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Liquid-Liquid Extraction Combined with Differential Isotope Dimethylaminophenacyl Labeling for Improved Metabolomic Profiling of Organic Acids

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ABSTRACT

A large fraction of the known human metabolome belong to organic acids. However, comprehensive profiling of the organic acid sub-metabolome is a major analytical challenge. In this work, we report an improved method for detecting organic acid metabolites. This method is based on the use of liquid-liquid extraction (LLE) to selectively extract the organic acids, followed by using differential isotope *p*-dimethylaminophenacyl (DmPA) labeling of the acid metabolites. The ^{12}C-/^{13}C-labeled samples are analyzed by liquid chromatography Fourier-transform ion cyclotron resonance mass spectrometry (LC-FTICR-MS). It is shown that this LLE DmPA labeling method offers superior performance over the method of direct DmPA labeling of biofluids such as human urine. LLE of organic acids reduces the interference of amine-containing metabolites that may also react with DmPA. It can also remove water in a biofluid that can reduce the labeling efficiency. Using human urine as an example, it is demonstrated that about 2500 peak pairs or putative metabolites could be detected in a 30-min gradient LC-MS run, which is about 3 times more than that detected in a sample prepared using direct DmPA labeling. About 95% of the 1000 or so matched metabolites to the Human Metabolome Database are organic acids. It is further shown that this method can be used to handle as small as 10 μL of urine. We believe that this method opens the possibility of generating a very comprehensive profile of the organic acid sub-metabolome that will be useful for comparative metabolomics applications for biological studies and disease biomarker discovery.

Keywords: mass spectrometry; liquid chromatography; isotope labeling; p-dimethylaminophenacyl bromide; acid-containing metabolites; metabolomics.

1. Introduction

Comprehensive and quantitative profiling of the metabolome of a biofluid or a biological sample is an analytical challenge, due to great diversity of chemical and physical properties of metabolites and a large difference of metabolite concentrations in a complex mixture. One strategy to address this challenge is to divide the metabolome into sub-metabolomes according to the presence of a specific functional group in a metabolite and then develop optimal analytical tools tailored to the analysis of the sub-metabolomes. For example, we have recently reported the use of dansylation chemistry to label all the amine- and phenol-containing metabolites, followed by liquid chromatography mass spectrometry (LC-MS) analysis of the labeled metabolites [1]. With enhanced chromatographic separation and improved ionization efficiency, this method can be used to generate a much more comprehensive profile of the amine- and phenol-containing metabolites in various biological samples [1-4], compared to conventional LC-MS methods. Several other isotope reagents offering enhanced analytical performance for sub-metabolome profiling have also been reported [5-11].

The success of this divide-and-conquer metabolome profiling strategy is very much dependent on the availability of robust chemical labeling methods that target at different groups of metabolites. An ideal labeling method should provide high labeling efficiency and alter the metabolite properties to enable better chromatographic separation and enhance ionization efficiency. In addition to dansyl labeling, we have developed an isotope labeling reagent *p*-dimethylaminophenacyl bromide (DmPA) for profiling the organic acid sub-metabolome [10]. This labeling reagent can be used to detect a large number of organic acids with signal enhancement of 10 to 1000 folds over the unlabeled counterparts. However, there are two major drawbacks of the current DmPA labeling method. One drawback is that the presence of water in

a sample, such as a urine sample, could affect the labeling reaction efficiency of the acid metabolites. For example, a urine sample prepared in 80% acetonitrile (volume/volume) can maintain high solubility of metabolites present in urine. However, 20% water is still present in the urine sample. Water is nucleophilic under the alkaline condition used for labeling and, therefore, can react with DmPA to form the hydrolyzed DmPA compound. Water can compete with organic acid metabolites, particularly the low abundance metabolites, resulting in no or reduced labeling of some acid metabolites present in urine. Previous studies suggested that even 5% water in the sample could affect the phenacyl reaction kinetics and product yield for the tested compounds [12]. The other drawback of the existing labeling method is that the amine metabolites, specially the high abundance amine metabolites, can suppress the signals of the acid metabolites. Even though the reaction condition used for DmPA labeling was optimized for labeling the acid metabolites, some amines, particularly the high abundance ones, could still be labeled.

To circumvent the above drawbacks, we have developed an improved acid labeling method based on the use of liquid-liquid extraction (LLE) for enriching the acid metabolites prior to carrying out the DmPA labeling. LLE is simple and rapid. It has been widely used for gas chromatography (GC) MS analysis of organic acids in clinical laboratories [13, 14]. In GC-MS analysis of organic acids, it typically uses a starting material of 0.5 to 2 mL of urine [15]. A recent report indicated the possibility of analyzing organic acids by GC-MS using 200 µL of urine as the starting material [16]. However, for metabolomic profiling work, it is desirable to use a smaller volume of a biofluid due to a limited sample available for many metabolomics studies. For example, in profiling the mouse urine metabolome, only tens of microlitres of urine are available. Fortunately, the DmPA labeling reaction offers much improved detectability for

profiling the acid metabolome [10]. Thus, in this work, we report the novel combination of liquid-liquid extraction and DmPA labeling for handling small volumes of samples (tens of microlitres). Using human urine as an example, we demonstrate that this method offers much improved performance for profiling the sub-metabolome of organic acids.

2. Experimental

2.1. Chemicals and Reagents

All chemicals and reagents, except those specifically noted, were purchased from Sigma-Aldrich Canada (Markham, ON, Canada). LC-MS grade water and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Edmonton, AB, Canada). Synthesis of ^{12}C- and ^{13}C- *p* -dimethylaminophenacyl bromide was performed according to the published procedure [10].

2.2. Urine and standard solution preparation

Urine samples were collected from healthy individuals. An informed consent was obtained from individual volunteers and ethics approval was obtained from the University of Alberta in compliance with the University of Alberta Health Information policy. The collected urine samples were stored at -80°C until further use. Urine was thawed on ice and centrifuged for 10 min at 13000 rpm. A standard solution of organic acid was prepared by dissolving each metabolite standard in water to give a concentration of 2 mM.

2.3. DmPA labeling using the existing protocol

For DmPA labeling, an existing protocol reported earlier (see Figure 1A for the workflow) [10, 17, 18] was adapted for labeling the urine and metabolite standards for

comparison with the new protocol (see below). An aliquot of 50 µL of urine or a metabolite standard prepared in 80% acetonitrile solution (volume/volume) was added into a vial with a screw cap. Then 50 µL of triethylamine (TEA) dissolved in fresh acetonitrile (20 mg/mL) were added. The vial was vortexed and spun down. Then a solution of either ^{12}C-DmPA or ^{13}C-DmPA in acetonitrile (20 mg/mL) was added into the vial. The vial was again vortexed and spun down. The vial was placed in an oven at 85°C for 55 min. After combining the ^{12}C-labeled sample with the ^{13}C-labeled sample, 2 µL of the mixture were injected into LC-MS for analysis.

2.4. Liquid-liquid extraction combined with DmPA labeling

For the new method of combining liquid-liquid extraction with DmPA labeling, Figure 1B shows the workflow using the 90 µL of urine as the starting material. In this case, 90 µL of urine or standard solution was aliquoted into a centrifuging plastic vial. Ten µL of 6M HCl solution was added, followed by adding 10 µL of saturated NaCl solution, and the vial was vortexed and spun down. The sample was extracted using 300 µL of ethyl acetate. Each vial was vortexed for 30 s and was then centrifuged at 8000 rpm for 5 min. The organic phase was transferred into another plastic vial with a screw cap. The pH of the organic phase was adjusted to 8 by adding 20 µL of TEA solution (180 mg/mL in acetonitrile). The sample was dried down by using SpeedVac. Then 60 µL of TEA solution in acetonitrile (20 mg/mL) were added. The vial was vortexed and spun down. A solution of either ^{12}C-DmPA or ^{13}C-DmPA in acetonitrile (20 mg/mL) was added into the vial. The vial was again vortexed and spun down. The vial was placed in an oven at 85 °C for 55 min. After combining the ^{12}C-labeled sample with ^{13}C-labeled sample, 2 µL of the mixture were injected into LC-MS. Injection of this small volume of sample in pure acetonitrile did not affect the chromatographic peak shape, as the flow rate used was

quite high, 0.18 mL/min, with a 2.1-mm × 100 column (see below). Similar peak shapes could be obtained from injection of a sample with reduced organic content (i.e., 20% acetonitrile).

The experimental workflows used for handling 20 μL and 10 μL of urine samples are shown in Figures 1C and 1D, respectively.

2.5. Measurement of extraction recovery rate

Three different concentrations of standard solutions (25 μM, 125 μM, and 250 μM) of four organic acids, phenylacetic acid, 3-hydroxybenzoic acid, hydrocinnamic acid, and phenylglyoxylic acid, were prepared. For each sample, 90 μL solution was extracted according to the LLE protocol described in Figure 1. After adding TEA to adjust pH to 8 and drying down by SpeedVac, the samples were redissolved in 50 μL of acetonitrile. The standard solutions before extraction and after extraction were injected into UPLC-UV for measuring the UV absorbance at 210 nm to determine the recovery rate. The recovery rate was obtained by comparing the peak area of a standard solution after extraction with the peak area of the same standard solution before extraction. Three experimental replicates were performed for each extraction.

2.6. Comparison of the overall extraction and labeling efficiency

To compare the overall extraction and labeling efficiencies of the original protocol and the new protocol, acid standards, lactic acid (250 μM), hydrocinnamic acid (125 μM), 3-hydroxybenzoic acid (125 μM) and phenylacetic acid (125 μM), were spiked into a urine sample individually. The urine sample with a spiked standard was processed and labeled by the original protocol and the new protocol. The labeled urine sample spiked with one standard was injected

into UPLC-UV for absorbance measurement at 338 nm. The chromatograms of the labeled standard obtained by using the two protocols were overlaid, and the peak area change was calculated to determine the overall efficiency difference of the two methods.

2.7. UPLC-UV

A Waters ACQUITY UPLC system with binary solvent manager and a photodiode array (PDA) detector was used for the detection and quantification of organic acid standards before and after extraction or after labeling. A Waters ACQUITY BEH C18 column (2.1 mm × 5 cm, 1.7 μm particle size, 130 Å pore size) was used. The flow rate was 0.42 mL/min, and the injection volume was 4 μL. Solvent A was 0.1% (v/v) formic acid in 5% (v/v) acetonitrile, and solvent B was 0.1% (v/v) formic acid in acetonitrile. The gradient was set as follows: 0 min, 15%B; 1 min, 15%; 8.5min, 50%B; 14.7min, 95%B; 15.2 min, 98%B; 16min, 15%B; 20min, 15%B.

2.8. LC-MS

An Agilent capillary 1100 HPLC system (Agilent, Palo Alto, CA) coupled with a Bruker Apex-Qe 9.4-T Fourier-transform Ion Cyclotron Resonance (FT-ICR) MS (Bruker, Billerica, MA, U.S.A.) with electrospray ionization (ESI) was used to analyze the labeled samples. The column used was a reversed-phase Eclipse C18 column (2.1 mm × 100 mm, 1.8 μm particle size, 95Å pore size). For the LC-MS work, solvent A was 0.1% (v/v) formic acid in 5% (v/v) acetonitrile (ACN), and solvent B was 0.1% (v/v) formic acid in ACN. The flow rate was 180 μL/min. The gradient elution profile was as follows: t=0 min, B%=20; t=9 min, B%=50; t=22 min, B%=65; t=26 min, B%=80; t=28 min, B%=98; t=30 min, B%=98; t=30.5 min, B%=20.

Injection of 1 µL of ACN was done during the column re-equilibrium time from 31 to 45 min at 20% B. The injection sequence was setup for each sample injection to wash the injection needle through the flushing port in order to minimize the sample carryover from the needle. The elute from the LC column was split at a ratio of 3:1, and about 60 µL/min was introduced into MS. All MS spectra were obtained in the positive ion mode. The MS conditions used for FTICR-MS were as follows: nitrogen nebulizer gas: 2.3 L/min, dry gas flow: 7.0 L/min, dry temperature: 190°C, capillary voltage: 4200 V, spray shield: 3700 V, acquisition size: 256 k, scan range: 200-1100, and ion accumulation time: 1 sec. Due to the slow scan speed of FTICR-MS, we optimized the 1.8-µm particle UPLC column separation conditions to achieve reasonably high efficiency while maintaining a sufficiently wide of peak. The typical peak width was about 18 s for relatively high abundant analytes and only one or a few spectra could be detected from the low abundant analytes.

2.9. Data processing and metabolite database search

All LC-FTICR-MS data were processed using an in-house developed software called IsoMS. First, the mass spectral peaks were picked using the Bruker DataAnalysis software 4.0. The $^{12}C/^{13}C$-ion pairs were found by their accurate mass difference of 2.0067 Da within the 2 ppm window. Redundant peaks of the same metabolite including natural isotope peaks, adducts peaks such as sodium adducts, dimer peaks, and multiply charged peaks were removed by the IsoMS software.

For putative metabolite identification based on accurate mass matches with the metabolites in a database, the Human Metabolome Database (HMDB) [19] was used. The mass accuracy window used for database search was set at 20 ppm. We note that, for targeted

metabolite or compound analysis where the analyte signal intensity is optimized at the optimal detection range of FTICR-MS (i.e., there is no signal saturation at the high end and the signal intensity is much higher than the detection limit at the low end), 2 ppm mass accuracy can be readily achieved. However, for metabolome profiling work where the analyte concentration differences in a complex sample can be quite large, not all analyte signals will fall within the optimal detection range of FTICR-MS. The high abundant ions may cause space charge effect that reduces the mass accuracy. For the low abundant ions, there may not be a sufficient number of ions to produce a perfect peak shape for peak detection. Mass errors of up to 20 ppm can be observed in these cases. Thus, in our work, we used 20 ppm mass window for database search.

3. Results and Discussion

3.1. Workflow for liquid-liquid extraction and DmPA labeling

Figure 1 shows the general workflows of the existing protocol and the new protocol using liquid-liquid extraction. Instead of preparing the urine sample in 80% acetonitrile in the old protocol, a new protocol was developed to separate the amine metabolites into the aqueous phase as well as remove the water in the sample by using an organic solvent to extract the acid metabolites. The starting volume of the urine sample was chosen to be 90 μL for the method development (Figure 1B). This volume of human urine is available for many metabolomics studies using samples typically collected for bio-banking. For the liquid-liquid extraction of organic acids from urine, the most commonly used organic solvent is ethyl acetate and urine is typically extracted under a very acidic condition (pH around 1) [20]. Under the very acidic condition, amine metabolites are protonated and dissolved readily in the aqueous phase. The organic acids are not ionized under this acidic condition and remain to be relatively hydrophobic

and, thus, they are more readily partitioned into the organic phase. A saturated NaCl solution is used to separate the organic phase from the aqueous phase better, since ethyl acetate is less soluble in a high ionic solution such as the saturated NaCl [21].

From our working experience in dealing with various metabolomic samples, metabolite sample loss can sometime be encountered during the sample drying down by SpeedVac [3]. Thus, in the liquid-liquid extraction protocol, before drying down the organic phase, TEA is added to adjust the pH to 8 in order to keep the organic acids more ionized to reduce volatility so that the loss of the acid metabolites can be minimized during solvent evaporation by SpeedVac. For the extraction, 300 μL of ethyl acetate is used to obtain an about 3:1 volume ratio of the organic phase to the aqueous phase, which is commonly used in organic acid extraction [15, 20].

For the DmPA isotope labeling reaction, the procedure used in the current protocol is the same as the one used previously [10]. We note that, after DmPA labeling, the labeled metabolites are stable and storage of the labeled samples over a period of several months (e.g., up to 6 months) did not alter the quantitative results. However, we have not investigated the stability of the carboxylic acids in stored urine. We will investigate this issue in detail in the future, particularly within the context of studying the effect of sample storage on biomarker discovery.

3.2. Method comparison for analyzing standard metabolites

To demonstrate the performance of the new protocol in comparison to the existing protocol, a mixture of two metabolite standards were analyzed. One was a common amine metabolite in urine, ethanolamine, and the other one was a common acid metabolite in urine, hippuric acid. Panels A and B of Figure 2 show the extracted ion chromatograms of ethanolamine and hippuric acid obtained by LC-FTICR-MS using the old and new protocols,

respectively. Figure 2A shows that an intense peak from DmPA labeled ethanolamine is detected in the old protocol without liquid-liquid extraction. Figure 2B shows that the new protocol with liquid-liquid extraction effectively removes the ethanolamine peak. Moreover, the peak intensity of DmPA labeled hippuric acid is increased by about 2-fold. The improved labeling efficiency offered by the new protocol can be very useful for detecting low abundance metabolites (see below). Several other organic acid standards including citric acid, succinic acid and other acids involved in the tricarboxylic acid (TCA) cycle of metabolism were tested using the new protocol and it was generally found that the new protocol improved the detectability of the organic acids compared to the old protocol. For examples, the signal of fumaric acid was found to be enhanced by 2.3-fold and the signal of oxoglutaric acid was enhanced by 5.9-fold. For these two acids each containing two COOH groups, two DmPA tags were attached to each molecule. In general, for a metabolite containing multiple carboxylic groups, the multiply labeled metabolite is the dominant product. The IsoMS program selects the peak pair of differentially labeled metabolites and calculates the peak ratio for relative quantification; other low abundance peaks from less than full labeling are not considered for quantification.

We also tested the new protocol for the standard amino acids that are usually present in high abundance in biofluids, such as urine. They can be readily analyzed using the dansylation labeling chemistry and thus removing these high abundance amino acids during the acid analysis process can be beneficial in detecting other organic acids. One example is shown in Figure 2C where the extracted ion chromatogram of asparagine labeled with 2 DmPA tags and asparagine labeled with 3 DmPA tags obtained by using the old protocol is displayed. However, by applying liquid-liquid extraction, asparagine was removed, prior to DmPA labeling, and thus it was not detected in LC-MS (see Figure 2D).

One potential concern for the new protocol is related to the analyte recovery rate during the sample extraction, drying and re-dssolution process. We have investigated this issue by determining the recovery rates for four standard acids, phenylacetic acid, 3-hydroxybenzoic acid, hydrocinnamic acid and phenylglyoxylic acid, prepared in three different concentrations (25 μM, 125 μM, and 250 μM). UPLC-UV measurements of the analyte before and after subjecting the standard solution to the LLE process were performed. Table 1 summarizes the results obtained. The average recovery rate was found to be about 84% ranging from 73% to 95%. The recovery rate is more consistent for a given acid at different concentrations, which suggests that, for relative quantification of a metabolite, the LLE process should not introduce a significant error.

We have compared the overall sampling processing and labeling efficiency of the original and new protocols for four standards, lactic acid, hydrocinnamic acid, 3-hydroxybenzoic acid and phenylacetic acid. The individual standard solution was spiked into a human urine sample where the endogenous concentration of the metabolite was much lower than that of the spiked solution (250 μM for lactic acid and 125 μM for other three acids),. After processing and labeling, UPLC-UV measurement of the labeled acid was carried out to determine the extent of improvement of the new protocol versus the old protocol. It was found that the improvement was 26-fold for lactic acid, 28-fold for hydrocinnamic acid, 5.6-fold for 3-hydroxybenzoic acid, and 4.4-fold for phenylacetic acid.

The above examples and results indicate that liquid-liquid extraction can be used to separate the amine metabolites from the organic acids and remove the water content, which can lead to improvement in the detection of organic acid metabolites by isotope labeling LC-MS.

3.3. Method comparison for analyzing urine samples

The new protocol was applied to analyze the metabolome of human urine. In this case, a urine sample was divided into two aliquots. One aliquot was subjected to liquid-liquid extraction and ^{12}C-DmPA labeling and another one was processed in the way except it was labeled with ^{13}C-DmPA. The ^{12}C/^{13}C-differentially labeled aliquots were mixed in 1:1 for LC-MS analysis. All the mass spectral peaks of the labeled metabolites would be shown in peak pairs in the mass spectra where background peaks would appear as singlet's. Using the IsoMS program, all the peak pairs can be picked up and then filtered by removing redundant peaks. The intensity ratios of the final list of peak pairs are calculated. Each peak pair with unique mass and retention time should represent a metabolite.

Panels A and B of Figure 3 show the ion chromatograms of human urine obtained using the old and new protocols, respectively. Figure 3C shows the overlaid chromatograms for direct comparison. There were 2491 peak pairs detected using the new protocol, while 874 peak pairs were found using the old protocol. The detection improvement is very significant, reflecting the ability of the new protocol to remove the amines and water that can interfere with the labeling and detection of the organic acids. As Figure 3C illustrates, amine metabolites such as ethanolamine, ethylamine and glycine in the urine sample were mostly removed, while at the same time the peak intensity was significantly increased for the organic acids such as citric acid, hippuric acid and lactic acid. In addition, the peak intensity from the hydrolyzed DmPA reagent was much reduced (i.e., 3.5% of the original peak intensity).

The above example demonstrates the new DmPA labeling protocol with liquid-liquid extraction can be used to improve the metabolome coverage of the organic acids greatly and also improve the specificity of the acid sub-metabolome analysis (see below; most matched peak pairs are belonging to the organic acids). It should be noted that, in a typical GC-MS

experiment, less than 200 organic acids can be detected [22, 23]. As it is shown in this work, the isotope labeling LC-MS method can detect ~2500 organic acids in urine. Thus, this method can be used to profile the organic acid sub-metabolome in a much more comprehensive manner.

3.4. Low volume sample analysis

While the above liquid-liquid extraction protocol developed for handling a 90 μL of urine as a starting material is adequate for metabolomics applications where relatively larger volumes of samples are available, it is often desirable and, sometimes, necessary to analyze a smaller volume of sample. For example, in some metabolomics work, the sample volume is limited, particularly in animal studies such as mice. In other cases, a valuable biofluid may be divided into small volume aliquots for multiple analyses including proteomics and genomics analysis, in addition to metabolome profiling. And even for metabolomics studies, a sample may be further divided into several aliquots for different isotopic labeling methods in order to increase the metabolome coverage. Therefore, the volume of an analysis sample can be 20 μL or even lower. We have explored the feasibility of scaling down the new protocol to handle 20 and 10 μL of starting materials.

One straightforward approach for scaling down to handle 10 μL of sample volume is to reduce the liquid-liquid extraction volume from 90 μL to 10 μL, and also the chemical reaction volume from 60 μL to 10 μL proportionally. However, this approach did not produce the same results as those with the 90 uL of starting materials; both the number of peak pairs detected and reproducibility were significantly lower. On the other hand, it was found that scaling down from 90 μL to 45 μL in extraction and from 60 μL to 30 μL in reaction did not sacrifice the analytical performance. When using this way of scaling down, after combining the ^{12}C-labeled sample with

the ^{13}C-labeled sample, the final volume would be as large as 120 μL, from which only 2 μL was injected into LC-MS. In order to offset the limitation of low volume starting material and hence overall low concentration of metabolites, the labeled mixture was concentrated using SpeedVac to a volume with an overall concentration equivalent to that produced using the 90 μL of starting material. Panels B, C and D of Figure 1 show the workflow for handling 90 μL, 20 μL and 10 μL of urine starting material and the difference in the three experiments are highlighted.

Figure 4 shows the comparison of the number of peak pairs detected from three volumes of starting material. For the 90 μL starting material, 2561 ± 27 (n=3) peak pairs were detected. The number of peak pairs obtained from the 20 μL starting material was 2548 ± 118 (n=3). The number of peak pairs from the 10 μL starting material was 2301 ± 249 (n=3). These results show that it is still possible to detect similar number of metabolites using low volume of samples even with 10 μL of urine. However, the number reproducibility from the 10 μL sample is not as good as those from the 90 or 20 μL samples. This may be due to sample loss encountered during the drying down by SpeedVac. The effect of sample loss is expected to be more pronounced for a small volume sample (10 μL), compared to large volume samples (20 or 90 μL). In other words, if the total amount of sample loss is the same for all samples (e.g., via the adsorption to the container walls), the percentage of sample loss is much higher for a small volume sample compared to a large volume samples.

Another important criterion to gauge the performance of the new protocol for handling various volumes of samples is the relative standard deviation (RSD) of the intensity ratio for all the detected metabolite peak pairs. It was found that the average RSD was 8.8% for the 20 μL sample and 12% for the 10 μL sample. Thus, the precision of the method should be sufficient for comparative metabolomics where the relative changes of the metabolomes from different cohorts

of samples are analyzed. The application of this method for comparative metabolomics is currently underway and will be reported in the future.

The above results indicate that the liquid-liquid extraction DmPA labeling method can be used to handle low volumes of urine samples. While the number of peak pairs detected and the precision of the peak intensity ratio for all the peak pairs are slightly reduced when 10 μL of starting volume was used, compared to 90 μL of volume, the use of 20 μL of urine does not affect the performance. We feel that for routine metabolomics applications 20 μL of urine can be conveniently used for acid metabolome profiling. However, if the sample volume is very limited, 10 μL of urine can still generate good results.

3.5. Putative metabolite identification

FTICR-MS can provide high mass measurement accuracy for measuring the masses of metabolite ions and the mass error is typically less than 2 ppm, although signal saturation from very high abundant ions or less than ideal peak shapes from low abundant ions can increase the mass error up to 20 ppm. The measured mass of an organic acid can be calculated from the detected m/z of the peak pair by subtracting the isotope tag. By matching the accurate mass with those of the metabolites in HMDB, a list of putatively identified organic acids in urine can be generated. Table 2 shows a partial list of putatively identified organic acids in a urine sample. The complete list of putatively identified organic acids in urine can be found in Supplemental Table S1. In the urine sample, there were 1004 matched metabolites out of 2527 detected peak pairs. About 95% of the 1004 matched metabolites have a carboxylic acid group; there are about 5% of them matched with acylcarnitines, diols, metabolites with hydroxyl group(s) only (e.g., sucrose), and other no-carboxylic-acid metabolites. The matched acids include many common

organic acids analyzed by GC-MS in clinical laboratories. Although these matched metabolites with HMDB need to be confirmed for positive identification by authentic standards, this list should still be useful to clinicians and biochemists to explore some unusual organic acids in some metabolism disorders that are detectable in urine [24]. To further broaden the metabolome coverage and increase the number of putatively identified organic acids, we are planning to use two-dimensional LC separation, combined with MS [25], to further expand the concentration dynamic range and detect more organic acids.

4. Conclusions

We have developed an improved isotope labeling method based on liquid-liquid extraction and *p*-dimethylaminophenacyl reaction for LC-MS metabolomic profiling of organic acid metabolites. LLE under acidic condition can effectively separate amine metabolites from organic acid metabolites, thereby increasing the detectability of organic acids using DmPA labeling LC-MS. LLE can also remove the water from a sample, which eliminates the water interference in DmPA labeling reaction and greatly reduces the water-induced hydrolysis product of the DmPA reagent that can interfere with the LC-MS detection of labeled organic acids. This method can be used to handle low volumes of urine samples; 2561 ± 27 (n=3), 2548 ± 118 (n=3), and 2301 ± 249 (n=3) peak pairs could be detected in human urine with a starting material of 90, 20 and 10 µL, respectively. These numbers are almost three times more than that detected using the original method without LLE. We envisage the routine use of this method for metabolomic profiling of organic acids for comparative metabolomics applications. In addition, we will combine this method with the isotope dansyl labeling amine- and phenol-metabolome

profiling workflow to extend the metabolome coverage. The applications of this method for handling other types of biofluids and biological samples will also be reported in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article (Supplemental Table S1) can be found, in the online version, at the ACA web site.

Table 1. Summary of the analyte recovery rate in the sample extraction, drying and re-dissolution procedure.

Organic acid	**Concentration**	**Recovery rate (n=3)**
phenylglyoxylic acid	25 μM	0.77 ±0.02
	125 μM	0.91 ±0.01
	250 μM	0.880 ±0.009
hydrocinnamic acid	25 μM	0.88 ±0.04
	125 μM	0.73 ±0.02
	250μM	0.75 ±0.02
phenylacetic acid	25 μM	0.88 ±0.02
	125 μM	0.82 ±0.02
	250 μM	0.86 ±0.03
3-hydroxylbenzoic acid	25 μM	0.95 ±0.02
	125 μM	0.85 ±0.04
	250 μM	0.84 ±0.05

Table 1. Partial list of organic acids putatively identified based on accurate mass match with the HMDB metabolites (the complete list is shown in Supplemental Table S1).

Peak pair series number	Retention time (min)	Intensity	Accurate Mass	Name of putative metabolite	HMDB ID
1	10.5	1.12E+06	46.0047	Formic acid	HMDB00142
2	16.7	6.20E+05	60.0203	Acetic acid	HMDB00042
				Glycolaldehyde	HMDB03344
3	24.8	3.48E+05	60.0204	Acetic acid	HMDB00042
				Glycolaldehyde	HMDB03344
4	20.3	4.06E+05	132.0413	Dimethylmalonic acid	HMDB02001
				(S)-2-Acetolactate	HMDB06855
				2-Acetolactate	HMDB06833
				Ethylmalonic acid	HMDB00622
				Glutaric acid	HMDB00661
				Methylsuccinic acid	HMDB01844
				Monoethyl malonic acid	HMDB00576
5	21.9	9.54E+04	141.0385	2-Aminomuconic acid semialdehyde	HMDB01280
6	21.4	7.01E+05	143.0540	Vinylacetylglycine	HMDB00894
7	20.2	1.41E+05	153.0371	3-Aminosalicylic acid	HMDB01972
				3-Hydroxyanthranilic acid	HMDB01476
8	25.6	1.90E+05	160.0691	Pimelic acid	HMDB00857
				3,3-Dimethylglutaric acid	HMDB02441
				3-Methyladipic acid	HMDB00555
9	18.0	8.52E+05	161.0623	Aminoadipic acid	HMDB00510
10	15.2	3.30E+05	162.0399	1,2-Dihydroxy-3-keto-5-methylthiopentene	HMDB12134
				3 Hydroxycoumarin	HMDB02149
11	18.5	2.98E+05	166.0321	Terephthalic acid	HMDB02428
				Benzoquinoneacetic acid	HMDB02334
				Phthalic acid	HMDB02107
12	22.8	1.80E+05	168.0374	Homogentisic acid	HMDB00130
				3,4-Dihydroxybenzeneacetic acid	HMDB01336
				3,4-Dihydroxymandelaldehyde	HMDB06242
				3-Hydroxymandelic acid	HMDB00750
				5-Methoxysalicylic acid	HMDB01868
				p-Hydroxymandelic acid	HMDB00822
				Uric acid	HMDB00289
				Vanillic acid	HMDB00484
13	16.0	1.81E+05	169.0293	L-2,3-Dihydrodipicolinate	HMDB12247
				2-Furoylglycine	HMDB00439
14	15.5	1.67E+05	171.0588	Tetrahydrodipicolinate	HMDB12289
15	27.7	1.22E+05	172.1534	Capric acid	HMDB00511
16	26.1	6.50E+05	173.1001	Isovalerylalanine	HMDB00747

				Hexanoylglycine	HMDB00701
				Isovalerylsarcosine	HMDB02087
				N-Acetylleucine	HMDB11756
17	25.7	1.25E+05	174.0854	Suberic acid	HMDB00893
				Demethylated antipyrine	HMDB06240
				Ethyladipic acid	HMDB02023
18	18.4	7.25E+04	176.0669	3-Isopropylmalate	HMDB12156
				2,3-Dimethyl-3-hydroxyglutaric acid	HMDB02025
				2-Isopropylmalic acid	HMDB00402
19	11.9	1.00E+06	90.0296	Dihydroxyacetone	HMDB01882
				D-Lactic acid	HMDB01311
				Glyceraldehyde	HMDB01051
				Hydroxypropionic acid	HMDB00700
				L-Lactic acid	HMDB00190
20	8.4	9.18E+05	90.0299	Dihydroxyacetone	HMDB01882
				D-Lactic acid	HMDB01311
				Glyceraldehyde	HMDB01051
				Hydroxypropionic acid	HMDB00700
				L-Lactic acid	HMDB00190
21	9.2	8.04E+06	90.0301	Dihydroxyacetone	HMDB01882
				D-Lactic acid	HMDB01311
				Glyceraldehyde	HMDB01051
				Hydroxypropionic acid	HMDB00700
				L-Lactic acid	HMDB00190

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Figure Legends

Figure 1. Workflow of an existing protocol involving direct DmPA labeling of a sample (A).Working flows of a new protocol using liquid-liquid extraction followed by DmPA labeling with a starting material of (B) 90 µL of human urine, (C) 20 µL of human urine and (D) 10 µL of human urine.

Figure 2. Extracted ion chromatograms of a mixture of DmPA labeled ethanolamine (m/z 384.2233) and DmPA labeled huppuric acid (341.1148) (the concentration was 2 mM for each standard and the injection volume was 2 µL) obtained by (A) the old protocol with direct DmPA labeling and (B) the new protocol with LLE and DmPA labeling. Extracted ion chromatograms of asparagine labeled with 2 DmPA tags and asparagine labeled with 3 DmPA tags (the concentration was 2 mM and the injection volume was 2 µL) obtained by (C) the old protocol and (D) the new protocol.

Figure 3. Base-peak ion chromatograms of DmPA labeled urine sample prepared using (A) the direct DmPA labeling method and (B) the new LLE DmPA method. (C) the overlaid ion chromatograms of (A) and (B) for comparison.

Figure 4. Number of peak pairs detected as a function of the volume of the starting material.

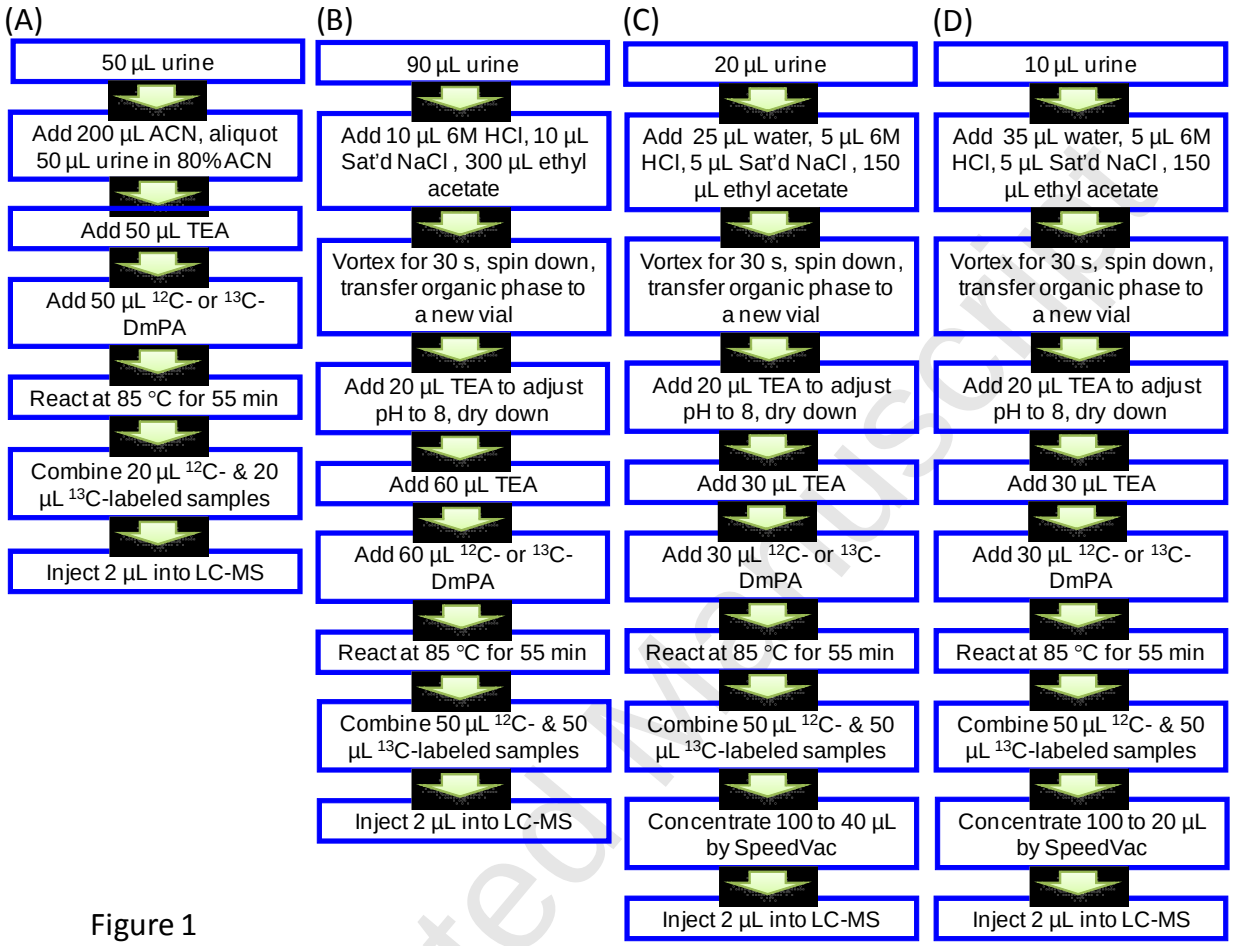

Figure 1

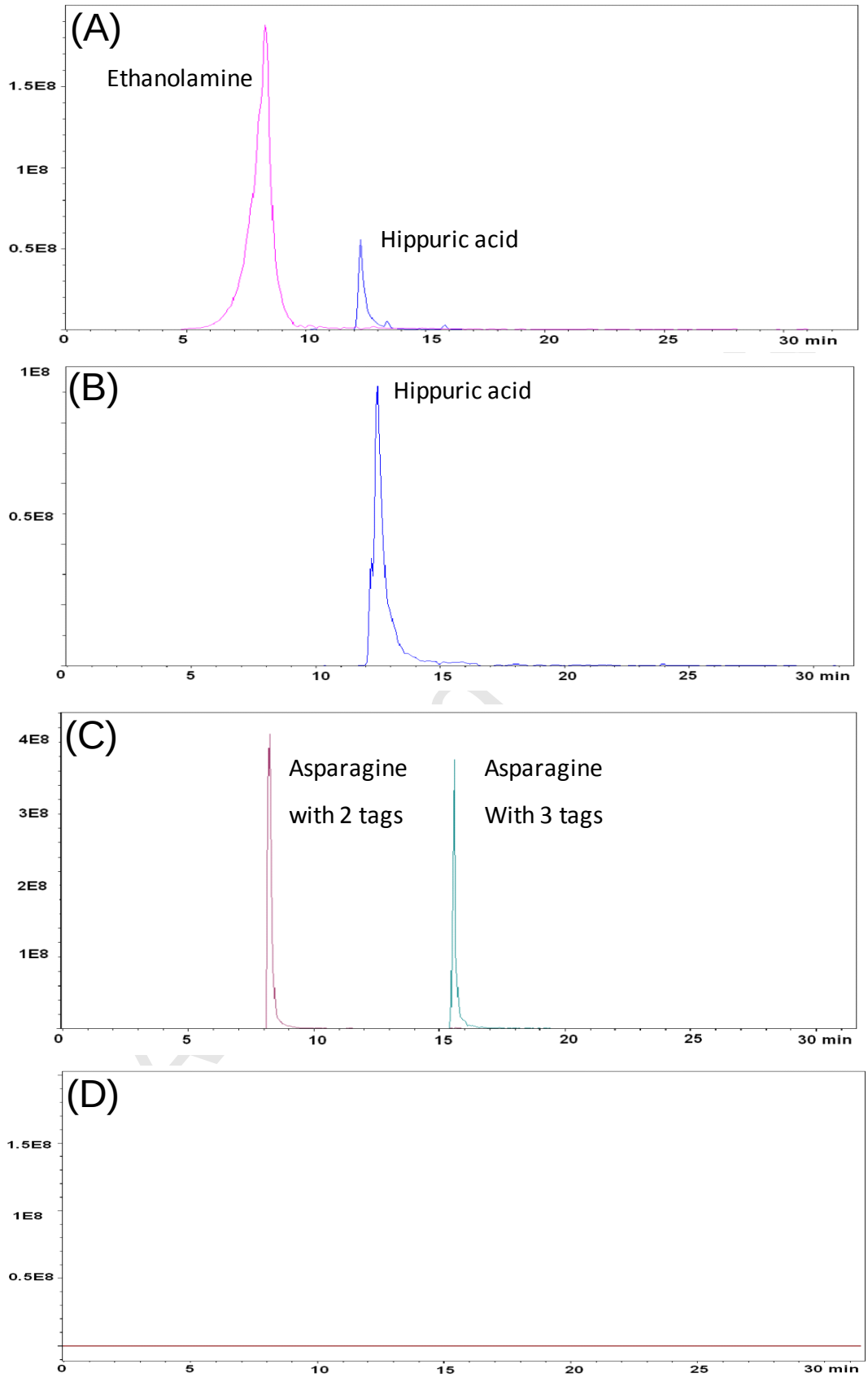

Figure 2

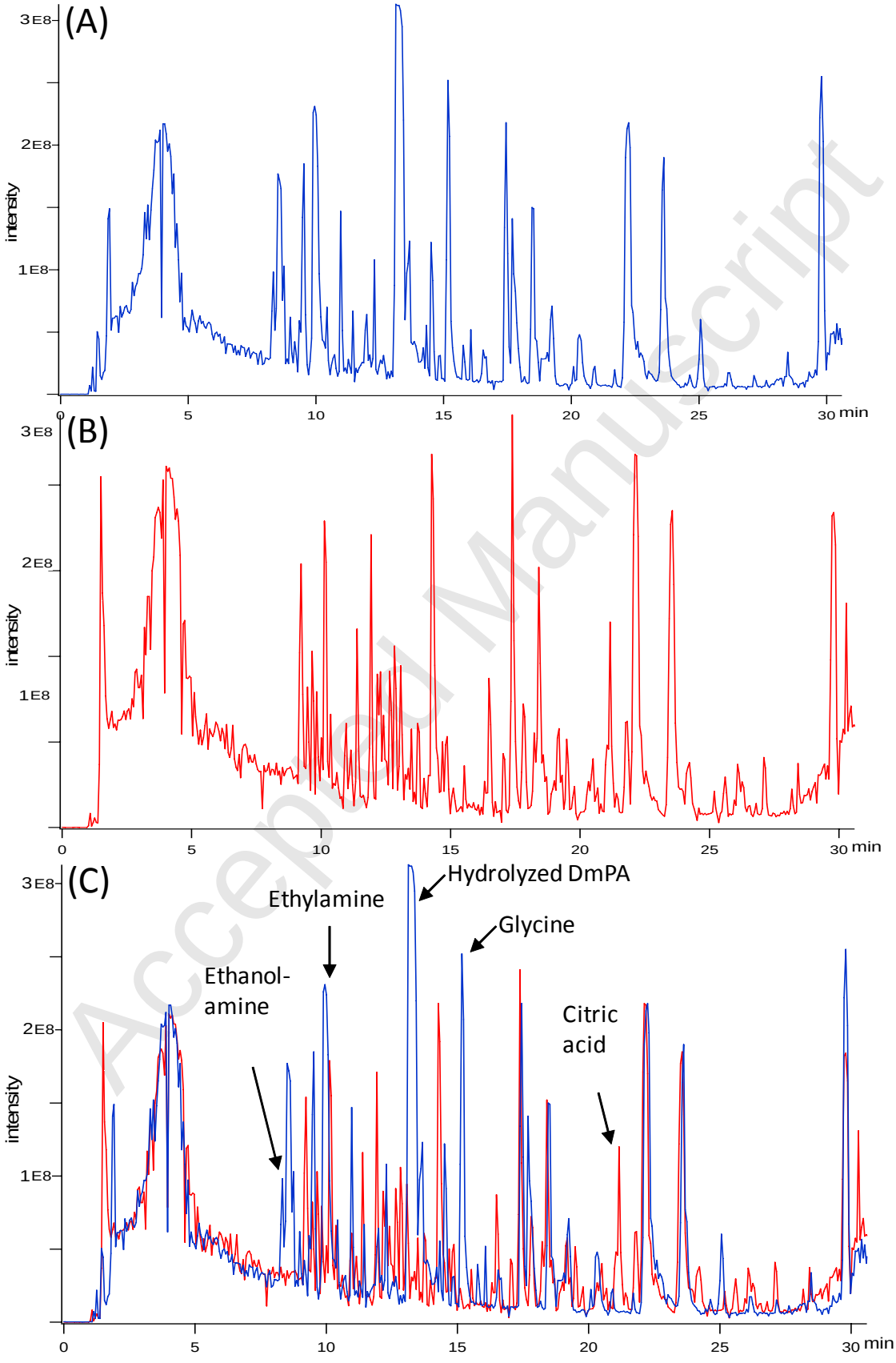

Figure 3

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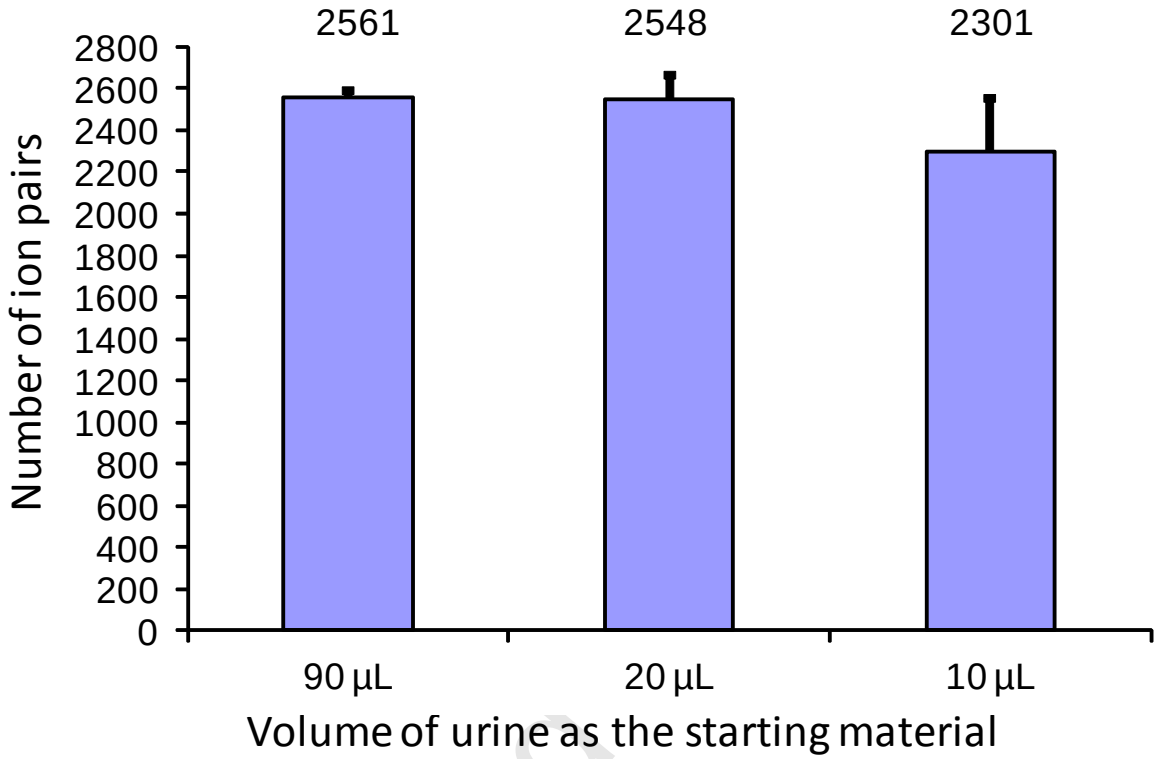

Figure 4

Highlights

An improved method for profiling the carboxylic acid sub-metabolome is reported.

Liquid-liquid extraction was used for separating the organic acids from the amines.

$^{12}C/^{13}C$-*p*-dimethylaminophenacyl (DmPA) labeling of the organic acids was carried out on the extract.

Detection interference by amines and labeling efficiency reduction by water were reduced.

About 2500 $^{12}C/^{13}C$-peak pairs or putative metabolites could be detected from 20 µL of human urine.