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Characterization of rapid extraction protocols for high-throughput metabolomics

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Abstract

RATIONALE—In the last five years, high throughput metabolomics has significantly advanced scientific research and holds the potential to promote strides in the fields of clinical metabolomics and personalized medicine. While innovations in the field of flow-injection mass spectrometry and three-minute metabolomics methods now allow investigators to process hundreds to thousands of samples per day, time-sensitive clinical applications, particularly in the emergency department, are limited by a lack of rapid extraction methods.

METHODS—Here we characterized the efficacy of fast liquid-liquid extractions for characterization of hydrophilic compounds through ultra-high pressure liquid chromatography-mass spectrometry. Internal stable isotope-labeled standards were used to quantitatively characterize markers of energy and oxidative metabolism in human whole blood, plasma and red blood cells – three common matrices of clinical relevance.

RESULTS—For all the tested matrices, vortexing time (4–60 min) did not significantly affect extraction yields for the tested hydrophilic metabolites. Coefficients of variations <<20% for all tested compounds, except for the redox sensitive metabolite cystine (accumulating over time). Internal standards and second extractions confirmed recoveries >80% for all tested metabolites, except for basic amino acids and polyamines, which showed reproducible yields ranging from 50–75%. Global profiling and absolute quantitation of 24 metabolites revealed similarities between the plasma and red blood cell metabolomes.

CONCLUSIONS—Rapid extraction (~4 min) of hydrophilic compounds is a viable and potentially automatable strategy to perform quantitative analysis of whole blood, plasma and red blood cells for research or clinical applications.

Keywords

Mass spectrometry; vortexing time; clinical metabolomics; plasma; blood; recovery

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Introduction

Over the past decade, the implementation of omics technologies to small molecule analysis has fostered significant strides in scientific research. In this view, mass spectrometry (MS)-based metabolomics has initially accompanied and more recently mostly replaced nuclear magnetic resonance (NMR), especially in the fields of cancer research^[1], immunity^[2] and blood research^[3], cardiovascular research^[4] and precision medicine^[5–7]. MS-based metabolomics holds the advantages of increased sensitivity and specificity when compared to classical NMR, resulting in the opportunity to analyze a fraction of the sample size required by routine metabolomics analyses via NMR while expanding the coverage of molecules detected and easing discovery-mode investigations^{[8][9]}. Until recently, MS-based metabolomics has been in part hampered by time-consuming analytical workflows and issues with robustness^[8]. However, the recent introduction of flow-injection MS-based workflow^[10] or ultra-high throughput liquid-chromatography has significantly improved the throughput of MS-based metabolomics experiments, at least at the analytical level, theoretically enabling investigators to run hundreds to thousands of samples per day. Ultra-high pressure liquid chromatography (UHPLC)-MS-based workflows that enable coverage of a significant portion of the metabolome (e.g. >5000 features, up to 500 named metabolites) in under 10^[11] or 3 minutes have been recently described^[12,13]. Additional methodology sacrifices chromatographic separation to favor the rapidity and throughput of the analysis using flow-injection coupled to time-of-flight MS (TOF-MS),^[14] which affords rapid real-time analytical readouts with a theoretical limit of 1,000 samples per day^[15–17].

Despite significant advancements in the field of analytical technologies, sample preparation strategies for MS-based metabolomics are time consuming, with standard workflows usually taking ~1h for the extraction of one batch of < 100 samples including at minimum 30 min for liquid-liquid extraction. This limitation represents a significant hurdle for the translation of metabolomics technologies to the clinic. The development of extraction strategies that are faster while maintaining robustness, in terms of reproducibility and metabolite extraction recovery, would ameliorate the lag time between sample collection and obtaining critical clinical information.

Historically, well-established extraction methods such as Bligh-Dyer and Folch methods and more recent variants^[18–22] have been used to recover water-soluble hydrophilic and hydrophobic metabolites through the sequential exposure of the matrix of interest to progressively less polar and more organic solvents (e.g. from water to methanol and chloroform). Building on recently established analytical capabilities allowing our lab to analyze hydrophilic compounds in a 3 min UHPLC-MS run,^[12] we will here focus on optimizing the extraction of water-soluble compounds such as amino acids and those involved in central carbon and nitrogen metabolism, as well as redox homeostasis. Though limited in scope by global metabolomics standards, hydrophilic metabolic markers of health and disease have been established in the most diverse biomedical fields, such as those of clinical biochemistry and intensive care medicine. For example, glucose has been historically established as a marker of diabetes,^[23] lactic acid^[24] and succinate^[25] as markers of systemic hypoxia and predictors of mortality in trauma patients, and metabolites

downstream of glucose, glutamine, and other key carbon sources have been recently associated with deregulated immunity,^[26] inflammatory responses^[27] and cancer^[28].

In the present study, we build on a widely established workflow for the liquid-liquid extraction of hydrophilic compounds to determine whether metabolic extraction reproducibility and recovery rates were affected by vortexing time.

Materials and Methods

Blood samples were collected at 9 am in the morning from fasting male healthy donor volunteers (age 27–33) in conformity with the Declarations of Helsinki.

Experiment 1 - Sample extraction as a function of vortexing time

Plasma and red blood cells (RBCs) were separated from whole blood through gentle centrifugation at 2,000g for 10 min at 4 °C. Whole blood, packed RBCs and plasma were extracted in ice cold extraction solution (Optima LC-MS grade methanol:acetonitrile:water 5:3:2) at 1:10 or 1:25 dilutions, as reported,^[29–31] prior to vortexing for either 4, 14, 26, 38, 49 and 60 minutes at 4 °C. Insoluble proteins and lipids were pelleted by centrifugation at 4 °C for 10 minutes at 10,000g and supernatants were collected and stored at –80 °C until subsequent analysis. Each extraction experiment was performed in technical triplicate.

To characterize the effect of vortexing time on extraction efficiency and reproducibility of quantitation, coefficients of variation (CVs: standard deviations divided by the mean of all measurements at any given time point) were determined for the relative and absolute quantitation against heavy labeled internal standards added to the matrix of interest prior to extraction. These standards included 15 uniformly ¹³C¹⁵N-amino acids at a final concentration of 2.5 μM, ¹³C₁-lactate (40 μM), 1,1,2,2-D-choline and 9-D-trimethylamine N-oxide (1 μM), and 2,2,4,4-D-citrate, ¹³C₅-2-oxoglutarate, ¹³C₄-succinate, ¹³C_{1,4}-fumarate, ¹³C₆-glucose, and ¹³C₂¹⁵N₁-Glutathione (heavy glycine-GSH) at a final concentration of 5 μM (Cambridge Isotopes Laboratories, Inc., Tewksbury, MA).

Experiment 2 – Metabolite yields and recovery rate after 60 min vortexing time

Samples were extracted as described above. After vortexing for 60 min, supernatants were removed and replaced with an equal volume of fresh lysis buffer with heavy labeled internal standards (as specified above), prior to additional vortexing (30 min) and centrifugation at 10,000g for 10 min at 4 °C. Supernatants of both the first and second round of extraction were collected to determine the residual concentration of extracted metabolites at the second 30 min extraction relative to the first 60 min extraction.

UHPLC-MS metabolomics analysis

Sample extracts were processed through UHPLC-MS, as previously reported^[12]. Briefly, analyses were performed on a Vanquish UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), as previously reported. Samples were resolved over a Kinetex C18 column, 2.1×150 mm, 1.7 μm particle size (Phenomenex, Torrance, CA, USA) at 25 °C

using an isocratic condition of 5% acetonitrile, 95% water, flowed at 250 μ l/min. Solvents for positive mode runs contained 0.1% (v/v) formic acid; solvents for negative mode runs contained NH_4OAc (5 mM). The mass spectrometer was operated independently in positive or negative ion mode scanning in Full MS mode (2 μ scans) at 60,000 resolution from 60 to 900 m/z, with electrospray ionization operating at 4 kV spray voltage, 15 sheath gas, 5 auxiliary gas. Calibration was performed prior to analysis using the PierceTM Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific). Acquired data was converted from .raw to .mzXML file format using Mass Matrix (Cleveland, OH, USA). Metabolite assignments, isotopologue distributions and correction for expected natural abundance of deuterium, ^{13}C and ^{15}N isotopes were performed using MAVEN (Princeton, NJ, USA) [32]. Extensive information on the compounds monitored in this study is provided in the Supplementary data files.

Statistical Analysis

Graphs, heat maps and statistical analyses (either T-Test or ANOVA) were performed with GraphPad Prism 5.0 (GraphPad Software, Inc, La Jolla, CA), Metaboanalyst 3.0^[33] and GENE-E (Broad Institute, MA).

Results

Routine extraction workflows (Figure 1.A) for hydrophilic (water-soluble) and mildly hydrophobic (methanol-soluble) compounds have been previously used by us and others [29–31]. This workflow involves resuspension of the biological matrix of interest in a -20°C cold extraction buffer containing methanol, acetonitrile and water at a 5:3:2 ratio v/v, prior to vortexing for 30 min at 4°C and centrifugation to remove pelleted proteins and insoluble, more hydrophobic compounds (e.g. diacylglycerols and triacylglycerols – Figure 1.A). Here we characterized whether extraction efficiencies would vary as a function of vortexing time (Figure 1.B). As a test matrix, we used whole blood, an easily accessible biological matrix of clinical relevance. Experiments were performed in technical triplicate (three independent extractions per time point) and results are extensively reported in Supplementary Table 1. Multivariate analysis, including hierarchical clustering analysis (Supplementary File 1) or Principal Component Analysis (PCA) of whole blood extracted for 4, 14, 26, 38, 49 or 60 min could not cluster the samples based on vortexing time ($n = 3$ per extraction time – Figure 1.C). Only a subset of sugar phosphates and basic compounds (polyamines spermidine and spermine) showed relative quantitative increase in CVs higher than 5% (black to red color scale) in comparison to normalized 4 min values as a function of vortexing time (Figure 1.D). Specifically, CVs (calculated as standard deviations divided by the mean of all measurements at any given time point) for whole blood metabolites were $<20\%$ for almost all tested hydrophilic metabolites independent from vortexing time, as shown in the box and whisker plots graphed in Figure 2.A. Only five metabolites showed significant vortexing-time dependency (*, repeated measures ANOVA, $p < 0.05$), though with minimal fold changes (<1.2 vs the median of 4 min values – Figure 2.B – top three panels). Only three compounds (Cys-Gly, dGMP and ADP) had CVs $> 20\%$ when merging all the triplicate data from any given vortexing time point (Figure 2.B – bottom three panels).

While whole blood is a readily available source for small molecule analysis of potential clinical relevance [34], most scientific research or clinical biochemistry literature focuses on the analysis of sorted cellular and biofluid fractions derived through gentle centrifugation of whole blood (Figure 3.A), such as plasma and red blood cells (RBCs). Metabolomics analyses of these matrices as a function of vortexing time revealed consistent time-dependent changes in the measured levels of cystine, a redox-sensitive metabolite deriving from cysteine oxidation (Figure 3.B and C, for RBCs and plasma, respectively). RBCs were also characterized by increased extraction of tryptophan as a function of vortexing time (CV > 20% - Figure 3.B). Absolute quantities of a subset of clinically relevant metabolites (listed with the relative results in Supplementary Table 1) were determined against heavy labeled internal standards spiked into the lysis buffer prior to the extraction of plasma, RBCs and whole blood. Absolute quantitation of these metabolites across whole blood, RBCs and plasma affords the direct comparability of these matrices. As a result, PLS-DA revealed comparability of the plasma and RBC metabolomes, at least with respect to the tested hydrophilic compounds (including clinically relevant glucose, lactate, glutathione and succinate – Figure 3.D). PLS-DA also showed that plasma and RBC extractions were in general more reproducible (higher degree of clustering) than whole blood (Figure 3.D).

We thus tested whether absolute quantitation of clinically-relevant compounds in plasma were affected by vortexing time. This information is relevant to determine more accurate CVs on extraction efficiency at different vortexing times. This analysis also provides information necessary to perform follow-up experiments on metabolite recovery efficiency (see below). Heavy labeled standards (Figure 4.A) were used as internal controls to test for recovery and stability of quantified compounds of interest over a vortexing time period from 4 min to 60 min. A representative spectrum is provided for the quantitation of endogenous plasma succinate vs the $^{13}\text{C}_4$ -succinate internal standard (Figure 4.B). The spectrum shows the monoisotopic peak of endogenous plasma succinate, the naturally-occurring M+1 (+1.0034, mass difference between ^{13}C and ^{12}C), representing ~4.4% of the monoisotopic peak for the 4 carbon atom succinate, and the M+4 (4x1.0034) peak of the exogenously introduced heavy-labeled internal standard. Quantification of endogenous metabolites was calculated as previously reported [12], according to the formula

$$[\text{Metabolite}] = (\text{Peak Area}_{\text{PARENT}} / \text{Peak Area}_{\text{HEAVY STD}}) \times [\text{Heavy STD}] \times \text{dilution factor}$$

Results for representative metabolites relevant to glycolysis, TCA cycle and redox homeostasis are graphed in Figure 4.C, showing plots of glucose, lactate, reduced glutathione (GSH) and succinate in plasma (n = 3) as a function of vortexing time during the extraction. In the case of lactate, where the internal heavy standard $^{13}\text{C}_1$ -lactate overlapped with the +1.0034 isotopologue of endogenous lactate, results were corrected for the natural abundance of lactate M+1 (3.3% of the monoisotopic peak of endogenous lactate). Results for a total of 24 metabolites quantified across all matrices (whole blood, plasma and RBCs) are reported in Supplementary Table 1, along with experimental CVs. Results confirm and expand on CVs determined through relative quantitation, with CVs << 25% for the tested metabolites, with the exception of fumarate in plasma (CV = 56%), with the highest absolute

concentrations determined in two out of three of the 4 min vortexing samples (Supplementary Table 1).

We thus moved on to determining metabolite recoveries at the end of the longest vortexing time cycle (60 min) in all three matrices. Briefly, after removal of supernatants at 60 min, pellets were incubated with an equal volume of fresh lysis buffer with heavy labeled internal standards, prior to a second cycle of extraction through 30 min vortexing and centrifugation (Figure 5.A). Recoveries were calculated by determining the residual concentrations of metabolites extracted after the second round of extraction in comparison to the absolute concentrations determined in the previous round. As a result, extraction recoveries >80% were confirmed for all the tested metabolites in whole blood, RBCs and plasma (Figure 5, Supplementary Table 1). Specifically, for all the tested metabolites, plasma extraction resulted in >90% recoveries (Figure 5.B). For RBCs and whole blood (Figure 5.C and D, respectively), basic amino acids arginine, lysine and histidine showed 50–75% recoveries upon 60 min vortexing.

Discussion

High-throughput metabolomics holds the potential to revolutionize the fields of clinical biochemistry and personalized medicine, paving the way for the emerging field of clinical metabolomics [35]. While advancements in analytical technologies now make it possible to analyze hundreds to thousands of samples per day, extraction procedures may represent a bottleneck in routine laboratory workflows described by us and others [29–31]. A critical step of this extraction protocol is represented by sample agitation in presence of ice cold lysis buffer at 4°C for 30 minutes. We hypothesized that shortening the duration of this step would not significantly affect extraction and recovery efficiencies at least for water-soluble hydrophilic metabolites of clinical relevance. Indeed, metabolites like glucose or lactate have been historically appreciated as clinical markers of diabetes or metabolic acidosis and mortality in the trauma population, respectively [23] [24]. Similarly, succinate has been recently established as a marker of mortality in the military [25] and civilian (*D'Alessandro et al. in press*) critically ill populations. Borrowing from the field of cancer metabolism and the concept of Warburg effect [28], recent advancements in the understanding of the metabolic reprogramming of immune cells [2,5,26], along with fibroblasts in pulmonary hypertension [4] have identified metabolites like lactate and succinate as key mechanistic regulators of proliferation, inflammation and responses to hypoxia. Metabolites like glutathione have long been appreciated for their centrality in redox homeostasis [36]. A rapid extraction workflow to make these compounds amenable to routine high-throughput analysis would be clearly valuable for clinicians and investigators alike. In this view, we here determined that reducing the vortexing time in our routine extraction workflow does not compromise extraction efficiency and metabolite recoveries for hydrophilic compounds. We tested this approach on a rather complex matrix (whole blood) and two sub-fractions of clinical relevance, plasma and RBCs. We did not observe any significant decline in metabolite recoveries even when the duration of sample vortexing during the extraction was as short as 4 min, compared to conventional (30 min) or longer extractions (up to 60 min). The use of internal standards afforded us to determine absolute concentrations of a total of 24 metabolites across plasma, RBCs and whole blood. This panel was used to inform PCA clustering of samples from

these matrices, a comparison that allowed us to suggest that plasma and RBC metabolomes are comparable with respect to the levels of the tested hydrophilic metabolites. Owing to the technical nature of this report, a limited number of biological replicates were here tested. Still, though further validation in larger cohorts will be necessary, results here suggest that whole blood is characterized by some peculiar metabolic features when compared to the other matrices, especially with respect to the levels of energy metabolism-related compounds, such as lactate, challenging the generally-accepted assumption that the whole blood metabolome can be assumed to be mostly influenced by RBC alone ^[34].

Recovery efficiency was determined by performing a second extraction on pellets from processed samples, revealing >90% recovery efficiency for all tested metabolites in plasma and generally >80% recoveries for RBCs and whole blood. However, 50–75% recoveries were observed for basic amino acids arginine, lysine and histidine in whole blood and RBCs. Since no significant differences were observed between 4 min and 60 min vortexing times for those basic compounds in whole blood and RBC extracts, buffer saturation rather than vortexing time are likely to account for the observed recovery issues. Adjusting the pH of the lysis buffer may help improve recoveries of these basic compounds, though likely at the expenses of more acidic ones ^[37].

The extraction method we described here is well suited for investigations aimed towards the hydrophilic water-soluble fraction of the metabolome as we did not evaluate performances for hydrophobic components. The protocol described here is not optimized for the extraction of hydrophobic compounds such as lipids, whose current standard extraction protocols requires stronger organic solvents (methanol, chloroform).^[18–22]

Conclusions

In the present study we investigated whether shorter protocols optimized for the extraction of hydrophilic compounds could be comparable to routine workflows previously described by us and others. We conclude that vortexing regimens as short as 4 min are sufficient to extract >80% hydrophilic metabolites with average CV << 20% (median 5%) from whole blood, plasma and RBCs. Quantitation of 24 metabolites of clinical and basic science relevance were quantified in all three matrices and used to conclude that RBCs and plasma hydrophilic metabolomes significantly overlap and compare to each other better than to whole blood. It will remain to be tested whether the current protocol can be further optimized to interface with robotized extraction procedures,^[38] a necessary step to improve throughputness of metabolomics analyses and their application to personalized medicine in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experiment 1 – design and results for whole blood

In **A**, an overview of the standard extraction protocol we previously described ^[12]. Here we characterized extraction efficiencies as a function of vortexing time (**B**). Principal component analysis (PCA) of whole blood extracted for 4, 14, 26, 38, 49 or 60 min (**C**) did not inform clustering of the samples on the basis of vortexing time ($n = 3$ per extraction time). Color code in PCA means: red: 4min; green: 14min; blue: 26 min; light blue: 38 min; purple: 49 min; yellow: 60 min. Only a subset of sugar phosphates and basic compounds (polyamines spermidine and spermine) showed relative quantitative increase in CVs higher than 5% (black to red color scale) in comparison to normalized 4 min values as a function of vortexing time (**D**).

Figure 2. Coefficients of variations for whole blood metabolites <20% for almost all tested hydrophilic metabolites independent from vortexing time

In A, box and whisker plots are shown for metabolites analyzed in this experiment (raw data and detailed reports are reported in the Supporting Table 1). Only five metabolites showed significant vortexing-time dependency (*, repeated measures ANOVA, $p < 0.05$), though with minimal fold changes (<1.2 vs the median of 4min values). Only three compounds (Cys-Gly, dGMP and ADP) had CVs > 20% when merging all the triplicate data from any given vortexing time point (B).

Figure 3. Vortexing time did not significantly affect extraction efficiency of plasma and red blood cells

Plasma and RBCs were separated from whole blood as summarized in A. Cystine, a redox sensitive metabolite derived from cysteine oxidation increased proportionally to vortexing time increases in both RBCs and plasma (B and C). RBCs were also characterized by increased extraction of tryptophan as a function of vortexing time (CV > 20%). Comparison of whole blood, plasma and RBCs on the basis of absolute quantitation of a subset of metabolites (Supplementary Table 1) against heavy labeled internal standards revealed the comparability of plasma and RBC metabolomes, at least with respect to the tested hydrophilic compounds (including clinically relevant glucose, lactate, glutathione and succinate).

Figure 4.

Stability of absolute quantitation of clinically relevant compounds independently from vortexing time of plasma, as determined against internal heavy labeled standards (**A**). A representative spectrum is provided for the quantitation of endogenous plasma succinate vs the $^{13}\text{C}_4$ -succinate internal standard (**B**). Graphs in **C** plot the quantitation of glucose, lactate, reduced glutathione (GSH) and succinate in plasma ($n = 3$) as a function of vortexing time during the extraction.

Figure 5.

Extraction recoveries >80% were confirmed for all the tested metabolites in whole blood, RBCs and plasma after a second 30 min extraction of residual pellets (A). For all the tested metabolites, plasma extraction resulted in >90% recoveries (B). For RBCs (C) and whole blood (D), a subset of basic amino acids were only partially extracted upon 60 min vortexing (recoveries from 50 to <80%).