

# Advantages of Tandem LC–MS for the Rapid Assessment of Tissue-Specific Metabolic Complexity Using a Pentafluorophenylpropyl Stationary Phase

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 Supporting Information

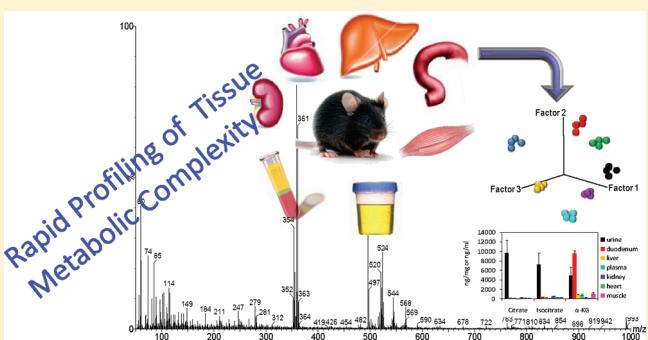
**ABSTRACT:** In this study, a tandem LC–MS (Waters Xevo TQ) MRM-based MS method was developed for rapid, broad profiling of hydrophilic metabolites from biological samples, in either positive or negative ion modes without the need for an ion pairing reagent, using a reversed-phase pentafluorophenylpropyl (PFPP) column. The developed method was successfully applied to analyze various biological samples from C57BL/6 mice, including urine, duodenum, liver, plasma, kidney, heart, and skeletal muscle. As result, a total 112 of hydrophilic metabolites were detected within 8 min of running time to obtain a metabolite profile of the biological samples. The analysis of this number of hydrophilic metabolites is significantly faster than previous studies. Classification separation for metabolites from different tissues was globally analyzed by PCA, PLS-DA and HCA biostatistical methods. Overall, most of the hydrophilic metabolites were found to have a “fingerprint” characteristic of tissue dependency. In general, a higher level of most metabolites was found in urine, duodenum, and kidney. Altogether, these results suggest that this method has potential application for targeted metabolomic analyzes of hydrophilic metabolites in a wide ranges of biological samples.

**KEYWORDS:** metabolomics, metabolic networks, metabolic complexity, hydrophilic metabolites, LC–MS, MRM, reversed-phase HPLC, C57BL/6 mice

## ■ INTRODUCTION

Metabolomics is the “systematic study of the unique chemical fingerprints that specific cellular processes leave behind”—specifically, the study of their small-molecule metabolite profiles.<sup>1</sup>

The metabolome represents an instantaneous snapshot of the physiology and pathology of a biological sample, as it is the collection of all metabolites in a biological cell, tissue, organ, organism, or biofluids.<sup>2</sup> There is still a need have a rapid broad based metabolomic profiling platform that accurately reflects the metabolic complexity seen in the tissues primarily used for metabolomic analyses. Comparative analyses of metabolic profiles are extremely useful to phenotype the physiological and pathological conditions induced by diseases, drug, food, environments, aging, strains, and genotypes.<sup>3–6</sup> Currently, two strategies in metabolomics/biomarker studies (targeted and nontargeted metabolite profiling) are widely used for analyzing metabolites.<sup>7–9</sup> In general, when using LC methods for targeting a more limited number of metabolite biomarkers, triple quadrupole (TQ) tandem mass spectrometry (MS) operating in multiple reaction monitoring (MRM) mode may be a preferred solution, with high mass-resolution full scan mass spectrometry (FT-ICR, Orbitrap and hybrid quadrupole time-of-flight (qTOF)) preferred for its potential ability for untargeted metabolite biomarker profiling.<sup>8,9</sup> TQ-MS



theoretically, has the highest sensitivity and linearity of LC–MS systems, but has the drawbacks of being able to detect a relatively limited number of known metabolites for which preoptimization must first be done to find the best MRM signature and collision energies. With the availability of UPLC and fast chromatographic runs, the newest tandem MS systems, such as the Waters Xevo used here, have overcome the need for relatively slow compound elution formerly needed to accommodate a large number of selected reaction monitoring scan events.

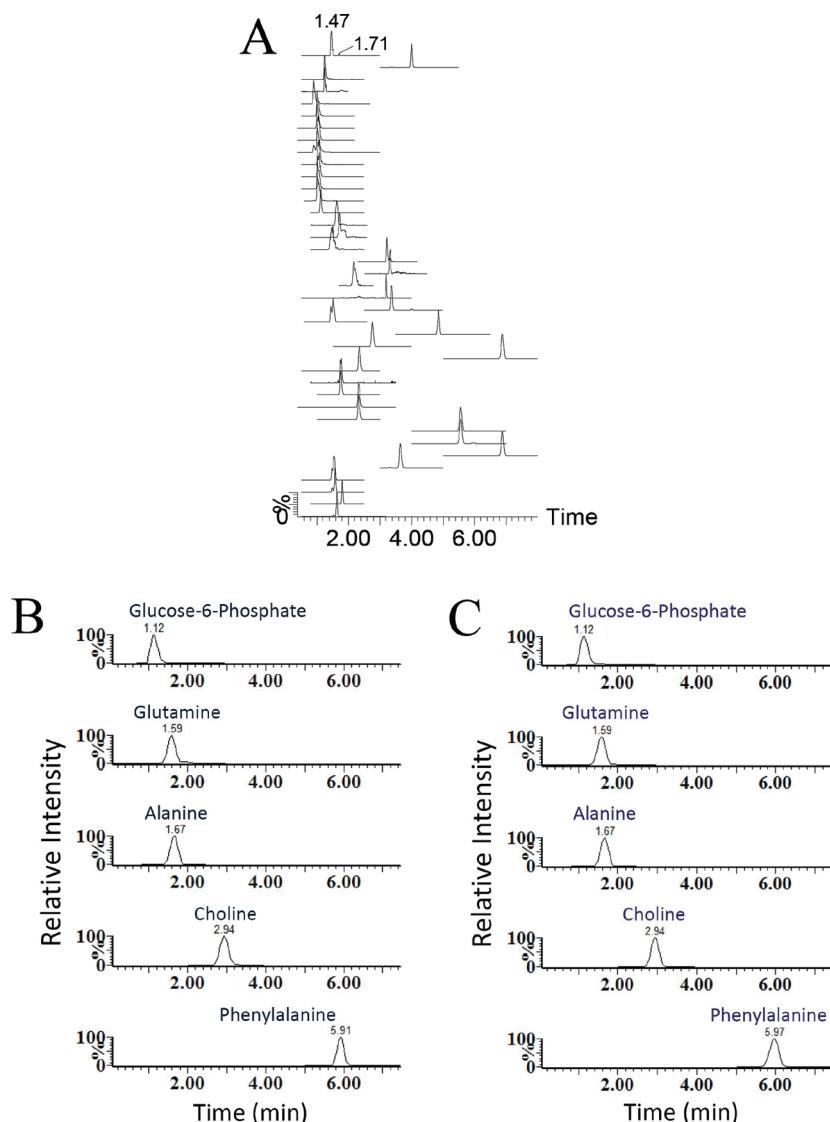
In vivo, the hydrophilic metabolites are involved in multiple metabolic pathways, as glycolysis, TCA cycle, pentose phosphate pathway, and amino acids.<sup>15–17</sup> Liquid chromatography is well suited to analyze polar, hydrophilic molecules typically encountered in biological samples without the need for prior derivatization.<sup>18</sup> The issue becomes which chromatographic method best allows a broad range of polar metabolites to be quantitatively assessed. Many have found hydrophilic interaction chromatography (HILIC) based methods to have great utility for targeted and untargeted metabolite biomarker profiling.<sup>10–12</sup> For example, Bajad et al.<sup>10</sup> compared the performance of nine different chromatography

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**Figure 1.** Heat map illustrating levels of 112 metabolites found in heart, liver, kidney, duodenum, skeletal muscle tissues, as well as plasma and urine from C57BL/6 mice (see Methods).



**Figure 2.** TIC obtained from the MRM screening mode of hydrophilic metabolites of the standards versus biological samples taken from C57BL/6 mice by HPLC-TQ MS. (A) Overview of MRM chromatograms of the standards used. (B) Representative MRM spectra of metabolite standards glucose 6-phosphate, glutamine, alanine, choline, and phenylalanine assessed. (C) Corresponding MRM spectra of glucose 6-phosphate, glutamine, alanine, choline, and phenylalanine from biological samples.

approaches involving seven different column chemistries and identified HILIC on an aminopropyl column as an effective method to separate a broad range of cellular metabolites including amino acids, nucleosides, nucleotides, coenzyme A derivatives, carboxylic acids and sugar-phosphates. Others have also found hydrophilic compounds are well suited for hydrophilic (HILIC) separation.<sup>13,14,27</sup> The use of HILIC overcame difficulties found with early versions of classic reversed-phase column methods, which can have poor performance with polar metabolites, as many polar compounds have poor retention, eluting near the void volume, and highly polar nucleotide triphosphate compounds like ATP do not elute as well-defined peaks.<sup>10</sup> However, great improvement for detection of hydrophilic compounds using reversed-phase chromatography has been seen when an amine ion pairing agent is used for separation of a broad range of negatively charged metabolites, including nucleotides, sugar phosphates, and carboxylic acids.<sup>7,10,19–21</sup> However, amine ion pairing reagents cause ion suppression in positive mode, leading to cumbersome metabolome coverage methods where a reversed-phase ion pairing method is used

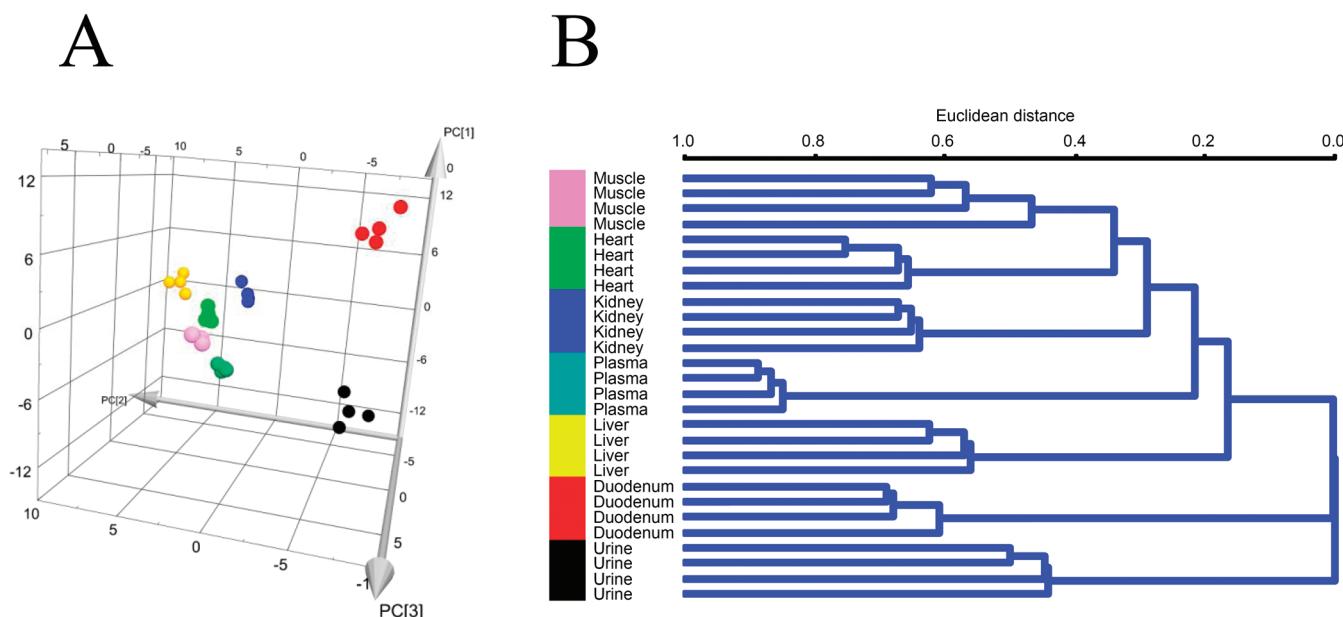
for the negative ion mode, and a HILIC column is used for the positive ion mode.<sup>7,22,23</sup>

In this study, we developed a rapid and simple LC–MS-based method on a reversed-phase pentafluorophenylpropyl (PFPP) column, which has been shown better separation than reversed-phase C<sub>18</sub> methods, without the need for an ion pairing reagent but with a comparatively slow elution time.<sup>28</sup> This PFPP method was optimized to work in positive and negative ion MRM modes and allows a rapid profiling of hydrophilic metabolites with high sensitivity and reliable reproducibility for assessment of metabolic complexity in a wide range of biological samples.

## MATERIALS AND METHODS

### Reagents

Acetonitrile (HPLC grade), formic acid (HPLC grade) and water (LC–MS grade) were purchased from Fisher Scientific (Fisher Scientific, Pittsburgh, PA); the standard compounds of amino acids lysine, valine, proline, alanine, tyrosine, histidine,



**Figure 3.** Multivariate principal component and hierarchical analyses using the normalized peaks areas of 112 hydrophilic metabolites detected in plasma, urine, duodenum, liver, kidney, heart, and skeletal muscle samples from C57BL/6 mice. (A) Three-dimensional PCA score plot of the different types of samples. (B) Hierarchical clustering plot of the different types of samples.

choline, serine, phenylalanine was purchased from Fisher (Fisher Scientific, Pittsburgh, PA); the standard compounds of lactate, glutamine, glutamate, pyruvate, fumarate,  $\alpha$ -ketoglutarate ( $\alpha$ -KG), succinate, malate, phosphoenolpyruvate (PEP), glyceraldehyde 3-phosphate (G-3-P), alpha-glycerol phosphate ( $\alpha$ -GP), isocitrate, citrate, erythrose 4-phosphate (E-4-P), ribulose 5-phosphate (R-5-P), glucose 6-phosphate (G-6-P), adenosine monophosphate (AMP), adenosine diphosphate (ADP), fructose 1,6-biphosphate (F-1,6-P), Fructose 6-phosphate (F-6-P), reduced glutathione (Gluta-red), oxidized glutathione (Gluta-Oxi) were purchased from Sigma (Sigma-Aldrich Corp., Saint Louis, MO). All other used reagents, such as pH adjusting solution, were all ACS grade reagents.

#### Animal Samples

Animal used in this study were kept in concordance with the USA National Research Council Guidelines and approved by the Subcommittee on Research Animal Care and Laboratory for Animal Resources of the Albert Einstein College of Medicine. Four *Mus musculus* C57BL/6 male mice (age: 5 weeks, body weight:  $20 \pm 2$  g) were purchased from the Jackson (The Jackson Laboratory, Bar Harbor, ME). The animals were housed individually in cages in a well-ventilated room with temperature:  $25 \pm 2$  °C, humidity:  $60 \pm 5\%$  and a 12 h dark to light cycle. Standard chow diet and water were provided *ad libitum*. The mice were sacrificed by exsanguination under isoflurane anesthesia. Liver, duodenum, kidney, heart and quadriceps muscles samples were collected and rapidly freeze-clamped, and then kept in liquid nitrogen until extraction. The bladder containing urine and blood samples were collected and immediately spun down to  $6500 \times g$  at 4 °C, then urine and plasma were transferred to new tubes and kept frozen at  $-80$  °C until processing.

#### Sample Processing

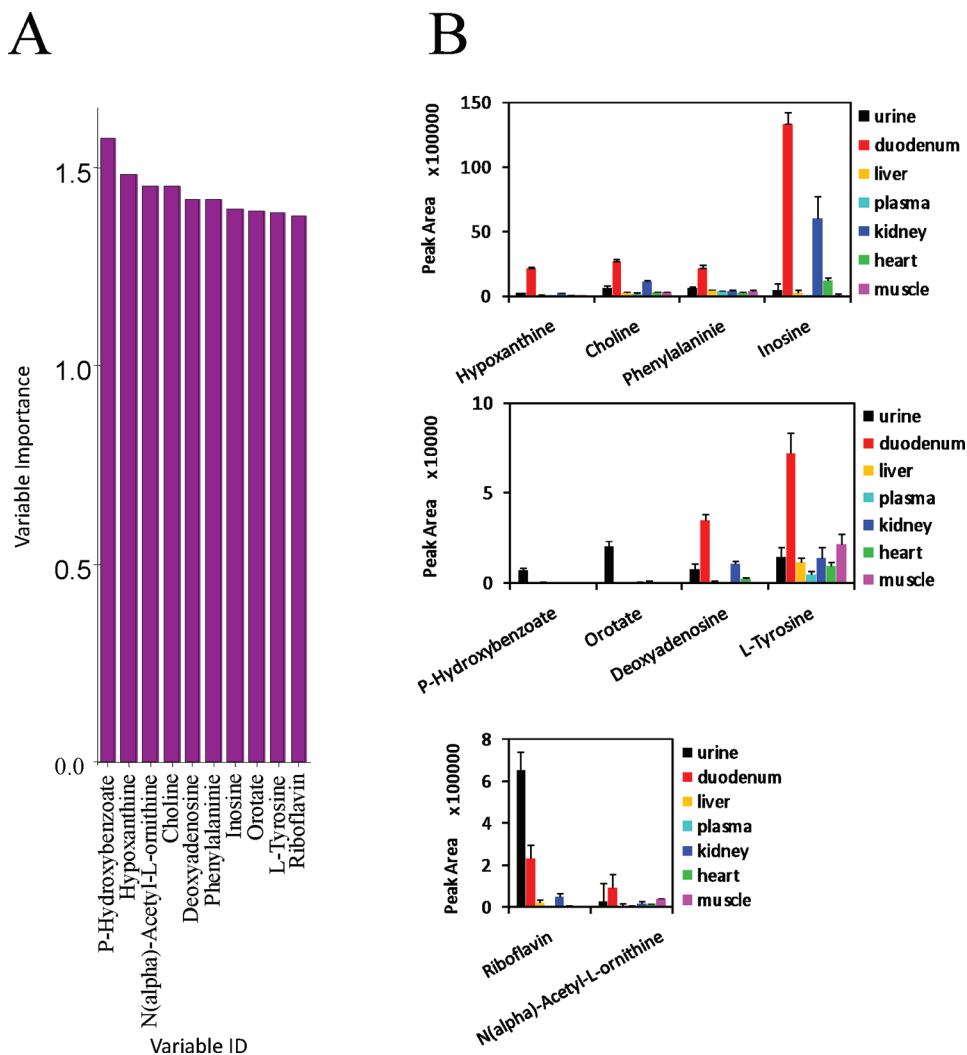
Urine samples were centrifuged to  $20\,000 \times g$  at 4 °C for 5 min and the supernatant diluted with 1 vol of water for LC–MS analysis. Plasma samples were prepared by mixing it with 2 vol of acetonitrile, then centrifuged to  $20\,000 \times g$  at 4 °C for 5 min and

the supernatant was separated for LC–MS analysis. Approximately 100 mg of tissue samples from heart, liver, skeletal muscle, kidney, and duodenum were weighed and placed into a 5 mL of glass tube, then the tissue was homogenized using 1 mL of 50% ice-cold methanol, after that and working in a fume hood 1 mL of chloroform was added and the mix was vortexed by 30 s and spun down to  $6500 \times g$  at 4 °C. Following, the supernatant was transferred to a new tube, mixed with 2 vol of acetonitrile, centrifuged to  $20\,000 \times g$  at 4 °C for 5 min, and then the supernatant was analyzed by LC–MS.

#### HPLC–MS

Chromatographic analysis was performed in a Waters Acuity UPLC system (Waters Corp., Milford, MA) using the below indicated columns. A flow rate of 0.3 mL/min was used for the pentafluorophenyl columns, 0.2 mL/min for the BEH and amino columns and 10  $\mu$ L injection volume were used for all cases. The column eluent was directed into the mass spectrometer without split. The columns used in this work were: Discovery HS F5 PFPP (150 mm × 2.1 mm, 3  $\mu$ m particle size) (Sigma-Aldrich Corp., Saint Louis, MI), PFP (150 mm × 2.1 mm, 2.6  $\mu$ m particle size) (Phenomenex, Torrance, CA), Luna NH<sub>2</sub> column (150 mm × 1 mm, 3  $\mu$ m particle size) (Phenomenex, Torrance, CA) and BEH C<sub>18</sub> (50 mm × 2.1 mm, 1.7  $\mu$ m particle size) (Waters Corp., Milford, MA). The flow rates and HPLC gradients for distinct columns were accordingly adjusted using the software, Waters UPLC Columns Calculator v1.1.1 (Waters Corp., Milford, MA).

Targeted analysis of metabolites from biological samples was performed using a linear gradient: 0–27% B over 8.0 min (A: 0.1% formic acid in water, pH 4.5; B: 100% acetonitrile). Mass spectrometry detection was performed using a Xevo Triple Quadrupole MS (Waters Corp., Milford, MA) equipped with an electrospray ionization source (ESI) operating simultaneously in positive and negative ionization mode. The desolvation gas flow rate was set to 900 L/h at a temperature of 500 °C, the cone gas flow rate was set at 50 L/h and the source temperature at 150 °C.



**Figure 4.** Levels of the 10 more significant metabolites determined from the variable importance plot of PLS-DA analysis, reflecting the metabolic complexity differentiating different types of biological samples. (A) Variable importance plot showing the 10 more significant hydrophilic metabolites contributing to the samples differential clustering pattern. (B) Relative levels of the 10 more significant metabolites contributing to the samples distinctive metabolomics patterns.

The capillary voltage was set to 3000 V for positive ion mode; 2800 V for negative ion mode; the cone voltage was set depending upon each specific MRM for each metabolite. Data was collected in MRM mode by screening parent and daughter ions simultaneously.<sup>10,11</sup> The dwell time was automatically set by the MassLynx software. By default, a minimum of 12 point per peaks were set to be collected; dwell times were automatically adjusted by the software depending on how many metabolites are being determined at any given time.

#### Calibration Curves

Eight concentrations of mixed standards were prepared by diluting concentrated stock solutions down to 10000, 8000, 5000, 1000, 100, 10, 1, 0.01 ng/mL for glutamine, glutamate, pyruvate, fumarate, α-KG, succinate, malate, PEP, G-3-P, α-GP, isocitrate, citrate, E-4-P, R-5-P, G-6-P, F-1,6-P, F-6-P, Glutathione reduced (GSH) and Glutathione oxidized (GSSG). Dilutions of 500, 400, 250, 50, 5, 0.5, 0.05, and 0.0005 ng/mL were prepared for AMP; 20000, 16000, 10000, 2000, 200, 20, 2, 0.02 ng/mL for ADP; 353, 282.4, 176.5, 35.3, 3.53, 0.353, 0.0353, 0.000353 nmol/mL for lactate; 2500, 1500, 1000, 500, 100, 10, 1, 0.02 ng/mL for α-KG.

0.1 ng/mL for the mix of amino acids. The chromatographic profiles based on the MRM screening mode of each targeted compounds were achieved by injecting 10 μL of each most concentrated of analytes solutions individually. Subsequently, the calibration curve for each targeted compounds were calculated in the software TargetLynx (Waters Corp., Milford, MA) using the individual determinations of each targeted compounds standards. It was illustrated that the linearity of calibration curves is remarkably good for all the targeted compounds and they have ample linear range (Supplemental Table 3, Supporting Information).

#### Data Processing and Statistical Analysis

The MS raw data from the biological samples runs was processed by TargetLynx, which integrated the metabolite peaks and generated a peak area list of each metabolite from every sample. The collected data was normalized by the tissue weight or volume of each sample. Furthermore, the normalized data was analyzed by the Ezinfo subprogram from MarkerLynx XS v4.1 (Waters Corp., Milford, MA).

The entire normalized data from all samples was imported into Ezinfo (MarkerLynx) to perform principal components analysis

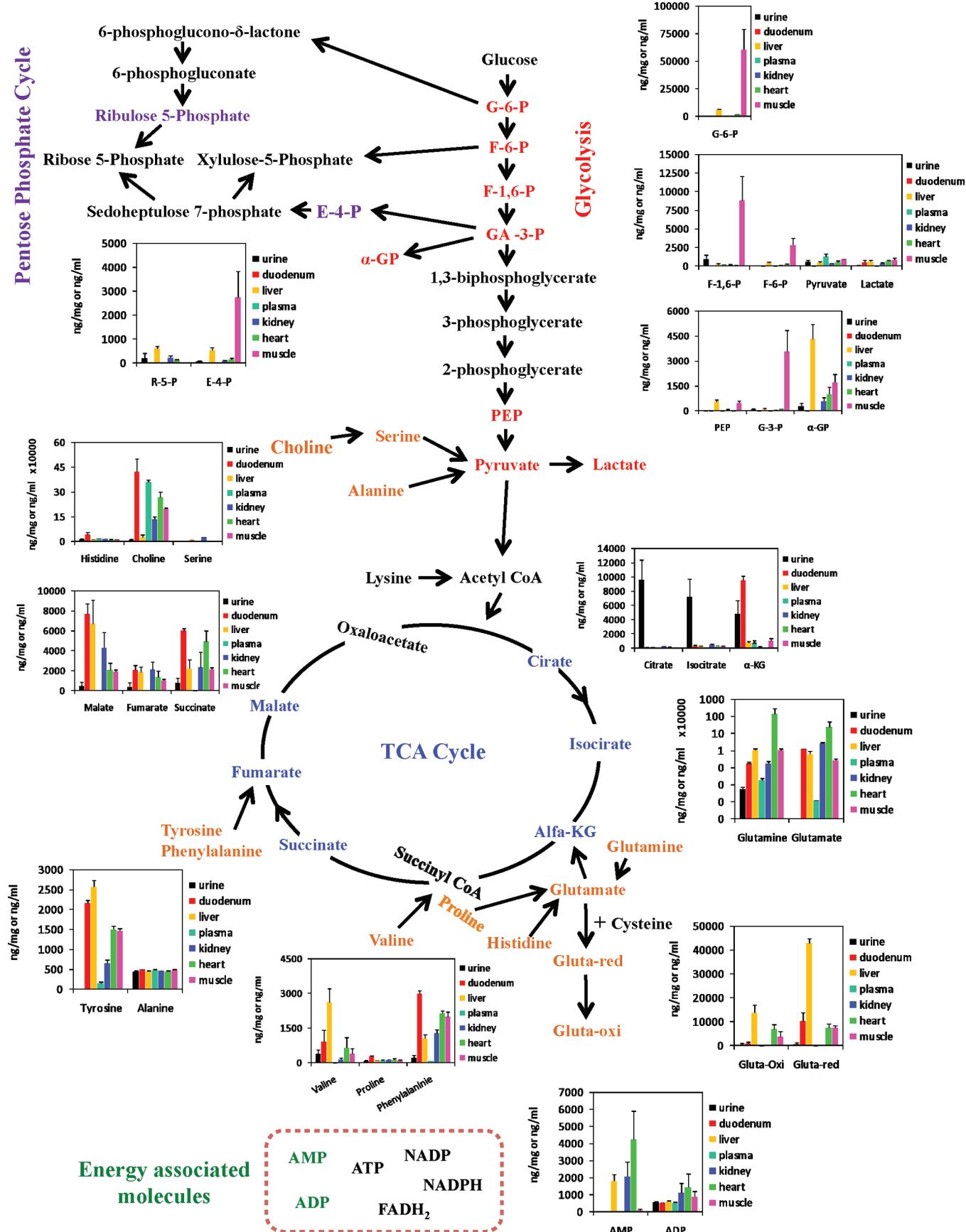


Figure 5. Metabolic pathway interdependence reflected by the quantitative levels of 30 hydrophilic metabolites including amino acids, the TCA cycle, the pentose phosphate cycle and glycolytic pathways. Colored words indicate that the metabolites were determined quantitatively; black words were metabolites analyzed in a relative manner.

(PCA) and partial least-squares discriminant analysis (PLS-DA).<sup>24</sup> Separately, hierarchical clustering analysis (HCA) was also performed by importing the normalized data into Pirouette software (Infometrix, Inc., Bothell, WA).<sup>25</sup> Using the complete metabolic profiles of the samples, significant differences in the targeted metabolites profiles was observed between the different types of samples. From PCA and PLS-DA analyses scores plots and variable importance plots were calculated. Scores plots showed clustering patterns of the samples based on their metabolite levels in qualitative and quantitative metabolomics data. The separation of clusters from different types of samples clearly reflects the differences in metabolite content from various metabolic pathways. The variable importance plot shows the contribution of each metabolite (biomarkers) in establishing the clustering patterns. Differences in the metabolites levels from different tissues had significant contributions to groups' classification in the scores plot. Furthermore, the levels of biomarkers is quantitatively characterized by this plotted methods (bar plot). HCA was another multivariate statistical analysis applied that also quantitatively reflected the samples clustering patterns similarly observed with PCA and PLS-DA analyses. The heatmap overview of the data was plotted by using excel function (2007v/QI Macros 2009, Microsoft Corporation, NY).

## RESULTS AND DISCUSSION

Profiling of hydrophilic metabolites is key to the characterization of metabolic complexity that phenotypically describes normal and disease states, or is seen in response to treatment.<sup>26</sup> Both positively and negatively charged hydrophilic metabolites are involved in multiple key metabolic pathways of biological organisms, such as the glycolytic, pentose and TCA pathways, as well as redox and energy charge. A robust profiling method that can assess both these positively and negatively charged hydrophilic metabolites in one rapid run is a primary challenge in analytical method development for metabolomics. A method for comprehensive metabolome coverage must be balanced versus run time and yet achieve robust quantitative performance. Optimization of HPLC column conditions is a vital step during development of LC–MS methodologies to optimally separate metabolites of interest.<sup>10,27,28</sup> In this work, various columns have been evaluated using the same type of biological samples. Below the different HPLC columns selected to analyze water-soluble metabolites are described.

- (1) Phenomenex PFP column (15 cm × 2.1 mm, 2.6  $\mu$ m particle size). The solvents composition (A, Water with 0.1% formic acid; B, acetonitrile) and chromatographic conditions were based specifications previously described.<sup>28</sup> The Supplemental Figure 1 (Supporting Information) shows the typical total ion chromatogram (TIC) of several mixed standards profiled by MRM screening mode of UPLC-TQ using this column. The figure illustrate that separation of targeted compounds was nonoptimal. Although some of acidic compounds were well profiled based on weak acidic eluent, most compounds could not been validated. Meanwhile, metabolites such as fumarate and pyruvate exhibited nonsymmetric peaks, likely attributed to the change pH that can not meet PKa adaption of most of the targeted analytes. When the elution conditions were modified (A, Water/Acetonitrile 95:5 with 20 mM ammonium acetate and 20 mM

ammonium hydroxide, pH 9.4–9.6; B, Acetonitrile),<sup>10</sup> some compounds peaks gained good shapes; however, the acidic compounds could not be profiled with a basic pH solvent system (Supplemental Figure 2, Supporting Information).

- (2) Waters BEH C<sub>18</sub> (5 cm × 2.1 mm, 1.7  $\mu$ m particle size). Solvents system (A, water with 0.1% formic acid; B, Acetonitrile) and separation conditions tested were the same described above.<sup>28</sup> Supplemental Figure 3 (Supporting Information) shows that  $\alpha$ -KG and many other metabolites could not be detected, and the retention times of most compounds revealed that the C<sub>18</sub> column has almost no retention capability for typical pentose, glycolytic and TCA metabolites. Therefore, this column can not be used for quantification of most of the targeted metabolites, due to its lack of separation resulting in significant peak interferences.
- (3) Phenomenex Luna NH<sub>2</sub> column (15 cm × 1 mm, 3  $\mu$ m particle size). Solvents system (A, Water/Acetonitrile 95:5 with 20 mM ammonium acetate and 20 mM ammonium hydroxide, pH 9.4–9.6; B, Acetonitrile) and separation condition used were previously described.<sup>10</sup> Supplemental Figure 4 (Supporting Information) illustrates that although some separation for TCA and pentose pathway compounds was achieved,  $\alpha$ -KG and other metabolites could not be analyzed at the solvents high pH.
- (4) Discovery HS F5 PFPP column (15 cm × 2.1 mm, 3  $\mu$ m particle size). The solvents and chromatographic conditions evaluated are described in.<sup>28</sup> The use of this column allows an adequate profiling of a total 112 hydrophilic metabolites from their specific MRM detection methods (Figure 1).<sup>10</sup> The Discovery HS F5 PFPP column was selected for qualification and quantification of the hydrophilic metabolites from the biological samples, due to its superior separation of metabolites. After testing various solvents systems, 0.1% formic acid versus 100% acetonitrile was found to be the most favorable solvent system. Using this combination of column and solvents, the TIC of most hydrophilic metabolites by MRM screenings typically showed high separation and excellent peak profiles in both standards (Figure 2A, B), and biological samples (Figure 2C). Furthermore, retention times and peak shapes proved to be very consistent, independent of whether analyzing standards or samples. Altogether, a LC–MS MRM screening method of 112 hydrophilic metabolites was developed and validated on the PFPP column (Figure 1 and Supplemental Table 1, Supporting Information). The urine, plasma, heart, liver, skeletal muscle, kidney, and duodenum samples from C57BL/6 mice were subjected to metabolomic profiling analyses using above-described LC–MS MRM method utilizing the PFPP column. Metabolite profiles containing 112 hydrophilic components from each of these seven types of biological samples were performed within 8 min, which is much faster than previous reports.<sup>7,10,11</sup> Targetlynx was used to integrate and normalize peaks areas of these 112 hydrophilic metabolites from all samples, and the method was characterized for the intersample

accuracy and precision (Supplemental Table 2, Supporting Information). Score plots from PCA analysis of the normalized samples shows that the different types of biological samples clearly cluster separately (Figure 3A). Additionally, the plasma, heart, liver, skeletal muscle and kidney clusters are relatively close together, and far away from the urine and duodenum groups. The cluster arrangement is reflects the higher normalized levels of metabolites in these last two types of biological samples. In addition, the clustering of these data by hierarchical analysis confirmed the principal component analysis results (Figure 3B). The variable importance plot obtained revealed that are many metabolites significantly contributing to the cluster separation, reflecting the metabolic complexity of these different sample types (Supplemental Figure 5, Supporting Information). The first 10 most important metabolites that contribute to the sample type distinction are shown in the Figure 4A, and the relative levels from the different types of samples is appreciated in Figure 4B. It appears that there is a common pattern for urine, kidney, duodenum samples, as these tissues contain higher levels of metabolites that contribute greatly to the distinct clustering of these samples. A total of 30 from the 112 targeted metabolites were completely quantified, based on the use of standard curves.

In general, metabolites detection was linear and sensitive for most molecules. The similarities of the results among different samples indicate that the interferences due to ion suppression were minimized with few exceptions, and even those were tissue dependent, by maintaining the samples at relatively low concentration, chromatographically separating the analytes as much as possible, and ensuring the optimal MS ionizing conditions for each molecule during their elution time. For example carnitine had a high variability in heart and skeletal muscle, but not in plasma, urine, liver, kidney and duodenum (see Supplemental Table 2, Supporting Information) Most of the quantified metabolites are intermediates of the glycolytic, TCA and pentose cycle pathways. The quantified metabolites were seen to have reproducible values, and a large dynamic range indicating high sensitivity for detection (Supplemental Table 3, Supporting Information).

These quantifiable hydrophilic components, in particular, are key to targeted metabolite biomarker studies for assessment of key interlocking metabolic pathways (Figure 5). Several studies indicate that amino acids and glycolytic, TCA cycle, and pentose phosphate pathway intermediates are involved in diverse mechanisms associated with the interpretation of various diseases.<sup>29–31</sup> This work suggests that the PFPP column method developed for analysis of hydrophilic metabolites using the Waters Xevo TQ tandem MS possesses a strong application potential in both diagnostics, and for mechanism based investigations of disease etiologies.

## CONCLUSION

The Tandem MS-pentafluorophenylpropyl (PFPP) stationary phase method described here offers the advantages of rapid, broad metabolomic profiling of positively and negatively charged hydrophilic metabolites in a single run, without the need for ion-pairing reagents. The suitability of this Tandem MS-PFPP stationary phase MRM method was demonstrated here by assessing the metabolic complexity seen in seven types of biological

samples (plasma, urine, heart, skeletal muscle, liver, kidney and intestine). A total of 112 hydrophilic metabolites were rapidly detected and profiled within 8 min of chromatographic separation. This rapid method can be used for quantification, as shown here, and multiparametric analyses (PCA, PLS-DA, and HCA), revealed reliable reproducibility. This optimized HPLC-PFPP-MS method offers selectivity, high sensitivity and reliable reproducibility for assessment of metabolic complexity in a wide range of biological samples, when combined with advanced Tandem MS (Waters XevoTQ).

## ASSOCIATED CONTENT

### Supporting Information

Supplementary figures and tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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