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Determination of Total Concentration of Chemically Labeled Metabolites as a Means of Metabolome Sample Normalization and Sample Loading Optimization in Mass Spectrometry-Based Metabolomics

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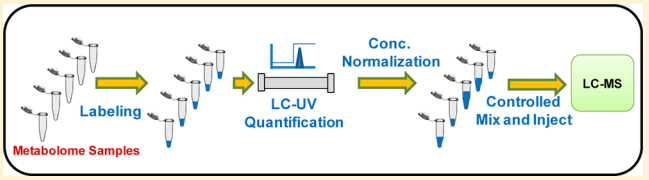
Supporting Information

ABSTRACT: For mass spectrometry (MS)-based metabolomics, it is important to use the same amount of starting materials from each sample to compare the metabolome changes in two or more comparative samples. Unfortunately, for biological samples, the total amount or concentration of metabolites is difficult to determine. In this work, we report a general approach of determining the total concentration of metabolites based on the use of chemical labeling to attach a UV absorbent to the metabolites to be analyzed, followed by rapid step-gradient liquid chromatography (LC) UV detection of the labeled metabolites. It is shown that quantification of the total labeled analytes in a biological sample facilitates the preparation of an appropriate amount of starting materials for MS analysis as well as the optimization of the sample loading amount to a mass spectrometer for achieving optimal detectability. As an example, dansylation chemistry was used to label the amine- and phenol-containing metabolites in human urine samples. LC-UV quantification of the labeled metabolites could be optimally performed at the detection wavelength of 338 nm. A calibration curve established from the analysis of a mixture of 17 labeled amino acid standards was found to have the same slope as that from the analysis of the labeled urinary metabolites, suggesting that the labeled amino acid standard calibration curve could be used to determine the total concentration of the labeled urinary metabolites. A workflow incorporating this LC-UV metabolite quantification strategy was then developed in which all individual urine samples were first labeled with ^{12}C-dansylation and the concentration of each sample was determined by LC-UV. The volumes of urine samples taken for producing the pooled urine standard were adjusted to ensure an equal amount of labeled urine metabolites from each sample was used for the pooling. The pooled urine standard was then labeled with ^{13}C-dansylation. Equal amounts of the ^{12}C-labeled individual sample and the ^{13}C-labeled pooled urine standard were mixed for LC-MS analysis. This way of concentration normalization among different samples with varying concentrations of total metabolites was found to be critical for generating reliable metabolome profiles for comparison.



Liquid chromatography mass spectrometry (LC-MS)-based metabolomic techniques have been widely applied to the qualitative and quantitative analysis of biofluids, cells, or tissue extracts for biological studies and biomarker discovery with high sensitivity, high resolution, and wide metabolite coverage.[1−4] However, for quantitative studies, variation in total metabolite concentration among different samples can complicate the relative quantification of the metabolome changes in comparative metabolomics.[5] This is particularly true for urinary metabolome profiling. Urinary metabolite concentrations can be governed by many factors, such as kidney filtration and water consumption, and up to 15-fold variations in urine volume can be observed for normal individuals.[6] This variation can be even greater due to disease or drug effects. The changes of the overall concentration can often obscure specific changes of metabolites that are of interest in metabolomic studies.[7] Although the collection of urine samples from an individual over a long period (e.g., 24 h) may account for the variation of total concentration, the collection and storage process would be inconvenient and cumbersome in practice. Therefore, a good sample normalization strategy is required to compensate for variations in the overall urine concentration. For other biofluids, such as bronchial lavage fluid and saliva, as well as metabolome extracts of various types of cells with unknown cell numbers, it is also desirable to normalize the sample concentration prior to sample workup and quantitative analysis.

For urine samples, various normalization methods have been reported in the literature. The most common one is creatinine normalization,[8−11] because the rate of creatinine formation and excretion is relatively constant under normal conditions. In this method, the concentrations of metabolite analytes are normalized relative to the concentration of creatinine. However, the assumption of constant creatinine excretion is

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often not valid because creatinine excretion does vary among individuals due to age, gender, and lean body mass differences, and even within the same individual, the urine creatinine level can change due to variables such as creatinine intake, time of day, level of exercise, and disease states.[12] Normalization to osmolality or specific gravity has also been used.[9,10,13,14] This method measures the total solute concentration of urine samples for normalization. However, the solute concentration includes contributions of all dissolving solids and may not directly reflect the total urinary metabolite output. Statistical normalization strategies have also been routinely used for NMR data[7,15] and in LC-MS studies, a similar normalization method based on the use of MS "total useful signal" (MSTUS) has been recently reported by Warrack et al,[16] which uses the total intensity of peaks that are present in all samples as the normalization factor. This method avoids contribution from xenobiotics and artifacts and has been demonstrated to be useful in detecting statistically significant changes in the endogenous metabolite profile of urine samples and reduces variation between biological replicates. However, this method does not allow the adjustment of relative sample amounts for mixing in the cases where two comparative samples (e.g., sample vs control) need to be mixed prior to MS analysis. Furthermore, this method does not offer a means of controlling the amount of samples to be injected into a mass spectrometer for analysis. The amount of sample injected can be very important for metabolite detection. If the injection amount is small, the low concentration metabolites will not be detectable because their concentration level becomes lower than the detection threshold of the instrument.[17] On the other hand, if a large injection amount is used, the electrospray ionization (ESI) source and the detector will be easily saturated with ions, and the high abundance peaks can obscure small peaks and make them undetectable. Because each normalization method has its strengths and limitations, there is no consensus on which normalization method works the best for LC-MS metabolome studies.

In this work, we present an alternative strategy for sample normalization while offering the possibility of controlling and optimizing the sample injection for optimal mass spectrometric metabolome detection. This method is based on the use of LC-UV for quantifying the total concentration of the chemically labeled metabolites to be analyzed in any type of biological sample including urine. We note that the use of LC-UV for quantification of the total peptides generated from a proteome digest has been reported earlier, using a probing wavelength of 214 nm that corresponds to the carbonyl group in the peptide backbone.[18] Unlike proteins and peptides, which have a relatively uniform backbone structure, metabolites have a wide variety of structures and thus very different UV absorptivity. As a result, it is very difficult to choose a single wavelength for detection. Most of the studies using LC-UV quantification of metabolites are focused on the analysis of a certain class of compounds.[19−21] To the best of our knowledge, there is no report of the use of LC-UV as a general tool for the quantification of a metabolome or a subset of the metabolome (e.g., all the metabolites with each containing an amine group, i.e., the amine-containing metabolome).

Although metabolites have very different structures, the use of labeling chemistry can somehow "unify" the metabolites by attaching the same functional group to each molecule. If this labeling group has a very specific absorption wavelength, then it is possible to quantify the labeled metabolites based on absorption at this wavelength. Recently, our group reported a $^{12}C/^{13}C$-dansylation labeling technique for absolute and relative quantification of the amine- and phenol-containing metabolome by LC-MS.[22] This labeling strategy allows separation of polar or ionic metabolites on a reversed phase (RP) column, as well as provides signal enhancement of 10- to 1000-fold over the unlabeled counterparts. Experimental variation can also be compensated for with the use of a pooled ^{13}C-labeled sample as the internal standard. Another advantage of this technique is that the aromatic ring structure of the dansyl group can also act as a good chromophore to facilitate UV quantification. In this work, we report a LC-UV method to quantify all of the labeled metabolites in urine samples using a fast step-gradient elution. The quantification results were then used to normalize the urine samples and to optimize the sample injection amount. We describe the procedures and rationales for selection of detection wavelength in LC-UV, appearance of chromatographic peak profiles, method of peak area integration in step-gradient LC, selection of calibration standards and calibration method for relative and absolute quantification of the total labeled metabolites, and strategy of optimizing the sample injection amount in LC-MS. Finally, integration of the LC-UV method into a differential isotope labeling LC-MS workflow for improved metabolome quantification is discussed.

EXPERIMENTAL SECTION

Chemicals and Reagents. ^{12}C-dansyl chloride (DnsCl) and amino acid standards were purchased from Sigma-Aldrich Canada (Markham, ON, Canada). The isotopic compound used to synthesize ^{13}C-dansyl chloride was purchased from Cambridge Isotope Laboratories (Cambridge, MA, USA). ^{13}C-dansyl chloride was synthesized in our lab as described previously,[22] and the other chemicals used to synthesize this isotope reagent were also purchased from Sigma-Aldrich. LC-MS grade water, acetonitrile (ACN), and formic acid were purchased from Thermo Fisher Scientific (Edmonton, AB, Canada).

Urine Sample Collection. Urine samples were collected from two individuals of each gender in three consecutive days. 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. On each day, three samples were taken with a collection interval of 1 h, denoted as A, B, and C. Between collection of A and B, the individuals were instructed to refrain from drinking water, while between collection of B and C, the individuals were asked to drink a large amount of water (1 L for individual 1 and 0.5 L for individual 2). The samples were stored at 4 °C immediately after collection. The urine samples were centrifuged at 4000 rpm for 10 min, and the supernatant was filtered twice through a 0.2 μm filter. The filtered urine was aliquoted and stored at −80 °C until further use.

Dansylation Labeling Reaction. The frozen urine samples were thawed in an ice-bath and then diluted 2-fold prior to the labeling reaction. Fifty microliters of urine or amino acid standard solution was mixed with sodium carbonate/sodium bicarbonate buffer and ACN. The solutions were vortexed, spun down, and mixed with 50 μL of freshly prepared ^{12}C-dansyl chloride solution (18 mg/mL) (for light labeling) or ^{13}C-dansyl chloride solution (18 mg/mL) (for heavy labeling). The reaction was allowed to proceed for 1 h at 60 °C. After 1 h,

NaOH was added to the reaction mixture to quench the excess dansyl chloride. The solution was then incubated at 60 °C for another 10 min. Finally, formic acid in 50/50 ACN/H_2O was added to consume excess NaOH and to make the solution acidic. The ^{12}C- or ^{13}C-labeled mixtures were centrifuged at 14 000 rpm for 10 min before injecting onto the ultrahigh performance liquid chromatography (UPLC) column for UV quantification. For MS analysis, the ^{12}C- and ^{13}C-labeled mixtures were combined in a ratio determined by the quantification results.

LC-UV Quantification. A Waters ACQUITY UPLC system with a photodiode array (PDA) detector was used for the quantification step. Two microliters of the labeled urine or amino acid solution was injected onto a Waters ACQUITY BEH C18 column (2.1 mm × 5 cm, 1.7 μm particle size, 130 Å pore size) for a fast step-gradient run. Solvent A was 0.1% (v/v) formic acid in 10% (v/v) acetonitrile, and solvent B was 0.1% (v/v) formic acid in acetonitrile. The gradient started with 0% B for 1 min and was increased to 95% within 0.01 min and held at 95% B for 1 min to ensure complete elution of all labeled metabolites. The gradient was restored to 0% B in 0.5 min and held at this condition for 3.5 min to re-equilibrate the column. The flow rate used was 0.45 mL/min.

LC-FTICR-MS Analysis. The labeled urine samples were analyzed using a Bruker 9.4 T Apex-Qe Fourier transform ion-cyclotron resonance (FTICR) mass spectrometer (Bruker, Billerica, MA) linked to an Agilent 1100 series binary HPLC system (Agilent, Palo Alto, CA). The samples were injected onto an Agilent reversed phase Eclipse Plus C18 column (2.1 mm × 10 cm, 1.8 μm particle size, 95 Å pore size) for separation. 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 chromatographic conditions were: t = 0 min, 20% B; t = 3.5 min, 35% B; t = 18 min, 65% B; t = 21 min, 95% B; t = 21.5 min, 95% B; t = 23 min, 98% B; t = 24 min, 98% B; t = 26.5 min, 99% B; t = 28.5 min, 99% B; t = 29.5 min, 20% B. The flow rate was 180 μL/min, and the flow from LC was split 1:3 before entering the electrospray ionization (ESI) source. All MS spectra were obtained in the positive ion mode. The resulting MS data were processed using R language program based on XCMS[23] written specifically for ^{12}C-/^{13}C-peak pair picking.[24] The program eliminated many false positive peaks, such as isotopic peaks, common adduct ions, and multiply charged ions, and only the protonated ion pairs were exported for further analysis.

Statistical Analysis. The extracted peak pair data for the two individuals' three-day urine samples was aligned by retention time and accurate mass, and only those peak-pair features shared by no less than 50% of the samples were retained for multivariate analysis. The resulting multivariate data set contains 108 observations (individual urine samples) and 467 variables (peak-pair features). The multivariate analysis was performed by Metaboanalyst[25] (www.metaboanalyst.ca) and SIMCA P+12 (Umetrics, Umeå, Sweden), and the data were mean-centered and autoscaled (unit variance) prior to analysis. Principal component analysis (PCA) was applied first to evaluate general clustering of normalized and un-normalized data for two different individuals. Supervised partial least-squares-discriminate analysis (PLS-DA) was then used to reveal subgroups within an individual.

RESULTS AND DISCUSSION

Wavelength Selection. Dansylation is a well-studied labeling chemistry that works for primary amines, secondary amines, and phenols. The aromatic ring structure of the dansyl group makes it a good chromophore with very characteristic absorptions. Supplemental Figure S1, Supporting Information, shows the absorption spectra of four standards, dansyl-tryptophan, dansyl-alanine, dansyl-putrescine, and dansyl-threonine, from 210 to 400 nm. The spectra features are very similar, suggesting that the dansyl group plays a major role for the absorption. As expected for aromatic hydrocarbons, three sets of bands were observed that originate from $\pi \rightarrow \pi^*$ transitions: one strong absorption band centered at ∼220 nm, one weaker band at ∼252 nm, and the weakest one at ∼338 nm.[26] The absorption spectra of the other 17 labeled amino acid standards as well as several labeled urine samples were also examined (data not shown). It was found that the peak wavelength could shift up to about 20 nm for more complex mixtures (urine), indicating that the presence of other functional groups can have minor effects on the overall absorption. For example, for the weakest band, the peak wavelength can vary from 326 to 349 nm. Since only one wavelength should be chosen for quantification, the median value of each set of bands was used for comparison.

There are several considerations when selecting a proper wavelength for quantification. First, the absorption should be specific to the dansyl group. Because UV absorbance is additive, absorption of other functional groups in a metabolite would also contribute to the measurement, which can affect accuracy of quantification. Many common organic chromophores, such as carbonyl, carboxyl, and phenyl, have absorption peaks under 300 nm, but very few functional groups absorb at higher wavelengths. This can be illustrated by the overlaid chromatograms of 17 unlabeled amino acids at 220, 252, and 338 nm (Supplemental Figure S2, Supporting Information). All amino acids elute out between 0.2 and 0.6 min. It is clear that significant absorption was observed at 220 and 252 nm but not at 338 nm. Thus, using a wavelength of 338 nm for detection can provide the least interference from other chromophores. Second, the high absorbance at 220 and 252 nm can easily saturate the UV signal at practically useful concentrations, such as for a 2-fold diluted urine sample, as shown in Supplemental Figure S3, Supporting Information. Finally, the rapid solvent change can lead to a nonflat background because of the differences in absorption coefficients and refractive indexes of different solvents. The background change would affect the accuracy for peak integration and should be kept as small as possible. Comparing the background change at these three wavelengths in Supplemental Figure S2, Supporting Information, it is apparent that the change in background is smallest at 338 nm. Taken together, 338 nm was chosen as the probing wavelength for all of the following quantification work.

Calibration of Labeled Amino Acid Standards. Since the main purpose of this method is to quantify all the labeled metabolites in a sample, a step-gradient was used to elute all compounds together. Using the UPLC system, it is possible to run at a high flow rate to increase the throughput. Figure 1A shows the overlaid elution profiles of the mixtures of 17 labeled amino acid standards (17-aa-std) at different concentrations. The early eluting peak corresponds to the quenched dansyl chloride (Dns−OH), which does not retain on the column well. The peaks between 1.4 and 2.0 min are from the labeled

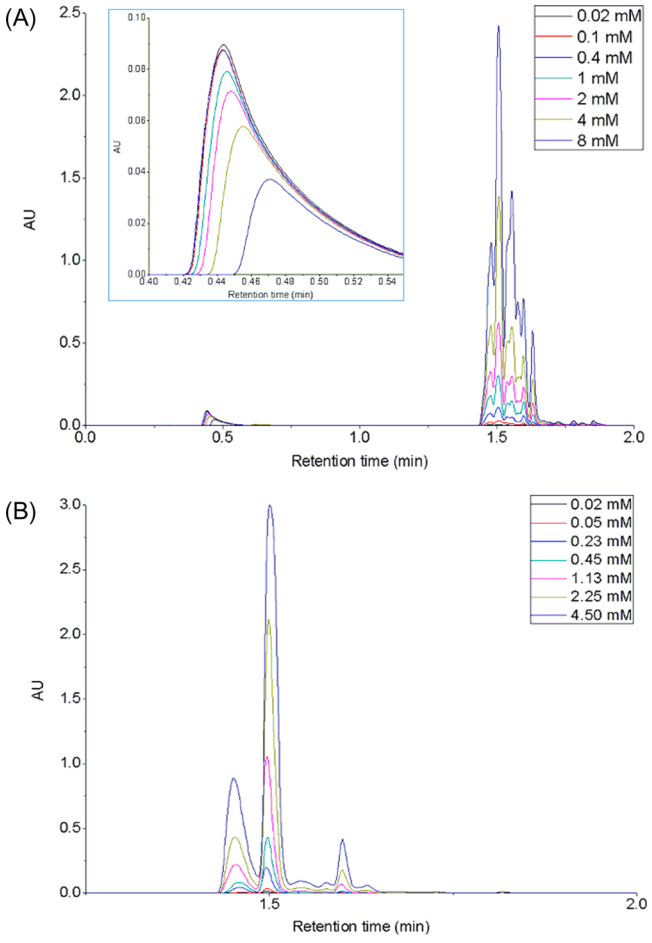

Figure 1. (A) Overlaid UV chromatograms of a mixture of 17 labeled amino acid standards (17-aa-std) at different concentrations. Inset: zoom-in region of 0.4−0.55 min. (B) Overlaid UV chromatograms of a labeled pooled urine sample at different concentrations.

amino acids. As shown in Figure 1A, the step-gradient allows fast elution of all labeled compounds in 2 min while separation of these compounds from the quenched DnsCl is achieved to avoid reagent interference in the UV measurement. The peak area increases accordingly with increasing amino acid concentration, and the elution profile is very similar for different concentrations. Figure 1A (inset) also shows that the peak area of the quenched DnsCl decreases with increasing amino acid concentration, because there is less excess DnsCl at higher concentrations of analytes. Even with a sharp solvent change in 0.01 min, there is still a slight separation of the amino acids, as evident from several peaks shown in each elution profile (see Figure 1A between 1.4 and 2.0 min). This is due to the wide variety of side-chains in amino acids that can interact differently with the column. Nevertheless, the total area of the labeled amino acid peaks can be readily integrated using the Empower software of the LC-UV instrument.

It should be noted that, although the baseline of the chromatograms is relatively flat at 338 nm, there is a very small negative peak that appears at 1.43 min due to the rapid solvent change. This system peak is very reproducible, with a retention time shift of less than 0.06% and a peak area variation of 2.32% in five replicate runs. Therefore, the peak area difference was used for quantification of the metabolites, which was calculated from the peak area measured for a given sample minus the system peak area measured in a blank run. Here, the system peak area has a negative value so the peak area difference is actually the sum of the sample peak area and the absolute value of the system peak. Integration was made from 1.43 to 2.0 min to ensure every peak from the sample has been included. While not tested in this work, other manufacturers' LC-UV instruments and LC columns may likely give different profiles. However, the strategy of integrating the entire elution peak including the system peak as described above should be applicable to other systems.

Figure 2A shows the calibration curve of peak area versus the concentration of 17-aa-std. The curve is linear from 0.02 to 6.25

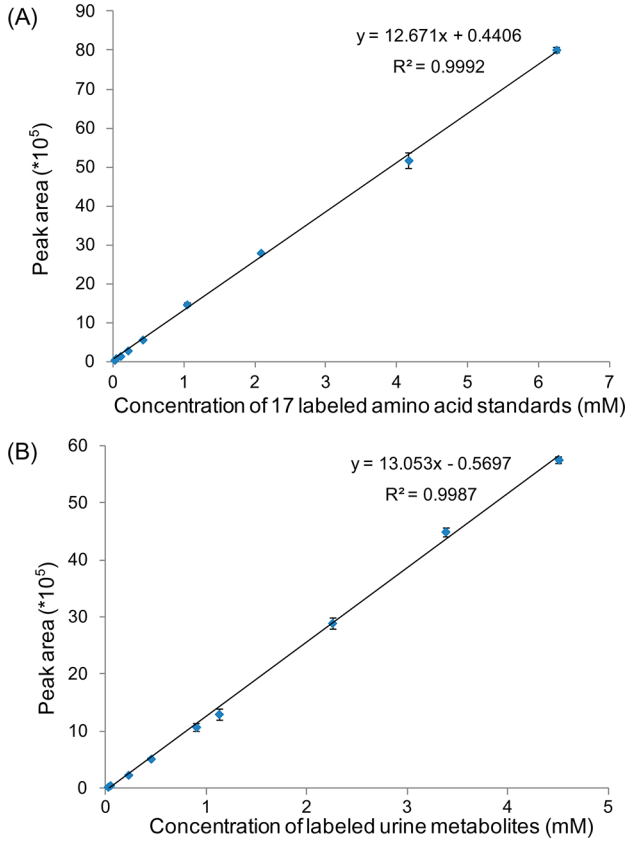

Figure 2. (A) Calibration curve of the mixture of 17 labeled amino acid standards (17-aa-std) from triplicate labeling experiments at each concentration. The labeled amino acid mixtures were diluted before dansylation. (B) Calibration curve of a labeled urine sample from a series of dilution of the highest urine concentration which was labeled in triplicate experiments. The concentration of the labeled metabolites in each diluted sample of the labeled urine was calculated from the undiluted labeled urine concentration determined from panel A by multiplying by the dilution factor at each data point.

mM amino acids with very good correlation (R^2 = 0.9992). In this case, a series of diluted 17-aa-std solutions were prepared and labeled individually. A similar curve was obtained when 6.25 mM standard solution was diluted after labeling, indicating that one can establish the calibration curve by preparing diluted solutions either before or after labeling. As dilution after dansylation is more convenient and consumes fewer reagents, this strategy was used for all the subsequent works. However, it should be noted that, if too high concentrations of analytes (>6.25 mM standard solution) were used to prepare the stock solution, a nonlinear response was observed due to the decreased dansylation efficiency as the relative amount of the

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dansyl chloride reagent was not sufficient. The dansyl chloride used for the reaction was 3.35 μmol, while the deviation from linearity became significant from 1.75 μmol of amino acids and no UV signal saturation was observed at these concentrations. Therefore, in order for the dansylation reaction to be complete, the amount of dansyl chloride needs to be more than ∼2-fold in excess. The low end of this linear range was limited by the formation of side products, such as Dns-NH_2 and Dns-$N(CH_3)_2$.[27] However, the acquired linear range of the calibration curve was adequate for quantification of most urine samples, as it will be discussed later.

Because the analyte composition of biological samples can be very different, it is important to investigate how well a calibration curve established from one sample can be used to quantify the amount of metabolites in another sample. To do this, we compared the calibration curves of the 17-aa-std and a mixture of 15 other labeled amine and phenol standards (15-std-mix) with varying structures (see Supplemental Figure S4, Supporting Information). These curves were obtained independently based on the actual concentration of standards used. Similarities in UV absorptivity were evaluated by comparing the slopes (sensitivity) of the two calibration curves using a modified student *t* test at 95% confidence level,[28] and the result showed that there was no statistical difference between these two slopes. In addition, if we use the linear regression equation obtained from the 17-aa-std to calculate the concentration of the 15-std-mix, the error was less than 4%. These results indicate that, although the absorptivity of individual dansyl metabolites at 338 nm can be different (some evidence are shown in Supplemental Figure S1, Supporting Information), the average absorptivity of a mixture of many dansyl labeled metabolites can be very similar, because the high absorption of some metabolites can be averaged out by other low absorption compounds. Finally, as it is shown below, there is no significant difference between the slopes of the 17-aa-std and the labeled urine curve. Therefore, we can use the calibration curve of the 17-aa-std to determine the absolute concentration of the total labeled metabolites in biological samples. We note that mixtures of amino acids can be purchased from chemical suppliers and readily prepared for dansyl labeling, providing a convenient means of establishing a calibration curve.

Quantification of Labeled Metabolites in Urine. Figure 1B shows the elution profile of the labeled urine, which is different from that of the labeled amino acids, because there are so many metabolites in the urine sample that elute out closely together, which tends to smooth the chromatographic peaks. As a result, fewer peaks were observed for urine. The calibration curve for urine samples was established from a pooled urine sample, with dilution from 1.3- to 200-fold (see Figure 2B). In this case, a pooled urine sample was labeled with dansyl chloride and the peak area of the labeled urine was measured by LC-UV. The total concentration of the dansylated metabolites in the labeled urine was determined using the calibration curve shown in Figure 2A, assuming that the absorptivity of the labeled urine is the same as that of the labeled 17 amino acid standards. The labeled urine was then diluted to produce a series of diluted samples for LC-UV measurements. The concentration of each diluted sample was calculated by taking the concentration of the undiluted sample and multiplying by the dilution factor or 1/dilution-fold.

As it is shown in Figure 2B, a linear relationship was observed between peak area and the labeled urine concentration. The slope of the curve is statistically indifferent from that of the 17-aa-std, proving that the average absorptivity of the labeled 17 amino acid standards is indeed the same as that of the labeled urine metabolites. Thus, the absolute concentration of the labeled urine metabolites can be calculated from the measured absorbance or peak area against the calibration curve of the labeled amino acid standards. In this case, the original urine concentration lies well within the linear range of the labeled amino acid standards. However, we note that, in the use of this method for absolute quantification of labeled urine metabolites, occasionally the original urine concentration of an individual sample could be slightly higher than 6.25 mM (the upper limit of the calibration curve shown in Figure 2A), which can result in incomplete labeling. Therefore, in our work, all of the original urine samples were diluted 2-fold prior to dansylation reaction to ensure the labeling was complete. For dansylation reaction, there was no matrix effect from the urine sample. A simple procedure to check the matrix effect on dansylation reaction is provided in Supplemental Note N1, Supporting Information.

It should be noted that chemical labeling to "unify" the absorptivity of a known metabolite mixture (used as the calibration standard) and the urine samples is critical for determining the total concentration of the urinary metabolites. Without labeling, total metabolite quantification by UV absorbance measurement is not possible. For example, it is anticipated that some metabolites in urine would have functional groups that can absorb at 338 nm. Indeed, if the chromatogram at 338 nm of unlabeled urine was examined, there was a small peak that has a similar retention time as the dansyl labeled peaks. Since this peak area was also proportional to urine concentration, we investigated the ability of the use of unlabeled urine for quantification. We compared the slopes of the calibration curves established at different wavelengths and retention time windows for three individuals' urine samples (Supplemental Figure S5, Supporting Information). We found there were several problems associated with direct quantification of the unlabeled urine. First, the slopes of curves depend heavily on the wavelength used. For example, for the peak at 1.43−1.5 min, the slopes at 338 nm were all similar for three individuals (Figure S5C, Supporting Information). However, at 280 nm, the slope for individual B becomes different from individual A and C (Figure S5B, Supporting Information), and at 254 nm, all three slopes are significantly different from each other (Figure S5A, Supporting Information). Likewise, for the peak at 0.22−1.3 min, the slopes at 254, 280, and 338 nm are also very different (Figure S5D−F, Supporting Information). As a result, it is very difficult to choose a wavelength at which the absorptivity is similar for all different individuals. Second, although the slopes at 338 nm and 1.43−1.5 min were similar for all three individuals, the peak area was much smaller compared to the dansyl peaks (<5%); therefore, the linear range was significantly reduced at the lower end. Moreover, since there is no good standard for calibration, it would be difficult to determine the absolute concentration of the metabolites. Thus, determination of total metabolite concentration cannot be done by direct UV analysis of urine samples.

Optimization of Sample Loading to LC-FTICR-MS. The ability of the LC-UV method for quantification of labeled metabolites allows us to control the amount of sample to be injected into the LC-ESI-FTMS instrument. In order to investigate the effect of sample injection amount on the FTMS results, the same sets of pooled urine samples (in

triplicate) used to establish the calibration curve were injected into the instrument with an injection volume of 2 μL. The urine samples were prepared by mixing equal amount of ^{12}C- and ^{13}C-labeled solutions, which will give peak pairs with a mass difference of 2.0067 in the FTMS run. The number of peak pairs obtained was plotted against the labeled urine concentration as shown in Figure 3. It is clear that the number

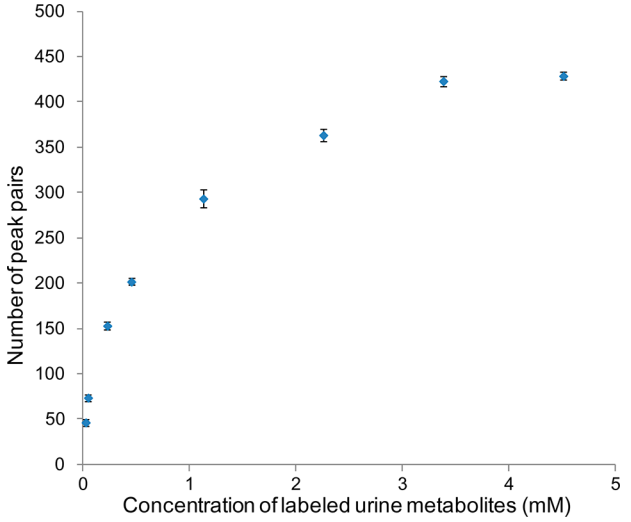

Figure 3. Number of peak pairs detected by LC-FTICR-MS vs labeled urine metabolite concentration (n = 3). The MS data was preprocessed to exclude peak pairs with ratios of larger than 1.5 or smaller than 0.67, as well as peaks with intensity of less than 25 000 counts (i.e., S/N < 10).

of peak pairs increases as the labeled urine concentration increases, because at lower concentrations, the low abundance peak pairs would be buried in the background and thus either become undetectable or be filtered out during the data processing step (i.e., S/N < 10).

As Figure 3 shows, when the labeled urine concentration is sufficiently high, the number of peak pairs levels off. This can be explained by considering the dynamic range of FTICR and the ion suppression effect of the ESI source. Compared to some other mass spectrometers, such as quadrupole MS, FTICR-MS has relatively small ion detection dynamic range.[29] If the sample concentration is too high, it is possible that the detection cell will be overloaded with ions, which can obscure the detection of small peaks from low abundance or less ionizable metabolites eluted out close together. The ESI source also poses an upper limit on the number of ions, because at high concentrations analyte competition for the limited space or charge on the droplet surfaces becomes important. In this case, the number of peak pairs comes to a plateau at a labeled urine concentration of 3.4 mM with 2 μL of injection volume. Therefore, in the subsequent metabolome profiling work, the urine amount was adjusted to be equal to this value for optimal sample injection.

Normalization of Urine Sample Concentrations for Differential Isotope Labeling LC-MS. The LC-UV quantification method was applied to the normalization of two individuals' three-day urine samples. For each day, three urine samples were collected and denoted as A, B, and C. Sample B was collected 1 h after sample A without drinking water. Sample C was collected 1 h after sample B, but a large amount of water was taken during this interval. It is therefore expected that the urine concentration of sample A should be similar to sample B, and sample C would be much diluted. Indeed, the chromatographic peak area of sample C was significantly smaller than that of A and B for all three-day samples of the two individuals (data not shown). Triplicate experiments of dansylation were done for each sample, and the peak area variation was found to be in the range of 0.2−6.6%, which indicates good reproducibility for the labeling reaction. The peak areas obtained from the LC-UV measurement were compared to creatinine assay and osmolality measurement results of the same set of urine samples (Supplemental Figure S6, Supporting Information). Since this is a relatively simple set of samples that only involve two healthy individuals in three consecutive days, it is unlikely to have a large variation in creatinine excretion. As Supplemental Figure S6, Supporting Information, shows, a good correlation was obtained in both cases, which is quite reassuring on the validity of the LC-UV method for sample normalization. However, LC-UV quantification is more reproducible than creatinine assay and osmolality measurement, as the relative standard deviation for those two methods can be as high as 20%. Moreover, the LC-UV method can potentially be applied to many different biofluids. As indicated in the introduction, the creatinine normalization method cannot be applied to samples where the concentration of creatinine itself varies due to biological processes or creatinine is totally absent in a sample (e.g., in cell extracts). The osmolality method also has shortcomings, such as inaccuracy due to salt content variations in samples.

We have incorporated this LC-UV sample normalization method into the differential isotope labeling LC-MS metabolome profiling workflow. The isotope labeling strategy allows us to use a ^{13}C-labeled pooled urine as the internal standard, while each individual sample is ^{12}C-labeled. For quantitative analysis, we can compare the metabolite concentration in two samples by comparing their ^{12}C-/^{13}C-peak ratio, if the same amount of ^{13}C-labeled pooled urine was used.[22] In a previous work, the pooled urine was prepared by adding equal volume of each individual sample. However, the problem of this pooling strategy is that the contribution of each sample would be different due to different concentrations, and some of the low abundance metabolites in low concentration samples may be lost. By taking advantage of the quantification method described here, we can prepare a pooled urine sample by adjusting the volume of individual samples so that an equal amount of each sample was aliquoted for pooling. This procedure can alleviate the bias toward high concentration samples. More significantly, when a ^{12}C-labeled individual sample is taken to mix with the ^{13}C-labeled pooled urine, the volume or concentration of the individual sample can be normalized based on its labeled urine concentration to ensure an equal amount of an individual sample and the pooled urine is mixed.

In order to examine the effect of normalization, two sets of samples were prepared. The first set of samples were un-normalized (denoted as UN). These samples were prepared by mixing equal volume of each ^{12}C-labeled urine sample with the ^{13}C-labeled pooled urine. The second set of samples was normalized (denoted as NOR) by measuring the concentration of the labeled urine metabolites in each sample with the use of the calibration curve shown in Figure 2B and the peak area of the eluted labeled metabolites in LC-UV and then adjusting the volume of each sample to ensure an equal sample amount was mixed with the pooled sample. At the same time, the volume was calculated to ensure the total amount of the ^{12}C- and ^{13}C-

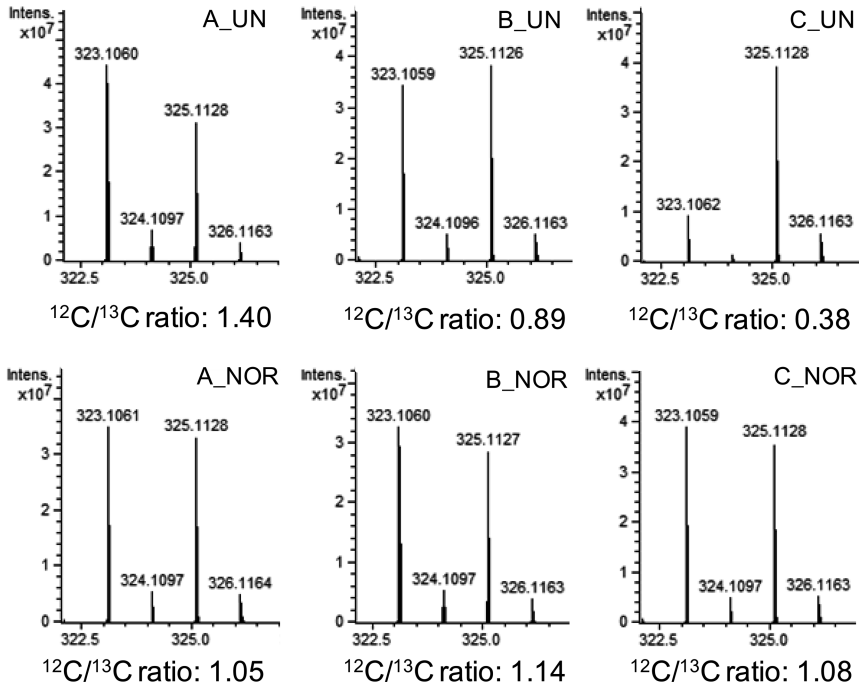

Figure 4. Representative mass spectra showing the ratios of a selected peak pair belonging to Dns-alanine.

labeled samples was the same before mixing. In both cases, the same pooled urine sample was used with a concentration of 3.4 mM. The injection volume was adjusted to account for the volume variation after mixing so that the injection amount in each case remained optimal.

Figure 4 shows the representative mass spectra of a selected peak pair and the calculated $^{12}C/^{13}C$ ratios. This peak pair was identified to be Dns-alanine by matching the accurate mass and retention time with the amino acid standard solution in our standard library. For the un-normalized samples, the ratio for sample C was much smaller than samples A and B, while the ratios were all similar for the normalized samples. Since the ^{13}C-labeled pooled urine amount was the same in each sample, we can calculate the ratios of A/B, B/C, and A/C from their $^{12}C/^{13}C$ ratios. For the un-normalized samples, the ratios were A/B = 1.57, B/C = 2.34, and A/C = 3.68, and the ratios for the normalized samples were A/B = 0.92, B/C = 1.06, and A/C = 0.97. Because the samples were collected in 2 h, one would expect that the amount of most metabolites would not change significantly, and therefore, the ratios should be close to 1. This was the case for the normalized samples, but for the un-normalized samples, the B/C and A/C ratios were clearly skewed due to the dilution of sample C.

Similar observations were obtained when we examined the intraday sample peak ratio distributions using box plots, as shown in Figure 5. The log ratios between the intraday samples should be close to 0 for most metabolites under the reasonable assumption that there are little biological variations during the 2 h collection interval, which should result in a box with a small spread around 0. This is illustrated to be the case in the box plot of the normalized samples (Figure 5A) for both individuals. For the un-normalized samples (Figure 5B), the log A/B ratio was still close to 0 because the concentrations of samples A and B were similar. However, the log ratios for A/C and B/C show significant deviation from 0, due to the dilution effect in sample C. Thus, there would be a large error, if we used these ratios for metabolite quantification.

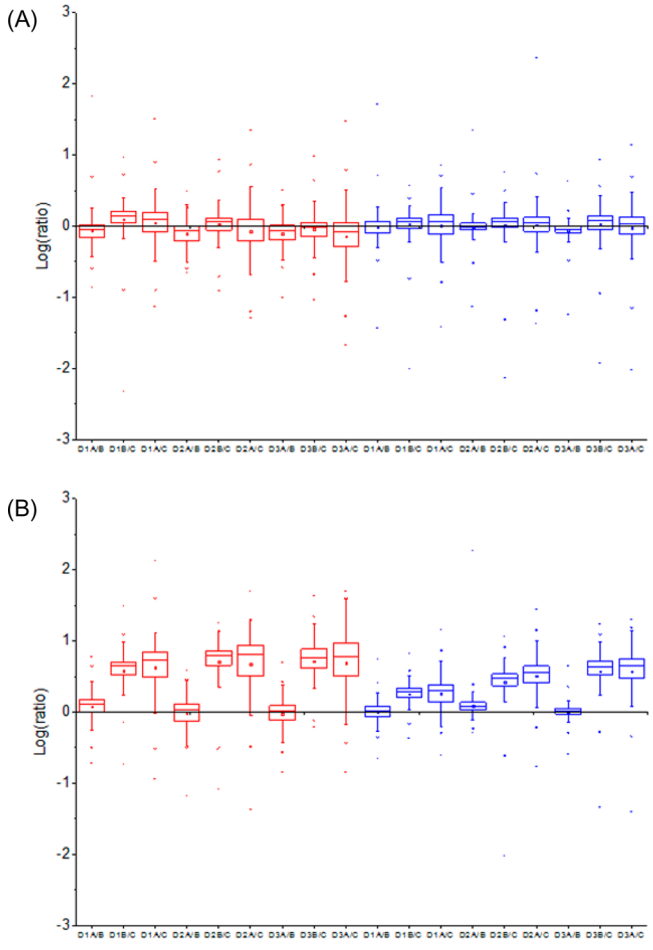

Figure 5. Box plots of the log intraday ratio for individuals 1 (in red) and 2 (in blue): (A) normalized data and (B) un-normalized data. The range of the box is 25 to 75 percentile. The line in the box represents the median value, and the mean value is shown as a dot in the box.

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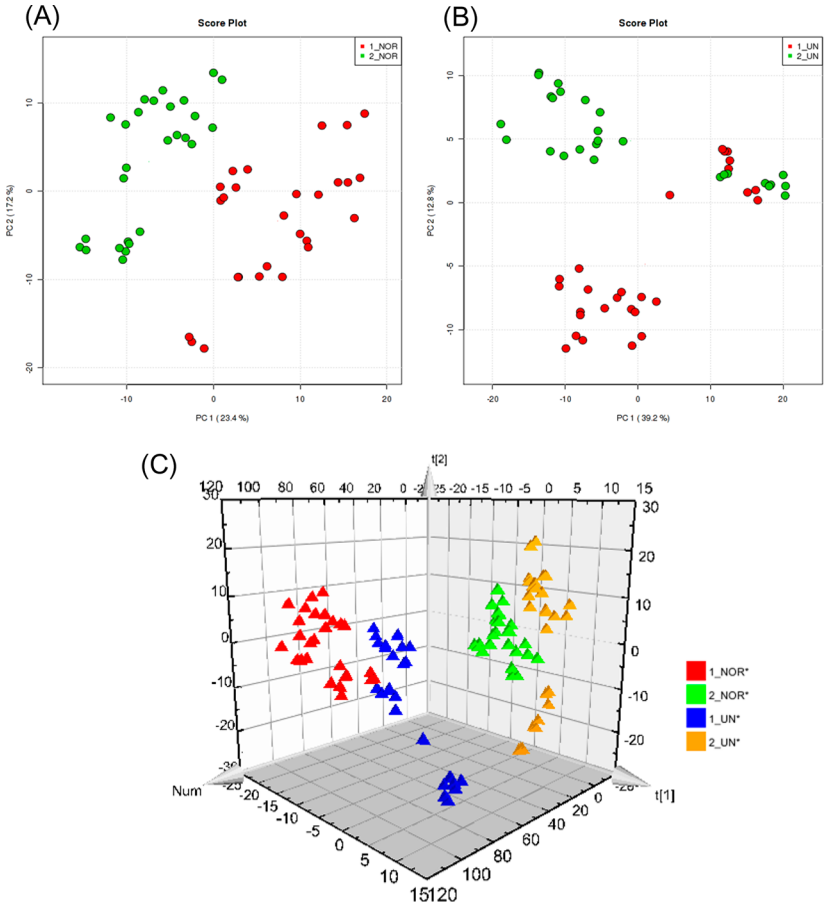

Figure 6. PCA plots for (A) normalized urine samples and (B) un-normalized urine samples: 1_NOR (in red), normalized urine samples from individual 1; 2_NOR (in green), normalized urine samples from individual 2; 1_UN (in red), un-normalized urine samples from individual 1; 2_UN (in green), un-normalized urine samples from individual 2. (C) PLS-DA plots of normalized and un-normalized urine samples: 1_NOR (in red), normalized urine samples from individual 1; 2_NOR (in green), normalized urine samples from individual 2; 1_UN (in blue), un-normalized urine samples from individual 1; 2_UN (in yellow), un-normalized urine samples from individual 2.

The whole set of data was analyzed by multivariate analysis. The unsupervised PCA was first applied to the normalized and un-normalized samples to generate an overview on how the data was scattered, as illustrated in panels A and B of Figure 6, respectively. It can be seen that separation between the two individuals was observed in both cases. However, for the normalized samples, the data was scattered more randomly, and the difference between the two individuals was mostly reflected by the first principal component. On the other hand, the separation between individuals of un-normalized samples was mainly attributed to the second principal component, and subgroups within each individual were observed due to different sample concentrations. In particular, the low concentration samples from the two individuals tend to gather together to form a third group, as shown on the middle right of Figure 6B. In order to better visualize these subgroups within each individual, supervised PLS-DA was utilized to make the classification of the two individuals. Figure 6C shows the 3D plot established using this model. The index number (Num) was used as one axis in order to separate the different sets for easier visualization. The PLS-DA fit criteria for the normalized samples were found to be $R^2 = 0.978$ and $Q^2 = 0.941$, indicating an excellent model, and overfitting is not a main concern here because this model is not forced to show separation of the subgroups.[30] It is clear from the plot that, without normalization (blue and yellow), different subgroups were artificially created based on sample concentrations. On the other hand, the normalized samples (red and green) only show separation of two individuals, with no further division into subgroups. These results illustrate that the LC-UV sample normalization strategy can overcome the problem of artificial separation caused by the variations of the original urine concentrations in relative quantification of urine metabolomes using differential isotope labeling LC-MS.

CONCLUSIONS

A strategy of determining the total concentration of chemically labeled metabolites and its incorporation into a differential isotope labeling LC-MS workflow has been developed and demonstrated for improved relative quantification of urine metabolomes. Quantification of the total concentration of labeled metabolites can be done using a fast step-gradient LC-UV method, in combination with chemical labeling to "unify" the UV absorptivity of diverse metabolites in a metabolome sample. In the present work, dansylation chemistry was used to label the metabolites containing amine and phenol groups, followed by LC-UV detection at 338 nm of the labeled sample in about 2 min. Knowing the total concentration of the labeled metabolite analytes, sample amount or concentration during the metabolome sample workup can be normalized to account for concentration variations in different metabolome samples. In addition, the amount of the sample injected into the mass

spectrometric detection system can be optimized and ultimately controlled to maximize the metabolite detectability to improve metabolome coverage. While this present work focuses on urinary metabolome profiling using isotope labeling LC-MS, this dansyl labeling LC-UV method, in principle, should be applicable to any other biological samples and MS platforms where knowing the total concentration of metabolites is desirable for optimal metabolome analysis.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Figures S1−S6 and Note N1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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