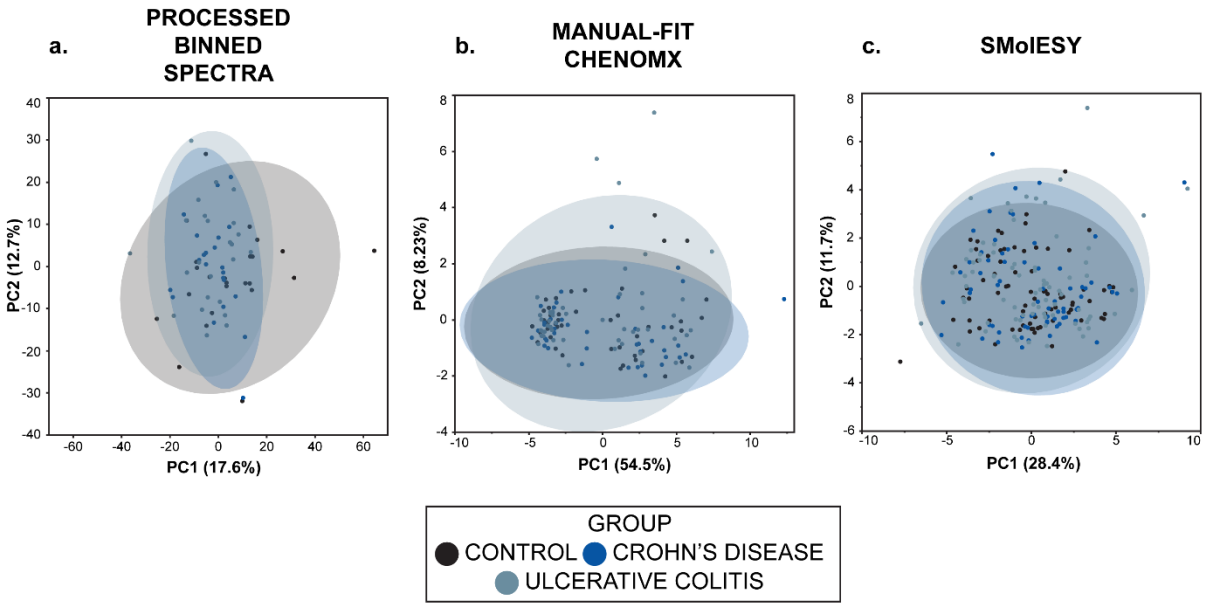

Figure S4. PCA group separation by clinical grouping.

PCA scores plots demonstrating the absence of group separation in a PCA model calculated with samples belonging to the control group (black), Crohn's disease (blue), or Ulcerative Colitis (blue-grey). PCA scores plot are obtained when the PCA models were generated with **a.** binned spectral data, **b.** exported metabolite concentrations derived from manual-fit Chenomx analysis or **c.** peak areas acquired from SMolESY analysis.

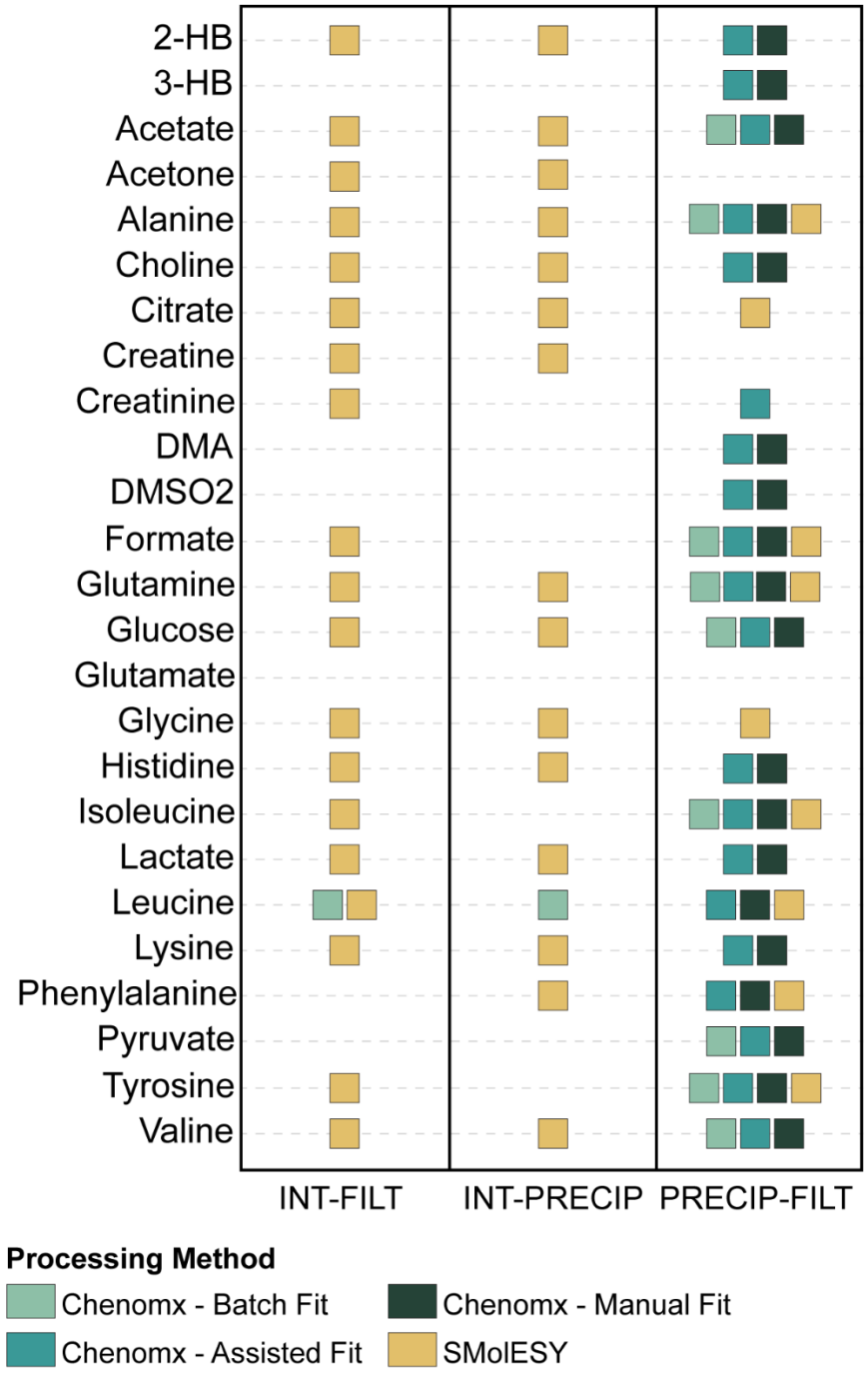

Figure. S5 Pairwise Statistical Significance Colorized Table

Color-coded table lists metabolites in alphabetical order from top to bottom with statistical significance noted by presence or absence of colored square in the corresponding row. Presence of a square in a metabolite row denotes that it was found to be statistically different ($p < 0.05$) when comparing intact

(INT) samples to ultrafiltered (FILT) samples (INT-FILT, left), intact samples to methanol-induced protein precipitated (PRECIP) samples (INT-PRECIP, middle), or methanol-induced protein precipitated to ultrafiltered samples (PRECIP-FILT, right). The color of the square denotes the processing method the metabolite was identified as being statistically different: Chenomx–Batch fit (light blue), Chenomx–assisted fit (teal), Chenomx–Manual fit (dark green), SMolESY (gold). Abbreviations: 2-HB: 2-hydroxybutyrate, 3-HB: 3-hydroxybuytrate, DMSO2: dimethyl sulfone, DMA: dimethylamine.

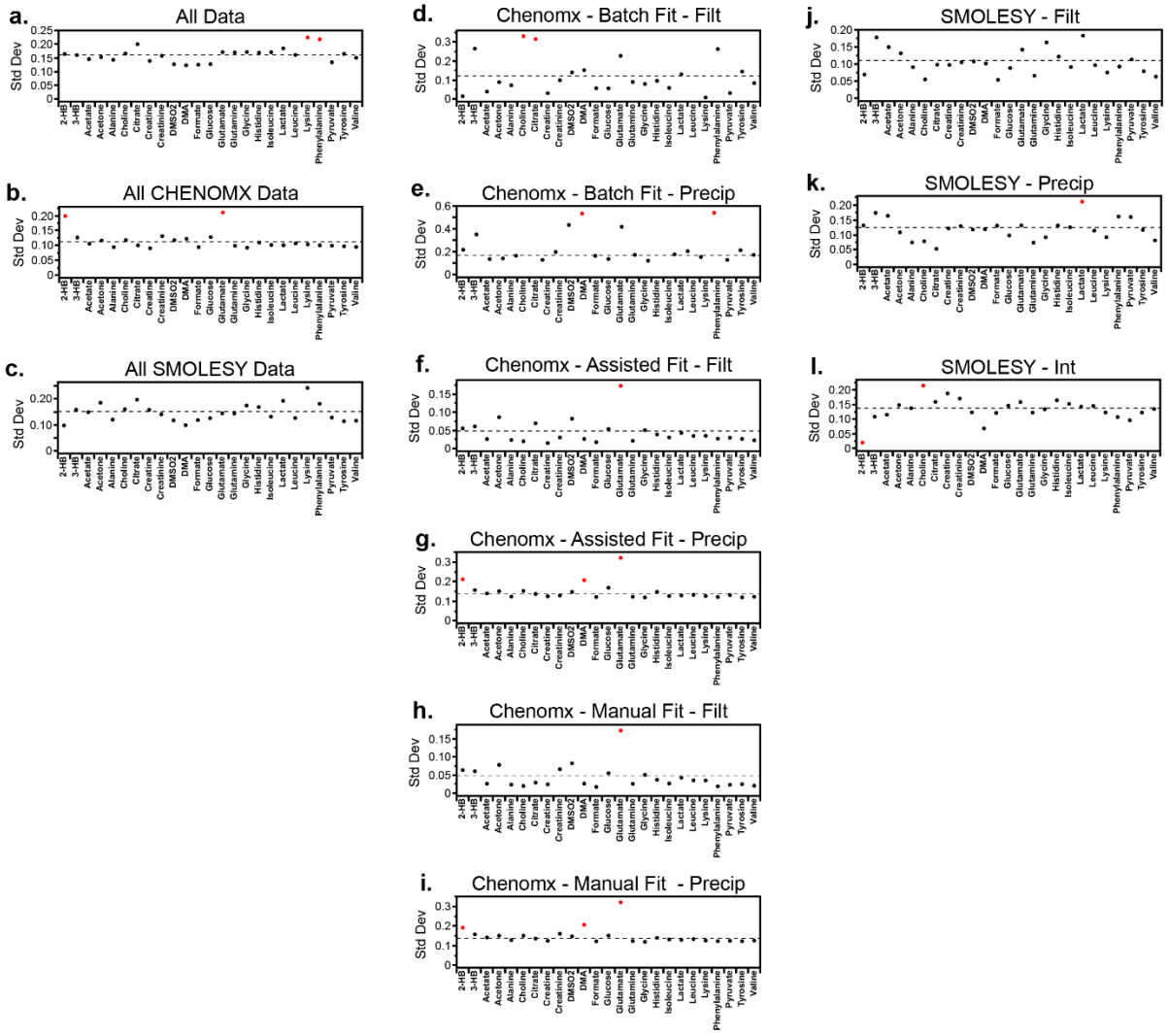

Figure S6. Metabolite Variance by Preparation and Processing Method

Scatterplots of standard deviations in metabolite concentrations as determined from each processing method. Outlier points are colored red. **a.** Total standard deviations from the combination of all data (Chenomx and SMolESY). **b.** Standard deviations from combined Chenomx assisted-fit and manual-fit data. **c.** Standard deviations from all SMolESY data. **d-i.** Standard deviations from individual Chenomx fit methods (**d-e**, batch-fit; **f-g**, assisted-fit; **h-I**, manual-fit). **j-l.** Standard deviations from SMolESY analysis of each sample preparation group (**j**, ultrafiltered; **k**, methanol-induced protein precipitated; **l**, intact).

Supplemental Information for
g Protocols for Reproducible Targeted Metabolomics

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Table S1: Metadata corresponding to the 69 individual plasma sample donors.

Sample #	Clinical Group	Age	Gender
5	Crohn's Disease	35.5	Male
9	Crohn's Disease	39.4	Male
12	Crohn's Disease	40.5	Female
16	Crohn's Disease	55	Female
18	Crohn's Disease	50.1	Male
20	Crohn's Disease	37.7	Female
28	Crohn's Disease	36.9	Male
34	Crohn's Disease	46.9	Male
40	Crohn's Disease	49.5	Female
44	Crohn's Disease	30.5	Female
46	Crohn's Disease	41.4	Female
47	Crohn's Disease	56.5	Female
48	Crohn's Disease	74.7	Male
53	Crohn's Disease	37.3	Male
56	Crohn's Disease	38.1	Male
61	Crohn's Disease	24.8	Female
62	Crohn's Disease	62	Male
64	Crohn's Disease	41	Male
65	Crohn's Disease	28	Male
66	Crohn's Disease	52.3	Male
69	Crohn's Disease	50	Male
2	Ulcerative Colitis	51.2	Male
4	Ulcerative Colitis	28.5	Female
6	Ulcerative Colitis	33.9	Male
8	Ulcerative Colitis	29.4	Male
10	Ulcerative Colitis	47.2	Female
11	Ulcerative Colitis	32.5	Male
15	Ulcerative Colitis	66.7	Male
19	Ulcerative Colitis	35.1	Female
21	Ulcerative Colitis	38.1	Female
23	Ulcerative Colitis	61	Male
24	Ulcerative Colitis	30.4	Male
25	Ulcerative Colitis	25.1	Male
27	Ulcerative Colitis	39.1	Female
29	Ulcerative Colitis	67	Male
31	Ulcerative Colitis	29.6	Female
32	Ulcerative Colitis	46.2	Male
35	Ulcerative Colitis	57.6	Male
36	Ulcerative Colitis	36.9	Male
38	Ulcerative Colitis	38.8	Female
42	Ulcerative Colitis	31.8	Female
43	Ulcerative Colitis	59.4	Male
52	Ulcerative Colitis	23.7	Male
57	Ulcerative Colitis	44.8	Male
68	Ulcerative Colitis	64.4	Male

1	Control	40.8	Female
3	Control	61.3	Female
7	Control	45.5	Male
13	Control	57.4	Male
14	Control	54.3	Male
17	Control	63.8	Female
22	Control	44.3	Male
26	Control	31.6	Female
30	Control	48.5	Male
33	Control	37.4	Male
37	Control	52.1	Male
39	Control	30.2	Female
41	Control	46.2	Female
45	Control	36.2	Female
49	Control	36.4	Male
50	Control	33.9	Male
51	Control	24.7	Female
54	Control	37.2	Female
55	Control	41	Male
58	Control	22.8	Male
59	Control	42.2	Female
60	Control	33.5	Male
63	Control	52.9	Male
67	Control	28.8	Female

Table S2: List of common names and human metabolome database ID numbers for the 25 targeted m

Common Name	HMDB ID
2-HYDROXYBUTYRATE	HMDB0000008
3-HYDROXYBUTYRATE	HMDB0000011
ACETATE	HMDB0000042
ACETONE	HMDB0001659
ALANINE	HMDB0000161
CHOLINE	HMDB0000097
CITRATE	HMDB0000094
CREATINE	HMDB0000064
CREATININE	HMDB0000562
DIMETHYL SULFONE	HMDB0004983
DIMETHYLAMINE	HMDB0000087
FORMATE	HMDB0304356
GLUCOSE	HMDB0000122
GLUTAMINE	HMDB0000641
GLUTAMATE	HMDB0000148
GLYCEROL	HMDB0000131
GLYCINE	HMDB0000123
HISTIDINE	HMDB0000177
ISOLEUCINE	HMDB0000172
LACTATE	HMDB0000190
LEUCINE	HMDB0000687
LYSINE	HMDB0000182
METHANOL	HMDB0001875
PHENYLALANINE	HMDB0000159
PYRUVATE	HMDB0000243
TYROSINE	HMDB0000158
VALINE	HMDB0000883

etabolites analyzed in this study

Table S3: Scaled Metabolite Concentrations for each Sample Preparation and Data Processir

	Chenomx - Manual Fit			Chenomx -	
	Mean	StDev	CV (%)	Mean	StDev
2-Hydroxybutyrate	0.37	0.19	51.70	0.39	0.21
3-Hydroxybutyrate	0.19	0.16	83.82	0.18	0.16
Acetate	0.15	0.14	96.53	0.14	0.14
Acetone	0.13	0.15	119.95	0.12	0.15
Alanine	0.11	0.13	112.22	0.11	0.12
Choline	0.18	0.15	86.16	0.17	0.15
Citrate	0.08	0.14	165.28	0.11	0.14
Creatine	0.06	0.12	196.46	0.06	0.12
Creatinine	0.28	0.16	57.84	0.16	0.13
Dimethyl sulfone	0.11	0.15	131.29	0.11	0.15
Dimethylamine	0.12	0.21	167.94	0.12	0.21
Formate	0.12	0.12	105.18	0.12	0.12
Glucose	0.19	0.15	78.69	0.19	0.17
Glutamate	0.56	0.32	57.56	0.56	0.32
Glutamine	0.15	0.12	81.52	0.12	0.12
Glycine	0.07	0.12	160.92	0.07	0.12
Histidine	0.20	0.14	69.74	0.19	0.15
Isoleucine	0.14	0.13	94.46	0.13	0.13
Lactate	0.14	0.13	91.79	0.14	0.13
Leucine	0.16	0.13	84.01	0.16	0.13
Lysine	0.18	0.13	70.22	0.18	0.13
Phenylalanine	0.14	0.12	87.18	0.16	0.12
Pyruvate	0.12	0.12	101.37	0.15	0.13
Tyrosine	0.14	0.12	88.83	0.13	0.12
Valine	0.12	0.13	105.06	0.11	0.12
Average	0.17	0.15	101.83	0.16	0.15
Number/Average positive					
Number/Average negative					

ng Method

Precipitated							
Assisted Fit		**Chenomx - Batch Fit**				**SMol**	
CV (%)	**% Diff**	**Mean**	**StDev**	**CV (%)**	**% Diff**	**Mean**	**StDev**
54.07	-5%	0.16	0.22	136.95	57%	0.14	0.17
87.51	5%	0.55	0.35	63.62	-194%	0.17	0.20
102.01	7%	0.14	0.14	96.57	4%	0.29	0.18
125.93	5%	0.07	0.14	195.57	42%	0.09	0.15
114.61	6%	0.28	0.17	58.92	-146%	0.18	0.08
88.02	1%	-	-	-	-	0.20	0.09
119.27	-39%	0.04	-	-	53%	0.11	0.05
208.24	5%	0.10	0.13	135.89	-51%	0.19	0.12
81.18	43%	0.24	0.20	81.10	12%	0.34	0.14
134.79	3%	0.22	0.43	194.22	-100%	0.04	0.12
176.95	5%	0.38	0.53	138.81	-213%	0.02	0.12
105.33	1%	0.28	0.16	59.62	-137%	0.19	0.14
90.58	3%	0.18	0.14	75.28	6%	0.20	0.10
57.56	0%	0.70	0.42	59.49	-26%	0.33	0.15
103.35	22%	0.35	0.17	49.35	-132%	0.28	0.07
160.57	0%	0.11	0.12	116.13	-42%	0.18	0.09
78.13	6%	-	-	-	-	0.31	0.13
96.01	6%	0.31	0.18	57.28	-122%	0.36	0.13
91.72	0%	0.29	0.21	71.70	-104%	0.39	0.21
84.81	2%	0.11	0.15	142.93	32%	0.35	0.12
70.68	0%	-	-	-	-	0.26	0.12
75.22	-14%	0.48	0.54	112.78	-240%	0.52	0.17
86.91	-23%	0.13	0.13	100.01	-5%	0.28	0.17
95.37	9%	0.43	0.21	49.61	-212%	0.28	0.13
111.16	8%	0.34	0.17	50.62	-185%	0.23	0.08
104.00	2%	0.27	0.23	97.45	-77%	0.24	0.13
19	7%			7	29%		
5	-16%			15	-98%		

lESY		Chenomx - Manual Fit				Chenomx -	
CV (%)	% Diff	Mean	StDev	CV (%)	% Diff	Mean	StDev
116.96	62%	0.08	0.06	76.90	78%	0.08	0.06
117.92	10%	0.05	0.06	113.82	72%	0.05	0.06
63.44	-98%	0.05	0.03	51.55	65%	0.05	0.03
163.27	29%	0.07	0.08	105.76	41%	0.08	0.09
42.32	-60%	0.05	0.02	44.59	53%	0.05	0.02
43.24	-16%	0.04	0.02	46.78	75%	0.04	0.02
50.11	-32%	0.06	0.03	49.80	28%	0.11	0.07
65.64	-194%	0.03	0.02	81.80	52%	0.03	0.02
41.06	-22%	0.15	0.07	44.48	46%	0.07	0.03
312.55	67%	0.05	0.08	166.50	56%	0.05	0.08
519.36	81%	0.02	0.03	141.67	85%	0.02	0.03
72.69	-60%	0.04	0.02	41.83	64%	0.04	0.02
49.61	-2%	0.10	0.06	56.79	49%	0.09	0.05
46.77	41%	0.29	0.17	58.68	47%	0.29	0.17
26.46	-84%	0.06	0.03	46.16	63%	0.04	0.02
51.88	-139%	0.05	0.05	107.56	36%	0.05	0.05
43.27	-54%	0.12	0.04	30.84	40%	0.13	0.04
36.44	-160%	0.06	0.03	47.63	59%	0.05	0.03
54.59	-175%	0.08	0.04	55.95	45%	0.08	0.04
34.64	-119%	0.07	0.04	51.92	57%	0.07	0.03
43.96	-47%	0.08	0.04	43.67	55%	0.08	0.04
32.98	-268%	0.04	0.02	44.96	70%	0.07	0.03
59.27	-131%	0.06	0.02	41.96	54%	0.07	0.03
44.17	-105%	0.05	0.02	55.23	67%	0.05	0.03
35.53	-92%	0.05	0.02	43.79	60%	0.05	0.02
86.73	-63%	0.07	0.04	66.02	57%	0.07	0.04
6	48%			25	57%		
19	-98%			0	-		

		Filtered					
Assisted Fit		Chenomx - Batch Fit				SMol	
CV (%)	% Diff	Mean	StDev	CV (%)	% Diff	Mean	StDev
69.51	78%	0.02	0.01	85.08	95%	0.10	0.07
124.87	74%	0.22	0.26	123.09	-14%	0.15	0.18
52.06	65%	0.07	0.04	59.61	55%	0.28	0.15
106.02	35%	0.08	0.09	108.62	35%	0.11	0.13
44.21	52%	0.16	0.07	44.74	-42%	0.28	0.09
46.78	75%	0.39	0.33	85.60	-120%	0.16	0.06
62.26	-36%	0.41	0.31	76.68	-399%	0.33	0.10
60.68	60%	0.06	0.03	47.26	-2%	0.14	0.10
43.26	74%	0.20	0.10	49.87	29%	0.29	0.11
170.59	57%	0.07	0.14	188.32	34%	0.05	0.11
142.21	85%	0.06	0.15	272.19	55%	0.02	0.10
41.83	93%	0.13	0.06	42.24	-16%	0.09	0.05
57.43	69%	0.12	0.06	47.02	38%	0.16	0.09
58.68	62%	0.28	0.23	81.36	50%	0.32	0.14
52.06	114%	0.19	0.09	46.92	-27%	0.23	0.07
105.51	43%	0.07	0.08	114.41	7%	0.45	0.16
30.52	44%	0.25	0.10	38.54	-24%	0.27	0.12
60.34	93%	0.08	0.06	71.56	41%	0.27	0.09
55.95	58%	0.23	0.13	56.63	-63%	0.38	0.18
51.92	81%	0.01	-	-	91%	0.26	0.10
43.63	76%	0.01	0.01	141.42	97%	0.23	0.08
41.61	72%	0.55	0.26	47.48	-294%	0.21	0.09
42.03	53%	0.06	0.03	52.67	52%	0.23	0.11
58.06	100%	0.29	0.14	49.21	-113%	0.22	0.08
48.43	87%	0.20	0.08	41.41	-69%	0.19	0.06
66.82	67%	0.17	0.12	82.16	-20%	0.22	0.10
24	71%			13	52%		
1	-36%			12	-99%		

lESY		Chenomx - Batch Fit-CPMG				SMolESY	
CV (%)	% Diff	Mean	StDev	CV (%)	% Diff	Mean	StDev
68.05	72%	0.02	-	-	96%	0.09	0.10
119.36	21%	0.18	-	-	6%	0.11	0.11
53.39	-91%	0.16	-	-	-9%	0.16	0.13
123.54	15%	0.06	-	-	49%	0.28	0.19
32.64	-145%	0.41	0.40	97.54	-257%	0.39	0.21
35.48	11%	0.00	-	-	100%	0.39	0.21
30.06	-297%	-	-	-	-	0.50	0.19
70.53	-119%	0.11	-	-	-72%	0.29	0.19
36.05	-5%	0.13	-	-	54%	0.34	0.17
208.30	54%	0.05	-	-	52%	0.07	0.13
510.70	84%	-	-	-	-	0.03	0.10
57.69	19%	0.16	-	-	-40%	0.30	0.19
56.26	18%	0.21	0.14	66.51	-10%	0.24	0.16
44.90	43%	-	-	-	-	0.22	0.15
28.22	-55%	0.25	-	-	-62%	0.45	0.17
36.11	-509%	0.08	0.07	88.32	-1%	0.34	0.18
45.00	-35%	0.43	0.44	102.95	-113%	0.48	0.18
34.30	-92%	0.51	0.69	134.73	-265%	0.42	0.22
47.56	-173%	0.15	-	-	-4%	0.21	0.16
36.76	-66%	0.61	0.06	10.65	-284%	0.37	0.20
32.45	-30%	1.00	-	-	-458%	0.54	0.24
43.32	-52%	0.44	-	-	-214%	0.22	0.11
49.18	-88%	0.03	-	-	77%	0.30	0.18
35.36	-63%	0.46	-	-	-231%	0.38	0.19
32.71	47%	0.53	0.49	92.72	-341%	0.40	0.21
74.72	-57%	0.27	0.33	84.77	-88%	0.30	0.17
10	38%			7	62%		
15	-121%			15	-158%		

Intact						
- NOESY				SMolESY - CPMG		
CV (%)	% Diff	Mean	StDev	CV (%)	% Diff	
117.05	77%	0.15	0.15	97.29	58%	
100.17	40%	0.05	0.12	239.77	73%	
82.64	-9%	0.13	0.17	130.20	12%	
66.48	-123%	0.20	0.20	96.15	-62%	
52.91	-245%	0.44	0.25	56.66	-285%	
53.46	-123%	0.42	0.20	46.98	-141%	
37.69	-512%	0.49	0.22	45.43	-496%	
63.15	-366%	0.28	0.18	65.07	-340%	
51.17	-23%	0.32	0.18	57.25	-15%	
180.56	36%	0.06	0.13	205.82	42%	
296.01	73%	0.04	0.12	322.85	70%	
62.82	-156%	0.37	0.20	54.48	-221%	
64.84	-26%	0.20	0.15	77.58	-3%	
66.06	60%	0.16	0.13	80.83	71%	
37.88	-195%	0.40	0.19	48.18	-166%	
52.11	-363%	0.38	0.21	54.36	-411%	
37.89	-139%	0.47	0.19	40.98	-136%	
53.47	-200%	0.48	0.26	54.13	-244%	
72.95	-52%	0.18	0.16	87.44	-30%	
54.48	-136%	0.40	0.24	61.90	-149%	
45.52	-200%	0.38	0.22	57.55	-110%	
50.20	-59%	0.20	0.14	70.68	-42%	
58.33	-148%	0.34	0.23	67.24	-175%	
50.80	-174%	0.44	0.22	51.18	-217%	
52.21	-230%	0.44	0.24	55.06	-269%	
74.43	-128%	0.30	0.19	89.00	-128%	
5	57%			6	54%	14
20	-174%			19	-185%	11

%Diff (SMoIESY)	p-values
-78%	0.0065
56%	0.0027
19%	0.2463
27%	0.0173
-12%	0.2055
-8%	0.3917
3%	0.7755
5%	0.7514
7%	0.5034
9%	0.6521
-10%	0.5957
-26%	0.0369
19%	0.1321
26%	0.0132
10%	0.1056
-10%	0.2317
1%	0.7514
-15%	0.1457
15%	0.2727
-6%	0.4265
30%	0.0001
10%	0.3524
-11%	0.2573
-16%	0.0887
-12%	0.2993
1%	
17%	
-19%	6

Table S4: p-values calculated from pairwise comparison of measured metabolite concentrations

			Sample Preparation Methods	
Pulse Sequence	**Fit Method**	**Metabolite**	**Reference**	**Comparison**
NOESY	ASSISTED FIT	2-Hydroxybutyrate	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	3-Hydroxybutyrate	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Acetate	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Acetone	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Alanine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Choline	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Citrate	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Creatine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Creatinine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Dimethyl sulfone	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Dimethylamine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Formate	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Glucose	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Glutamate	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Glutamine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Glycine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Histidine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Isoleucine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Lactate	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Leucine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Lysine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Phenylalanine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Pyruvate	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Tyrosine	PRECIPITATED	FILTERED
NOESY	ASSISTED FIT	Valine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	2-Hydroxybutyrate	INTACT	FILTERED
NOESY	BATCH FIT	2-Hydroxybutyrate	PRECIPITATED	FILTERED
NOESY	BATCH FIT	2-Hydroxybutyrate	PRECIPITATED	INTACT
NOESY	BATCH FIT	3-Hydroxybutyrate	INTACT	FILTERED
NOESY	BATCH FIT	3-Hydroxybutyrate	PRECIPITATED	FILTERED
NOESY	BATCH FIT	3-Hydroxybutyrate	PRECIPITATED	INTACT
NOESY	BATCH FIT	Acetate	INTACT	FILTERED
NOESY	BATCH FIT	Acetate	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Acetate	PRECIPITATED	INTACT
NOESY	BATCH FIT	Acetone	INTACT	FILTERED
NOESY	BATCH FIT	Acetone	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Acetone	PRECIPITATED	INTACT
NOESY	BATCH FIT	Alanine	INTACT	FILTERED
NOESY	BATCH FIT	Alanine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Alanine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Choline	INTACT	FILTERED
NOESY	BATCH FIT	Choline	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Choline	PRECIPITATED	INTACT
NOESY	BATCH FIT	Citrate	INTACT	FILTERED
NOESY	BATCH FIT	Citrate	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Citrate	PRECIPITATED	INTACT
NOESY	BATCH FIT	Creatine	INTACT	FILTERED

NOESY	BATCH FIT	Creatine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Creatine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Creatinine	INTACT	FILTERED
NOESY	BATCH FIT	Creatinine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Creatinine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Dimethyl sulfone	INTACT	FILTERED
NOESY	BATCH FIT	Dimethyl sulfone	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Dimethyl sulfone	PRECIPITATED	INTACT
NOESY	BATCH FIT	Dimethylamine	INTACT	FILTERED
NOESY	BATCH FIT	Dimethylamine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Dimethylamine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Formate	INTACT	FILTERED
NOESY	BATCH FIT	Formate	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Formate	PRECIPITATED	INTACT
NOESY	BATCH FIT	Glucose	INTACT	FILTERED
NOESY	BATCH FIT	Glucose	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Glucose	PRECIPITATED	INTACT
NOESY	BATCH FIT	Glutamate	INTACT	FILTERED
NOESY	BATCH FIT	Glutamate	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Glutamate	PRECIPITATED	INTACT
NOESY	BATCH FIT	Glutamine	INTACT	FILTERED
NOESY	BATCH FIT	Glutamine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Glutamine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Glycine	INTACT	FILTERED
NOESY	BATCH FIT	Glycine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Glycine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Histidine	INTACT	FILTERED
NOESY	BATCH FIT	Histidine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Histidine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Isoleucine	INTACT	FILTERED
NOESY	BATCH FIT	Isoleucine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Isoleucine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Lactate	INTACT	FILTERED
NOESY	BATCH FIT	Lactate	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Lactate	PRECIPITATED	INTACT
NOESY	BATCH FIT	Leucine	INTACT	FILTERED
NOESY	BATCH FIT	Leucine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Leucine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Lysine	INTACT	FILTERED
NOESY	BATCH FIT	Lysine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Lysine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Phenylalanine	INTACT	FILTERED
NOESY	BATCH FIT	Phenylalanine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Phenylalanine	PRECIPITATED	INTACT
NOESY	BATCH FIT	Pyruvate	INTACT	FILTERED
NOESY	BATCH FIT	Pyruvate	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Pyruvate	PRECIPITATED	INTACT
NOESY	BATCH FIT	Tyrosine	INTACT	FILTERED
NOESY	BATCH FIT	Tyrosine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Tyrosine	PRECIPITATED	INTACT

NOESY	BATCH FIT	Valine	INTACT	FILTERED
NOESY	BATCH FIT	Valine	PRECIPITATED	FILTERED
NOESY	BATCH FIT	Valine	PRECIPITATED	INTACT
NOESY	MANUAL FIT	2-Hydroxybutyrate	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	3-Hydroxybutyrate	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Acetate	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Acetone	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Alanine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Choline	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Citrate	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Creatine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Creatinine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Dimethyl sulfone	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Dimethylamine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Formate	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Glucose	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Glutamate	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Glutamine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Glycine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Histidine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Isoleucine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Lactate	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Leucine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Lysine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Phenylalanine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Pyruvate	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Tyrosine	PRECIPITATED	FILTERED
NOESY	MANUAL FIT	Valine	PRECIPITATED	FILTERED
NOESY	SMOLESY	2-Hydroxybutyrate	INTACT	FILTERED
NOESY	SMOLESY	2-Hydroxybutyrate	PRECIPITATED	FILTERED
NOESY	SMOLESY	2-Hydroxybutyrate	PRECIPITATED	INTACT
NOESY	SMOLESY	3-Hydroxybutyrate	INTACT	FILTERED
NOESY	SMOLESY	3-Hydroxybutyrate	PRECIPITATED	FILTERED
NOESY	SMOLESY	3-Hydroxybutyrate	PRECIPITATED	INTACT
NOESY	SMOLESY	Acetate	INTACT	FILTERED
NOESY	SMOLESY	Acetate	PRECIPITATED	FILTERED
NOESY	SMOLESY	Acetate	PRECIPITATED	INTACT
NOESY	SMOLESY	Acetone	INTACT	FILTERED
NOESY	SMOLESY	Acetone	PRECIPITATED	FILTERED
NOESY	SMOLESY	Acetone	PRECIPITATED	INTACT
NOESY	SMOLESY	Alanine	INTACT	FILTERED
NOESY	SMOLESY	Alanine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Alanine	PRECIPITATED	INTACT
NOESY	SMOLESY	Choline	INTACT	FILTERED
NOESY	SMOLESY	Choline	PRECIPITATED	FILTERED
NOESY	SMOLESY	Choline	PRECIPITATED	INTACT
NOESY	SMOLESY	Citrate	INTACT	FILTERED
NOESY	SMOLESY	Citrate	PRECIPITATED	FILTERED
NOESY	SMOLESY	Citrate	PRECIPITATED	INTACT
NOESY	SMOLESY	Creatine	INTACT	FILTERED

NOESY	SMOLESY	Creatine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Creatine	PRECIPITATED	INTACT
NOESY	SMOLESY	Creatinine	INTACT	FILTERED
NOESY	SMOLESY	Creatinine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Creatinine	PRECIPITATED	INTACT
NOESY	SMOLESY	Dimethyl sulfone	INTACT	FILTERED
NOESY	SMOLESY	Dimethyl sulfone	PRECIPITATED	FILTERED
NOESY	SMOLESY	Dimethyl sulfone	PRECIPITATED	INTACT
NOESY	SMOLESY	Dimethylamine	INTACT	FILTERED
NOESY	SMOLESY	Dimethylamine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Dimethylamine	PRECIPITATED	INTACT
NOESY	SMOLESY	Formate	INTACT	FILTERED
NOESY	SMOLESY	Formate	PRECIPITATED	FILTERED
NOESY	SMOLESY	Formate	PRECIPITATED	INTACT
NOESY	SMOLESY	Glucose	INTACT	FILTERED
NOESY	SMOLESY	Glucose	PRECIPITATED	FILTERED
NOESY	SMOLESY	Glucose	PRECIPITATED	INTACT
NOESY	SMOLESY	Glutamate	INTACT	FILTERED
NOESY	SMOLESY	Glutamate	PRECIPITATED	FILTERED
NOESY	SMOLESY	Glutamate	PRECIPITATED	INTACT
NOESY	SMOLESY	Glutamine	INTACT	FILTERED
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NOESY	SMOLESY	Glycine	INTACT	FILTERED
NOESY	SMOLESY	Glycine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Glycine	PRECIPITATED	INTACT
NOESY	SMOLESY	Histidine	INTACT	FILTERED
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NOESY	SMOLESY	Histidine	PRECIPITATED	INTACT
NOESY	SMOLESY	Isoleucine	INTACT	FILTERED
NOESY	SMOLESY	Isoleucine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Isoleucine	PRECIPITATED	INTACT
NOESY	SMOLESY	Lactate	INTACT	FILTERED
NOESY	SMOLESY	Lactate	PRECIPITATED	FILTERED
NOESY	SMOLESY	Lactate	PRECIPITATED	INTACT
NOESY	SMOLESY	Leucine	INTACT	FILTERED
NOESY	SMOLESY	Leucine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Leucine	PRECIPITATED	INTACT
NOESY	SMOLESY	Lysine	INTACT	FILTERED
NOESY	SMOLESY	Lysine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Lysine	PRECIPITATED	INTACT
NOESY	SMOLESY	Phenylalanine	INTACT	FILTERED
NOESY	SMOLESY	Phenylalanine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Phenylalanine	PRECIPITATED	INTACT
NOESY	SMOLESY	Pyruvate	INTACT	FILTERED
NOESY	SMOLESY	Pyruvate	PRECIPITATED	FILTERED
NOESY	SMOLESY	Pyruvate	PRECIPITATED	INTACT
NOESY	SMOLESY	Tyrosine	INTACT	FILTERED
NOESY	SMOLESY	Tyrosine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Tyrosine	PRECIPITATED	INTACT

NOESY	SMOLESY	Valine	INTACT	FILTERED
NOESY	SMOLESY	Valine	PRECIPITATED	FILTERED
NOESY	SMOLESY	Valine	PRECIPITATED	INTACT

FDR P-value
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$p < 0.001$
$p < 0.001$
NS
$p < 0.001$
$p < 0.001$
NS
$p < 0.05$
$p < 0.001$
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NS
$p < 0.001$
$p < 0.001$
NS
$p < 0.001$
NS
$p < 0.001$
NS
NS
NS
$p < 0.001$
$p < 0.01$
$p < 0.001$
$p < 0.001$
$p < 0.001$
$p < 0.001$
$p < 0.001$
NS
$p < 0.001$
$p < 0.001$
$p < 0.001$
NS
$p < 0.001$
NS
$p < 0.001$
$p < 0.001$
$p < 0.001$
NS
$p < 0.001$
NS
$p < 0.001$
$p < 0.05$
$p < 0.001$
$p < 0.001$
NS
NS
NS
$p < 0.001$
$p < 0.01$
NS

$p < 0.001$
NS
$p < 0.001$

Table S5: P-values from pairwise comparison of CV values

		Chenomx - Manual Fit
Precipitated		
	Chenomx - Manual Fit	#DIV/0!
	Chenomx - Assisted Fit	0.4084
	Chenomx - Batch Fit	0.6055
	SMolESY	0.4514
Filtered		
	Chenomx - Manual Fit	0.0001
	Chenomx - Assisted Fit	0.0001
	Chenomx - Batch Fit	0.0815
	SMolESY	0.1465
Intact		
	SMolESY-NOESY	7.87E-13
	SMolESY-CPMG	7.83E-13

Precipitated			
Chenomx - Assisted Fit	**Chenomx - Batch Fit**	**SMolESY**	**Chenomx - Manual Fit**
0.4084	0.6055	0.4514	0.0001
#DIV/0!	0.3672	0.3802	8.1744E-06
0.3672	#DIV/0!	0.8984	0.0018
0.3802	0.8984	#DIV/0!	0.2232

8.1744E-06	0.0018	0.2232	#DIV/0!
2.9622E-05	0.0045	0.2415	0.5483
0.0509	0.2327	0.6216	0.0344
0.1083	0.4721	0.0165	0.5807

2.27E-13	4.94E-09	5.33E-04	1.37E-09
2.26E-13	4.96E-09	5.33E-04	1.37E-09

Filtered			Int
Chenomx - Assisted Fit	**Chenomx - Batch Fit**	**SMolESY**	**SMolESY-NOESY**
0.0001	0.0815	0.1465	7.87E-13
2.9622E-05	0.0509	0.1083	2.27E-13
0.0045	0.2327	0.4721	4.94E-09
0.0001	0.6216	0.0165	5.33E-04
0.5483	0.0344	0.5807	1.37E-09
#DIV/0!	0.0815	0.6166	1.43E-09
0.0391	#DIV/0!	0.6398	2.19E-07
0.6166	0.6398	#DIV/0!	9.96E-04
1.43E-09	2.19E-07	9.96E-04	#DIV/0!
1.43E-09	2.20E-07	9.95E-04	6.86E-01

act
SMolESY-NOESY
7.83E-13
2.26E-13
4.96E-09
5.33E-04

1.37E-09
1.43E-09
2.20E-07
9.95E-04

6.86E-01
#DIV/0!

Table S6: Comparative FC values

	METHOD/STUDY Precip/Filt FC			
Metabolite	**CHENOMX - BATCH FIT**	**CHENOMX - MANUAL FIT**	**Gowda**	**Madid-Gambin**
2-Hydroxybutyrate	9.28	4.49	1.01	1.84
3-Hydroxybutyrate	2.57	3.54	1	0.99
Acetate	2.15	2.85	1.63	1.09
Acetone	0.89	1.7		0.71
Alanine	1.74	2.12	1.05	0.81
Choline	-	4.04	1.24	1.02
Citrate	0.09	1.39	0.61	0.44
Creatine	1.48	2.1	1.11	0.96
Creatinine	1.24	1.87	1.05	0.86
Formate	2.04	2.75	1.25	0.72
Glucose	1.52	1.98	1.11	0.89
Glutamate	2.52	1.9	0.99	-
Glutamine	1.83	2.67	1.01	0.94
Glycine	1.53	1.57	1.12	0.97
Histidine	-	1.66	0.93	0.88
Isoleucine	3.8	2.45	1.15	0.9
Lactate	1.25	1.83	0.96	0.86
Leucine	7.62	2.31	1.1	0.92
Lysine	-	2.22	1.14	0.89
Phenylalanine	0.86	3.3	1.16	0.88
Succinate	1.47	2.24	-	1.02
Tyrosine	1.47	3.06	1.12	0.9
Valine	1.69	2.51	1.14	0.88
AVG	2.35	2.46	1.09	0.93
STD	2.23	0.80	0.18	0.24

SMOLESY	AVG(Gowda, Madid-Gambin,SMOLESY)	STDEV(Gowda, Madid-Gambin,SMOLESY)
1.28	1.38	0.42
1.06	1.02	0.04
1	1.24	0.34
0.73	0.72	0.01
0.66	0.84	0.20
1.27	1.18	0.14
0.34	0.46	0.14
1.33	1.13	0.19
1.14	1.02	0.14
1.94	1.30	0.61
1.25	1.08	0.18
1.01	1.00	0.01
1.18	1.04	0.12
0.4	0.83	0.38
1.13	0.98	0.13
1.34	1.13	0.22
1.01	0.94	0.08
1.3	1.11	0.19
1.1	1.04	0.13
2.39	1.48	0.80
-	1.02	-
1.24	1.09	0.17
1.18	1.07	0.16
1.15	1.05	0.22
0.44	0.21	0.19

%CV(Gowda, Madid-Gambin,SMOLESY)	%Diff (MANUAL-FIT,AVG)	%Diff (BATCH-FIT,AVG)
31%	-226%	-574%
4%	-248%	-153%
27%	-130%	-73%
2%	-136%	-24%
23%	-152%	-107%
12%	-243%	-
29%	-200%	81%
16%	-85%	-31%
14%	-84%	-22%
47%	-111%	-57%
17%	-83%	-40%
1%	-90%	-152%
12%	-156%	-75%
46%	-89%	-84%
13%	-69%	-
20%	-117%	-236%
8%	-94%	-33%
17%	-109%	-589%
13%	-113%	-
54%	-123%	42%
-	-120%	-44%
16%	-182%	-35%
15%	-135%	-58%
0.20	-135%	-113%
0.14	52%	174%

	METHOD/STUDY/Precip/IntFC			
Metabolite	**Madrid-Gambin**	**SMOLESY**	**%Diff**	**AVG**
2-Hydroxybutyrate	1.68	5.04	-200%	3.36
3-Hydroxybutyrate	1.14	0.81	29%	0.98
Acetate	1.97	1.37	30%	1.67
Acetone	0.6	0.21	65%	0.41
Alanine	1.14	0.56	51%	0.85
Choline	1.23	0.55	55%	0.89
Citrate	0.74	0.22	70%	0.48
Creatine	1.02	0.59	42%	0.81
Creatinine	1.54	0.9	42%	1.22
Formate	0.93	0.86	8%	0.90
Glucose	0.9	0.68	24%	0.79
Glutamate	-	1.08	-	1.08
Glutamine	1.23	0.57	54%	0.90
Glycine	0.87	0.59	32%	0.73
Histidine	0.93	0.63	32%	0.78
Isoleucine	1.06	1.02	4%	1.04
Lactate	1.29	1.55	-20%	1.42
Leucine	1.08	0.97	10%	1.03
Lysine	1.88	0.36	81%	1.12
Phenylalanine	1.12	1.95	-74%	1.54
Succinate	1.39	-	-	1.39
Tyrosine	1.33	0.88	34%	1.11
Valine	1.11	0.67	40%	0.89
AVG	1.19	1.00	19%	1.10
STD	0.34	0.99	60%	0.58

STDEV	CV %
2.38	71%
0.23	24%
0.42	25%
0.28	68%
0.41	48%
0.48	54%
0.37	77%
0.30	38%
0.45	37%
0.05	6%
0.16	20%
-	-
0.47	52%
0.20	27%
0.21	27%
0.03	3%
0.18	13%
0.08	8%
1.07	96%
0.59	38%
-	-
0.32	29%
0.31	35%
0.43	38%
0.50	25%

Metabolite	AVG (PRECIP/FIT))
2-Hydroxybutyrate	1.38
3-Hydroxybutyrate	1.02
Acetate	1.24
Acetone	0.72
Alanine	0.84
Choline	1.18
Citrate	0.46
Creatine	1.13
Creatinine	1.02
Formate	1.30
Glucose	1.08
Glutamate	1.00
Glutamine	1.04
Glycine	0.83
Histidine	0.98
Isoleucine	1.13
Lactate	0.94
Leucine	1.11
Lysine	1.04
Phenylalanine	1.48
Tyrosine	1.09
Valine	1.07
AVG	1.05
STD	0.21

Combined Data Sets			
STDEV (PRECIP/FIT)	**%CV (PRECIP/FIT)**	**AVG (PRECIP/INT)**	**STDEV (PRECIP/INT)**
0.42	31%	3.36	2.38
0.04	4%	0.98	0.23
0.34	27%	1.67	0.42
0.01	2%	0.41	0.28
0.20	23%	0.85	0.41
0.14	12%	0.89	0.48
0.14	29%	0.48	0.37
0.19	16%	0.81	0.30
0.14	14%	1.22	0.45
0.61	47%	0.90	0.05
0.18	17%	0.79	0.16
0.01	1%	1.08	-
0.12	12%	0.90	0.47
0.38	46%	0.73	0.20
0.13	13%	0.78	0.21
0.22	20%	1.04	0.03
0.08	8%	1.42	0.18
0.19	17%	1.03	0.08
0.13	13%	1.12	1.07
0.80	54%	1.54	0.59
0.17	16%	1.11	0.32
0.16	15%	0.89	0.31
0.22	20%	1.09	43%
0.19	14%	0.59	50%

%CV (PRECIP/INT)	p-value
71%	0.2232
24%	0.7697
25%	0.2912
68%	0.1268
48%	0.972
54%	0.0808
77%	0.9336
38%	0.2288
37%	0.4964
6%	0.4444
20%	0.1647
-	-
52%	0.6319
27%	0.7625
27%	0.2671
3%	0.6229
13%	0.0234
8%	0.6258
96%	0.8977
38%	0.9345
29%	0.9304
35%	0.4391
38%	0.43
25%	0.50

Table S7: Standard Deviation IQR Outlier Analysis

Metabolite	All Data	Chenomx	CNX - BATCH - FILT
2-Hydroxybutyrate	0.164	0.199	0.015
3-Hydroxybutyrate	0.160	0.127	0.265
Acetate	0.146	0.106	0.039
Acetone	0.153	0.117	0.089
Alanine	0.143	0.094	0.072
Choline	0.165	0.118	0.331
Citrate	0.200	0.100	0.315
Creatine	0.139	0.090	0.031
Creatinine	0.158	0.131	0.099
Dimethyl sulfone	0.127	0.118	0.140
Dimethylamine	0.123	0.122	0.152
Formate	0.125	0.094	0.057
Glucose	0.127	0.128	0.056
Glutamate	0.171	0.210	0.227
Glutamine	0.170	0.099	0.090
Glycine	0.171	0.092	0.079
Histidine	0.169	0.110	0.096
Isoleucine	0.170	0.101	0.058
Lactate	0.185	0.101	0.130
Leucine	0.160	0.107	-
Lysine	0.224	0.103	0.008
Phenylalanine	0.217	0.101	0.263
Pyruvate	0.134	0.099	0.031
Tyrosine	0.164	0.097	0.144
Valine	0.150	0.095	0.084

CNX - BATCH -PRECIP	CNX - ASST - FILT	CNX - ASST - PRECIP
0.218	0.056	0.212
0.351	0.061	0.156
0.136	0.026	0.140
0.142	0.086	0.151
0.165	0.024	0.123
-	0.020	0.152
-	0.070	0.137
0.130	0.015	0.125
0.198	0.031	0.129
0.434	0.082	0.147
0.534	0.027	0.207
0.164	0.018	0.121
0.137	0.054	0.169
0.419	0.172	0.321
0.173	0.022	0.122
0.123	0.051	0.119
-	0.039	0.148
0.177	0.031	0.126
0.205	0.043	0.129
0.154	0.035	0.132
-	0.035	0.126
0.540	0.027	0.121
0.129	0.030	0.131
0.214	0.027	0.120
0.173	0.023	0.122

CNX - MAN - FILT	CNX - MAN - PRECIP	SMOLESY	SMOLESY - FILT
0.064	0.192	0.098	0.070
0.060	0.158	0.158	0.178
0.027	0.142	0.149	0.150
0.078	0.151	0.185	0.131
0.024	0.128	0.120	0.091
0.020	0.151	0.160	0.055
0.030	0.136	0.196	0.098
0.025	0.124	0.158	0.098
0.066	0.160	0.140	0.105
0.082	0.147	0.117	0.108
0.027	0.206	0.099	0.102
0.018	0.122	0.118	0.054
0.055	0.151	0.126	0.089
0.172	0.321	0.144	0.142
0.026	0.124	0.143	0.066
0.051	0.119	0.174	0.163
0.037	0.140	0.168	0.122
0.027	0.132	0.131	0.092
0.043	0.129	0.192	0.183
0.036	0.133	0.126	0.097
0.035	0.126	0.241	0.076
0.019	0.123	0.181	0.093
0.024	0.125	0.128	0.114
0.025	0.123	0.114	0.079
0.021	0.126	0.116	0.064

SMOLESY - PRECIP	SMOLESY - INT
0.132	0.020
0.174	0.109
0.165	0.116
0.109	0.149
0.075	0.138
0.079	0.215
0.053	0.160
0.122	0.188
0.130	0.171
0.118	0.124
0.119	0.068
0.131	0.122
0.098	0.147
0.132	0.159
0.074	0.123
0.092	0.134
0.132	0.165
0.126	0.153
0.212	0.143
0.114	0.145
0.092	0.123
0.162	0.108
0.160	0.097
0.117	0.123
0.081	0.135