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Dansylation Metabolite Assay: A Simple and Rapid Method for Sample Amount Normalization in Metabolomics

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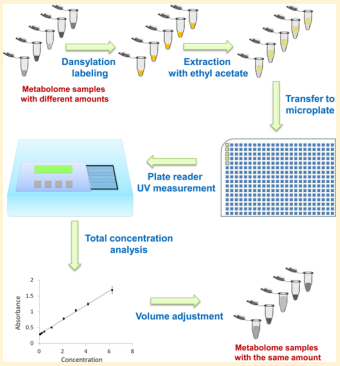
Supporting Information

ABSTRACT: Metabolomics involves the comparison of the metabolomes of individual samples from two or more groups to reveal the metabolic differences. In order to measure the metabolite concentration differences accurately, using the same amount of starting materials is essential. In this work, we describe a simple and rapid method for sample amount normalization. It is based on dansylation labeling of the amine and phenol submetabolome of an individual sample, followed by solvent extraction of the labeled metabolites and ultraviolet (UV) absorbance measurement using a microplate reader. A calibration curve of a mixture of 17 dansyl-labeled amino acid standards is used to determine the total concentration of the labeled metabolites in a sample. According to the measured concentrations of individual samples, the volume of an aliquot taken from each sample is adjusted so that the same sample amount is taken for subsequent metabolome comparison. As an example of applications, this dansylation metabolite assay method is shown to be useful in comparative metabolome analysis of two different *E. coli* strains using a differential chemical isotope labeling LC-MS platform. Because of the low cost of equipment and reagents and the simple procedure used in the assay, this method can be readily implemented. We envisage that, this assay, which is analogous to the bicinchoninic acid (BCA) protein assay widely used in proteomics, will be applicable to many types of samples for quantitative metabolomics.



In recent years, liquid chromatography–mass spectrometry (LC-MS)-based metabolomics techniques have become a popular choice for the study of biological processes and biomarker discovery.[1−3] In LC-MS-based metabolomics, individual samples from two or more groups are analyzed to study the metabolome profile differences among these samples. Because the total concentration of metabolites can vary significantly from sample to sample,[3−5] sample amount normalization to equalize the amounts of individual samples prior to quantitative analysis is required in order to generate accurate and precise results. In this paper, the term "normalization" refers to adjust either the volume or concentration of an individual sample so that the same amount is taken from all the individual samples used in a metabolomics study.

Ideally, a good normalization strategy for metabolome analysis should have the following features. First, it should be convenient to perform and should not add too many extra steps or cost to the overall sample processing procedure. Second, it is desirable to carry out normalization after the initial sample preparation steps so that any variations during the sample workup process can be taken into account. Third, it is preferable to perform sample normalization before LC-MS analysis to ensure that similar instrumental responses are obtained for all samples. Because of nonuniform responses of individual metabolites, analyte signals obtained from different concentrations of samples cannot be linearly scaled. Thus, using the same concentration of samples for LC-MS will produce more-accurate results. Fourth, normalization can provide information on the absolute concentration of the samples, relative to a standard.[6] This would allow a user to control the sample injection amount into LC-MS precisely to ensure that an optimal amount is injected. This is important for detecting low concentration metabolites in a sample and avoiding overinjection that can cause problems such as column saturation and sample carryover from one run to another. Finally, the normalization method should be universally applicable to all biological media.

There are several normalization methods reported in the literature for metabolomics. Normalization to creatinine or osmolality has been used for urine samples.[7−10] However, in some cases, creatinine itself may vary, according to the disease state.[11] Normalization to cell counts, total protein concentration or DNA concentration has been described for cultured cells;[12−14] the cell counts or protein/DNA amounts were shown to be useful as sample amount indicators. However, these methods were targeted at specific biological media and cannot be readily extended to other biological samples such as saliva, cerebrospinal fluid (CSF), and fecal samples. For these types of samples, using the same volume or weight does not guarantee that the same total amount of metabolites is taken from each sample. Post-analysis data normalization strategy has also been reported in recent years. The advantages of this strategy are that it is convenient to perform (i.e., no extra experimental procedures required) and widely applicable. Various forms of this strategy has been reported, including

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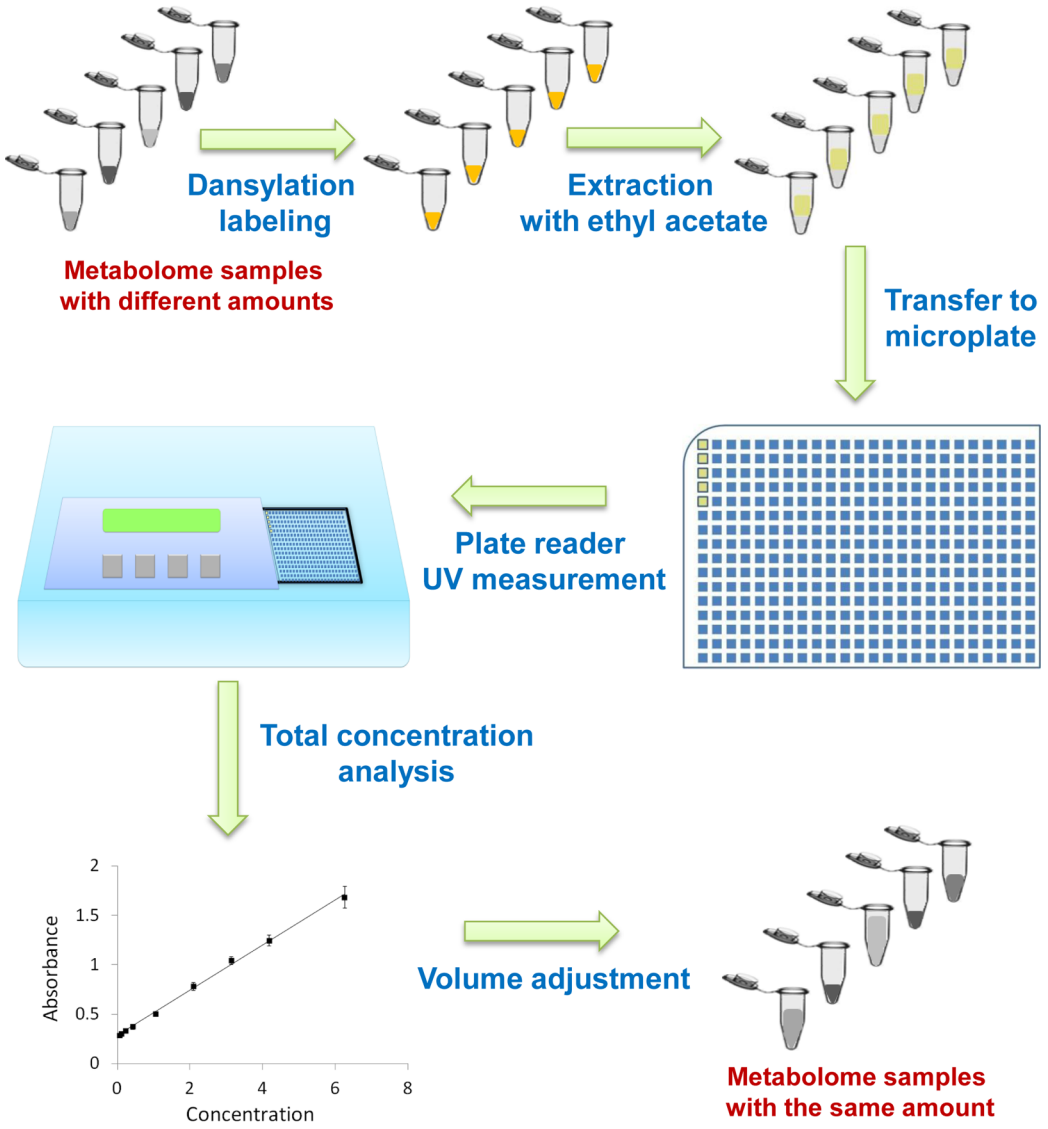

Figure 1. Dansylation metabolite assay (DMA) workflow for sample amount normalization.

normalization to the sum of all metabolite abundance,[15] normalization to the MS "total useful signal",[16] as well as normalization to specific metabolic markers.[4,17] However, the major disadvantage of this strategy is the lack of control of the sample amount injected into a mass spectrometer. As a result, uneven LC-MS responses may be generated from samples of different concentrations, which can compromise the accuracy and precision of metabolite quantification, as well as the metabolome coverage.

We have recently reported a sample normalization method based on the use of liquid chromatography−ultraviolet analysis (LC-UV) for quantifying the total concentration of chemically labeled metabolites.[6] In this method, metabolites were first labeled with ^{12}C-dansyl chloride and the absorbance was measured at 338 nm, targeting the dansyl chromophore; dansylation labeling is a robust and proven method used in techniques such as LC-UV, LC-fluorescence, and MS.[18−22] A fast step-gradient was applied to allow coelution of all labeled metabolites, and the total absorption was measured to determine the sample concentration. This method can be readily applied to any type of biological samples and has been demonstrated to be useful as a sample normalization strategy in various applications.[23−27] However, one major drawback of this method is that it requires an expensive LC-UV system to perform the analysis. The cost per analysis can be relatively high, considering the high consumption of high-performance liquid chromatography (HPLC)-grade solvents (and columns). Another drawback is that sample throughput is not high. For a large-scale metabolomics study, this can be a concern.

In this work, we report a dansylation metabolite assay sample normalization method that measures absorbance of labeled metabolites using a microwell plate reader, instead of an LC-UV system. Microwell plate readers are relatively inexpensive and commonly used in biological laboratories for measuring total concentration of proteins or DNA. The dansylation metabolite assay allows simultaneous measurement of multiple samples within a very short period, which greatly increases the throughput. It also requires less laboratory consumables and instrument maintenance.

EXPERIMENTAL SECTION

Supplemental Note N1 in the Supporting Information provides information on chemicals and reagents, *E. coli* cell culture and harvest conditions, determination of the protein amount of cell

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extracts, metabolite extraction, dansylation labeling,[28,29] and LC-UV quantification. The other key experimental procedures are described below.

Microplate Quantification of Labeled Metabolites. To extract the labeled metabolites into the organic layer, three volumes of ethyl acetate were added to the labeled solution, followed by treatment in a vortex for 30 s and centrifugation at 5220*g* for 2 min. For quantification of labeled amino acid standards, 25 μL of the organic layer was pipetted into a Greiner UV-Star 384-well microplate (Monroe, NC, USA) and absorbance measurement was made at 340 nm on a SpectraMax 340PC plate reader from Molecular Devices (Sunnyvale, CA, USA), while 50 μL of the organic layer was added into the plate for quantification of labeled metabolites in *E. coli*.

LC-MS Analysis and Data Processing. For non-normalized analysis, the ^{12}C- and ^{13}C-dansyl labeled solutions were combined in a 1:1 volume ratio. For normalized analysis, the ^{12}C- and ^{13}C-labeled metabolites were mixed in a ratio determined by the quantification results. The combined mixture was analyzed using a Bruker Maxis Impact QTOF mass spectrometer (Billerica, MA, USA) linked to an Agilent 1100 series binary HPLC system (Palo Alto, CA, USA). The LC-MS conditions were the same as those reported.[30] For each LC-MS run, masses were calibrated to the spectrum that contained the dansyl-amine peaks at m/z 242.57160 (two tags, two charges), m/z 484.13592 (two tags, one charge) and m/z 971.27799 (dimer), using the Data Analysis software, and the calibration result was applied to all the other spectra in the same LC-MS run. The resulting MS data were processed using a peak-pair picking software, IsoMS.[30] The level 1 peak pairs[30] were aligned from multiple runs by retention time within 30 s and accurate mass within 5 ppm. Only the common peak pairs were retained for statistical analysis. Principal component analysis (PCA) and volcano plot analysis were performed by Metaboanalyst (www.metaboanalyst.ca).[31] The data were mean-centered and autoscaled (unit variance) prior to analysis.

RESULTS AND DISCUSSION

Figure 1 shows the overall workflow of the dansylation metabolite assay (DMA) for normalization of biological samples using a microplate reader. The assay involves three key steps: dansylation labeling of amines and phenols in a sample, extraction of the labeled metabolites using ethyl acetate, and UV absorbance measurement of the organic extract. Based on the measured total concentration of the labeled metabolites, the same sample amount is taken from all of the samples. To measure the absolute concentration of labeled metabolites relative to a standard in a sample, a calibration curve of a standard (e.g., a mixture of 17 dansyl labeled amino acid standards or 17-Dns-aas) can be used. In developing this assay, several experimental parameters and procedures were considered, which are described below. The assay was then applied to a cellular metabolomics study to evaluate its performance.

Extraction of Labeled Metabolites. After the dansylation labeling reaction, excess dansyl chloride is quenched with sodium hydroxide to form the hydrolyzed product (Dns−OH). To quantify the labeled metabolites, Dns−OH must be removed from the labeled solution prior to quantification. In LC-UV,[6] Dns−OH elutes at the high aqueous phase and the labeled metabolites elute at the high organic phase; thus, a step-gradient elution can be used to separate them. This LC retention behavior also indicates that Dns−OH is much more hydrophilic, compared to other labeled metabolites, suggesting the possibility of their separation by using a simple liquid−liquid extraction (LLE) method. We evaluated the performance of LLE with ethyl acetate, a commonly used extraction solvent with moderate insolubility, relatively high boiling point, and low toxicity (see Supplemental Note N2 in the Supporting Information). The optimized extraction protocol involves a one-time extraction with 3 volumes of ethyl acetate and 1 volume of labeled solution, which gives an extraction efficiency of ∼93% for the labeled amino acid standards.

Absorbance Measurement. The absorbance of dansyl-labeled metabolites was measured at 340 nm, which is the smallest detection wavelength available on the SpectraMax 340PC plate reader. This wavelength is close to the wavelength used in the LC-UV sample normalization method (338 nm).[6] Supplemental Figure S1 in the Supporting Information shows the absorption spectrum of 17-Dns-aas; the absorbance starts to decrease at wavelengths higher than 340 nm. The measurement was done using a UV-transparent 384-well plate with low background absorbance at 340 nm. This plate also shows good resistance to ethyl acetate with an optimal working volume of 15−110 μL, which matches with our applications.

We prepared a series of diluted 17-aas solutions and labeled them separately to evaluate the linearity and linear range of this quantification method. In this case, 10 μL of labeled amino acid solutions were extracted with 30 μL of ethyl acetate, and 25 μL of each extracted solution was added into the 384-well plate. Supplemental Figure S2A in the Supporting Information shows the calibration curve generated, which is linear from 0.04 mM to 6.25 mM with good correlation (R^2 = 0.9981); note that the three points at the lower end depart from this linear curve, to some extent. As in the case of our LC-UV work,[6] the upper end of this linear curve was limited by the decreased dansylation labeling efficiency at higher analyte concentrations. Increasing the reagent amount relative to the analyte amount can extend the upper limit, but this is not needed, since the upper limit shown in Figure S2A in the Supporting Information is already sufficient for normalization of metabolomic samples. The lower end was limited by the formation of side products that produced the background signals. We note that the lower end of this linear curve (0.04 mM) was higher than that which could be achieved with LC-UV (0.02 mM), since the sensitivity (slope) of the current method was lower; the absorbance of 0.02 mM standard was close to the absorbance of a labeled blank solution. Nevertheless, the linear range of this calibration curve should be sufficient for quantification of most real biological samples. Decreasing the detection limit of the assay is not necessary, as the total concentration of labeled metabolites in most metabolomic samples would be in the range of high μM to mM. In addition, the sensitivity can be improved by increasing the path length (i.e., increasing the volume of solution added into a well). From our working experience with different types of samples, we suggest that the volume of a solution used for the microplate reader measurement be adjusted, depending on the type of biological samples analyzed. For example, we found that 25 μL was generally sufficient for quantification of human urine samples, while a larger volume (e.g., 50 μL) was optimal for analyzing the extracts of bacterial cells in which the total metabolite concentration is lower than that of urine.

Method reproducibility was evaluated in terms of experimental reproducibility and run-to-run reproducibility. Supplemental Table T1 in the Supporting Information shows a

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summary of the results obtained. In this case, experimental reproducibility was determined from three experimental replicates that should account for variations during labeling, extraction, solution transfer, and absorbance reading. As Supplemental Table T1 in the Supporting Information shows, the percent relative standard deviation (%RSD) values at different 17-Dns-aas concentrations are all below 10%, indicating a good experimental reproducibility. Run-to-run reproducibility was determined by measuring absorbance at 0, 5, and 10 min after the samples were added into the plate. In practice, there will be a time interval between the first added sample and the last added one. Although ethyl acetate has a relatively high boiling point, compared to other organic solvents, some evaporation might still occur during this time period. Therefore, we evaluated the run-to-run reproducibility within a 10-min period to determine whether solvent evaporation would affect the measurement. As shown in Supplemental Table T1 in the Supporting Information, the % RSD values within a 10-min period were only 1%−3%, indicating that solvent evaporation would not cause a problem for absorbance measurement of samples added within a 10-min interval. This usually allows ∼30 samples to be processed and measured at the same time. Using a multichannel pipet should increase the number of samples handled in a 10-min interval.

Quantification of Labeled Metabolites in *E. coli*. As an example of applications of DMS for sample amount normalization, we applied this method for metabolomic profiling of *E. coli* cells. We first determined the linearity between the measured absorbance and the cell amount. *E. coli* cells from the same culture medium were aliquoted into 0.25-, 0.5-, 1-, 2-, 4-, and 8-mL portions, and metabolites in each portion were extracted and labeled under the same conditions. A good linear relationship ($R^2 > 0.99$) was observed between the absorbance of the labeled metabolites and the volume of the cell suspensions (Supplemental Figure S2B in the Supporting Information) (note that the last point at the lower end departs from the linear curve to some extent), indicating that the total amount of extracted metabolites correlates very well with the cell amount. Thus, in real-world applications where the number of cells is not known, the DMA quantification results can serve as a surrogate of the cell amount. To validate this, we compared the metabolite measurement results obtained by our assay to the protein amounts measured using a well-established BCA quantitative method.

In this work, two different *E. coli* strains (ATCC 47076 and ATCC 9637) were each grown on five agar plates. During harvest, different amounts of cells were collected from each plate. Because of the small size of *E. coli* cells, cell counting using a microscope was difficult. Instead, we measured the protein concentrations as the cell amount indicator and then compared the absorbance values of the labeled metabolites measured by the dansylation assay with the measured protein amounts. The results are shown in Supplemental Figures S3A and S3B in the Supporting Information for *E. coli* ATCC 47076 and ATCC 9637, respectively. Figure S3 in the Supporting Information shows that there is a good linear correlation between the absorbance and the protein amount for both strains. Supplemental Note N3 in the Supporting Information provides more discussion on comparison of the dansylation assay and the BCA assay.

Sample Amount Normalization in *E. coli*. To evaluate whether the labeled metabolite absorbance measurement could serve as a valid normalization strategy for metabolomics, we compared the metabolomic profiles of the two *E. coli* strains with and without normalization. A differential isotope dansyl labeling LC-MS platform[24,28,30] was used to profile the amine/phenol submetabolome differences of the two strains. The non-normalized set of samples was prepared by mixing equal volumes of each ^{12}C-labeled sample with a ^{13}C-labeled pool, while the second set of samples was normalized based on the absorbance values of ^{12}C-labeled individual samples. It is important to note that there is background absorbance from a labeled solution, which is likely caused by the presence of byproducts of the labeling reagent (e.g., dimerization). Therefore, a blank subtraction step must be carried out first, which can be done using a predetermined calibration curve, where the *y*-intercept reflects the background absorption (see Supplemental Table T2 in the Supporting Information for more details).

Metabolome Comparison of Two *E. coli* Strains. One prerequisite for a fair comparison between different sample groups is that the variation within each group should be small.[4] In this example of comparing the metabolome profiles of two *E. coli* strains, we first applied a PCA model to the two metabolome datasets (see Figure 2). As Figure 2A shows, for the non-normalized sample dataset, separation between the two strains is attributed to the second principal component (19.7% of the total variation), while the most important variation reflected by the first principal component is the cell amount (65.8% of the total variation). In contrast, for the normalized sample dataset (Figure 2B), the two strains are clearly separated on the first principal component, which represents 43.2% of the total variation, indicating that metabolic difference between the two strains is the major variation in the dataset. These PCA score plots illustrate that our sample normalization strategy is effective in reducing the artificial variation caused by different sample amounts used in the non-normalized dataset.

We further analyzed the two datasets using the volcano plot statistical analysis. Supplemental Figure S4 in the Supporting Information shows the volcano plots generated by examining the differentiating metabolites with the criteria of fold change (FC) > 2 or FC < 0.5, and $p < 0.01$. Only 29 metabolites were found to be differentially expressed using these criteria in the non-normalized samples, while there were 145 metabolites at significantly different levels in the normalized dataset. The much-lower identification rate in the non-normalized dataset is mainly due to the large variations within each strain caused by the sample amount differences. Supplemental Figure S5 in the Supporting Information shows the %RSD values of relative metabolite quantities measured from multiple samples within each strain. In the normalized data, the %RSD values were reduced by almost 50%. These results again confirm that the variations within each strain have been reduced through this normalization process to allow identification of a larger number of differentiating metabolites between the two strains.

The above example demonstrates that sample amount normalization is very important for comparative metabolomics and the dansylation metabolite assay can be used as a simple and rapid normalization method. It should also be noted that this assay is not destructive if the dansyl-labeled sample is used for metabolome profiling, as in the case of using differential dansyl labeling for profiling the amine/phenol submetabolome. After metabolite quantification is finished, the solution can be recollected for further analysis. Although this assay only measures the total concentration of labeled amine/phenol submetabolome, the large diversity of amines and phenols in a

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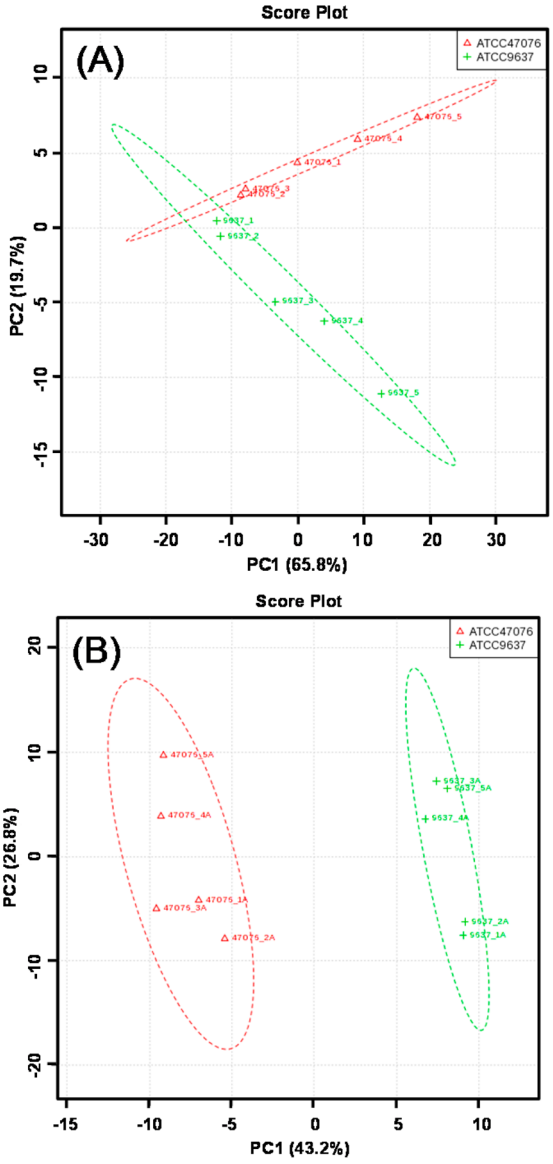

Figure 2. PCA score plots for (A) non-normalized dataset and (B) normalized dataset. Red triangles and green crosses represent *E. coli* strains ATCC 47076 and 9637, harvested from five different agar plates, respectively.

metabolome sample ensures that the measured concentration is a good representation of the total metabolome concentration. This assay should be applicable to many types of biological samples, including biofluids. One potential limitation of this assay is that the UV measurement is done at 340 nm, and, thus, if a sample contains high concentrations of chemicals that absorb at 340 nm, interference from these chemicals may cause errors in the measurement of the labeled metabolites. However, considering that the total concentration of labeled metabolites is in the millimolar (mM) range for many samples, lower concentrations of 340-nm absorbing compounds present in a sample should not affect the quantitative results significantly.

CONCLUSIONS

We have developed a dansylation metabolite assay for sample amount normalization in quantitative metabolomics. It uses a microplate reader to measure the absorbance of labeled metabolites at 340 nm in a sample after dansylation labeling of the metabolites and ethyl acetate extraction to remove the quenched excess dansyl reagent. This method is simple, rapid, and easy to implement. We envisage the application of this dansylation metabolite assay, which is analogous to the widely used BCA assay in quantitative proteomics, as a robust sample normalization method in metabolomics.

ASSOCIATED CONTENT

Supporting Information

Supplemental materials are provided, as indicated in the text. 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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