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Sample Normalization Methods in Quantitative Metabolomics

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Abstract

To reveal metabolomic changes caused by a biological event in quantitative metabolomics, it is critical to use an analytical tool that can perform accurate and precise quantification to examine the true concentration differences of individual metabolites found in different samples. A number of steps are involved in metabolomic analysis including pre-analytical work (e.g., sample collection and storage), analytical work (e.g., sample analysis) and data analysis (e.g., feature extraction and quantification). Each one of them can influence the quantitative results significantly and thus should be performed with great care. Among them, the total sample amount or concentration of metabolites can be significantly different from one sample to another. Thus, it is critical to reduce or eliminate the effect of total sample amount variation on quantification of individual metabolites. In this review, we describe the importance of sample normalization in the analytical workflow with a focus on mass spectrometry (MS)-based platforms, discuss a number of methods recently reported in the literature and comment on their applicability in real world metabolomics applications. Sample normalization has been sometimes ignored in metabolomics, partially due to the lack of a convenient means of performing sample normalization. We show that several methods are now available and sample normalization should be performed in quantitative metabolomics where the analyzed samples have significant variations in total sample amounts.

Keywords: metabolomics; quantitative metabolomic profiling; metabolite quantification; sample normalization; liquid chromatography; mass spectrometry.

1. Introduction

Quantitative metabolomics involves the application of analytical, statistical and bioinformatic techniques to profile the metabolomes of comparative samples, examine the metabolomic changes associated with phenotypes of biosystems from which the biological or clinical samples are taken, and investigate the roles of significant metabolic changes in the biosystems including functional studies and/or discovery of chemical biomarkers of phenotypes. NMR and mass spectrometry are two primary tools for metabolomic analysis [1,2]. However, compared to NMR, MS is increasingly being used for metabolomics due to higher detection sensitivity and larger metabolome coverage [3,4]. MS, in combination with isotope standards or isotope labeling of samples, can also produce quantitative information on metabolites [5-7]. While absolute quantification is important for targeted analysis of individual metabolites of interest in applications such as validation of a potential metabolite biomarker, only relative quantification of metabolites is available in high-coverage discovery metabolomics with a focus on discovering one or set of metabolite biomarkers of a phenotype such as a disease and functional metabolomics with a focus on investigating the functional roles of metabolites in a biological system. Relative quantification measures the relative quantity differences of an individual metabolite as expressed by the metabolite's peak intensity variations in comparative samples. It does not require the use of standards and thus can be used for untargeted analysis without knowing the identities of the metabolites *a priori* [8-10]. In general, a much larger number of metabolites can be profiled by relative quantification, particularly in liquid chromatography (LC) MS-based techniques [10].

Figure 1 shows the general workflow for quantitative metabolome profiling. There are three major steps: pre-analytical work, analytical work and data analysis. In pre-analytical work, processes such as sample collection, sample pre-processing and sample storage need to be carefully carried out in order to maintain the sample integrity prior to chemical analysis [11]. For example, proper storage of samples such as immediate freezing and storing at -80°C for biofluids is important to minimize concentration changes of metabolites due to enzyme activities or other physicochemical processes after sample collection [12,13]. In the analytical workflow, there are several steps involved, including, in no particular order, sample processing such as removal of interfering components (e.g., proteins), chemical derivatization if needed, spiking reference standards or reference control-sample, buffering or adjusting solvents or pH of the sample solution, and analyzing the processed samples using NMR or MS. After data acquisition from all the samples, data processing and analysis are employed to extract information on identity and absolute or relative quantity of individual metabolites.

Each step or process listed in Figure 1 needs to be carefully carried out or controlled in order to produce accurate and precise quantitative data that reflect the true concentration differences of individual metabolites in the samples. This review focuses on the discussion of sample normalization. Sample normalization refers to a process to adjust the sample volume or concentration prior to data acquisition or adjust the acquired signals after data acquisition to equalize the total signals of metabolites in individual comparative samples (see Figure 2). For the latter, the total signals detected are assumed to be related to the sample amount injected and thus normalizing the signal intensity is thought to be equivalent to normalizing the sample amount. In this review, the importance of performing sample normalization in quantitative metabolomics is first discussed. Several considerations in the development and usage of a proper sample

normalization strategy for metabolomic profiling are described. A survey of methods reported for sample normalization including their relative merits for metabolomics is presented. Finally, because there are no unified views on how best one can perform sample normalization in quantitative metabolomics, we comment on implementing sample normalization in current practices, which may help future development of a more unified sample normalization method as part of standard operational procedure (SOP) for metabolomic profiling.

2. Importance of Sample Normalization

Because the major goal of quantitative metabolome profiling is to determine the concentration differences of individual metabolites in two or more comparative samples, the size, weight or volume of individual samples can significantly affect the relative quantification results. For example, in a biofluid such as urine, the total concentration of metabolites in a sample herein denoted as sample concentration can differ by more than 14-fold [14]. The term, concentrated or diluted urine (or any other type of sample), refers to a urine sample with high or low "total concentration" of metabolites, respectively. Even for the same individual, the urine sample concentration can vary greatly, depending on water intake, diet and excretion via, for example, sweating. Because urine concentration can be influenced by external factors, in clinical field, this varying factor is often controlled by using a reference analyte inherently present in all urine samples. Creatinine is the most commonly used reference and the assumption is that creatinine concentration reflects the urine concentration [15]. A diluted urine would have a lower creatinine concentration and vice versa. The measured absolute concentration of an individual metabolite (e.g., glucose) is divided by the creatinine concentration which can be readily measured in urine using a commercial assay kit. The concentration of the metabolite is reported as x mM (or

μM)/creatinine [16]. In other words, the metabolite concentration is normalized to the creatinine concentration and the normalized concentration is then compared to a reference range to determine whether the metabolite concentration is within the normal range or not. Thus, sample concentration or amount normalization is a well recognized and commonly practiced process in urinary metabolite measurement in clinical field. As many metabolomics studies are in the area of discovering potential biomarker(s) of a disease, it makes sense to conform the practice of metabolome measurement to the clinical norm to produce clinically acceptable and useful results.

For urinary metabolomics, creatinine measurement could be used for sample normalization, although other methods have been developed for the same purpose (see below). However, for many other biofluids or biological samples, there is no known compound that is widely accepted as a reference for sample normalization. The sample concentration of feces, sweat, bronchial lavage fluid, etc., can vary greatly. The cell extracts or tissue extracts can also have different total amounts of metabolites, depending on several factors including the number of cells sampled, cell lysis efficiencies, composition of cells, and cell density in tissue for tissue samples. If only a fixed volume (e.g., 5 μL of sweat), weight (e.g., 1 mg of dried feces), or size (e.g., a tissue of 2 mm long × 2 mm wide × 10 μm thick) is taken from each individual sample for metabolomic comparison, it is impossible to delineate the contributing factors in the measured concentration differences of individual metabolites. The measured differences can be due to the sample concentration differences, the nested underlying differences caused by a true differentiating factor such as a biological event (e.g., a mutation in cells or a disease in clinical samples), or a combination of the two. Because we want to determine the metabolite concentration changes caused only by a biological event, not by a non-biological factor, sample

normalization prior to analyzing all the individual samples should be considered as a critical and necessary step in the overall metabolomic profiling workflow.

3. Performance Considerations in Sample Normalization

Compared to genomics and proteomics, sample normalization in metabolomics is much more challenging because of the greater diversity of metabolite structures. To date, there is no standard method for measuring the total amount of metabolites directly in a way similar to that of total protein amount measurement commonly used in proteomics (e.g., bicinchoninic acid assay). Alternatively, other indirect or partial physical or chemical property measurement methods have been reported to relate the measured values or quantities to the total metabolite amount for sample normalization (see next section). In developing or applying a sample normalization method for metabolomics, the overall performance of the method is often assessed by the ability of the method to minimize variations within the same group of samples. The easiest and most common way of evaluating the variations is through principal component analysis (PCA) score plot. An optimal sample normalization method should display the shortest distance between samples within the same biological group and the greatest separation between samples from different groups. Comparing the intra-group's relative standard deviations (RSD) or the number of discriminant features found can also be used to evaluate the method performance. Smaller RSD or less discriminant features within a group is desirable. For targeted metabolome profiling, a series of standard compounds can be spiked at different concentrations into the comparative samples while maintaining the same total spike-in amount. The ability of a sample normalization method to detect the differences in spike-in data can then be evaluated. This approach has been used in a NMR-based metabolomics study [17].

Besides intra-sample variability comparison, the normalization accuracy should also be assessed. This can be done by normalizing a series of samples with known dilution, and then evaluating whether signals of a particular compound in diluted samples have been brought back to the original value and how accurate the adjusted value is. In developing a new normalization method, it is also important to establish a correlation between the new method and the conventional normalization approaches, as a part of method validation. It is assumed that under normal circumstances (e.g., healthy individuals), results obtained from different normalization methods should show positive correlations with each other. For example, in the methods to be discussed later, correlation between MS "total useful signal" (MSTUS) and osmolality normalization factors was determined to be $R^2 = 0.71$ [14], correlations between UV absorption of dansyl labeled metabolites and creatinine or osmolality were $R^2 = 0.95$ and 0.90, respectively [18], and correlation coefficients for matrix-induced ion suppression (MIIS) method with creatinine and osmolality were 0.93 and 0.99, respectively [19].

In addition to method validity, there are several other considerations that also need to be taken into account before selecting an appropriate normalization method for a particular application, as discussed below.

It is important to decide whether the normalization step is performed pre-acquisition or post-acquisition (see Figure 2) [20]. In pre-acquisition methods, the volumes of biological samples are adjusted according to the measured quantities (e.g., creatinine concentrations) to equalize the total sample concentration for all samples. By taking the same volume of aliquot from each sample, the same amount is used for analysis by NMR or MS. Alternatively, instead of adjusting the sample volume, different volumes of samples according to the measured quantities are taken to ensure the same amount used for metabolome analysis. In contrast, in

post-acquisition methods, the sample amounts are not experimentally normalized and thus the total injection amounts used for metabolomic profiling are different, resulting in different overall NMR or MS signals. The individual metabolite signals are then adjusted or normalized for different samples based on a certain criterion such as the total ion signal intensity of a chromatogram from a sample.

Because the same amount is used for sample analysis, a major advantage of pre-acquisition normalization is that similar instrumental responses can be obtained for all samples. This is important for MS-based methods. In electrospray ionization (ESI) MS, responses of individual metabolites are often non-uniform at different concentrations because of different ionization efficiencies and different degrees of ion suppression. As a result, the analyte signals may not necessarily be linearly scaled with the metabolite concentration in a complex biological matrix. Thus, by adjusting all the comparative samples to the same total concentration, more accurate quantitative results will be produced. Another advantage of pre-acquisition method is that the measured quantities from the samples can be used to control the sample injection amount in NMR or MS analysis. In this way, an optimal sample amount can be used for metabolomic profiling. For example, in LC-MS, it is important to keep the injection amount optimal in order to increase the probability of detecting low concentration metabolites [21,22], while avoiding the over-injection problem that can cause signal saturation and sample carryover.

Comparisons between pre-acquisition and post-acquisition methods have been reported. For example, Chen et al. showed that for five serially diluted rat urine samples, post-acquisition normalization methods failed to overcome urine variability because of the nonlinear response to sample dilution caused by ion suppression or saturated metabolites. On the other hand, pre-acquisition correction was effective for reducing variations introduced by different urine

concentrations. They also demonstrated that pre-acquisition injection volume calibration is superior in reducing intra-group bias in the presence of biological variations [23]. Similarly, the study performed by Edmands et al. suggested that pre-acquisition normalization is a better choice for biological information recovery as it identified the largest number of discriminant MS features when compared to three post-acquisition normalization methods [20]. However, the downside of pre-acquisition normalization is that an additional experimental step is required in the overall workflow. In addition, an extra amount of sample may be required for measuring the quantities, as this sample aliquot may not be used for metabolome analysis. Post-acquisition methods avoid these pitfalls and are relatively more convenient and quicker to perform, as long as a software program to properly extract the quantitative information from the acquired data is available. Nevertheless, it should be kept in mind that the performance of post-acquisition may be compromised by variations in detection responses, as described above.

Another factor to be considered is the ease of operation, particularly for pre-acquisition normalization, since an extra step is required. In metabolomics, using a large number of samples is preferred in order to create valid statistical models and to obtain more accurate biological information [24]. However, the number of samples to be analyzed is often not only limited by the availability of biological samples, but also the overall analytical time and efforts required. The latter is affected by the complexity of sample preparation protocols as well as the length of analysis time per sample. In this regard, it is desirable to keep the normalization method simple, quick and convenient to perform so that it would not restrain the use of a large sample set for metabolomics.

The selection of a proper normalization method also depends on the type of biological sample to be analyzed. Some methods are only applicable to specific biological media while

others are more generic. For example, normalization to creatinine is based on the relatively constant excretion rate of creatinine through glomerular filtration, and is therefore only applicable to urine specimens. On the other hand, normalization to MS "total useful signal" (MSTUS) uses the total intensity of peaks that are present in all samples under study as the normalization factor, and it is thus more universally applicable. While a specific method targeting one type of samples may be more efficient on occasions for its targeted biological medium, a generic normalization method should be more desirable because of its adaptability to various sample types and the ease of method transfer from one type of biological matrix to another.

From the above general discussion, it is clear that, when we judge the utility of a sample normalization method, several factors including applicability of the method for the biological samples on hand, normalization accuracy, convenience, speed and cost of performing normalization need to be considered. In the following section, we will review the reported normalization methods for MS-based metabolomics studies and discuss their performance according to these factors.

4. Methods for Sample Normalization

Over the past several years, a number of sample normalization methods have been reported for metabolomics applications. Table 1 provides a summary of the reported methods for sample normalization, while Table 2 summarizes the advantages and disadvantages of each method, with a focus on the instrument availability, time and convenience to perform measurement, as well as the method validity.

Table 1. Summary of sample normalization methods used in MS-based metabolomics.

Sample Type	Normalization Methods	Pre- or Post-acquisition	Recommended method	Reference
Cattle urine	Specific gravity Freeze-drying MSTUS[1]	Pre Pre Post	Similar performance for all three methods	Jacob et al. [25]
Rat urine	Urine volume Osmolality Creatinine MSTUS	Post Post Post Post	Osmolality and MSTUS	Warrack et al. [14]
Rat urine	Total intensity Median fold change Quantile LOESS[2]	Post Post Post Post	Median fold change	Veselkov et al. [26]
Rat urine	MSTS[3] MSTUS MSGUS[4] Urine volume	Post Post Post Post	Urine volume	Godzien et al. [27]
Rat urine	All MS signals MSTUS Creatinine value Creatinine peak area Creatinine value Creatinine value + all MS signals Creatinine value + MSTUS	Post Post Post Post Pre Pre + Post Pre + Post	Creatinine value + all MS signals and Creatinine value + MSTUS	Chen et al. [23]
Human urine	Specific gravity Specific gravity Median fold change Urine volume	Pre Post Post Post	Pre-acquisition normalization to specific gravity	Edmands et al. [20]
Human urine	MSTUS	Pre	-	Mattarucchi and Guillou [21]
Human urine	Conductivity LOESS	Post Post	LOESS	Gonzalez-Dominguez et al.[28]
Human urine	MIIS[5]	Pre	-	Chen et al. [19]
Human urine	UV absorbance	Pre	-	Wu and Li [18]
Adherent cell line (OVCAR-8)	Total protein Cell count Total DNA	Post Post Post	Total DNA	Silva et al. [29]
Adherent cell	Protein content	Post	Similar	Cao et al. [30]

line (MDCK)	Metabolic markers	Post	performance for both methods	
Adherent cell line (MCF-7)	Cell count Sum of peak areas	Post Post	Similar performance for both methods	Hutschenreuther et al. [31]
Human cell lines	MIIS	Pre	-	Chen et al. [32]
Cyanobacterial strains	Chlorophyll *a* Total protein Glycogen sIC[6] sIC.90[7] sIC.AA[8]	Post Post Post Post Post Post	Similar performance for all six methods	Huege et al. [33]
E. coli	UV absorbance	Pre	-	Wu and Li [34]
Sweat	Na concentration	Pre	-	Appenzeller et al. [35]
Sweat	UV absorbance	Pre	-	Hooton and Li [36]
EBC[9]	Urea	Post	-	Esther et al. [37]
EBC	MSTUS IS response IS response + collected EBC volume IS response + expired EBC volume	Post	MSTUS	Peralbo-Molina et al. [38]
EBC	Sum of all areas	Post	-	Fernandez-Peralbo et al. [39]
Dog fecal samples	Dry weight	Post	-	Guard et al. [40]
Human fecal samples	UV absorbance	Pre	-	Xu et al. [41]
Saliva	UV absorbance	Pre	-	Zheng et al. [42]

1. MSTUS: MS total useful signal; 2. LOESS: Locally weighted scatter plot smoothing; 3. MSTS: MS total signal; 4. MSGUS: MS group useful signal; 5. MIIS: Matrix-induced ion suppression; 6. sIC: selected ion count for all metabolites; 7. sIC.90: selected ion count for 90% of the non-extreme metabolite pools; 8. sIC.AA: selected ion count for the total amino acid content. 9. EBC: exhaled breath concentrate. 10. IS: internal standard.

4.1. Normalization of Urine Samples

Urine is one of the most commonly investigated biofluids in metabolomics, because it can be easily and non-invasively collected in large quantities, and the sample is relatively clean, requiring a simple pre-treatment procedure [15]. Unfortunately, urinary solute concentrations often vary widely depending on hydration status, time since previous urination, dietary intake or other physiological factors [43,44]. Thus, it is not surprising that the majority of research efforts on normalization method development actually target specifically at urine samples. The most widely accepted approach for urine volume correction is to express metabolite levels relative to creatinine concentration [45-47], because the rate of creatinine excretion through glomerular filtration is relatively constant under normal conditions within or across individuals [48]. However, the assumption of constant creatinine excretion is often invalid, as creatinine excretion was found to vary across individuals due to age, gender, race and muscle mass differences or disease states [49-52]. Even within the same individual, the urine creatinine level may change depending on diet, time of day, level of exercise, and physiological conditions [53-55]; the number of subjects used for these studies ranged from 4 individuals over 13 days [53], 7 individuals over 90 days [54], to 11 individuals over several months [55]. As a result, the validity of using creatinine concentration as the normalization factor is often challenged [56]; in the work of Alessio et al [56], 376 individuals were studied. In another example, Burton et al. showed that normalization of urinary pteridines to creatinine did not improve differentiation between benign and malignant breast cancer samples, and the authors suggested that alternative renal dilution factors are needed (urine samples of 25 individuals were used in the study) [57]. In addition, it is also questionable whether it is sufficient to normalize the wide range of metabolites based on a

single compound [58]. Future work is still needed to determine whether the use of a large number of subjects per cohort in a metabolomic study could overcome some creatinine-level-abnormalities associated with a fraction of the samples to produce reliable metabolomic profiling results. Nevertheless, because of the convenience and low cost in creatinine measurement, creatinine normalization prior to LC-MS acquisition can still be useful in controlling injection volumes to ensure that urine samples are injected at comparable levels, and that similar instrumental response is warranted for all samples. If creatinine normalization alone is not sufficient due to variations in creatinine excretion rate, post-acquisition remedy can be applied to further improve the results. As an example, in a recent study of rat urine (n = 5 in the absence of biological variation and n = 32 in the presence of biological variation), it was demonstrated that injection volume calibration based on creatinine value prior to LC-MS analysis is effective in adjusting urinary solute concentrations to similar levels. This pre-acquisition normalization method significantly reduces intra-group variations as indicated by better clustering in principle component analysis (PCA) score plots and reduced peak area relative standard derivations (RSDs) in intra-group comparisons [23].

Urine osmolality is a direct measure of the total urinary solute concentration that is only affected by the number of particles in urine. Therefore, it is often considered as a gold standard for estimating urinary concentration [59], and has been used as a valid scaling factor for urinary solutes [60,61]. Application of osmolality normalization in urinary metabolomics analysis has been reported, which showed better separation between different biological groups as well as reduced variations within biological replicates when compared to no normalization or normalization to urine volume and creatinine [14]. However, the procedure of measuring osmolality is often not practically available, and thus specific gravity is usually measured,

instead, as an estimation of osmolality [59]. Urine specific gravity is the ratio between the density of urine and that of pure water at a constant temperature, which can be measured either directly by gravimetry or indirectly by refractometry. Specific gravity has been used as a normalization method for urinary metabolites in many applications [43,62,63]. Recently, the feasibility of using specific gravity as a normalization strategy in urine metabolomics has been assessed by Jacob et al. [25] in comparison with freeze-drying, a valid normalization strategy for anabolic practices in cattle [64,65]. The authors have showed that normalization by specific gravity improved separation between two study groups and revealed the same differentiating ions as the freeze-drying method, and they thus proposed that specific gravity can be used as an alternative to the time-consuming freeze-drying for urine metabolome normalization. Besides creatinine, osmolality and specific gravity, other conventional urine normalization methods include normalization to 24-hour urine volume, [14,66] conductivity [28] and flow rate correction [67]; but these methods are used less often and will not be further elaborated here.

Post-acquisition or data-driven normalization approaches have been used for some metabolomics studies in recent years. Warrack et al. have proposed the idea of using the total intensity of peaks that are common to all samples, known as MS “total useful signal” (MSTUS), as the scaling factor [14]. This concept is similar to the use of total integrated proton signal for normalization in proton NMR-based metabolomics analysis [68]. Incorporation of only “useful signals” ensures that contributions from xenobiotics and artefacts are minimized. The authors have compared MSTUS normalization to other common normalization approaches in the non-targeted metabolomic profiling of rat urine from different dose groups. They recommended both MSTUS and osmolality for detection of significant metabolic changes as these methods are most

efficient in differentiation between high and low dose groups (see Figure 3). Since reported in 2009, MSTUS normalization has been employed in several applications [21,27,69].

In addition to MSTUS, more sophisticated statistical strategies have been introduced. For example, Veselkov et al. have compared four normalization techniques [26]. The first two methods, based on total intensity and median fold change, respectively, assume that metabolite peak intensities vary linearly with concentration, while the other two approaches, quantile and locally weighted scatter plot smoothing (LOESS), consider peak-intensity-dependent scaling factors (i.e., in the presence of ion suppression or saturation). They found that for the majority of urinary metabolites, the peak intensities did not respond differentially to dilution, which supports validity of the first two methods (i.e., measuring total intensity and median fold change). In terms of normalization performance, all four methods perform equally well in the absence of biological variation. However, when biological variation is considered, the performance of total intensity normalization is slightly inferior due to variations in total metabolite output between samples. Despite the comparative effectiveness of the other three methods, the authors recommended the median fold change approach because of its relaxed assumption with regard to the proportion of asymmetrical metabolite changes. The effect of median fold change normalization in the presence of biological variation is illustrated in Figure 4.

More comprehensive evaluations of statistical treatments have been discussed by Ejigu et al. [70]. In general, the advantage of data-driven approaches over conventional urine normalization methods is that the methodology is not restricted to urine samples and can be readily adapted to other types of biological matrices. However, since the scaling factor is based on MS data, the normalization process has to be performed after LC-MS analysis. As discussed before, post-acquisition normalization cannot control the amount of sample injected into the

mass spectrometer, and therefore cannot be used to alleviate problems associated with varying injection amount such as signal saturation, ion suppression and missing values from low abundance or not easily ionizable metabolites. Although pre-acquisition MSTUS has been proposed by Mattarucchi et al. to improve the quality of extracted LC-MS data [21], it requires LC-MS acquisitions to be performed for all samples prior to metabolomics analysis in order to obtain MSTUS values. This would increase the workload considerably and is not practical for analyzing a large number of samples.

Recently, Chen et al. have proposed a novel idea of using MS signals for pre-acquisition urine normalization [19]. The method is based on measuring the extent of ion suppression induced by urine matrix on a spiked indicator compound (hexakis), using flow injection analysis electrospray ionization mass spectrometry (FIA-ESI-MS). The authors have shown that the level of ion suppression is proportional to urine concentration, and therefore a regression equation can be established to estimate the relative concentration of unknown urine samples. Application of this method has been demonstrated on the urine metabolomics study on breast cancer. Overall, this matrix-induced ion suppression (MIIS) method provides high sample throughput and can also be fully automated. The applicability of this approach has also been demonstrated on cell cultures (see below), but remains to be investigated for other biological matrices. One advantage of this method over other methods such as osmolality or specific gravity measurement is that no additional instrument is required as the same MS used for metabolomic profiling is employed for ion suppression measurement (see Table 2). On the other hand, this is also a disadvantage, because an expensive MS instrument is used for measuring the ion signals for sample normalization.

Another concept for sample normalization is to determine the UV absorbance of the sample solution as a measure of the total concentration of solutes that absorb at the specific wavelength. This type of normalization approach is more representative of the overall sample composition compared to the use of a single compound such as creatinine, and is independent of the biological medium. In addition, UV measurement can be performed prior to LC-MS analysis to allow injection amount adjustment. Kemperman et al. has reported normalization of urine samples to the area under the curve at 214 nm (AUC_{214}) and showed that this method is preferred to creatinine normalization for minimizing peak area and intensity variations of peptides [58]. However, for metabolites with varying structures, the 214 nm measurement may not reflect the total concentration of all metabolites in a sample.

We have developed a method of determining the total metabolite concentration based on the use of chemical labeling to attach a UV absorbing dansyl moiety to amines and phenols, followed by a rapid step-gradient LC-UV detection of the labeled metabolites at 338 nm [18]. The concept is shown in Figure 5. Although a derivatization step is required in this method, the derivatization reaction is simple to perform and takes about one hour. It can also be performed simultaneously for labeling multiple samples. Derivatization is widely used for other normalization methods such as BCA assay and Bradford assay in protein quantification and creatinine measurement in urine. Because labeling efficiencies of amines and phenols in different samples of the same matrix such as urine are similar, accurate relative quantification of amines and phenols from the UV measurement of the labeled products can be achieved [71]. In addition, the labeled metabolites can be directly analyzed by LC-MS, if dansylation isotope labeling LC-MS is used for profiling the amine/phenol submetabolome. One disadvantage of this method is

that it requires an LC-UV equipment. However, in a recent work, microwell plate reader was used for measuring UV absorbance (see below) [72].

We have shown that this normalization strategy effectively corrects for the dilution effect in intra-day urine samples and minimizes artificial separation caused by the variations in the original urine concentrations. Although parallel comparison with other normalization methods was not performed, we did observe a good correlation between the UV absorbance and creatinine or osmolality values. It should be noted that this dansylation LC-UV normalization method is based on the assumption that the total concentration of labeled metabolites composed of mainly amines and phenols is linearly related to the total concentration of all the metabolites in a sample. Whether this assumption is generally true for all the samples remains to be determined. Although, at this stage, we do not know with certainty any disease that would cause a biased change in the total concentration of amines and phenols, it may happen under special circumstances such as proteinuria and unusual diet, and such a biased change would affect the accuracy of the normalization method. However, if there are only a few exceptions (e.g., due to the presence of one dominant compound [58]) out of a large cohort of samples where the determined sample amount is slightly different from the rest of the samples, a post-acquisition method can be applied to improve the overall data quality. For example, Chen et al. have illustrated that the combination of pre-acquisition injection amount calibration based on creatinine values and post-acquisition MSTUS normalization provides the best results in overcoming urine sample variability [23].

4.2. Normalization of Cell Extracts

Cellular metabolomics plays an important role in systems biology and has found applications in many areas such as toxicology and preclinical drug testing in which *ex vivo* models are required [73,74]. In cellular metabolomics, it is often of interest to investigate quantitative metabolic changes in response to different environmental stimuli. Unfortunately, it is difficult to control the amount of cells harvested from different culture mediums or plates due to variations in seeding density and/or treatment conditions [30]. For example, exposure to toxic compounds such as 2,3,7,8-tetrabenzodi-p-dioxin (TCDD) may lead to decreased cell proliferation rate and subsequently lowered overall metabolite level [75]. The same problem is also encountered in microbial metabolomics where comparative metabolomic analysis of bacterial species under different environmental conditions is studied or a metabolome profile is used for microorganism differentiation and identification. Therefore, sample normalization of mammalian and microbial cell extracts is important.

For mammalian cells, cell counting using a hemocytometer is commonly used for cell amount normalization. In the case of adherent cells, detachment of the cells from the culture flask is required, typically by using trypsinization or direct scraping. Trypsinization is considered less suitable for metabolomics because it can lead to changes in the cellular metabolome profile [76] or introduce metabolomic artifacts [77]. Direct scraping is more labor-intensive and can often result in loss of cells. In addition, the accuracy of cell counting normalization method is often impaired by inhomogeneity of the cell suspension, and random variations introduced during aliquoting and transfer process [31]. Moreover, since this method is performed at the time of harvest, it would delay subsequent quenching procedure and may result in alterations in the metabolic profile. For bacterial cells, direct counting of the cell number is difficult to implement because of their small sizes. Alternatively, optical density at 600 nm (OD_{600}) is often determined

as a measure of the light scattered by a culture, and the OD_{600} value is then correlated to a known colony number to estimate the amount of cells. As an example, Marcinowska et al. have shown that normalization to OD_{600} values provides a robust basis for quantitative analysis and differentiation of clinically relevant bacterial cells [78]. While this method is applicable to homogeneous cell suspensions, it may not be as convenient for adherent cell cultures. Besides, similar to direct cell counting, additional time is required to perform the measurement before cell sample preparation.

Other conventional approaches for cell amount determination include measurement of the dry weight of cell debris and quantification of the total protein content [79]. The dry weight method is time-consuming and is not preferred for metabolomics studies in which a large number of samples need to be processed. Also, relatively large errors may be introduced in dry weight measurement when the sample amount is small [33]. The protein amount can be readily determined using well-established colorimetric methods such as BCA assay and Bradford assay, and has been used in several applications for studying cellular metabolic changes [80,81]. However, Silva et al. have shown that the protein concentrations of both the metabolite extraction solution and the remaining cell pellet failed to produce expected correlations with seeded cell number, due to poor protein recovery in metabolomics-compatible solvents and incomplete protein re-solubilization from the pellet [29]. Therefore, assaying the protein amount would require a separate experiment to be performed in order to obtain accurate quantitation results, which is not desirable as it consumes part of the samples and prolongs the sample preparation process. Instead, the authors have proposed the use of DNA concentration as the normalization factor for metabolomic data, as they have demonstrated good correlation between DNA concentration of the cell pellet and seeded cell number for four adherent cell lines. Despite

the reported efficiency and robustness of this method, examples of how normalization to DNA concentration could improve statistical analysis in metabolomics were not given in this work.

In recent years, several data-based normalization strategies have also been introduced for cellular metabolomics. One approach involves the use of specific metabolic markers as the cell amount indicator [30,33,75], with the assumption that the concentrations of these selected metabolites are directly proportional to the cell number and are independent of the treatment conditions under study. For example, Cao et al. has identified pantothenate and inositol as the best candidate markers for normalization of Madin–Darby canine kidney (MDCK) cells based on three criteria: good linearity between the metabolite signal intensity and the cell amount for serially diluted cell suspensions, high linear correlation between the metabolite abundance and the protein content for cells seeded at different concentrations, and improved separation between two cell lines and closer clustering within each cell line. In general, the use of single or a few metabolic markers is simple, fast and convenient. However, it should be noted that the same metabolite markers are not necessarily applicable in other cellular systems, as their cell line and treatment independence may not always hold true. Therefore, the validity of these metabolic markers for other studies requires further assessment, which poses a limitation to the utility of this normalization method.

Alternatively, the use of total metabolite intensity is often considered as a more robust way of estimating the cell number. Huege et al. have evaluated the normalization effects of three total intensity parameters, namely the intensity sum of all metabolites, the intensity sum of 90% non-extreme metabolites and the intensity sum of amino acids and their conjugates, in cyanobacterial metabolomics [33]. It was concluded that these three normalization factors are essentially equivalent in terms of their influence on sample cohesion within strain groups and

separation among different strains. Hutschenreuther et al. have also evaluated the performance of peak area sum normalization in comparison to cell count [31]. They observed a good linear correlation between sum of peak areas and cell count within a specified linear range. However, they noted that this normalization method should only be applied when the cell extract concentration in two comparative samples differs by less than two-fold, as otherwise the number of "false significants" would increase to over 10%. This is likely attributed to the fact that not all metabolites exhibit linear response with concentration (e.g., presence of borderline metabolites and ion suppression effect). Therefore, the authors suggested that similar extract concentrations should be used for comparison in cellular metabolomics. This conclusion is in accordance with our earlier discussion on the importance of pre-acquisition normalization.

As we discussed in Section 4.1, normalization to UV absorbance of the sample solution is independent of the biological matrix and is performed prior to MS acquisition, which makes it a promising approach for cell amount adjustment. In addition, when incorporated into the LC-MS metabolome profiling workflow, this normalization step is usually carried out immediately before LC-MS (i.e., after all initial sample preparation steps). This is particularly advantageous to cellular metabolomics for two reasons. Firstly, there is no extra procedure during the cell harvest stage so that metabolite quenching can be performed without any delay. Secondly, metabolite extraction from cells often involves more steps compared to biofluids, which makes it more prone to experimental errors. The UV normalization can be used to correct for concentration variations introduced during the sample workup process. Application of the dansylation LC-UV normalization method on microbial metabolomics has been demonstrated on bacterial differentiation and the study of butanol tolerance in *Staphylococcus warneri* SG1 [34,82].

More recently, we have adapted this UV absorption method into a dansylation metabolite assay (DMA), which measures absorbance of labeled metabolites using a microwell plate reader, instead of the more expensive LC-UV system [72]. It was noted that excess dansyl chloride reagent would interfere with UV measurement if the sample concentration is low. Therefore, the excess reagent was first quenched with NaOH to convert to the highly hydrophilic product dansyl-OH, and was then removed by liquid-liquid extraction. The DMA improves throughput by allowing simultaneous measurement of multiple samples within a short period, and can be readily implemented because of the low cost and simple procedures. We showed good linear relationships between the UV absorbance values and the cell suspension volume or the protein amount. The validity of this normalization strategy for metabolomics has been demonstrated by the improved separation between two *E. coli* strains as well as decreased %RSD values within each strain. This UV absorbance normalization approach has also been applied to mammalian cells, HEK 293 cell line [83] and yeast cells [84].

As mentioned in Section 4.1, the use of matrix-induced ion suppression (MIIS) method for normalization of cultured cell lines has also been demonstrated [32]. The principle is similar to that used for urine normalization, i.e., to estimate the sample concentration by measuring the extent of ion suppression induced by the sample matrix. However, because metabolite extracts from 1×10^6 cells represent a more diluted matrix compared to urine samples, the drying gas temperature had to be lowered in order to reveal differences in the amount of non-volatile components in different cell extracts. The authors then applied this MIIS method on comparative metabolomics study of two lung cancer cells, CL 1-0 and CL 1-5, with the same cell numbers. Normalization based on the MIIS measurement reduced the mean metabolite fold change between two cell lines from 2.01 to 1.01, a value that is more reasonable considering the

homeostasis of cell biology. Overall, this study demonstrated the validity of using this MIIS method for estimation and adjustment of total metabolite concentrations in cellular metabolomics studies. Nevertheless, the applicability of this method depends on the FIA-ESI-MS conditions, which suggests that optimal experimental parameters have to be evaluated for different cell amounts (e.g., 1×10^5 cells) or for different biological samples. In addition, as indicated earlier, an expensive MS equipment is needed for ion signal measurement, while other methods such as dansylation UV measurement use simpler equipment.

4.3. Normalization of Other Biological Samples

Compared to urine and cellular samples, the need for sample normalization in other biological media is far less explored. While blood and cerebrospinal fluid (CSF) are considered homeostatically regulated, there are still a number of sample types in which proper control of the solute concentration is absent. For example, the sample volume of sweat can often vary depending on the water content, which can be affected by several factors such as ambient temperature and relative humidity. There is also large variation in sweat production for different individuals [85]. As commented in a recent review on sweat metabolomics, the absence of proper normalization methods to account for the sample volume variations presents a major drawback in the quantitative analysis of sweat [86]. To address this problem, Appenzeller et al. has proposed the use of sodium and potassium concentrations as normalization factors for sweat, which is similar to normalization of urine samples to their creatinine content [35]. They found that the potassium concentration was highly variable within both the female group (i.e., sweat samples collected from all females) and the male group (i.e., sweat samples collected from all males), making this species unsuitable for determination of the sweat volume. In contrast, the authors

recommended the use of sodium as an internal standard for sweat volume correction because of its low inter-individual variability. However, similar to the limitations of creatinine normalization, this method is also subject to disease states such as cystic fibrosis [87]. Even in healthy individuals, the sodium concentration may vary, for example, with sweat rate and salt intake [88]. In addition, the use of a single species for normalization may not be appropriate in metabolomics, as discussed previously. Recently, the dansylation LC-UV method has been successfully applied to normalize sweat samples for sweat metabolomics [36].

Exhaled breath condensate (EBC) is a biospecimen that is not commonly used in metabolomics, but can be a promising medium for clinical analysis because of its non-invasive nature. However, the formation of respiratory droplets is not constantly related with the production of water vapor, which can lead to markedly variable dilution of the respiratory solutes in EBC [89]. Several efforts have been made to assess the extent of dilution, most of which uses the concentration of one or more non-volatile, hydrophilic respiratory solutes as the dilution factor. This is based on the assumption that these solutes are diluted to the same factor by the condensed water vapor [90]. The most commonly used dilution marker is urea because of its even distribution in the body, relatively high stability and low volatility [91]. The use of urea as a dilution indicator has been applied in several studies [37,92,93]. Other developed parameters for evaluating dilution include total cation, conductivity [90] and protein concentration [94]. Nevertheless, there is still lack of convincing evidence on whether normalizing EBC data with such dilution markers can provide better reproducibility among metabolomic samples, and the assumption that the change in solute levels only depends on dilution may not be valid [95]. Alternatively, data-based normalization methods, such as normalization to MSTUS [38] or to the total signal of all compounds detected per sample [39], have been applied in metabolomic

analysis of EBC samples. In particular, Peralbo-Molina et al. have compared MSTUS with other three proposed normalization methods for EBC, namely the internal standard (IS) response and the combination of IS response with the collected EBC volume or the expired EBC volume. They concluded that MSTUS is the most suited strategy because it minimizes both intra-individual variability and methodological variability [38].

Fecal samples represent another biological medium in which proper normalization is required, because they contain both solid and liquid materials with varying proportions [41]. Even with the same sample weight or volume, the metabolite concentration can be different depending on the liquid content and solid density. One approach to normalize the metabolite concentration is by using dry weight, but this procedure is quite time consuming which requires overnight drying of the samples and individual sample weighing [40]. Alternatively, a universal approach such as post-acquisition statistical methods or pre-acquisition UV absorbance measurement can be applied. As an example, Xu et al. has applied the dansylation LC-UV normalization method for the profiling of human fecal metabolome and also described a step-gradient LC-MS method for sample normalization [41]. They observed a wide distribution of the total labeled metabolite concentration, which ranged from 0.60 to 6.37 mM. Even for the fecal samples collected from the same individual at three different days, the total concentration of labeled metabolites can vary by more than 3-fold. This result highlights the importance of sample normalization in quantitative fecal metabolomics. They found that similar normalization results could be obtained from LC-UV and step-gradient LC-MS, but the latter was more sensitive as expected (i.e., >10-fold improvement). In case that LC-UV is not available, step-gradient LC-MS can be used as an alternative means for sample normalization.

In addition to the biological samples described above, there are still a number of matrices, such as saliva, tears and bronchial lavage fluid, which may require proper normalization in their metabolomic applications. Unfortunately, there is very limited discussion on normalization of these biofluids. A more universally applicable normalization method should be applied. An example of applying the universal dansylation LC-UV method on human saliva samples has been demonstrated in the study of metabolome changes associated with mild cognitive impairment [42]. This method has also been applied for sample normalization in plant metabolomics (Ginseng Roots) [96], milk metabolomic profiling [97] and wine metabolomic analysis [98].

5. Implementing Sample Normalization

As discussed earlier, the main objective of sample normalization is to minimize variation caused by differences in sample concentration so that the overall variability within the same sample group is lowered, and consequently inter-group differences can be more readily measured. Variability among metabolomic samples mainly come from two sources: variations introduced at the pre-analytical, analytical and data processing work (technical variability) and variations that are inherent in the samples (biological variability). Typically the acceptable technical variability (expressed as RSD) should not exceed 20% [99] for metabolite quantification. The biological variability can be different depending on the biological samples of interest. For example, for cultured cell lines or bacterial cells the biological variability within a group is relatively small [100], while for human biofluids such as urine or serum the biological variability can be much greater due to differences in genetics, age, body weight, diet and many other factors [101]. The actual observed variability is a combination of technical and biological variability. However, if

the sample concentration varies considerably, the observed variability can be "artificially" boosted. Thus, sample normalization needs to be performed in a quantitative metabolomics study, if variations in sample concentrations among the comparative samples are greater than the analytical technical variation (e.g., $\geq \pm 20\%$).

Because of the lack of a simple method to measure the total concentration of all the metabolites present in a complex sample, as discussed above, a number of methods based on the measurement of a selected physical quantity (e.g., osmolality) or chemical quantity (e.g., total concentration of dansyl labeled amines and phenols) from a sample have been reported for sample normalization with varying degrees of performance. We envisage that research in the development of improved or new methods for sample normalization will continue in the foreseeable future. As analytical techniques are being advanced to become more precise and more accurate (i.e., $< \pm 20\%$) for quantitative metabolome profiling, rapid and accurate sample normalization method will be required in most, if not all, metabolomics studies. Practically a measured physical or chemical quantity that can truly reflect the total concentration of all the metabolites in a sample will serve well as a normalization factor, with due consideration of special circumstances that may lead to inaccuracy (e.g., administration of un-usual diet that causes abnormal increases of dietary metabolites in some biofluid samples within a biomarker discovery study, engineered metabolite expression that leads to abnormal increase of one or more metabolites in a comparative cell sample in a cellular metabolomics study, etc.). Until such a measurement can be performed routinely for all types of samples, we would like to recommend the reader to select a proper method for sample normalization using Table 2 as a reference. There are now several options for performing sample normalization, as reviewed above. The use of a proper normalization method for a given project depends on the sample type and the availability

of equipment or software for performing either pre-acquisition normalization or post-acquisition normalization or both.

In selecting a sample normalization method, in general, pre-acquisition normalization is preferred if such a method is readily available in hand, because it improves the overall information recovery, especially when sample concentration variation is large (e.g., urine, sweat, cell extracts, etc.). The information loss by post-acquisition methods depends on the percentage of metabolites that fail to respond linearly with concentration in a particular sample set and with a specific analysis method. As an example, Chen et al. have shown that close to 25% of the total detected features display unsatisfactory linearity for urine samples diluted from 1 to 20 fold based on their LC-MS platform [23]. Nevertheless, we would like to recommend a post-acquisition method to be incorporated into the data pre-processing steps even when a pre-acquisition method is used, because it can correct small errors not addressed by pre-acquisition sample normalization (e.g., errors introduced during sample processing or detection). For example, a quick inspection of the total ion chromatograms generated from LC-MS runs of all comparative samples can spot any abnormality such as an outlier or more than expected signal variations; applying a post-acquisition normalization may recover the information to a level satisfactory for performing quantitative metabolome analysis.

6. Concluding Remarks

In this review, we have discussed the rationale of performing sample normalization in quantitative metabolomics and the factors to be considered when selecting an appropriate normalization method for specific applications. We have reviewed a number of normalization methods reported mainly for MS-based metabolomics and commented on their performances in

terms of the ability to reduce sample amount variations, the applicability to different types of biological samples and the convenience to perform. We believe that sample normalization should be part of the overall metabolomic profiling workflow for quantitative metabolomics, if the sample amount variation is greater than the analytical variation (e.g., $\geq \pm 20\%$). We hope this review could serve as a reference to assist in the selection of a proper sample normalization method for a given metabolomics application.

Table 2. List of instrument used, advantages and disadvantages of normalization methods.

Sample Type	Normalization Methods	Instrument	Advantages	Disadvantages
Urine	Creatinine	Colorimetric assay	- Quick and easy to perform - Relatively inexpensive - Low sample consumption	- Creatinine excretion may not be constant, especially in the case of renal impairment
	Osmolality	Osmometer	- Provides the closest measure to the physiology of urinary concentration	- Requires specific instrument that is not commonly used in clinical or research labs - Result may be affected by sample inhomogeneity
	Specific gravity	Refractrometer	- Instrument is less expensive and more easily accessible - Quick and easy to perform - Provides a fair estimation of urine solute concentration	- Measurement accuracy can be influenced by many factors such as presence of heavy molecules, temperature and pH
	Dry mass	Freeze dryer	- Samples are adjusted to the same mass concentration before injecting into MS to reduce variations of matrix effects - Easy to perform	- Time consuming, at least 48 h required - Drying-reconstitution process may lead to loss of metabolites
	Urine volume	None	- No additional equipment/procedure required	- Time consuming, requires 24 h collection - Collection and storage process is inconvenient and cumbersome
	MIIS	MS	- No additional equipment is needed; the same LC-MS instrument is used for both normalization and metabolomics analysis - Relatively quick and easy to perform	- Measurement accuracy can be sensitive to contaminant buildup in ESI source, which requires more frequent source cleaning and maintenance - requires MS instrument time, which may decrease overall throughput.

	Dansylation LC-UV	LC-UV	- High accuracy of measurement - Low sample consumption - Compatible to dansylation LC-MS metabolome profiling	- Requires LC-UV equipment - Accuracy may be reduced if a disease could cause large change in total concentration of amines and phenols (at this stage, there is no known disease causing this effect)
	Total metabolite signal	MS	- Universally applicable - No additional experimental procedure required	- Requires MS equipment - Cannot adjust LC-MS injection amount - May not be valid if different number of metabolites with increased and decreased levels
Cell extract	Cell count	Hemocytometer	- Cell number is the most direct measure of the cell amount	- Performed at time of harvest, which can delay subsequent sample preparation - Generally have larger variations, especially with small cell numbers
	Dry weight	Freeze-dryer	- Relatively easy to perform	- Drying process is time consuming - Large errors may be introduced with small sample amount
	Total protein	Colorimetric assay	- High accuracy of measurements - Relatively quick to perform	- A separate experiment is required for protein quantification, which consumes part of the sample and takes extra time
	Total DNA	Spectrophotometer	- High accuracy of measurements - Uses the same sample as the metabolomics study	- Requires specific reagents and apparatus for measuring DNA - Relatively time consuming (overnight solubilization)
	Housekeeping metabolites	MS	- Fast and convenient to perform - No additional experimental procedure required	- The utility of the housekeeping metabolites has to be validated for each cell line - Cannot adjust LC-MS injection amount

	MIIS	MS	- Same as for urine (above)	- Same as for urine (above) - May require method optimization for different cell numbers or cell lines.
	Dansylation LC-UV	LC-UV	- Same as for urine (above)	- Requires LC-UV equipment
	Dansylation Metabolite Assay	Colorimetric assay	- High accuracy of measurement - Low sample consumption - Compatible to dansylation LC-MS metabolome profiling	- Requires performing liquid-liquid extraction which can be relatively time-consuming
	Total metabolite signal	MS	- Same as for urine (above)	- Same as for urine (above)
Sweat	Sodium concentration	Capillary zone electrophoresis	- Only requires very low sample volume and a short time for analysis	- Requires specific instrument - The assumption of constant sodium concentration may not be valid
	Dansylation LC-UV	LC-UV	- Same as for urine (above)	- Requires LC-UV equipment
Exhaled breath condensate (EBC)	Urea	MS or urease	- Most widely used dilution indicator for EBC - No additional experimental procedures if use MS measurement	- MS data is obtained post-acquisition - Measurement by urease incubation is inconvenient to perform
	Total cation	Ion chromatography	- Provided comparable dilution estimation to urea	- Requires specific and expensive instruments
	Conductivity	Conductivity meter	- Less expensive and more convenient to perform compared to total cation measurement	- Requires lyophilisation to remove NH_4^+
	Protein concentration	Micro BCA protein assay	- Standard assay kit readily available at reasonable prices	- Correlations between protein and metabolite dilution/condensation remain to be investigated

	Total metabolite signal	MS	- Same as for urine (above)	- Same as for urine (above)
Fecal samples	Dry weight	Heating oven	- Relatively easy to perform	- Drying process is time consuming
	Dansylation LC-UV	LC-UV	- Same as for urine (above)	- Requires LC-UV equipment
	Dansylation LC-MS	Step-gradient LC-MS	- High accuracy of measurement - Very sensitive - Compatible to dansylation LC-MS metabolome profiling	- Requires LC-MS equipment
Other samples such as plant extracts, milk, wine	Dansylation LC-UV	LC-UV	- Same as for urine (above)	- Requires LC-UV equipment

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Figure captions

Figure 1. General workflow for quantitative metabolome profiling.

Figure 2. Typical workflow of sample normalization. Normalization can be performed either pre-acquisition or post-acquisition.

Figure 3. Comparison of MSTUS normalization to other commonly used normalization strategies. Non-targeted metabolomic analysis was performed on two groups of 12 Sprague-Dawley rats, with six males and six females in each group. One group received a low dose (non-toxic) and the other group received a high dose (toxic) of a compound that induces phospholipidosis in this species. This figure shows the resulting PCA score plots of (A) without normalization; (B) normalized to urine volume; (C) normalized to osmolality; (D) normalized to creatinine concentration and (E) normalized to MSTUS. Open squares: low dose males; open triangles: low dose females; solid squares: high dose males; solid triangles: high dose females. Normalization to osmolality (C) and MSTUS (E) show the best separation between four biological groups and smallest variation within the biological replicates. Adapted from reference [14] with permission.

Figure 4. Box plots showing peak intensity distributions in (A) non-normalized and (B) median fold change normalized urine metabolomics data, collected from 20 male (red) and 20 female (blue) Wistar rats, as well as serially diluted QC urine samples (green). QCD2 to 8 corresponds to 2 to 8-fold dilution of the QC samples. The box represents 25^{th} to 75^{th} percentile and the median is shown as a black diamond in the box. The y-axis is the log2 ratio of metabolite peak intensities to the median value calculated across the

data set. For non-differentially expressed metabolites the log2 ratios should have a small spread around zero. In the absence of normalization a large variation in log2 fold change is observed across different biological samples, which is caused by differences in overall sample concentration. Median fold change normalization corrects for such concentration variations by adjusting the median peak intensities across samples to be approximately equal. As a result, near zero log2 ratios were observed for the non-differentially abundant metabolites. Adapted from reference [26] with permission.

Figure 5. Schematic illustration of dansylation LC-UV quantification. (A) The metabolome samples first undergo derivatization with dansyl chloride to attach the dansyl group to the amine- and phenol-containing metabolites. (B) The dansyl moiety is a good chromophore that "unifies" the absorptivity of labeled metabolites. As a result, UV absorption can be measured at the specific absorption wavelength of the dansyl group. A step gradient is used to allow co-elution of all labeled metabolites, and the integrated peak area is correlated to a fraction of the metabolome that reacts with dansyl chloride (amines + phenols). (C) The metabolome sample concentration or volume can be adjusted according to LC-UV quantification results in order to inject the same amount from all individual samples into LC-MS for metabolomic profiling in a conventional LC-MS workflow. For differential chemical isotope labeling LC-MS workflow, the LC-UV quantification results can be used to prepare an equal amount mixture from a labeled sample and a differential isotope labeled control sample, followed by injecting the same amount of mixture into LC-MS for the comparative samples.

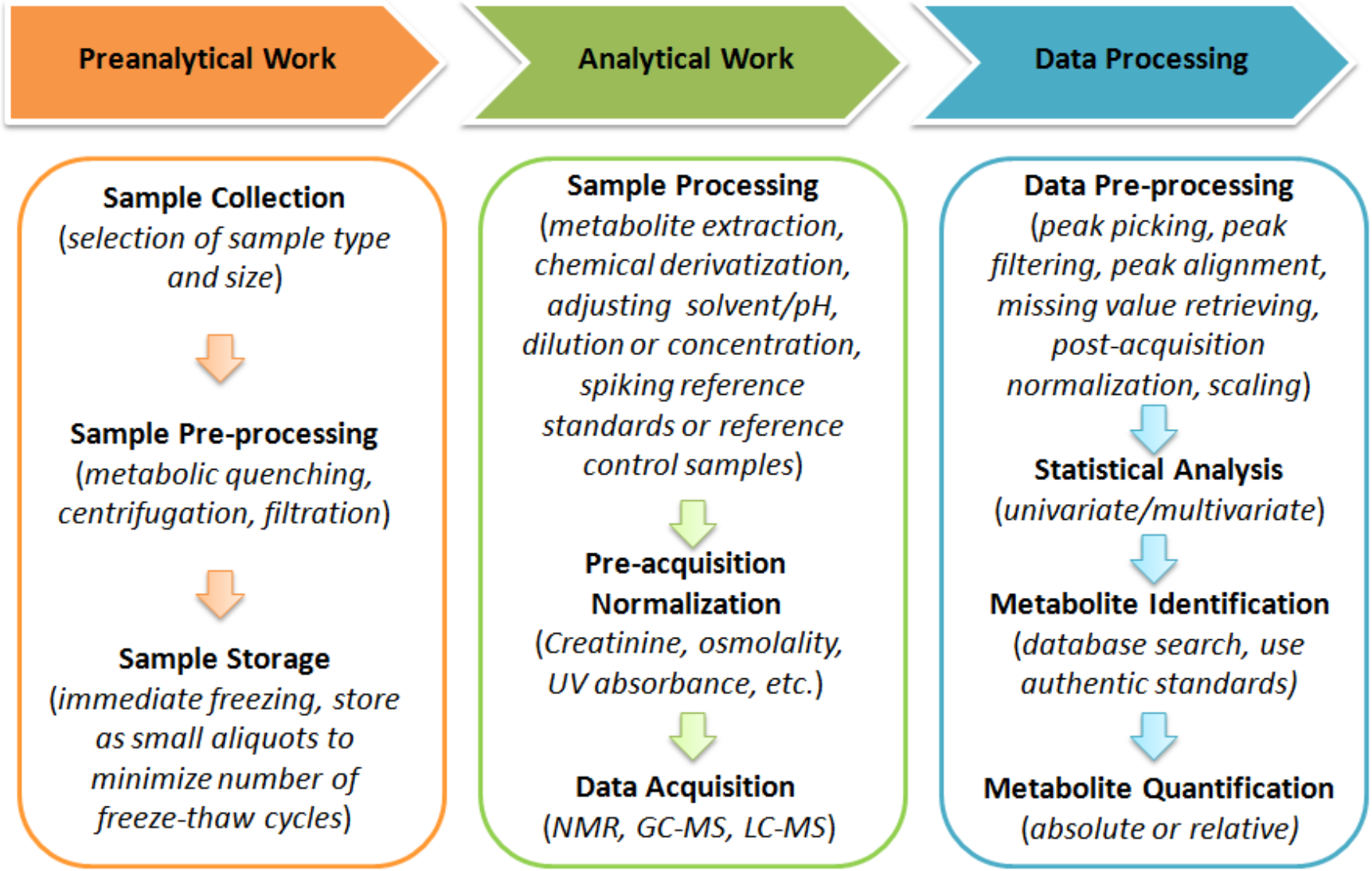
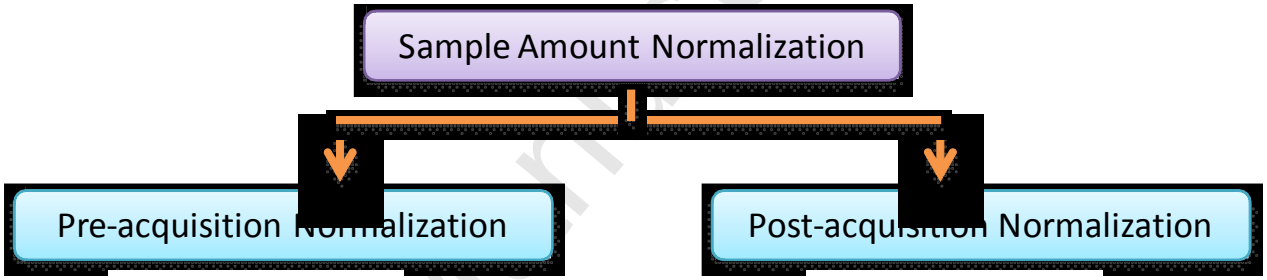

Preanalytical Work
Analytical Work
Data Processing
Sample Collection
(selection of sample type and size)
Sample Pre-processing
(metabolic quenching, centrifugation, filtration)
Sample Storage
(immediate freezing, store as small aliquots to minimize number of freeze-thaw cycles)
Sample Processing
(metabolite extraction, chemical derivatization, adjusting solvent/pH, dilution or concentration, spiking reference standards or reference control samples)
Pre-acquisition Normalization
(Creatinine, osmolality, UV absorbance, etc.)
Data Acquisition
(NMR, GC-MS, LC-MS)
Data Pre-processing
(peak picking, peak filtering, peak alignment, missing value retrieving, post-acquisition normalization, scaling)
Statistical Analysis
(univariate/multivariate)
Metabolite Identification
(database search, use authentic standards)
Metabolite Quantification
(absolute or relative)

Figure 1



Metabolome samples with different concentrations or amounts

Volume/concentration adjustment

Metabolome samples with same concentration or amount

Data acquisition

Sample	1	2	3	4	5
Metabolite 1	1.04	1.02	0.99	0.94	1.12
Metabolite 2	0.25	0.25	0.29	0.3	0.31
Metabolite 3	1.14	1.12	1.19	1.21	1.18

Metabolome samples with different concentrations or amounts

Data acquisition

Sample	1	2	3	4	5
Metabolite 1	1.04	0.61	0.47	1.57	2.27
Metabolite 2	0.24	0.16	0.14	0.46	0.6
Metabolite 3	1.16	0.65	0.63	1.59	2.15
Total intensity ($*10^5$)	5.19	3.03	2.55	7.75	10.9

Adjust signal according to acquired data

Sample	1	2	3	4	5
Metabolite 1	1.04	1.05	0.96	1.05	1.08
Metabolite 2	0.24	0.27	0.28	0.31	0.29
Metabolite 3	1.16	1.11	1.28	1.06	1.03

Figure 2

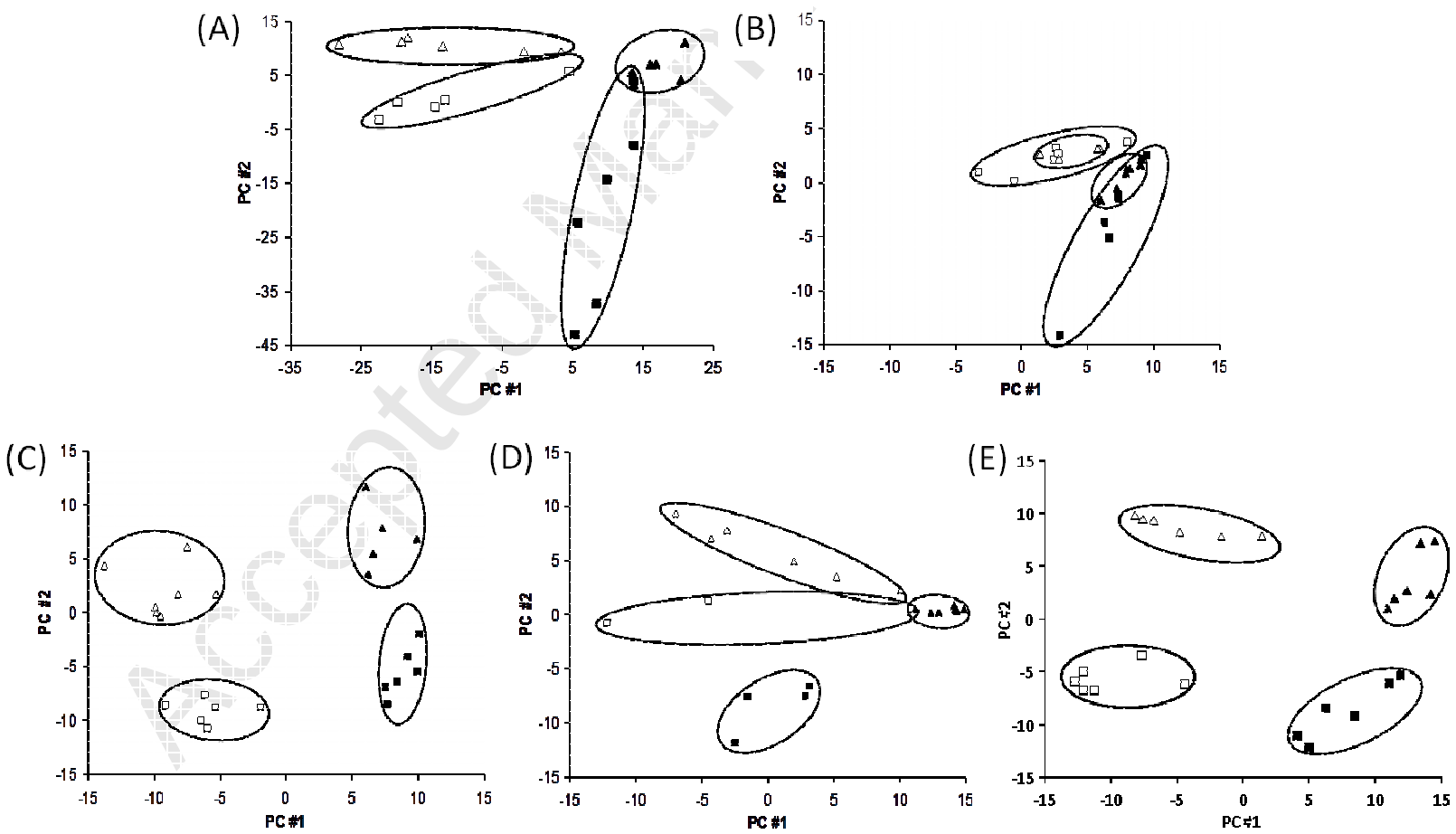


Figure 3

Adapted with permission from Warrack et al. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2009**, *877*, **547**.

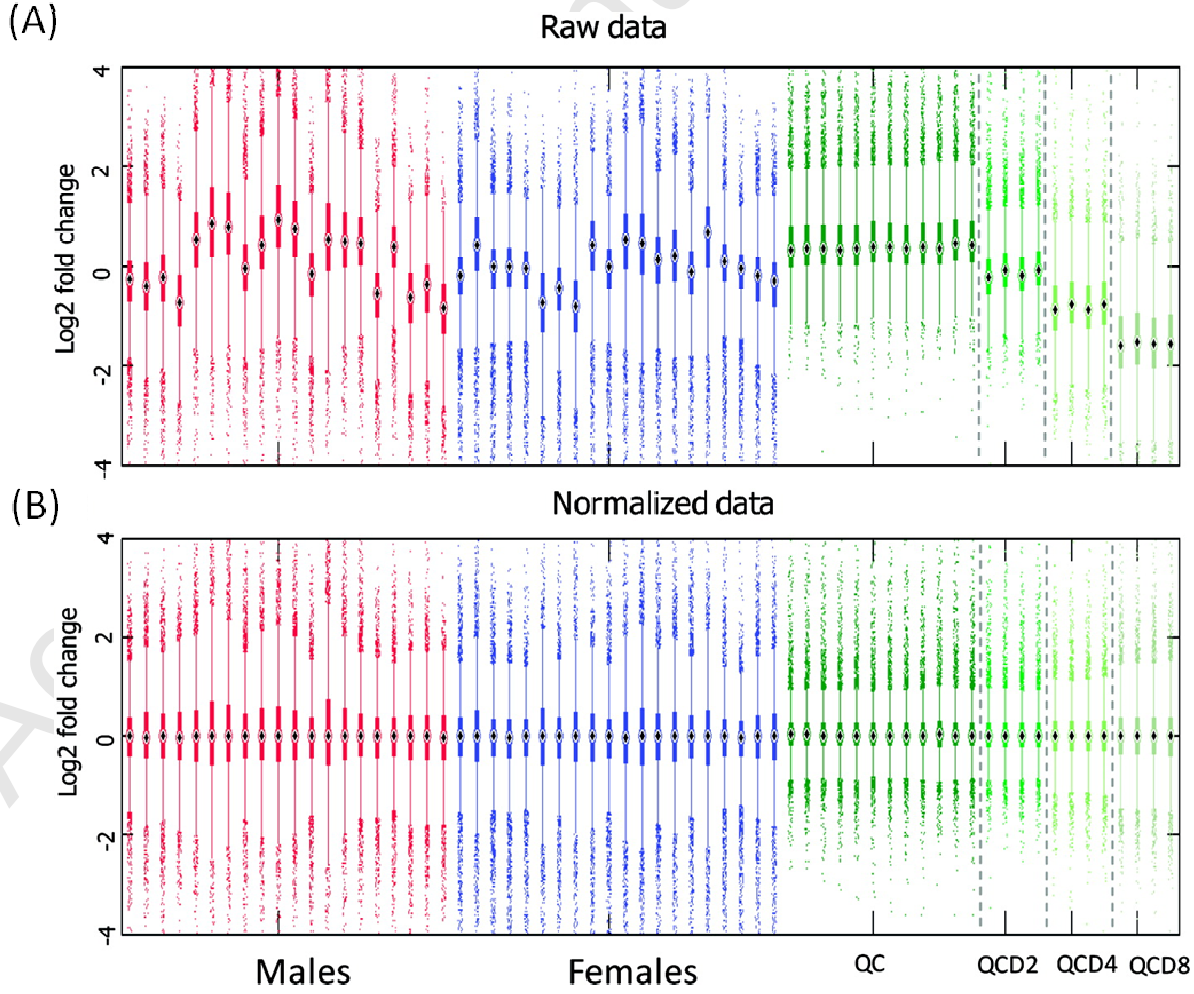


Figure 4

Adapted with permission from Veselkov et al. *Analytical Chemistry* **2011**, *83*, 5864.

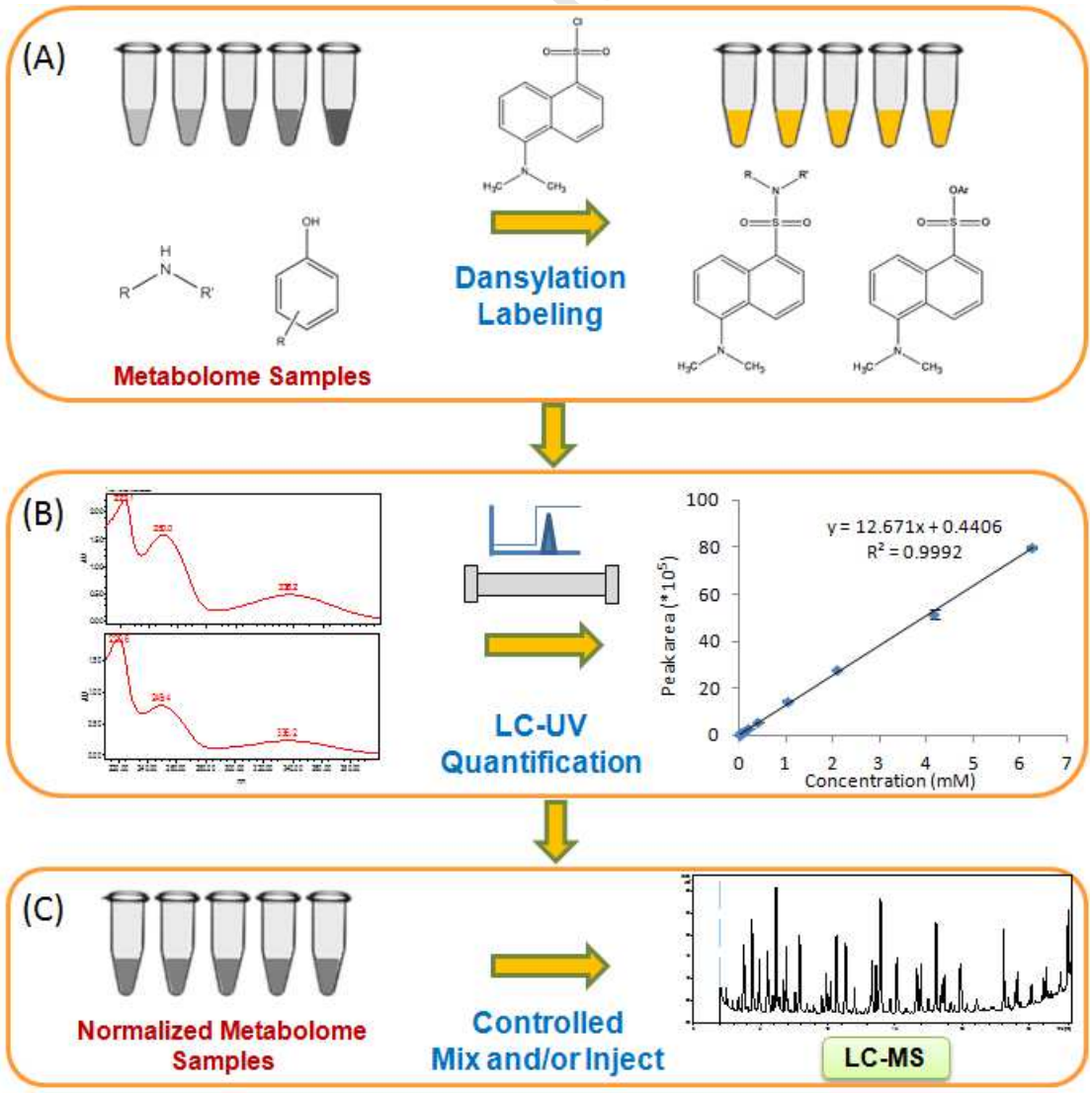

(A)
Metabolome Samples
Dansylation
Labeling
(B)
LC-UV
Quantification
y = 12.671x + 0.4406
R² = 0.9992
Peak area (*10⁵)
Concentration (mM)
(C)
Normalized Metabolome
Samples
Controlled
Mix and/or Inject
LC-MS

Figure 5

Highlights

- Sample-to-sample amount variation could be larger than analytical variation.
- Sample normalization is a critical step in quantitative metabolomics.
- Sample normalization should be incorporated into a metabolomic profiling workflow.
- There is no unified method; but a number of methods have been reported.
- The performance of sample normalization methods needs to be carefully considered to select a method.