Normalization strategies for metabonomic analysis of urine samples

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ABSTRACT

Unlike plasma and most biological fluids which have solute concentrations that are tightly controlled, urine volume can vary widely based upon water consumption and other physiological factors. As a result, the concentrations of endogenous metabolites in urine vary widely and normalizing for these effects is necessary. Normalization approaches that utilized urine volume, osmolality, creatinine concentration, and components that are common to all samples ("total useful MS signal") were compared in order to determine which strategies could be successfully used to differentiate between dose groups based upon the complete endogenous metabolite profile. Variability observed in LC/MS results obtained from targeted and non-targeted metabonomic analyses was highly dependent on the strategy used for normalization. We therefore recommend the use of two different normalization techniques in order to facilitate detection of statistically significant changes in the endogenous metabolite profile when working with urine samples.

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1. Introduction

Mass spectrometry (MS)-based metabonomic studies have been performed in order to determine qualitative and quantitative differences between the endogenous metabolite pools of study groups [1–12]. Most matrices, such as plasma, serum, and cerebral spinal fluid, are physiologically controlled [13–16]; however, urine volumes can vary widely based upon water consumption and other physiological and pathophysiological factors, and, as a result, the concentrations of endogenous metabolites in urine also vary. It is not uncommon to encounter up to 15-fold variations in urine volume [17] in a metabonomic study. Even greater variations may be observed if a dosed compound is a renal toxicant [18]. It can often be difficult to obtain an accurate measure of urine volume for rats which are housed in metabolic cages, particularly since contamination from food, water and feces can dramatically affect fluid levels in the collection vessel. Furthermore, urine volume is only a useful parameter if 24-h collections are performed. Reliable methods are needed to evaluate the amount of a specific endogenous metabolite not only in terms of absolute amount per sample but also relative to the total amount of all endogenous species. For urine, this necessitates the normalization of samples and data to a common denominator in order to minimize variation that results from individual urine output. Normalization performed post-sample analysis provides a way to investigate urine samples without requiring an absolute measurement of urine volume. Under normal conditions, urinary creatinine output is relatively constant and measurable. As a result, it has become common practice to normalize urinary analyte levels to this endogenous metabolite [19–22]. However, creatinine production does vary [22] and excretion can be impacted by an external stressor such as kidney impairment. In these cases normalization to creatinine is obviously not warranted. Osmolality is another factor that has been used to normalize urine measurements based on the premise that osmolite concentration is a direct measure of total endogenous metabolic output [23–25], and can be independently measured. Finally, we introduce the concept of MS "total useful signal" (MSTUS) [26], which uses the total intensity of components that are common to all samples, thus avoiding xenobiotics and artifacts that would not be appropriate measures of urine concentration. This latter approach is similar to the common practice used in proton NMR-based metabonomics analyses wherein each spectrum is normalized to the total integrated proton signal, after excluding regions corresponding to xenobiotics, internal standards and artifact-prone water and urea regions [27,28].

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Phospholipidosis is a lipid storage disorder which results in increased numbers of foamy macrophages in lung or liver tissue following a toxicological insult [29,30]. Phenylacetylglycine (PAG) has been reported to be a potential urinary marker for this condition [31–34]. These previous metabonomic studies employed either nuclear magnetic resonance (NMR) or MS to monitor PAG in urine samples and utilized either internal standards or computational techniques for normalization. In our current work, an agent that consistently induced phospholipidosis in rats was chosen as a model in vivo toxicity system. With this agent, large differences in urine volumes produced by different dose groups and the potential for sample loss during collection required normalization of the raw data prior to quantitative analysis. Using this model, we evaluated the common strategies of normalization described above, including urine volume, osmolality, creatinine concentration, and MSTUS. Other techniques employed for the normalization of MS data sets include the utilization of multiple internal standards, and custom or vendor-supplied software packages [35–39].

2. Materials and methods

2.1. Chemicals, reagents, and solvents

Mobile phases and standards were prepared using 18 MΩ deionized (DI) water (Purelab Plus, US Filter, Lowell, MA). High performance liquid chromatography (HPLC) grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ). Formic acid and sodium azide were from Sigma–Aldrich (Milwaukee, WI). D5-hippuric acid (d5-HA) was from CDN Isotopes (Quebec, Canada). Phenylacetylglycine (PAG) was from Bachem (King of Prussia, PA). Metabonomics Test Mixture was purchased from Waters Corp. (Milford, MA).

2.2. Osmolalities

Freezing-point depression was used to determine osmolalities of *in vitro* samples. Measurements were made using an Advanced Instruments Osmometer Model 2020 (Norwood, MA).

2.3. In vivo and sample collection

The study consisted of two groups of 12 Sprague–Dawley rats (Charles River Laboratories, Inc., Raleigh, NC), with six males and six females in each group receiving either a low (non-toxic) or a high (toxic) dose of a compound that is known to cause phospholipidotic changes in this species [unpublished result]. Urine was collected on wet ice over a 24 h period on day 1 and day 14. Urine volumes were recorded following collections. On day 14, the animals were euthanatized by intraperitoneal injection of sodium pentobarbital and subsequent exsanguination. The study was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and was approved by the Bristol-Myers Squibb Site Institutional Animal Care and Use Committee.

2.4. Sample preparation

Rat urine samples were diluted by either 1:8 or 1:200 with 98:2 water:acetonitrile (v/v) containing 0.1% sodium azide and 8 ng/μL d5-HA as an internal standard. Sodium azide was used as a bacteriostatic agent to stabilize the samples for LC/MS analyses. Sodium azide was not added when the urine samples were collected since, as a salt, it would have directly contributed to osmolality as measured by freezing-point depression. The internal standard was used for quantitative measurements in the targeted analysis; however, in the non-targeted analysis it was only utilized to ascertain that
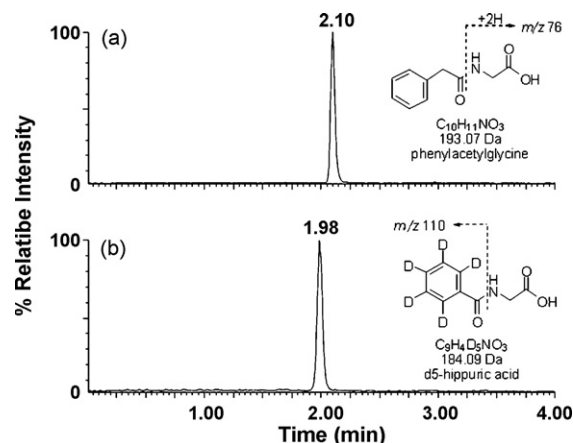

Fig. 1. Extracted ion chromatograms, structures and product ions used for the targeted metabonomic analysis. (a) *m/z* 194 to *m/z* 76 for phenylacetylglycine. (b) *m/z* 185 to *m/z* 110 for d5-hippuric acid.

mass spectrometer performance was stable during the analysis of the sample set.

The Metabonomics Test Mixture was reconstituted in 1 mL 98:2 DI water:acetonitrile, according to label directions. A 50 μL aliquot was further diluted with 200 μL 98:2 DI water:acetonitrile containing 8 ng/μL d5-HA internal standard for use as a Quality Control sample for the non-targeted analyses.

2.5. Liquid chromatography/mass spectrometry

2.5.1. Targeted analysis

The 1:200 dilution set was analyzed by LC–MS/MS using selected reaction monitoring (SRM) on a Quattro Ultima (Waters/Micromass, Manchester, UK) triple quadrupole mass spectrometer interfaced to a Waters 2790 HPLC (Milford, MA). Chromatographic separations were achieved employing a 2.0 mm × 50 mm, 3 μm, Luna C18-2 column (Phenomenex, Torrance, CA) with gradient elution at 1.0 mL/min. The column temperature was maintained at 20 °C. Mobile phase A was 98:2 water:acetonitrile with 0.1% formic acid and mobile phase B was 98:2 acetonitrile:water with 0.1% formic acid. The initial mobile phase composition, 98:2 mobile phase A:mobile phase B, was held for 1 min. A linear gradient was then formed from 2% to 100% mobile phase B over 2 min. The final composition was held for 0.5 min before returning to the initial conditions. A single 5 μL injection was made for each sample. The sample order was randomized.

The LC column effluent was split so that the flow into the electrospray interface was ∼250 μL/min. Analysis of the chromatographic eluent was carried out using positive electrospray ionization (ESI). Analytes were detected by monitoring the following transitions: *m/z* 194 to *m/z* 76 for PAG and *m/z* 185 to *m/z* 110 for d5-HA (Fig. 1). Instrumental settings follow: collision energy 10 eV; argon as collision gas at ∼4.3e−3 bar; sample cone voltage 40 V; source temperature 130 °C; nebulizer gas temperature 400 °C.

2.5.2. Non-targeted analysis

The 1:8 dilution set was analyzed by LC/MS on an LTQ-FT (Thermo Scientific, San Jose, CA) interfaced to a Waters Acquity ultra performance liquid chromatograph (UPLC) (Milford, MA). Chromatographic separations were achieved employing a 2.0 mm × 100 mm, 1.7 μm, Acquity BEH-C18 column (Waters, Milford, MA) with gradient elution at 0.6 mL/min. The column temperature was 40 °C. Mobile phase A was 98:2 water:acetonitrile with 0.1% formic acid and mobile phase B was 98:2 acetoni-

trile:water with 0.1% formic acid. A two step gradient was formed from 0% to 20% mobile phase B over 6 min then to 95% mobile phase B over 2 min. The final composition was held for 1.5 min before returning to the initial conditions.

Positive and negative ESI Fourier transform mass spectrometry (FTMS) data were acquired from separate injections from m/z 85 to m/z 850. A single 5 μL injection was used for each ionization mode and samples were run in randomized order. Metabonomics Test Mixture was injected at the start of the analysis and after every 16 injections (i.e. after every 8 samples) to ascertain that the mass spectrometer performance was stable during the analysis of the sample set. The instrument was operated at 12,500 resolution. Instrumental settings follow: capillary temperature 320 °C, capillary voltage 25 V for positive ion mode, 15 V for negative ion mode; tube lens voltage 70 V; ESI metal needle option; ESI needle voltage 5 kV for positive ion mode, 6 kV for negative ion mode; sheath gas 100 arbitrary units (arbs); auxiliary gas 15 arbs; sweep gas 0.8 arbs.

2.6. Software

Data files were acquired using vendor provided MS operation software (Waters MassLynx or Thermo Scientific Xcalibur). The SRM data files obtained from the targeted analysis of PAG were processed using the QuanLynx module of the MassLynx software to obtain peak areas for PAG and d5-HA. Data files from non-targeted analyses were processed with custom software using an approach similar in concept to others previously published [26,40]. Our software consists of two modules. The first module was used for all normalization strategies. The second module was used only for normalization to MSTUS. For normalization to urine volume, creatinine concentration, and osmolality, ions were extracted using a 10 ppm window and those with signal-to-noise (S:N) > 10:1 were summed to generate a base peak chromatogram. Normalization to creatinine was achieved for each sample by calculating the MS area response for m/z 114.0662, $(M+H)^+$ for creatinine, and then scaling the base peak chromatograms to this measurement. Similarly, the base peak chromatograms were normalized to urine volume and osmolality. The second module of the software uses a proprietary algorithm to combine related ions into molecular components and then sums all of the integrals for all peaks that were common among all of the samples to generate the base peak chromatogram. In doing this, the MSTUS approach attempts to limit the contributions of xenobiotics and artifacts to the normalization factor by including only those peaks that are present in all samples, including the controls. Multivariate analysis and visualization of the results were performed with Partek-Pro 6.0 software (Partek, St. Louis, MO).

3. Results and discussion

Fifteen-fold variations in urine volume are common in studies where no renal impairment occurs [17]. Table 1 illustrates the variations observed for 24-h urine collections in the present study. Within the low dose female group the urine volumes varied from 1.7 to 16.8 mL on day 14, corresponding to a 10-fold difference. When volumes were compared across the entire sample set, the change was greater than 15-fold. The corresponding changes in osmolality values change by less than 3.5-fold within a group and by less than 5-fold across the entire set.

Each sample, regardless of the original volume, was analyzed following the protocol described in Section 2 and the obtained data were then normalized. For the targeted analysis, samples were normalized to urine volume and to osmolality. For the non-targeted analysis, separate evaluations were conducted on the data normalized to urine volume, osmolality, creatinine concentration, or MSTUS.

Table 1
24-h urine collections and corresponding osmolality values from 4 rat study groups.

	Day 1		Day 14	
	Volume (mL)	Osmolality (osmoles/kg)	Volume (mL)	Osmolality (osmoles/kg)
Low dose male	11.5	1035	11.8	773
	17.5	655	18.7	550
	8.0	601	10.6	808
	6.5	986	7.7	982
	6.5	1303	10.1	1256
	7.5	1412	11.7	928
Low dose female	12.5	905	16.8	676
	5.5	960	8.1	702
	9.0	721	12.4	563
	2.0	1625	3.0	1110
	3.5	1412	1.7	1025
	5.5	959	12.1	653
High dose male	23.5	458	20.1	676
	14.0	772	17.9	863
	11.5	1263	25.5	700
	23.5	578	14.9	699
	6.5	1499	10.7	743
	3.5	1099	28.7	428
High dose female	8.0	864	16.2	578
	7.0	608	26.5	340
	9.0	960	25.2	389
	5.5	1018	19.5	490
	9.1	749	21.0	442
	3.8	835	25.1	326

3.1. Normalization to urine volume and to osmolality for targeted analyses

Urine samples were diluted 1:200 for targeted analysis to avoid saturation of the MS detector. A single LC–MS/MS analysis was performed on each sample and the ratio of the PAG peak area to the internal standard, d5-HA, area was calculated. Without normalization, the relative standard deviation (RSD) within a group ranged from 25.1% to 53.8% (Fig. 2a). The results from day 1 and day 14 for the different study groups overlapped and no statistically significant changes could be identified. Normalization to urine volume increased the RSDs by approximately 2-fold in all study groups (Fig. 2b), and changed the relative amounts of PAG such that the
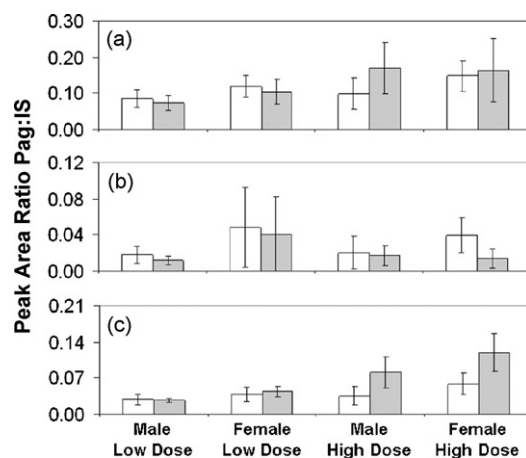

Fig. 2. The impact of normalization on the peak area ratio of phenylacetylglycine to d5-hippuric acid internal standard. (a) No normalization. (b) After normalization to urine volume, no statistically significant changes were observed. (c) After normalization to osmolality. Day 1: white rectangles; day 14 (necropsy): gray rectangles. Error bars represent 1 standard deviation.
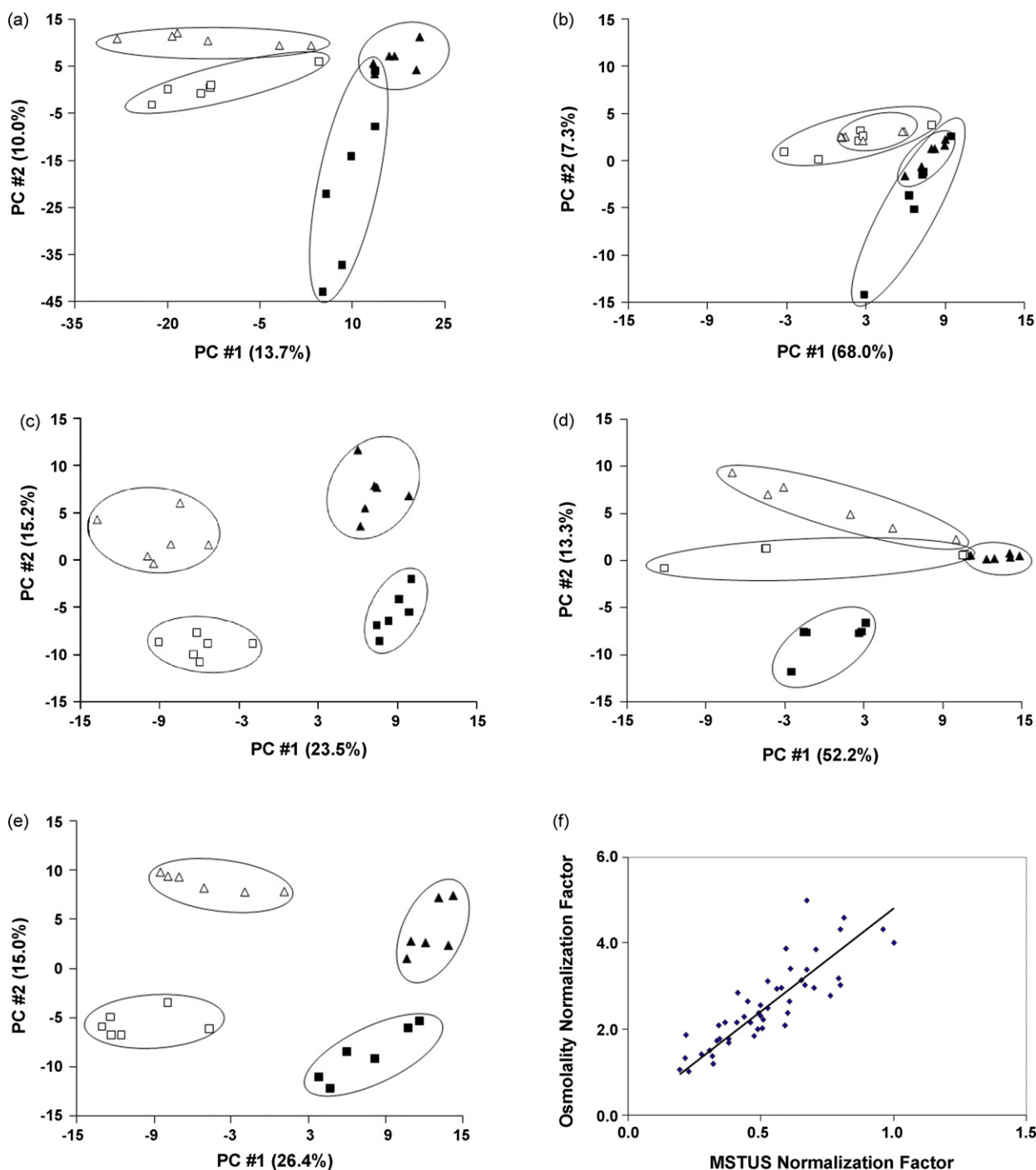
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Fig. 3. PCA plots for day 14 LC/MS data (a) without normalization; (b) normalized to urine volume; (c) normalized to osmolality; (d) normalized to creatinine concentration; (e) normalized to TUS; males represented by squares; females by triangles; low dose represented by open square or triangle; high dose by solid square or triangle. (f) Correlation between MSTUS and osmolality normalization factors; R2 = 0.71.

putative biomarker actually decreased in dosed animals where it would have been expected to increase.

Osmolality is representative of the concentration of solutes in a fluid and is determined by measurement of freezing-point depression. It has been used for normalization of urine in clinical settings and has been reported to offer advantages over normalization to creatinine concentration [23]. Osmolality is not normally influenced by diurnal rhythms, diet, activity, gender, age, stress or health as creatinine levels are [23]. The effect of normalization to urine osmolality is best illustrated by the female high

dose group where the RSD was reduced from 53.8% to 30.9% on day 14. Fig. 2c shows results from all four groups after normalization to osmolality. Statistically significant increases ($p < 0.01$) were observed for both male and female high dose groups on day 14. Histopathology of lung tissue on day 14 confirmed the presence of phospholipidosis in all high dose animals through the use of Nile Red staining techniques [41]; therefore, the increased PAG levels observed in the high dose animals are consistent with the idea that PAG can be used as a marker for phospholipidosis.

3.2. Normalization strategies for non-targeted metabonomic analysis

The results from the targeted analysis confirmed a statistically significant change between the study groups so these samples were then utilized as a model set for a non-targeted metabonomic analysis to evaluate the effect of different normalization strategies. In order to provide sufficient signal for data acquisition over an 800 Da mass range, the samples needed to be more concentrated than those used for the targeted analysis; therefore, the original urine samples were diluted 1:8 prior to positive and negative ESI accurate mass LC/FTMS analyses. In the following section, the non-targeted accurate mass LC–FTMS data were analyzed by principal components analysis (PCA) with univariate scaling after application of the different normalization strategies discussed previously. For clarity, only data from day 14 from the four dose groups are presented in Fig. 3.

3.2.1. No normalization

The principal components calculated from the non-normalized data are shown in Fig. 3a. Although there is some separation between the dosing groups, the male and female high dose groups overlap. Significant variation between the biological replicates is observable.

3.2.2. Normalization to urine volume

The principal components calculated following normalization to urine volume are shown in Fig. 3b. The separation between the study groups is reduced when compared to the non-normalized data (Fig. 3a). There is separation between the high and low dose groups; however, within each, the males and females overlap.

3.2.3. Normalization to osmolality

As mentioned previously, osmolality is indicative of solute concentration and is a direct measure of the total endogenous metabolic output. The principal components calculated from the data normalized to osmolality are shown in Fig. 3c. The dosing groups are better separated and variation between the biological replicates is reduced when compared to non-normalized data (Fig. 3a).

3.2.4. Normalization to creatinine

When data is normalized to creatinine, a single component is used as a surrogate for all endogenous metabolites in the sample. Its utility has been demonstrated for applications in clinical chemistry and NMR metabonomic studies [19–21]. Typical biological variation for creatinine is small (2-fold) within a homogenous population such as Sprague–Dawley rats on a fixed diet; however, 4–5-fold changes have been reported for humans over a 30 day period due to the influence of stress, diet, activity, age, race, gender, and/or health [22]. Diurnal influences have also been observed [42]. Fig. 3d illustrates the principal components calculated from the data after normalization to creatinine. This result is similar to that obtained for the non-normalized data, Fig. 3a. There is less separation between the groups, overlap of the male low dose group with the female high dose group, and large variations between the low dose biological replicates.

3.2.5. Normalization to MSTUS

Data files from non-targeted analyses were processed using custom software which uses post-acquisition computational data processing to identify component signals in the raw data and, by summing these signals, determine a total useful signal value for each sample. For each sample, approximately 1800 ions were detected. After background subtraction, the result was approximately 1100 components. Of these, about 800 were common to all samples and were used to generate the MSTUS normalization factor. The number of observed components was similar between ion modes and the overlap was about 17%. All 1100 component signals in each sample were then normalized to an equivalent MSTUS value prior to principal components analysis. The MSTUS processing method is similar to that used for proton NMR-based metabonomics analyses [27–28] and utilizes features similar to those reported in other metabonomic and proteomic LC/MS processing methods such as mzmine [43], metAlign [44], Xalign [45], MSFACTS [46] and MMSR [40].

Fig. 3e depicts the principal components after normalization to MSTUS. As was observed for normalization to osmolality (Fig. 3c), this strategy also provides separation between the dosing groups and reduces the variation between biological replicates. A correlation between the MSTUS normalization factors and osmolality is shown in Fig. 3f.

4. Concluding remarks

A targeted metabonomic study was performed to determine the levels of PAG in rat urine samples. Normalization to osmolality was required in order to observe statistically significant changes for male and female high dose groups. These samples were then utilized as a model set for a non-targeted metabonomic analysis to evaluate the effect of different normalization strategies on the complete endogenous metabolite profile.

One might anticipate that urine volume would be inversely correlated with the overall urine concentration; however, based upon the dramatic differences seen when normalizing to volume versus other techniques (compare Fig. 3b vs. Fig. 3c–e), this is clearly not the case. For the targeted study, normalization to urine volume caused the putative biomarker for phospholipidosis to decrease in the presence of verified lesions. When we evaluated the complete endogenous metabolite profile, normalization to creatinine concentration (Fig. 3d) was comparable to no normalization (Fig. 3a). Both provided some differentiation between the dose groups, but a large variation between the biological replicates was observed. Normalization to both osmolality (Fig. 3c) and MSTUS (Fig. 3e) improved the differentiation between the dose groups nearly equivalently (Fig. 3f). We therefore recommend the use of two different normalization techniques in order to facilitate detection of statistically significant changes in the endogenous metabolite profile when working with urine samples. Osmolality can be used in cases where it is possible to get this independent measurement, and a MSTUS or related approach can be used when osmolality measurements are not convenient or not valid.

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