# A Three-Minute Method for high-throughput quantitative metabolomics and quantitative tracing experiments of central carbon and nitrogen pathways

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# SUPPLEMENTARY MATERIAL

## TABLE OF CONTENTS

# SUPPLEMENTARY INFORMATION – MATERIALS and METHODS EXTENDED

## Experimental Methods

### UHPLC-MS Setup

The analytical setup on which these experiments were performed consists of a Vanquish UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA), scanning in Full MS mode (2 μscans) at 70,000 resolution from 60 to 900 m/z, with 4 kV spray voltage, 15 shealth gas, 5 auxiliary gas, and operated independently in positive or negative ion mode. Calibration was performed prior to analysis using the Pierce™ Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific). Samples were resolved over a Kinetex C18 column, 2.1 x 150 mm, 1.7 μm particle size (Phenomenex, Torrance, CA, USA) equipped with a guard column (SecurityGuard™ Ultracartridge – UHPLC C18 for 2.1 mm ID Columns – AJO-8782 – Phenomenex, Torrance, CA, USA) at 25ºC using an isocratic condition of 5% acetonitrile, 95% water, and 0.1% formic acid flowed at 250 μl/min. MS data acquired by the Q Exactive was converted from .raw to .mzXML file format using Mass Matrix (Cleveland, OH, USA). Every ~5000 runs guard column cartridges were replaced with either new ones or regenerated through 1h sonication in 50% methanol. Metabolite assignments were performed using MAVEN (Princeton, NJ, USA).[1] The MAVEN software platform provides tools for peak picking, feature detection, metabolite assignment using the KEGG pathway database, as well as isotopologue distributions and correction for expected natural abundance of deuterium, $^{13}C$ and $^{15}N$ isotopes. Metabolite assignments were further confirmed by a process of chemical formula determination using isotopic patterns and accurate intact mass.[1] Analyte retention times were confirmed by comparison with external standard retention times, as described below.

Relative and absolute quantitation was performed by exporting integrated light and heavy isotopologue peak areas values into Excel (Microsoft, Redmond, CA, USA) and

comparing the data to external calibration curves and internal heavy labeled standards (Cambridge Isotopes Laboratories, Inc., Tewksbury, MA) as detailed below. Graphs, heat maps and statistical analyses (including T-Test and ANOVA with significance thresholds for p-values < 0.05) were performed with GraphPad Prism 5.0 (GraphPad Software, Inc, La Jolla, CA) and GENE-E (Broad Institute, MA).

## Metabolite standard characterization

### *Coverage and selectivity*

To optimize isocratic run duration and to strengthen metabolite identification assignments, metabolomics coverage and selectivity, experimental retention times were determined over a four and three minute isocratic method (Kinetex C18 column - 5% acetonitrile in water + 0.1% formic acid, 250 µl/min – see above for further details) using a ~400 standard compound library (MLSMS, IROATech, Bolton, MA, USA – **Supplementary Table 1**). Compounds were resuspended to 20.8 µg/ml in 5% methanol (v/v), according to the manufacturer's instructions (**Supplementary Table 1**). As the library is packaged in 96-well plates, dissolved compounds were then mixed across rows to make 54 pools. These pools were diluted 1:9 (v/v) into either 0.1% formic acid or a metabolite extraction solution consisting of methanol, acetonitrile, water (5:3:2, v/v/v). Injection volumes for each pool dilution of 10 µl in both positive and negative ion mode were used to determine compound retention times and peak areas with MAVEN.

### *Robustness of chromatography and quantitation*

To assess robustness of chromatographic pressure and retention times, pressure was monitored over 1500 non-consecutive runs spanning across six days of analysis of packed red blood cells and supernatants (see below for details about the samples). Technical mixes were prepared by combining 4 individual red blood cell extracts from storage day 2 (the earliest time point in our sample set). Technical mixes were injected every 15 runs, and 10 non-consecutive technical mixes from day 1 to day 2 or from day 4 to day 5 were analyzed to assess technical variability, expressed as coefficients of variation (CV = mean/standard deviation). These data were used to confirm (i) inter-day reproducibility of peak shape, intensity, and retention time of 20 non-consecutive runs over 6 days of analysis; and (ii) reproducible deviation from expected $^{13}C/^{12}C$ monoisotopic peak ratios for a representative

metabolite (e.g. reduced glutathione, 10 carbon atoms: expected ratio 11.1%). Blank injections of extraction solution were assessed at the beginning and the end of the sample set and every 100 runs to ensure the absence of any sample carry-over. Mass accuracy after 1500 runs was tested to ensure mass a ccuracy of less than 5 ppm error.

***Linearity of quantitation of light and heavy standards over 4 orders of magnitude in presence and absence of matrix***

To determine linearity and sensitivity of this method, we weighed and progressively diluted a subset of standards to concentrations ranging over 4 orders of magnitude, from 0.5 to 5000 pmol per injection. This standard subset (Sigma Aldrich, St. Louis, MO, USA) included compounds in glycolysis (D-glucose, glucose 6-phosphate, fructose 1,6-bisphosphate, lactate), TCA cycle (citrate, α-ketoglutarate, fumarate, malate) and glutaminolysis/redox homeostasis (L-glutamate, glycine, reduced and oxidized glutathione). Inter-day reproducibility, linearity of absolute quantitation, and minimization of ion suppression effects were determined with the use of uniformly labeled $^{13}C_6$-glucose that was spiked into extraction solution at four different concentrations ranging over four orders of magnitude (0.1, 1, 10, 100, 1000 µM) prior to plasma extraction (described below in Applications). To ensure accuracy of quantitation in the presence of a complex matrix, in addition to assessing the extent of matrix ion suppression, extracted plasma samples harvested from 3 control rats (see below) were diluted 1:1 (v/v) with extraction solution, and 10 µl diluted and undiluted samples were analyzed with the three-minute method.

***Reproducibility of extraction efficiency, chromatographic and MS technical stability***

To further characterize stability and reproducibility, CVs were determined for the quantitation of heavy labeled and xenometabolite standards added to plasma prior to extraction. These standards included 18 $^{13}C^{15}N$-amino acids at a final concentration of 0.25 µM (Cambridge Isotopes Laboratories, Inc., Tewksbury, MA) and 5-fluorouracil at a final concentration of 2.5 µM (Sigma Aldrich, St. Louis, MO, USA).

**Applications**

***Rat model of trauma/hemorrhagic shock***

Animal experiments were performed under protocol approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver. All rats were maintained in the accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. Rats were housed under barrier-sustained conditions with 12 hour light-dark cycles and allowed free access to food and water before use.

***Biofluids***

Rat biofluid samples were kindly provided by Dr. Anthony Bacon (University of Colorado Denver). Briefly, nonlethal trauma/hemorrhagic shock was induced in Sprague Dawley rats (n = 4) by laparotomy and controlled hemorrhage from the femoral artery until the rats achieved mean arterial pressure (MAP) equal to 30 mm Hg (MAP 30), as previously described.[2] Plasma, mesenteric lymph and bronchoalveolar lavage fluid (BALF) samples (20 µl) were collected before hemorrhagic shock (Shock) and immediately after (30 minutes of shock at MAP 30), either in presnce or absence of mesenteric lymph diversion (MLD) through cannulation of the mesenteric artery – a surgical procedure that mitigates the insurgence of adverse sequelae to trauma/hemorrhagic shock, such as multiple organ failure or acute lung injury. Sham control animals (Sham, n = 4) remained normotensive throughout the duration of the experiment. Shams were anesthetized and underwent tracheostomy, blood vessel cannulation, and blood sampling at identical time points to those of shocked animals.

For metabolite extraction, 20 µl of biofluids (plasma, mesenteric lymph, or BALF) were extracted in 480 µl of ice cold extraction solution, and vortexed for 30 minutes at 4 ºC, as reported.[1] Insoluble proteins and lipids were pelleted by centrifugation at 4ºC for 10 minutes at 10,000g and supernatants were collected and stored at -80°C until subsequent analysis.

***$^{13}C^{15}N$-glutamine in vivo labeling experiment and lung biopsies***

Heavy labeled lung biopsies from Shock/Sham (n = 8 per group) rats were kindly provided by Dr. Annie Slaughter (University of Colorado Denver). Sham and Shock rats were generated as described above. At 15 min into shock, Shock and Sham rats received a 1 mL/kg intravenous infusion of $^{13}C_5{}^{15}N_2$-glutamine (Product no. CNLM-1275-H-0.1, Cambridge Isotope Laboratories, Inc., Tewksbury, MA) in physiological solution (35 mM) at a rate of 0.5 mL/min. This dose was determined to have minimal effect on steady-state

metabolism while still ensuring detection of trace isotopologue signal. No additional blood was drawn from injection of labeled glutamine until the 30 min time point to allow for drug circulation (duration of 15 min). Animals remained in shock for a total of 45 min then sacrificed before harvesting of 10 mg of lung biopsies after sacrifice.

Rat lungs samples were milled with a mortar and pestle in the presence of liquid nitrogen, then weighed to the nearest 0.1 mg and extracted in ice-cold lysis/extraction buffer at a concentration of 10 mg/mL. Samples were agitated vigorously at 4°C for 30 min and centrifuged at 10,000g for 15 min at 4°C. Protein and lipid pellets were discarded, while supernatants were stored at -80°C prior to batch metabolomics flux analyses.

***Packed red blood cell collection and storage***

Packed red blood cell (RBC) units, kindly provided by Dr. Tatsuro Yoshida (New Health Sciences, Inc., Cambridge, MA) were collected and stored as previously reported. One unit of whole blood (500 ± 50 ml) was collected from four healthy donors per AABB/FDA guidelines and in compliance with the Declaration of Helsinki, using CP2D in the Nutricel Pall Medical collection bag system (Braintree, MA, USA). Plasma was separated from RBCs by centrifugation followed by manual expression, and AS-3 (Nutricel) was added to a final hematocrit of approximately 60%. The estimated amount of residual plasma was 5–10 ml/unit. RBC units were pre-storage leukoreduced via filtration using the in-line Haemonetics RC2D leukoreduction filter and stored at 1–6°C. Samples (0.5 ml) were obtained through sterile couplers at day 2, 7, 14, 21, 28, 35 and 42 (the last day a unit can be transfused). The supernatant was separated from RBCs via centrifugation (1,500g for 10 min at 4°C) followed by a second spin at 12,500g for 6 min to sediment residual cellular material and contaminating platelets. RBCs and supernatants were immediately extracted in ice-cold metabolite extraction solution at 1:9 and 1:24 (v/v) dilutions, as previously described.[4] Samples were then agitated at 4°C for 30 min and then centrifuged at 10,000g for 10 min at 4°C. Protein and lipid pellets were discarded, while supernatants were stored at -80°C prior to metabolomics analyses.

***Method Comparison to gradient-based C18 and HILIC methods***

To compare the three minute method to a conventional chromatographic separation used for metabolomics analyses, 10 µl of RBC and 20 µl of AS-3 supernatant extracts were analyzed with the three minute method and two gradient-based methods using a C18 or

HILIC.[19,20] The two alternative chromatographic methods consisted of (i) a 9 minute method on a Kinetex C18 column, 2.1 x 150 mm, 1.7 µm particle size (Phenomenex, Torrance, CA, USA) run at 400 µl/min and 30ºC with the following gradient: 0-2 min 5% B hold; 2-5 min 5-95% B; 5-6 min 95-5% B; 6-9 min 5% B hold (mobile phases – A: H2O, 0.1% formic acid; B: acetonitrile, 0.1% formic acid); or (ii) a 15 min method on an Acquity UPLC BEH Amide Column, 2.1 x 100 mm, 1.8 µm particle size (Waters, Milford, MA, USA) run at 350 µl/min and 35ºC with the following gradient: 0-1.5 min 100% B hold; 1.5-10 min 100-40% B; 10-12.3min 40% B hold; 12.3-13 min 40%-100% B; 13-15 min 100% B hold (mobile phases – A: 5% acetonitrile, 10 mM ammonium acetate, pH 10.0; B: 95% acetonitrile, 10 mM ammonium acetate, pH 10.0). The Q Exactive parameters were identical to the previously described settings for the three-minute method, and all samples were analyzed independently in positive and negative ion mode.

***Ex vivo labeling experiments***

Packed RBCs (n =4) were collected, processed and stored in CP2D-AS-3, as described above. Before processing, AS-3 (containing 55 mM dextrose in 100 ml of solution) was supplemented with 11 mM $^{13}C_{1,2,3}$-glucose (Product no. CLM-4673-PK - Cambridge Isotope Laboratories, Inc., Tewksbury, MA). Samples were collected on a weekly basis at storage day 2, 7, 14, 21, 28, 35 and 42, and cells and supernatants were isolated and extracted as detailed above.

***Quantitative flux analysis***

Packed RBCs incubated with $^{13}C_{1,2,3}$-glucose (see previous paragraph) were extracted with the addition of with a standard mixture containing eighteen $^{13}C$,$^{15}N$-labeled amino acids to a final concentration of 0.25 µM (Product no. MSK-A2-1.2 - Cambridge Isotope Laboratories, Inc., Tewksbury, MA). As a proof of principle for the feasibility of quantitative flux analysis, we utilized the peak for $^{13}C_3$,$^{15}N_1$-alanine, which is not obtainable through the metabolism of $^{13}C_{1,2,3}$-glucose, to absolutely quantify endogenous $^{12}C_3$,$^{14}N_1$-alanine and *de novo* synthesized $^{13}C_3$-alanine that is derived from the transamination of $^{13}C_3$-pyruvate in RBCs and AS-3 supernatants. The formula used to perform this qauntitation is described in the results section.

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