Reproducibility and Stability of Aqueous Metabolite Levels in Extracted Serum by NMR Spectroscopy

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Background: Metabolomics offers the potential of correlating a macroscopic view of an organism to measured levels of small molecule reporters of metabolic pathways. Despite strong growth in metabolomics studies, questions on reproducibility and sample stability deserve a closer look.

Objective: This work measured acetonitrile extractions of the aqueous components of fetal bovine serum (FBS) by ^{1}H NMR spectroscopy to determine the stability and reproducibility of metabolite levels over time at storage temperatures of 20, 4, -30, and -80 °C.

Method: First, mock sera, spiked sera, and pooled human sera were used to find the measurement precision and detection limits of the instrumentation used here (600 MHz, room-temperature triple resonance probe). Next, using four replicates at each of four storage temperatures, 48 metabolites extracted (2:1 acetonitrile to serum) from FBS samples were profiled over several time scales.

Results: Although most metabolites were found to be more stable than expected at room temperature, ca. two weeks, allantoin, creatinine, and glutamine degraded much more rapidly than others at both room temperature and 4 ˚C, measurably decreasing over a few hours or 1 day, respectively. Storing samples at 4 °C dramatically improves the lifetime of all metabolites, while the fidelity of extracted samples over very long term storage at -30 and -80 ˚C is supported by this work. Slight degradation of the cryogenically stored serum extracts is linked to freeze-thaw cycles.

Conclusion: The poor stability of a few metabolites for short times supports vigilance in minimizing and standardizing room temperature handling and refrigeration of extracted samples, as inconsistent sample storage even on short time scales would introduce variation that would confound clustering.

Keywords: Metabolomics, metabonomics, NMR, metabolic profiling, reproducibility, stability, serum

1. INTRODUCTION

The ability to comprehensively measure absolute and relative concentrations of small molecules (<1500 Da) in biofluids, tissues, and organs*[1]* provides researchers and clinicians "snap shots" into complex metabolic pathways, facilitating investigations in diverse fields including personalized medicine*[2]* , nutrition*[3]* , botanicals*[4, 5]* , pharmacotherapy*[6]* , and natural products*[7]* . Broadly, the methodological challenges in conducting metabolomics include the ability to detect, unambiguously assign, and accurately quantitate the greatest number of metabolites. Also, since unknown metabolite signals frequently appear in data sets, expanding libraries of known metabolite signals is a high priority*[8-11]* , while *de novo* structure solving may complement library development. *[12]* Encompassing such methodological issues is the need to better understand the sources and magnitudes of non-interventional variation in metabolomic data at all stages of a study.

Establishing a reproducible sample preparation method reduces unwanted confounding variation in metabolomics data. Variation in sample preparation could occur in 1) collection, 2) shipment, 3) storage and 4) processing of the sample. Stages 1-3 have begun to receive increased scrutiny. Variation in sample collection could be observed within an individual and across a cohort, and can depend on whether serum or plasma is collected and how it is collected. For example, clotting time has been found to have a larger effect on metabolic profiles than short periods of room temperature exposure and the freeze-thaw cycle, *[13]* and freeze-thaw cycles of rat serum can produce instability in results.*[14]*

Since sample collection and storage can introduce variation, there is a need to minimize these variables in sample handling. *[15]* Biofluids such as serum may be minimally processed, such as by filtration, or they can be extracted using both organic and inorganic solvents.

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The stability of the accessible metabolome over time for a given patient has been considered in both 14 day*[16]* and 4 month*[17]* windows, as well as among a cohort of similar patients*[8]* , where low non-interventional variation was noted in each case and supports the broader thesis that the metabolome is sufficiently stable to detect effects of treatments, disease progression, lifestyle changes, etc.. *[8, 16, 17]* Some results have suggested that slightly more reliable

measurements can be obtained from serum than plasma for some metabolites*[18]* , while the use of cold packs for storage/shipping after collection enhanced reliability when samples could not be immediately frozen at -80 ˚C. *[16]* Whereas recent work has characterized reproducibility with emphasis on sample collection and the stability of the metabolome, the reproducibility and stability of metabolite levels after the initial sample processing steps such as metabolite extractions have not been fully explored.*[19]*

Extractions remain common in metabolomic studies since they inactivate viruses, are effective at suppressing protein levels, and are simple to implement in reproducible ways. Increasing efforts have been devoted to improving the extraction of aqueous metabolites from biofluids, where acetonitrile or methanol extractions have yielded the most promising number of metabolites for quantification. *[12, 20-23]* Methanol shows potential over acetonitrile for recovering metabolites from serum and leading to improved quantitation and number of identified metabolites, *[22, 23]* but it is not yet clear if methanol precipitation is also sufficient for stripping viral membranes and inactivating samples. Other methods for enhancing spectral data include the use of ultrafiltration for protein background reduction. *[1, 23]* This work considers the stability and reproducibility of targeted metabolomics specifically in extracted serum samples.

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are used to measure metabolite levels*[24-28]* , with increasing focus on the complementary roles of MS and NMR data in probing the metabolome. *[20, 29, 30]* Despite being perceived as an insensitive technique, NMR accesses increasing numbers of metabolites*[31]* as higher static magnetic fields, cryogenic and reduced coil-diameter probes, and improved experimental methods become available. Further, NMR is paired with relatively simple, non-destructive options for sample preparation of tissues or biofluids. *[27]* NMR also provides unique chemical fingerprints for unambiguous metabolite identification, and straightforward quantification without calibration curves.*[28]*

This study assessed the reproducibility and time-dependent stability at several common storage temperatures of extracted aqueous metabolites found in fetal bovine serum (FBS) by NMR-based metabolomic methods. To accomplish this, we first investigated fundamental performance characteristics of the NMR measurement itself. Specifically, we considered limits of detection, accuracy, and precision in mock and human sera while recording spectra on a room temperature inverse probe operating at 600 MHz. These assessments then informed the design of the reproducibility and stability studies conducted on extracted FBS as the primary focus of this work.

2. MATERIALS AND METHODS

2.1 MOCK SERUM AND DETECTION LIMIT

A series of proline standards were created for testing precision and the detection limit. Standards solutions were extracted and subsequently dried on a SpeedVac (Thermo Scientific Savant SPD131DDA SpeedVac Concentrator with RVT4104 Refrigerated Vapor Trap) for reconstitution in NMR buffer (100 mM phosphate at 7.4 pH; 0.1 mM DSS standard; 100% D_2O) for quantification. Final concentrations of standards in NMR tubes spanned 10 mM to 1 μM. For example, to obtain the 1 uM proline sample (**Figure 1**), a 150 uL mock serum standard was prepared to 4 μM proline, extracted, dried, and reconstituted in 600 μL NMR buffer (see 2.2 Extraction Protocols).

2.2 EXTRACTION PROTOCOLS

Two methods of extraction, chloroform:methanol (1:1) and acetonitrile, were evaluated for retention of metabolite concentrations with mock serum standards. Standards were extracted by either (i) combining 150 μL of sample and 800 μL of chloroform:methanol (1:1) or by (ii) combining 150 μL of sample and 300 μL of acetonitrile. Next, samples were vortexed for 1 minute at 4 °C, and then centrifuged at 10,000 g for 10 minutes at 4 °C (IEC Micromax RF). The upper aqueous supernatant layer was removed by manual pipetting for the chloroform:methanol protocol, while the entire supernatant was taken for the acetonitrile method. The supernatants were then placed in a SpeedVac for concentration (ca. 3 hr). Samples were reconstituted in 600 μL of the NMR buffer with 0.1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS).

2.3 SERUM EXTRACTION

Fetal bovine serum was obtained as a large pooled sample from Sigma Aldrich. One randomly selected, de-identified sample (1 mL) of human serum was purchased from Bioserve Inc. (Beltsville, MD) under IRB consent and divided in to 5 aliquots for the reproducibility data shown in **Fig 3-4**. The FBS samples were taken from the single pooled stock from a -80 °C freezer and thawed. Sixteen FBS samples then underwent the acetonitrile extraction protocol to provide four replicate samples for each storage condition (20 ˚C, 4 ˚C, -30 ˚C, and -80 ˚C). After data acquisition on the NMR spectrometer, samples were stored in temperature-controlled environments. Room temperature (20 °C) and 4 °C samples were stored in the NMR tubes while the -30 and -80 ˚C samples were pipetted from the NMR tubes and stored in microcentrifuge tubes. Samples stored in the -30 and -80 °C freezers were thawed only to take NMR data, to help focus on observing any degradation solely on time. Room temperature samples were stored in a climate controlled room at 20 ± 1 ˚C, while a walk-in cold room was used for storage at 4 ± 2 ˚C. The freezer operating at -30 ˚C was frequently accessed, and its temperature was observed to fluctuate by -30 ± 5 ˚C. The freezer operating at -80 ˚C was infrequently used and generally stable within ± 2 ˚C , but did experience a small number of observed temperatures reaching -72 ˚C during the storage times considered in this study.

2.4 ^{1}H NMR SPECTROSCOPIC ANALYSES

Standardized mock serum samples and FBS samples following SpeedVac concentration were reconstituted in 600 μL NMR buffer, and then transferred to a 5 mm NMR tube (Norell, Inc., Marion, NC). One-dimensional ^{1}H NMR experiments were performed on all samples using a 600 MHz NMR spectrometer (Varian Inc.) equipped with a 5 mm triple resonance (HCN) inverse probe at 298 K using VnmrJ software. A presaturation NOESY sequence was used for water suppression. Acquisition parameters for the standard proline spectra were 8 transients for 10 – 0.08 mM, 32 for 0.06 mM, 128 for 0.03 mM, and 512 for 0.01 – 0.001

mM. Parameters for the comparison of extraction protocols and FBS spectra were 8 and 64 transients, respectively. Profiling of ^{1}H NMR signals was performed by MMM using Chenomx NMR Suite 8.1 (Chenomx, Canada) following the Human Metabolome Database. *[11]*

As will be noted throughout the manuscript, we report the profiled concentrations of the final solutions used for NMR spectroscopy, not of their parent stocks (see protocol in section 2.1 for example). Since 150 uL of serum was extracted and diluted to a final NMR volume of 600 μL, concentrations should be multiplied by four to obtain their values in the parent serum.

3. RESULTS AND DISCUSSION

Prior to assessing the impact of storing extracted samples of FBS at different temperatures (Section 3.3), we conducted a preliminary study to assess detection limits on the instrumentation, efficacy of extraction, and reproducibility of measured metabolite levels. While this study (section 3.1 and 3.2) was sufficient to delineate protocols for the stability study (section 3.3), we note that accuracy, precision, and reproducibility in metabolomics NMR are just receiving more detailed investigation and more work is warranted.*[32-35]*

3.1 DETECTION LIMITS AND EXTRACTIONS

We characterized the detection limit of the spectrometer system (600 MHz, room temperature 5mm inverse probe) for a representative metabolite, proline, which is a fairly challenging metabolite for profiling due to complex multiplets which result in decreased peak intensities relative to metabolites which give singlets. One dimensional ^{1}H NMR spectra were obtained on decreasing concentrations of the representative metabolite (e.g. proline) for determining a concentration limit. Profiled proline concentrations spanning 0.8 mM to 1 μM are shown in (Fig. **1**). It is noted that Chenomx NMR Suite 8.1 allows for profiling metabolites to concentrations as low as 0.1 μM. The maximum number of transients used was 512 (2 hrs spectrometer time), which is reflective of the largest number that would be used for a serum sample given time and stability constraints. It is possible to detect metabolites at a lower concentration by NMR if more transients were used, or if cryogenic probes and higher fields were considered, but this was outside the scope of the present study.

Inspection of the results in **Fig. 1** shows agreement of the profiled concentrations with their standard values, where lines of best fit (solid line, **Fig. 1**) for profiled concentrations agree closely with the theoretical slope of 1 (dashed line, **Fig. 1**). The detection limit is established by identifying both the lowest concentration sample that can be profiled, and the highest concentration sample that could not. For this study, those values were the 3 μM and 1 μM standard samples. Specifically, when using 512 transients, the spectrum of the 3 μM proline sample exhibits clearly identifiable signals, and can be profiled, yielding a value of 3.4 μM for a sample that was prepared to be 3.0 μM (open circle, panel c of **Fig. 1**). In contrast, the 1 μM spectrum could not be profiled as the proline signals, even with line broadening techniques, could not be distinguished from the noise. With the benefit of prior knowledge that the 1 μM sample contains proline, and the advantage of the sample being pure in proline, a rater can estimate a concentration of 1.2 μM for this sample (filled star, panel c of **Fig. 1**). However, lacking this knowledge, an unbiased rater would not be able to profile the 1 μM proline spectrum. To see if the detection limit results in **Fig. 1a-c** translate in to a real serum sample, we then spiked fetal bovine serum with a series of caffeine concentrations at very low concentrations (< 10 μM) prior to extraction. Using a blinded rater profiling the resulting spectra, it was found that concentrations as low as 1 μM could be profiled, although more scatter is observed below about 5 μM (**Fig. 1d**).

Proline signals are distributed over multiplets that occur in crowded regions of serum spectra, while caffeine gives several methyl singlets, one of which in a less crowded region was chosen as the signal from which all the concentrations were profiled. Not surprisingly, caffeine could be profiled, albeit with poorer precision, below 3 uM, whereas proline was a more challenging metabolite to detect.

In summary of this part, clearly detection limits will vary with metabolite identity and spectral overlap, but these results indicate that about 8-10 μM (resulting in about 2-3 μM in the NMR tube) is the practical limit for the instrumentation considered here.

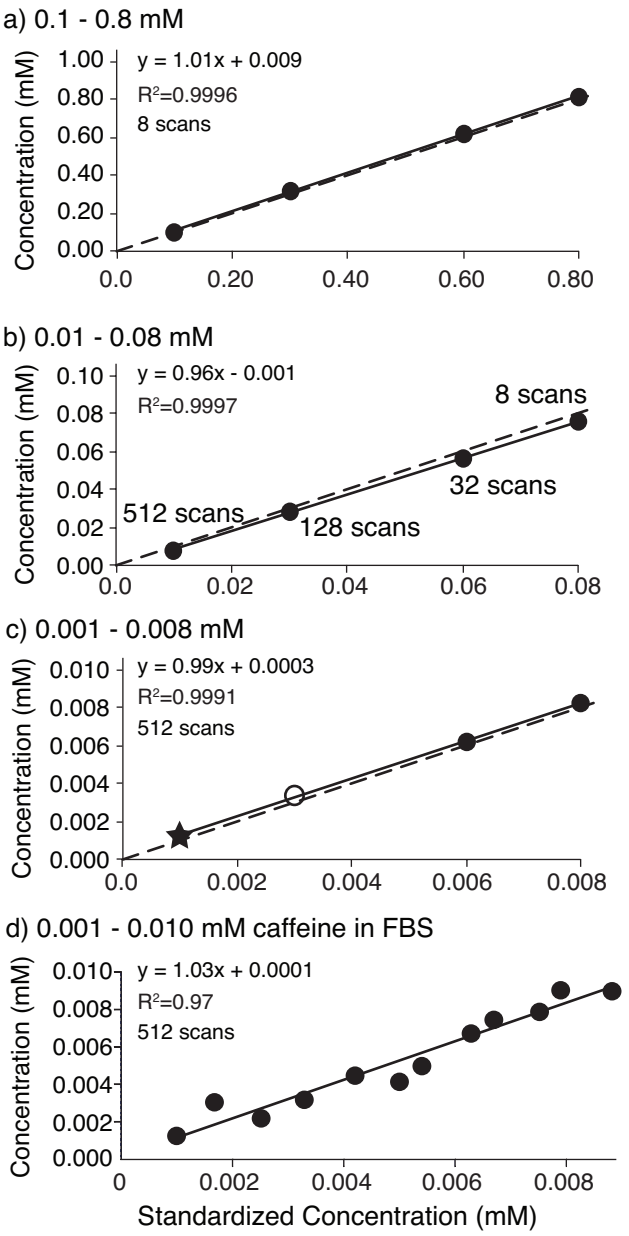

Fig. (1). Profiled concentrations obtained from standardized solutions. (a-c): profiled concentration of proline in standards using Chenomx NMR Suite for spectra obtained with 1D-^{1}H-prNOESY (600 MHz, RT inverse probe). Note the actual concentrations of the samples in the NMR tube are displayed; the original mock sera are prepared at four times each concentration. The open circle in panel (c) distinguishes 3 μM as the lowest concentration that could be profiled (which is 12 μM in the original mock serum), whereas the starred symbol represents the highest concentration (1 μM in the NMR tube, 4 μM in the original mock serum) that could not be profiled (see text for discussion).

Next, two commonly used extraction methods were compared. Proline and glucose mock serum standards were prepared to characterize the recovery of metabolites when using the chloroform/methanol and acetonitrile extraction methods. For verification, non-extracted mock sera were also analyzed as control standards. Whereas chloroform/methanol has been widely employed in prior literature, we have noted increasing utilization of acetonitrile in extractions, and so we sought a comparative analysis of the methods. Final concentrations of these metabolites in the NMR buffer from 0.1 to 10 mM for proline and 1 to 10 mM for glucose were profiled and compared to expected concentrations as shown in (**Fig. 2**).

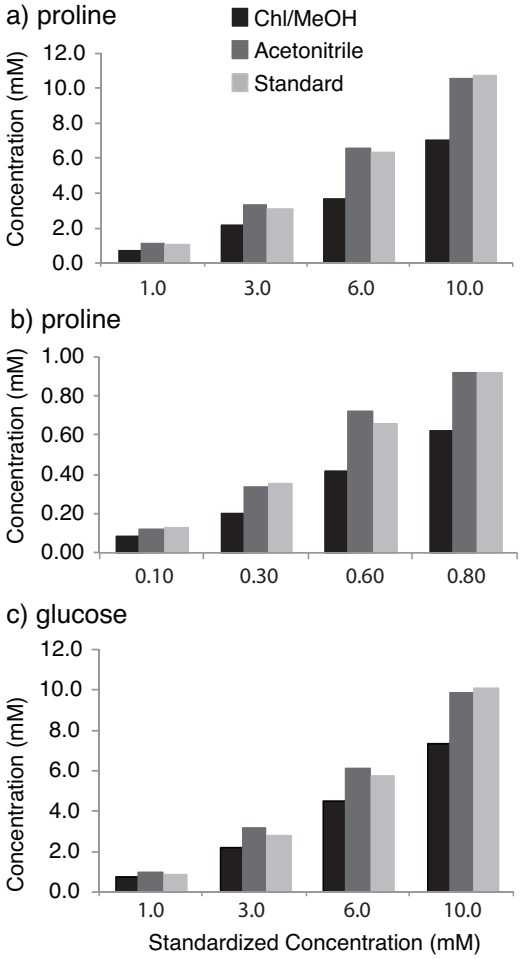

Fig. (2). Bar graphs representing the profiled concentration of proline and glucose using Chenomx NMR Suite 8.1 (vertical axis). The standard concentrations are labeled on the horizontal axis. Chloroform/methanol (1:1), acetonitrile, and the standard are represented as black, gray, and light gray, respectively. Note that the value of the standard concentrations is obtained by diluting the original mock serum standard four-fold and then profiling the subsequent NMR spectrum (i.e. not extracted).

Strong agreement between the profiled and non-extracted standardized values were obtained (light grey, **Fig. 2**) confirming the accuracy of the standards. Next, profiled concentrations in proline and glucose samples extracted by chloroform/methanol (black bars, **Fig. 2**) consistently yielded lower concentrations compared to standards, while profiled concentrations obtained from acetonitrile extracted samples (dark grey bars, **Fig. 2**) were more accurate.

The degree of loss when utilizing chloroform/methanol extraction may owe not only to partitioning of aqueous metabolites between the aqueous and organic layers during extraction, but also to physical limitations in removing the entire upper aqueous layer. Thus in **Fig. 2**, the partitioning effect could cause some of the proline concentration (non-polar) to shift to the less-polar layer. *[36]* However, the depressed glucose concentration measured in chloroform/methanol extracts indicates that partitioning is not the only factor and supports concerns over isolating the entire supernatant in the chloroform/methanol procedure.

While extraction of serum reduces protein background levels, ultrafiltration improves removal of these backgrounds and may be warranted for future studies*[23, 37]* .

3.2 REPRODUCIBILITY IN HUMAN SERUM

We used pooled human serum (commercially acquired) to examine the reproducibility (precision) of profiled metabolites from extractions performed on five aliquots. Fig. **3** displays a stacked plot of a region of the NMR spectra for the five aliquots, which demonstrates conservation of the spectral structures and intensities among them. We profiled 46 metabolites in the five human serum aliquots (**Fig. 4**). The averages among the 5 replicates of the concentrations of the 46 profiled metabolites are shown in **Fig. 4a**, with error bars representing the standard deviations. The magnitude of the standard deviations reflects the combination of intrinsic variation among the 5 spectra and intra-rater profiling variation.

Trend lines in **Fig. 4b** indicate that 10% error characterizes the variation among the profiled concentrations for these five replicates, with a median percent error of 12%. However, percent errors give an incomplete description of the uncertainties among all profiled metabolites. The median standard deviation of metabolite concentrations computed from the 5 replicates, omitting glucose, lactate and acetate, was just 5 μM; the average standard deviation was 8 μM. And for metabolites less than 50 μM, the uncertainty was often just a few micromolar (**Fig. 4b**). For metabolites at the detection limit, which is about 2-3 μM in the NMR tube, even low uncertainties of a few micromolar can create a misleading impression of the percent uncertainties. Note that glucose (1.4 ± 0.2 mM), lactate (3.8 ± 0.4 mM), and acetate (0.63 ± 0.08 mM) are present at much higher concentrations. Reporting absolute uncertainties appears more informative to characterizing the precision of NMR metabolite measurement. As expected, metabolites with simple spectral features in less crowded regions were more reproducibly profiled, whereas metabolites exhibiting complex splittings in crowded spectral regions led to more variation in determining concentrations. These results support that improving spectral resolution and increasing automation in NMR metabolite profiling will decrease uncertainties.

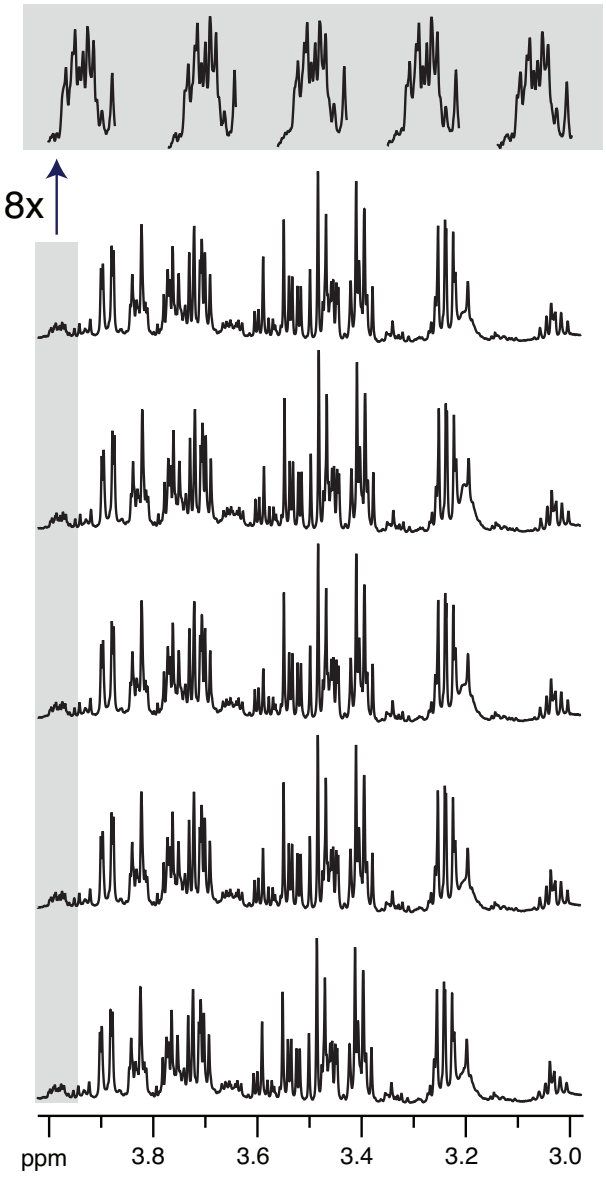

Fig. (3). ^{1}H NMR (presat. NOESY) stack plot of a dense spectral (3.0 to 4.0 ppm) that is useful for identifying amino acids. Acetonitrile extractions were performed on five human serum aliquots selected from a single vial. Strong conservation of intensity and resolution support reproducibility for independent sample preparation and measurements.

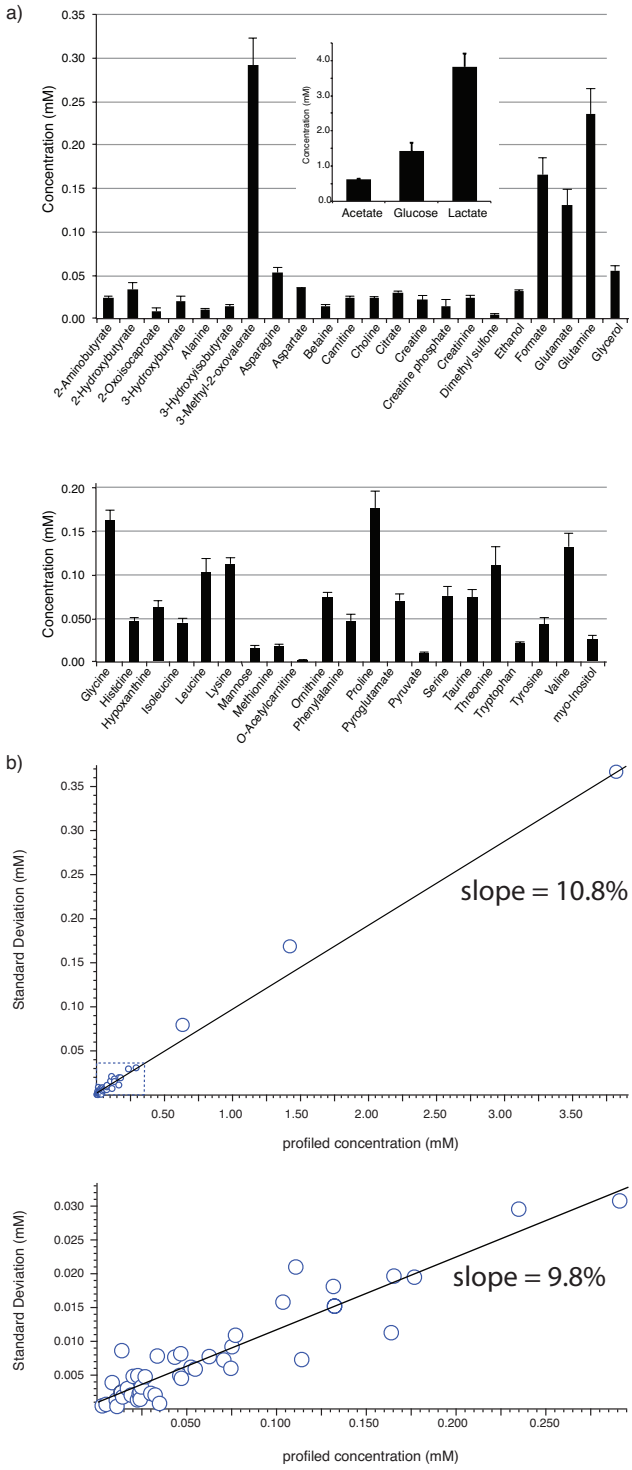

Fig. (4). Average profiled (by a single rater) concentrations of extracted metabolites in the NMR sample (concentrations should be multiplied by four to obtain their values in the parent serum) and uncertainties (standard deviations) for five aliquots of extracted human serum. A summary of labeled profiled concentrations and their standard deviations is illustrated in panel (a) while the relationship between the standard deviation and the value follows a 10% trend line over a broad dynamic range of profiled concentrations.

3.3 STABILITY AND REPRODUCIBILITY WITH STORAGE AT 20, 4, -30, -80 ˚C

Although there are a several protocols for preparing serum samples for NMR metabolomics*[22]* , the use of extractions to isolate aqueous metabolites remains a common practice and offers a number of compelling benefits: virus inactivation, effective reduction of background proteins, simplicity and therefore reproducibility. For this work, FBS was investigated using ^{1}H NMR spectroscopy to determine proper handling of samples based on stability and degradation rates at common storage temperatures. We utilized FBS as a surrogate for human serum in part for cost reasons, as FBS presented a route to perform four replicates at each of four different temperatures, eliminating sample variation by basing the entire study off of one large pooled sample. Additionally, FBS gives sharp NMR lines and demonstrated low protein backgrounds. Quantifying 48 metabolites, this study provides insights into the proper handling of extracted serum samples, while supporting a broader observation that NMR is an intrinsically reproducible technique.*[38]*

Often serum extracts are stored at 4 and -80 °C, so degradation at these temperatures was of particular interest, but we also investigated storage at -30 and 20 °C. Based on the studies in Section 3.2, which supported the utility of measuring replicates, four replicates for each temperature were decided upon as a manageable set.

The temperature stability results for room temperature (20 ˚C) and refrigeration (4 ˚C) storage of 48 profiled metabolites are summarized in **Fig. 5**. (see table in Supporting Information with full data) Three metabolites, present at much higher concentrations, are summarized for 20 and 4˚C storage in the inset of **Fig. 5**.

Many metabolites were found to be extremely stable over short periods of time, even at room temperature. A few metabolites (e.g. allantoin, creatinine, and glutamine) decreased in concentration at room temperature more rapidly than the other metabolites, with the onset of degradation detectable after just 2 hours, emphasizing the need to minimize and standardize room temperature handling of extracted sera. These three metabolites also showed less stability at 4 ˚C relative to the remaining metabolites profiled, such that refrigeration for a few days alters their observed levels. (Fig. 5). The variation over short times at room or refrigeration temperatures can exceed the precision error, meaning that inconsistent time handling of samples could introduce unwanted variation that confounds clustering. Whereas the majority of metabolites showed substantial degradation over 3 weeks at room temperature, sera kept at 4 °C showed substantially longer stability of their metabolites. For example, the relative stabilities of acetate, alanine, formate, glutamine, lysine, and xanthine (to name a few) varied considerably between 4 ˚C vs. 20 ˚C in **Fig 5**.

Certain metabolites proved difficult for metabolic profiling. Glucitol is found in a crowded region near 3.8 ppm, making quantification challenging. Only one profiler (rater) was used for all data in this report, where simple, rater-specific rubrics aided consistency in profiling signals in crowded regions.

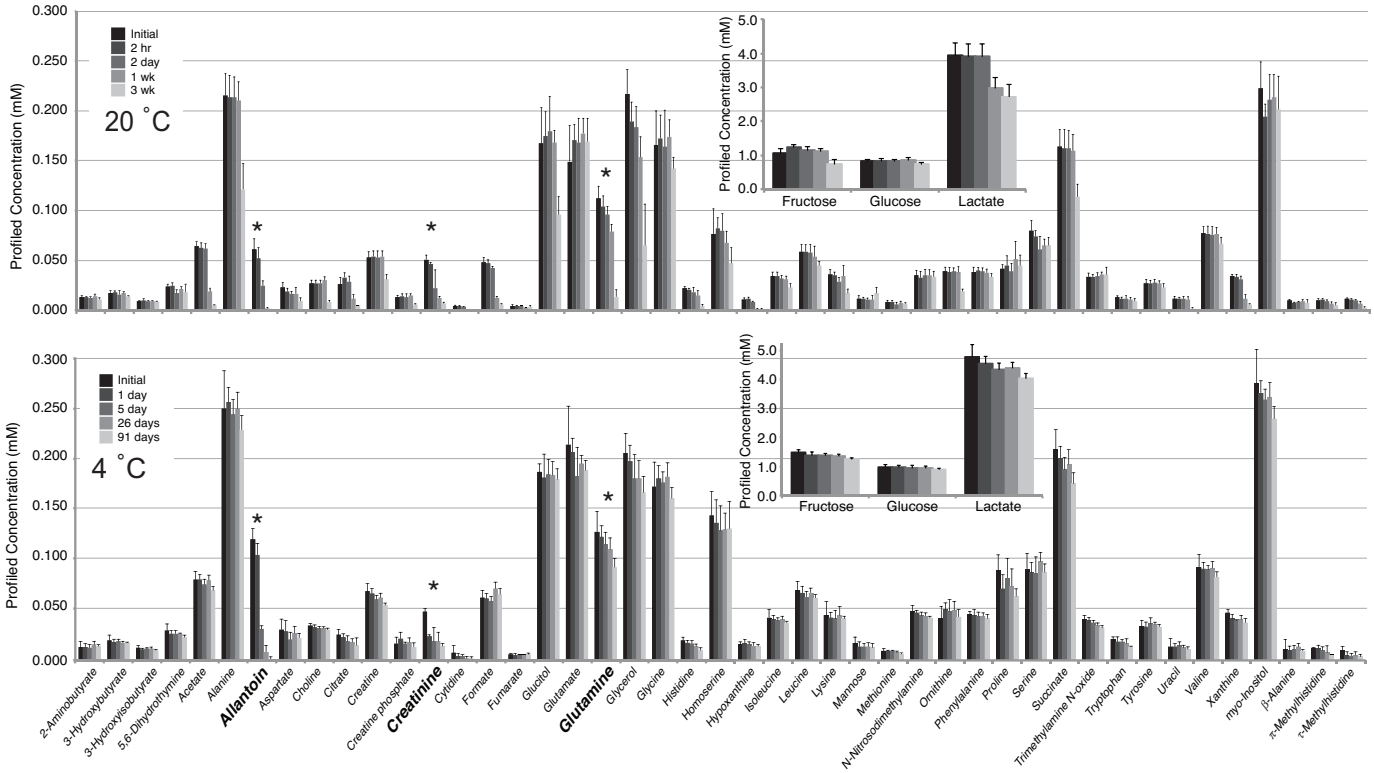

Fig. (5). The average concentrations and their standard deviations over four replicates of 45 of the 48 profiled metabolites are summarized, with time scales indicated in the accompanying legends. As before, these concentrations are of the final solutions used for NMR spectroscopy, and should be multiplied by four to obtain the concentrations in the original serum. The room temperature study followed 4

replicates over a three week period, whereas the refrigeration study followed four replicates over a three month period. Uncertainties represented by whiskers on metabolite columns are calculated standard deviations between the four aliquots.

When multiple raters are considered, uncertainties will increase and we note a general trend in NMR metabolomics studies to date of using single raters, effectively rendering rater error as systematic. Rater error would increase the uncertainties for all metabolites and represents one of the larger concerns for utilizing NMR metabolomics more broadly, such as in clinical settings. Further, we reasonably anticipate that rater errors likely increases the most for profiling peaks that occur in crowded regions. Single-rater NMR metabolomics preserves the ability to interrogate biomarker trends with the use of multivariate analysis techniques such as PCA and PLS-DA. *[39, 40]*

Percent errors were typically found between 6-12 %. For several metabolites that are present at micromolar concentrations, percent errors can exceed 20% and would be much larger than wanted. We encountered this issue in our preliminary studies, which showed that percent errors are necessarily high for those metabolites with concentrations near the limit of detection; in such cases, the absolute errors are more informative metrics of precision. This also implies that it is important to profile multiple aliquots of a sample and average the data to enhance the precision of metabolomics studies.

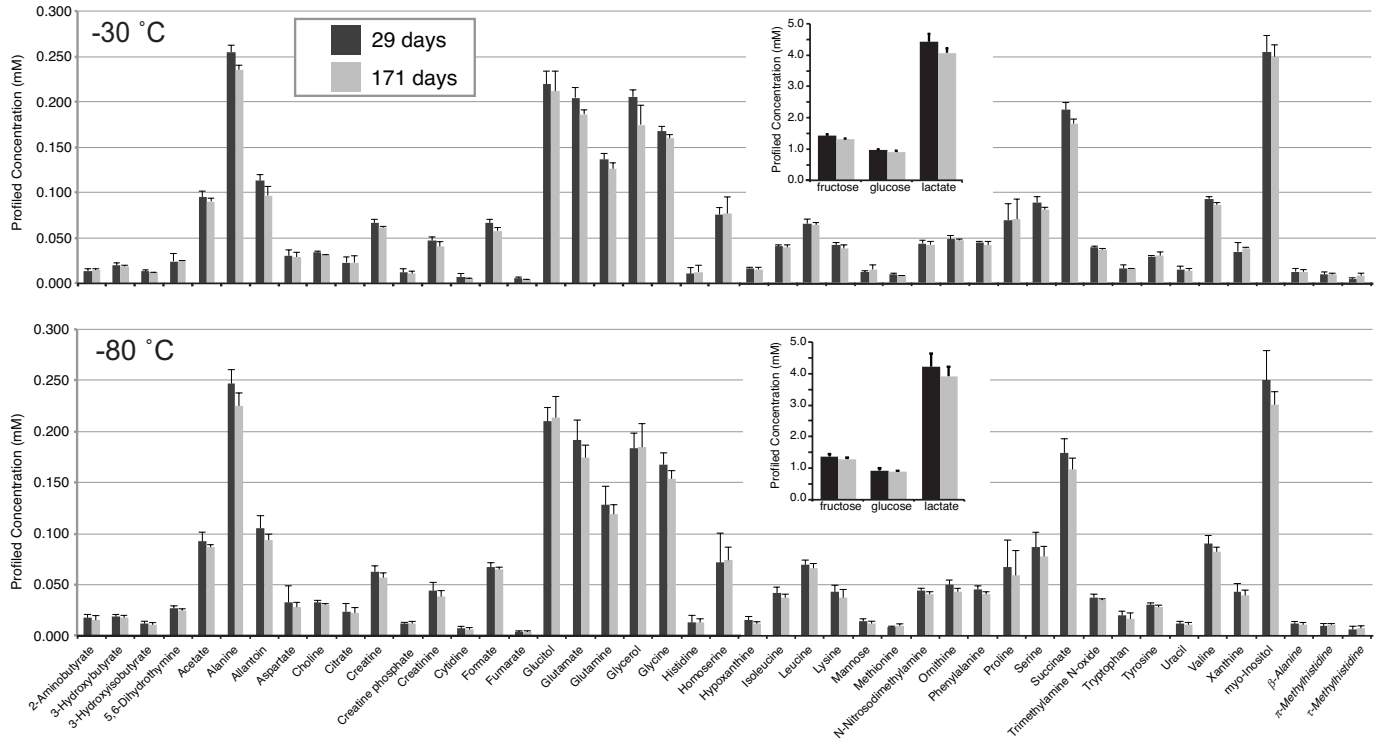

Fig. (6). The average concentrations and their standard deviations over four replicates of 48 profiled metabolites are summarized, with time scales indicated in the accompanying legends. Concentration shown are for the NMR solutions and should be multiplied by 4 to obtain their values in the serum. The -30 and -80 °C studies followed 4 replicates over an almost 6 month period. Errors represented by whiskers on metabolite columns are standard deviations between the four aliquots.

The temperature stability results for samples stored under cryogenic conditions of -30 and -80 ˚C are summarized in **Fig. 6**. (see table in Supporting Information with full data) Three metabolites, present at much higher concentrations, are summarized in the inset. As expected, metabolites were more stable over longer periods of time when refrigerated at colder temperatures such as -30 and -80 °C. In **Fig. 6**, a few metabolites (i.e. glucitol and homoserine) exhibit an increase in concentration which we attribute to difficulties in profiling those metabolites, such as spectral crowding or low concentrations. However, there appears to be a systematic degradation by about 15% of metabolites at the same rate when comparing the -30 and -80 °C data. Given the prior results at warmer storage temperatures in **Fig. 5**, we postulate that the degradation exhibited by the metabolites in the extracted serum samples stored under freezing conditions in **Fig. 6** are due to the freeze/thaw cycle rather than thermal degradation over time. To minimize the effects of the freeze/thaw cycles, we only collected two measurements on these samples. These data (**Fig. 6**) support that freeze/thaw cycles of frozen samples stored over long times must be minimized.

Representative spectra help to illustrate some complex differences observed in the temperature stability of different metabolites, particularly for room temperature (20 ˚C) and refrigeration (4 ˚C) conditions. Many metabolites were stable over long times at both of these temperatures, (representative examples in **Fig. 8**), but several metabolites degraded over a variety of timescales. As noted, out of the 48 metabolites observable in this work, three showed significantly less stability than the others under common handling and storage temperatures (**Fig. 5**, **Fig.8**). But more complex degradation trends were also observed. For example acetate is stable up to about 2 days at room temperature, and then rapidly degrades (**Fig. 8**). However acetate was stable for at least three weeks under refrigeration (**Fig. 8**). Intermediate stability similar to acetate was also observed for several other compounds such as homoserine, citrate, and xanthine, which all showed improved long term stability under refrigeration (**Fig. 5**). Refrigeration is directly observed to enhance the stability of a number of metabolites over longer storage times up to three weeks.

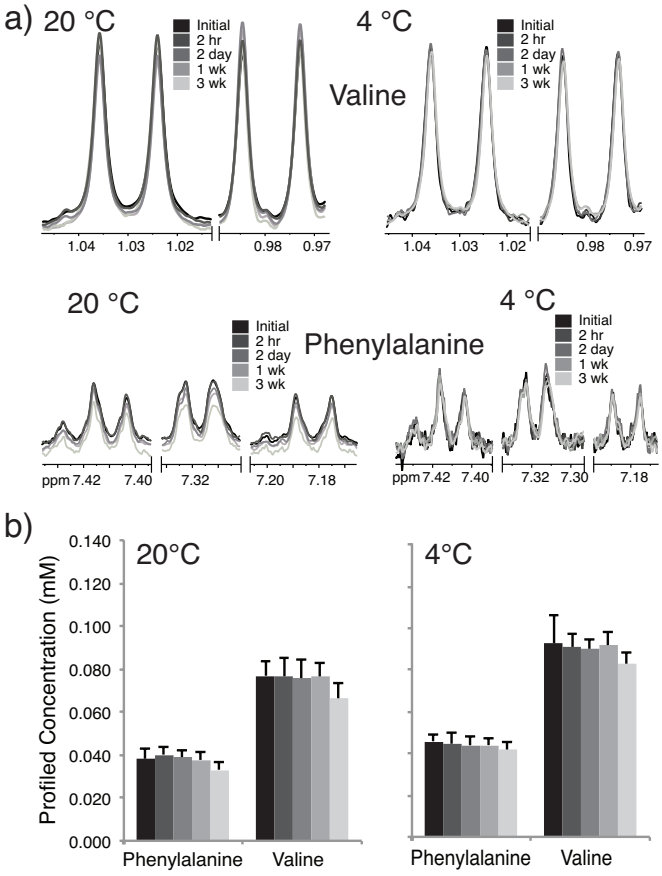

Fig. (7). The strong conservation of spectral features (a) for metabolites, such as valine and phenylalanine shown here, in extracts of serum that demonstrate remarkable stability over storage at room temperature and refrigeration. Profiled concentrations in (b are reproduced from **Fig. 5**.

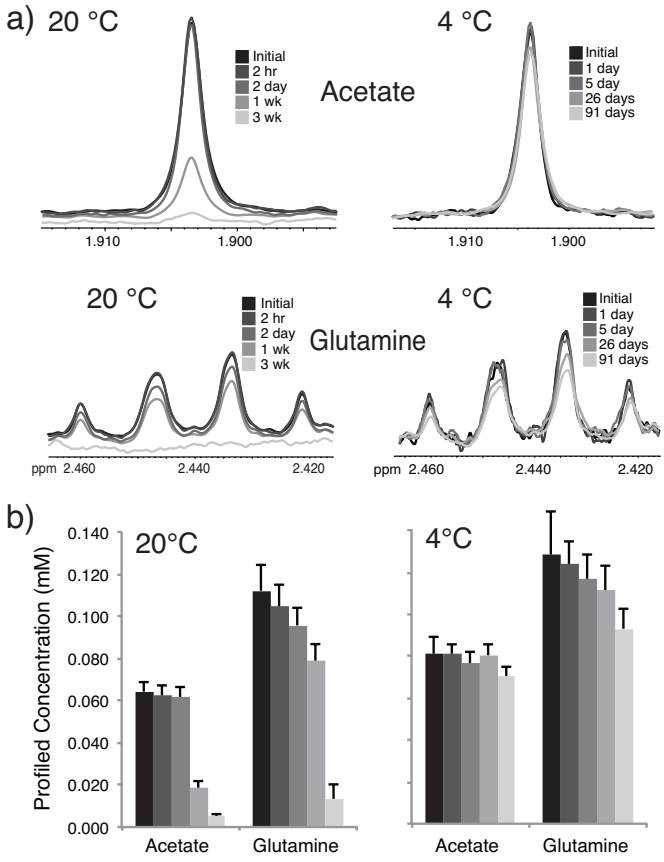

Fig. (8). Some metabolites degrade detectably at room temperature and also under refrigeration, even over relatively short time periods, such as acetate and glutamine. Profiled concentrations are reproduced from **Fig. 5**.

CONCLUSION

Whereas the stability of sera and plasma have been examined previously, this work characterized the stability of samples extracted with acetonitrile and resuspended in an aqueous buffer. Despite treatment with acetonitrile, significant metabolite degradation was measured over short time scales at room temperature and even during refrigeration.

Nearly all of the 48 metabolites profiled in FBS in this study were found to be stable at room temperature for about 2 hours, when allantoin, creatinine, and glutamine began to degrade detectably. Storage at 4 °C significantly improved the stability of metabolites over time, although degradation of the three noted above became measurable after about a day. As expected, extracted serum samples can be stored for extended periods at -30 or -80 ˚C, but should be thawed only once for analysis.

This work mandates minimizing and standardizing room temperature handling and refrigeration even of extracted sera. It is noteworthy that refrigeration following extraction does not guarantee stability for all metabolites even on time scales as short as one day. While 48 detected metabolites are appropriate for the instrumentation used here, they represent a small fraction of the metabolome. Having demonstrated here that some metabolites can have poor stability in extracted samples at both room temperature and refrigeration, it is likely that other metabolites not observed here are also unstable under similar conditions.

CONFLICT OF INTEREST

The authors report no conflict of interest in this work.

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SUPPLEMENTARY MATERIAL

Tables of the numerical data represented in Figure 5-6 are provided in supplemental information.

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