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ARTICLE *in* ANALYTICAL CHEMISTRY · AUGUST 2007

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Experimental and Analytical Variation in Human Urine in ^{1}H NMR Spectroscopy-Based Metabolic Phenotyping Studies

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^{1}H NMR spectroscopy potentially provides a robust approach for high-throughput metabolic screening of biofluids such as urine and plasma, but sample handling and preparation need careful optimization to ensure that spectra accurately report biological status or disease state. We have investigated the effects of storage temperature and time on the ^{1}H NMR spectral profiles of human urine from two participants, collected three times a day on four different days. These were analyzed using modern chemometric methods. Analytical and preparation variation (tested between −40 °C and room temperature) and time of storage (to 24 h) were found to be much less influential than biological variation in sample classification. Statistical total correlation spectroscopy and discriminant function methods were used to identify the specific metabolites that were hypervariable due to preparation and biology. Significant intraindividual variation in metabolite profiles were observed even for urine collected on the same day and after at least 6 h fasting. The effect of long-term storage at different temperatures was also investigated, showing urine is stable if frozen for at least 3 months and that storage at room temperature for long periods (1−3 months) results in a metabolic profile explained by bacterial activity. Presampling (e.g., previous day) intake of food and medicine can also strongly influence the urinary metabolic profiles indicating that collective detailed participant historical meta data are important for interpretation of metabolic phenotypes and for avoiding false biomarker discovery.

^{1}H NMR spectroscopy has been used since the early 1980s to effect multivariate metabolic profiling in man and animals including diseases such as type II diabetes mellitus and hyperlipidemia.[1−3] Even these early studies showed good analytical reproducibility and quantitative accuracy of NMR measurements on defined compounds with reference to established HPLC or enzymatic assays for a variety of metabolites.[2,3] The applications of pattern recognition methods to analyze NMR data in the late 1980s[4] showed that effective classification of samples based on their NMR-derived metabolic properties could be achieved and that a wide range of toxic and disease states could be mapped and modeled.[5−10] Plasma and urine samples give complementary "global" representations of the integrated metabolic state of an organism, i.e., patterns related to metabolic processes under the control of many cell and tissue types. Plasma gives an "instantaneous" (i.e., representing the metabolic content at the exact time of collection) readout of systematically controlled metabolites and lipoproteins, whereas urine gives a "time-averaged" pattern for polar metabolites that are excreted in variable amounts according to variations in whole-body homeostatic control. Metabonomic and metabolomic approaches to biomarker discovery are now widespread, and understanding analytical and biological variation is critically important in any metabolic profiling study. In particular, it is necessary to quantify and separate artifactual and analytical variation from the biological variations of interest and, at a higher level, to understand the combined sources of biological variation that may confuse the analysis and lead to erroneous biological conclusions.

Sources of variation in metabolic studies that must be considered include the following: (i) sample collection and storage; (ii) sample pretreatment prior to analysis; (iii) instrumental variation and calibration; (iv) intraindividual variation due to physiological factors and temporal patterns in exposure to environmental factors such as nutrients or stress; (v) interindividual variation due to genetic and environmental factors; (vi) interindividual variation related to biological hypothesis, e.g., presence of a particular disease state. Metabolites vary considerably in their chemical

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10.1021/ac070212f CCC: $37.00
Published on Web 06/08/2007

stability, which is related to conditions they experience during sample preparation (i and ii). Variations in temperature will alter spontaneous reactions such as decarboxylation of acetoacetate to acetone and hydrolytic reactions such as ester cleavage. It should also be noted that most biofluids have some level of intrinsic biochemical activity, e.g., esterase, transaminase, and peptidase, which may be altered markedly in disease states, hence complicating the sample handling problem in the clinic.

Several reports have addressed issues concerning variation in metabolite profiles unrelated to the hypothesis being tested. For example, diurnal variation has been documented in urine from rats[11] and mice,[12] as a result of differences in behavioral activity at different times of the day. Different strains of normal rats have been shown to exhibit different characteristics such that statistical analysis can clearly distinguish them on the basis of their urinary metabolic profiles.[13] The ^{1}H NMR profiles of urine from healthy humans are typically dominated by metabolites such as creatinine, lactate, alanine, citrate, dimethylamine, glycine, hippurate, and trimethylamine-*N*-oxide (TMAO)[4,14] Dietary intake has been shown to modulate these profiles in a mild but predictable fashion, depending on the meal eaten; for example, a fish meal will result in increased TMAO[4,14,15] Physical activity prior to collection of urine in humans can influence resultant ^{1}H NMR metabolite profiles.[16] Moreover, it has been shown that population differences are also expressed in human metabolic profiles,[17] which is important to understand as part of molecular epidemiological studies.[18] In a recent study, first morning urine was collected without dietary restrictions in Swedish and British subjects, which were found to be phenotypically different.[19] Principal components analysis (PCA) was used to facilitate identification of specific molecules such as TMAO and taurine as being part of a distinguishing phenotypic biomarker separating the two populations. Of course it is well-known that bias can be introduced into blood plasma samples collected for metabonomic analysis as a result of collection and postcollection handling procedures, such as freeze−thaw cycles and room-temperature storage.[20] However, there have been no comprehensive analyses on handling and pretreatment of urine samples for large population or epidemiological studies. By definition, these are the most challenging, as many samples have to be collected and handled in the field, so simple and robust procedures are required. One such study currently underway is molecular phenotyping to accelerate genomic epidemiology (MolPAGE), within which, careful consideration will be paid to optimization and standardization of sample collection and storage procedures with a view to minimize sample sizes, e.g., the volume of blood collected from a participant, while maximizing the throughput rate for sample analysis.

The high reproducibility and information richness of ^{1}H NMR spectroscopy lends itself to metabolic phenotyping studies and chemometric analysis.[21,22] Recent advances in pattern recognition methods such as orthogonal projections to latent structures discriminant analysis (O-PLS-DA)[23] and *s*tatistical *to*tal *c*orrelation *s*pectroscopy (STOCSY)[24] have greatly improved the prospects for biomarker discovery, in that large data sets of full-resolution NMR spectral data can now be routinely analyzed.

In this paper, we present an investigation of the influence of sample collection and handling procedures such as storage temperature, and length of storage (prior to freezing at −40 °C), on the metabolic profile of urine samples collected at various times of the day as measured by ^{1}H NMR spectroscopy and analyzed by modern chemometric methods. In this, the inter- and intraindividual metabolic variation could also be assessed. The aims of this study were to assess the consequences of delayed freezing of urine samples collected for metabonomic analysis, compare and contrast the metabolic profiles of first morning urine with urine collected at other times of the day after fasting, and identify the metabolites responsible for the variations detected.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma (St. Louis, MO). D_2O (99.9%) was from Goss Scientific Instruments Ltd. (Essex, UK).

Sample Collection and Handling. The study design is summarized in Scheme 1. The study participants were two healthy participants, one male, one female, aged 27 and 29 years, respectively. Midstream urine samples were collected on 2 days (from participant 1) and 4 days (from participant 2) over 1 month. The participants were given instructions not to consume food 7 h prior to collection and not to consume any alcohol the night before. On each day, three samples were collected. The first, collected at home, was left at room temperature for 2 h (over which time it was transported into the laboratory) and then stored at −40 °C. The second and third collections were taken on arrival after fasting, and late afternoon, after 7-h fasting, respectively. Each were aliquoted into five tubes subject to the following conditions: kept at room temperature for 2 h and stored at −40 °C; kept at room temperature for 24 h and stored at −40 °C; refrigerated at 4 °C for 2 h and stored at −40 °C; refrigerated at 4 °C for 24 h and stored at −40 °C; stored immediately at −40 °C.

It is anticipated that in some studies within the MolPAGE project, participants will be requested to collect urine for a period of time prior to a research visit. Part 1 of this study was intended to mimic a situation in which a participant was requested to collect urine on the morning of, and the morning prior to, a research visit. For this reason, each "on arrival" sample was aliquoted into

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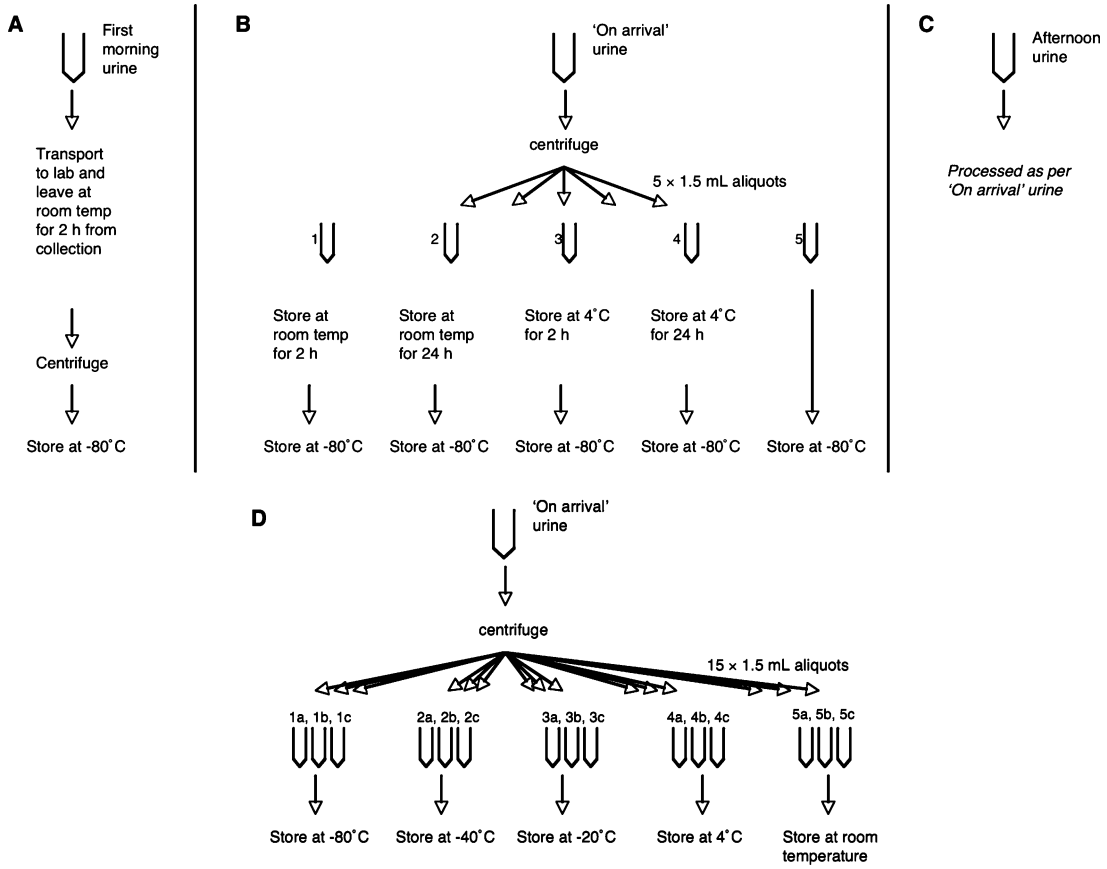
Scheme 1. Outline of Collection and Handling for Each Sample[a]



[a] In part 1, three urine samples were collected from the same participant on each day of the study. The first was collected as "first morning urine" (also called T_{FM}) after overnight fasting, Subsequent collections on the same day were "on arrival" (T_{AM}) and afternoon (T_{PM}), which were both split 5 ways and handled as shown. in part 2, One on arrival sample was selected and split 15 ways, each being stored overnight (1a, 2a, etc.), for 1 month (1b, 2b, etc.) or 3 months (1c, 2c, etc.) at the temperatures indicated.

five separate containers: one immediately frozen at −40 °C, assumed to be representative of the true metabolic urinary profile at the time of collection, two stored at 2 and 24 h at 4 °C, to mimic the situation in which a participant collected urine and stored it in their refridgerator, and two samples stored for 2 and 24 h at room temperature, to mimic the situation which the participant did not refrigerate the sample.

The second part of the study examined the effects of long-term storage on urine samples. One sample was and aliquoted 15 times, each aliquot being stored at one of five temperatures: −80, −40, −20, and 4 °C and room temperature (18−23 °C), for 3 months, 1 month or overnight (0 months).

NMR Spectroscopy. Thawed samples were centrifuged at 13000*g* for 10 min. The 200-μL aliquots were then added to 96-well plates containing 200 μL of H_2O and 200-μL solution of 0.2 M phosphate buffer (pH 7.4) 1 mM sodium trimethylsilyl [2,2,3,3-$^2H_4$]propionate (TSP), and 3 mM NaN_3 in 20% D_2O. All samples were run on a 600-MHz Bruker DRX spectrometer fitted with a 5-mm flow injection probe connected to a Bruker Efficient Sample Transfer system (Bruker Biospin, Karlsruhe, Germany). 1D ^{1}H NMR spectra were acquired with a pulse sequence of the form d1−$\pi/2$−t1−$\pi/2$-tm−$\pi/2$, where $\pi/2$ represents a 90° hard pulse, d1 is a relaxation delay (2 s), t1 is a short delay (4 μs), and tm is the mixing time (100 ms). The resonance of H_2O ($\sim\delta$4.7) was selectively irradiated with a continuous wave low power pulse during d1 and tm. For each sample, 128 transients were collected into 32k data points. Prior to Fourier transformation, the free-induction decay was multiplied by an exponential function corresponding to a line-broadening factor of 1 Hz in the frequency domain. Spectra were phased, baselined, and referenced to the TSP resonance (δ0.00) using iNMR (v1.1, G. Balacco).

PCA and O-PLS-DA. Prior to chemometric analysis, the spectral data region corresponding to TSP (to δ0.50) was removed, along with residual water (δ4.50−δ5.00) and urea (δ5.50−δ6.10), the latter two to eliminate variations associated with presaturation. The data were then normalized such that the total integral of the remainder of each spectrum was a constant. PCA and O-PLS-DA[24] models were constructed using in-house software (MetaSpectra, O. Cloarec, Imperial College London). Prior to PCA, the data were mean-centered, and prior to O-PLS-DA, the data were scaled to unit variance. O-PLS-DA models, visualized using the method described elsewhere,[24] were plotted as a function of the ^{1}H chemical shift; the intensity corresponds to the covariance, and the color corresponds to the correlation between each variable and its defined class. STOCSY was performed on the data set to detect statistical correlations between resonances within the spectra (as described elsewhere[24]) and to facilitate assignment. Visualization of these correlated resonances was facilitated by coloring each variable according to the correlation between it and the variable selected. Unless otherwise stated, one orthogonal component was calculated for each O-PLS-DA model.

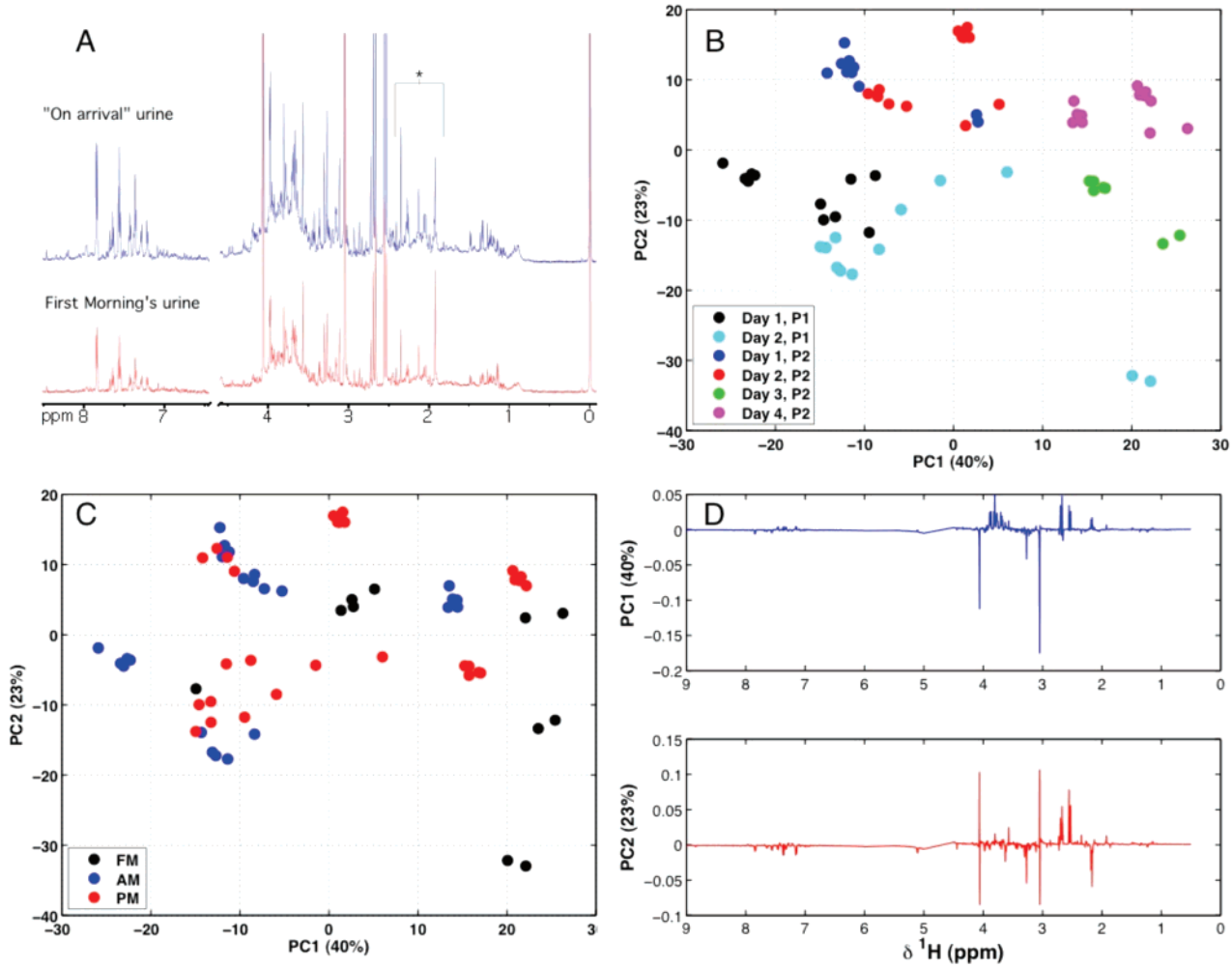


Figure 1. (A) Characteristic ^{1}H NMR spectra of first morning (red) and "on arrival" (blue) urine collected on the same day from the same participant (after fasting) and stored for 2 h at room temperature. Spectra were referenced to TSP at δ0.00. The bracketed region (*) highlights a series of resonances that exemplify relative changes from one collection to the next. (B) PCA scores plot for the entire data set, colored according to day of collection. Each color represents a different day, with P1 and P2 denoting paritcipant 1 and participant 2, respectively. (C) PCA scores plot for entire data set (same as B) but colored according to time of day of collection. (D) PCA loadings plot for the first two principal components plotted as a function of chemical shift. Numbers in brackets are the total variance explained within each principal component.

RESULTS

Preliminary Inspection of NMR Spectra. Figure 1A compares the ^{1}H NMR spectra from a typical first morning urine sample to that of a sample collected on arrival to the laboratory on the same day after an overnight fast. Both samples were stored at room temperature for 2 h prior to being stored at −40 °C. The spectra are dominated by methyl and methylene resonances from creatinine at δ3.05 and δ4.06, respectively. On visual inspection, both spectra were very similar, notable exceptions being a shift in the citrate (from δ2.685 to δ2.671) and an ∼2-fold decrease in hippurate resonances for "on arrival" urine. The complex nature of the metabolic profile of urine, manifested by the presence of overlapping resonances, renders chemometric analyses essential in the interpretation of results from experiments in which the number of samples is large. Thus, further analyses of the urine samples were conducted by PCA and O-PLS-DA.

Preliminary Principal Components Analysis of ^{1}H NMR Data. All samples taken on arrival or after 7-h fasting and first morning samples were analyzed by PCA. This type of analysis is designed to highlight systematic variation across series of NMR spectra. It results in the calculation of a series of principal components (PCs) for each sample, which can be plotted to reveal trends in the spectra.

After normalizing the spectra to constant sum and mean-centering all variables, the scores for PC1 and PC2 were calculated. Figure 1B shows the plot of PC1 against PC2, with points colored according to the day of collection. Participant 1 was seen to separate out from participant 2 in the combined "space" of the first two PCs. Clustering was also evident for urine collected on the same day. Systematic variation was observed within each collection day according to the time of day in which the sample was collected, for example, the clustering of "AM" urine from participant 1 at very negative PC1 values (Figure 1C), is separated from the rest of the urine collected on this day. Figure 1D plots the corresponding loadings as a function of the ^{1}H chemical shift. These indicate which resonances are most influential in the calculation of the PCA scores. It was seen that creatinine and various resonances between δ3.0 and δ4.0 were most influential in the calculation of PC1, while PC2 was

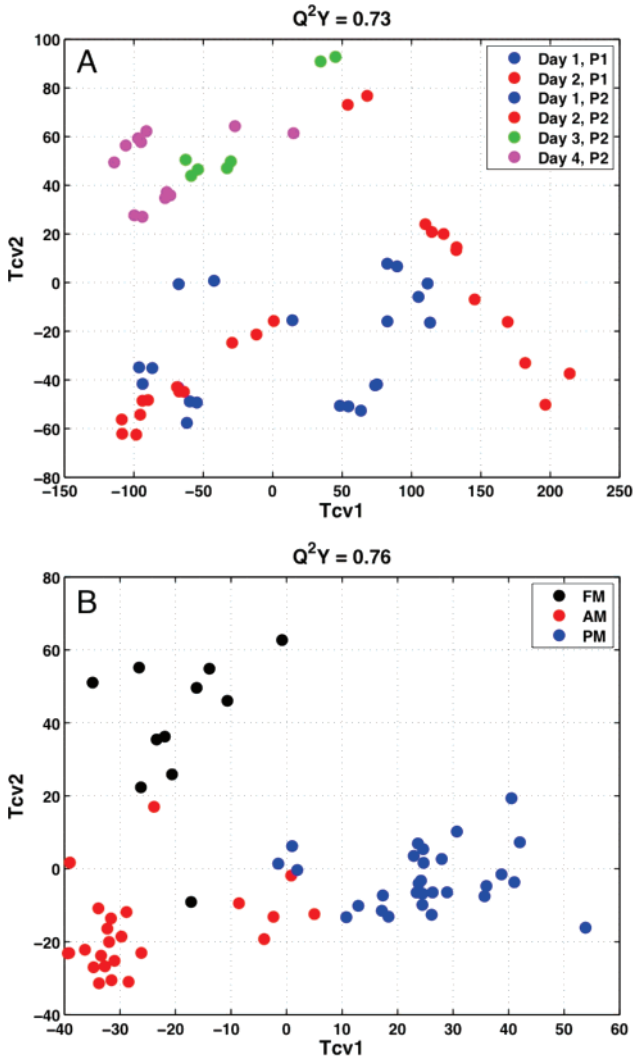


Figure 2. O-PLS cross-validated scores for the discrimination of the urine spectra. (A) O-PLS-DA model in which the classes were defined as the day of collection. $Q^2\hat{Y} = 0.73$ (after computing 1 orthogonal component), (B) O-PLS-DA model in which the classes were defined as the time of day of collection. $Q^2\hat{Y} = 0.76$ (after computing 5 orthogonal components). Color coding is given in the legend.

dominated by resonances from citrate and several peaks around δ2.16, and peak positional variation of creatinine resonances. Peak position variation of creatinine was manifested as "dispersive" loadings for those resonances in PC2, due to differences in salt concentration in the samples.

Preliminary O-PLS-DA of ^{1}H NMR Data. Modeling by O-PLS-DA also showed separation based primarily on the day of collection. In this modeling technique, the systematic variation in the spectra due to noise does not affect the output. Figure 2 shows the cross-validated score plots from PLS analysis in which the data were grouped into classes of day of collection or time of day of collection. Clustering was observed for urine samples collected on the same day (Figure 2A), in agreement with the above PCA analysis. When samples were instead classed according to time of day of collection (T_{FM}, T_{AM}, T_{PM}) regardless of the day of collection, clustering was also observed (Figure 2B). O-PLS-DA models also were constructed for the same data set defining classes according to postcollection storage time and storage temperature, but no clustering was evident, with $Q^2\hat{Y}$ values of <0 (not shown).

STOCSY-Based Metabolite Identification Using Multiple NMR Spectra. It was apparent from the PCA plot that within each day of collection there were systematic changes in the ^{1}H NMR data associated with time of day, temperature, etc. To identify metabolites that were responsible for this, O-PLS-DA was used to identify resonances significantly different between the groups, and these were assigned using a combination of the recently developed STOCSY methods[24] and published literature.[25]

When urines were classed according to participant, pairwise comparison of all the urines from the two participants showed significant differences in their metabolic profiles, as seen Figure 3A . A large resonance at δ3.27 was most significant in discriminating participant 1; as this had no significant statistical correlation to other resonances in the spectra, this was assigned to be TMAO. Participant 1 was also found to have higher levels of *N*-methylnicotinic acid. Participant 2 was seen to have higher levels of citrate, glycine (uncorrelated singlet at δ3.57), and acetate at δ1.92.

^{1}H NMR urinary data were then grouped according to the day of collection and compared pairwise to identify molecules that change on a "day-to-day" basis within an individual. In Figure 3B, the urine taken on two different days from participant 1 was compared by O-PLS-DA. A singlet at δ3.23 was seen to significantly discriminate the two data sets. STOCSY analysis of the entire data set showed this resonance to be correlated statistically to a multiplet at δ3.42 (Figure 3C), and careful inspection of the ^{1}H NMR spectra indicated the resonance at δ3.42 to be a doublet subject to peak positional variation. This spectral pattern corresponds to betaine aldehyde, an intermediate in choline metabolism.[26] Another resonance significant in the discrimination was a singlet at δ2.35, statistically correlated with a doublet of doublets at δ7.25. Resonances that discriminated day 2 were doublets at δ7.16, δ7.32, δ7.37, and δ7.46 and multiplets at δ3.63 and δ2.17, all of which were statistically correlated with each other. These latter resonances are indicative of the glucuronide, sulfate, and *N*-acetyl-L-cysteinyl derivatives of acetaminophen—a common painkiller (paracetamol).[27] For participant 2, urinary ^{1}H NMR data were similarly grouped according to day of collection and each day compared to the data from the other 3 days. The O-PLS analysis showed urine collected from participant 2 was different on each day, with different specific resonances significantly discriminating between them. For example, on day 1, doublets at δ1.42, δ1.46, and δ1.50 and singlets at δ2.82, δ3.49, and δ3.53 were seen to discriminate this data set from the others, and on days 2 and 3, acetaminophen metabolites were detected in the urine from participant 2, assigned from STOCSY analysis and the literature.[27] On day 4, a complex series of resonances between δ3.6 and δ3.9 (Figure 3D) were seen to discriminate urine collected on this day. From assignment tables, this was determined to be mannitol. Inspection of the NMR spectra showed this to be present in all the urine samples collected from this participant on this day only.

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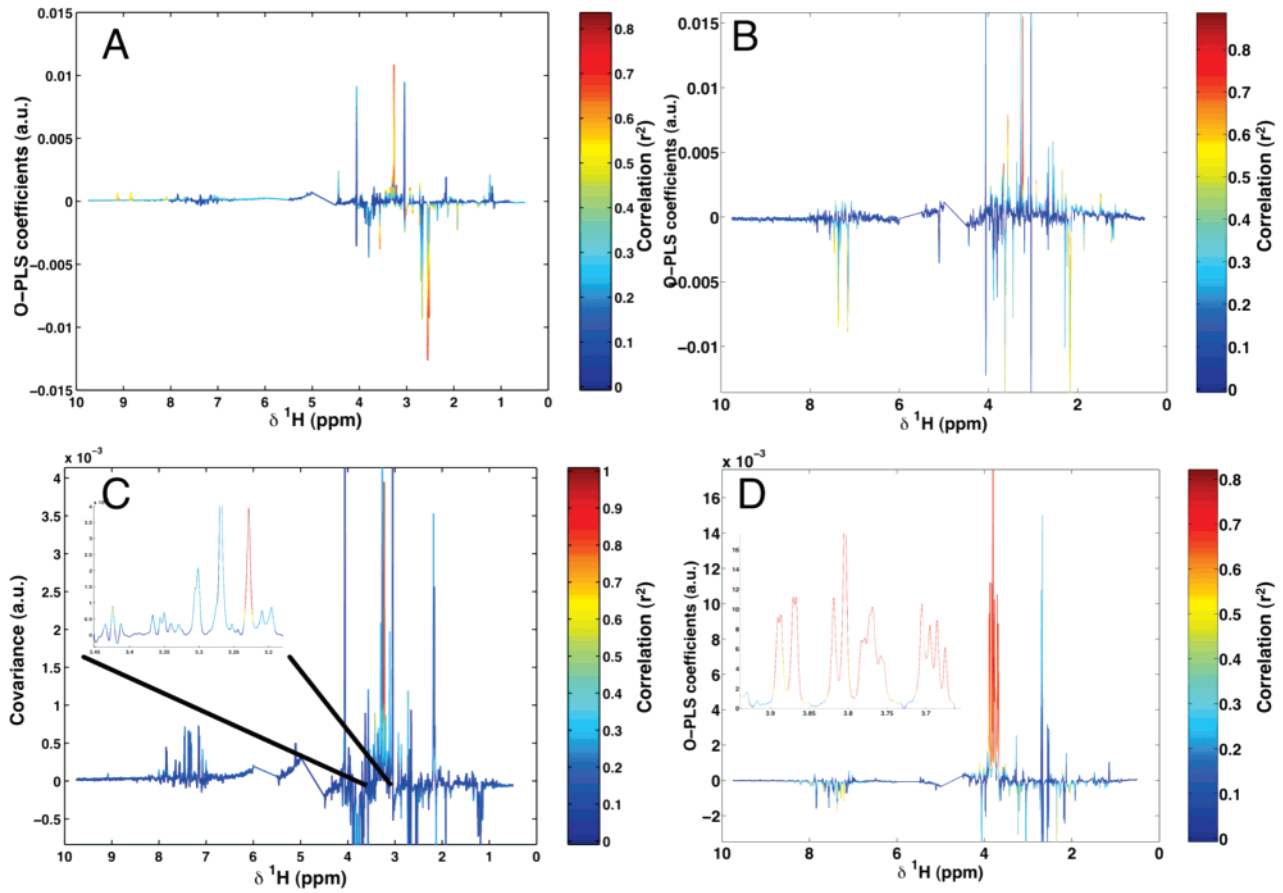

Figure 3. Results from O-PLS models in which urine was classed according to participant or to day of collection. O-PLS coefficients plotted as a function of ^{1}H chemical shift and colored with respect to their respective significance in discriminating the two data sets. (A) Participant 1 V participant 2. $Q^2\hat{\mathbf{Y}} = 0.82$. (B) O-PLS coefficients plot from model where urine from day 1 from participant 1 was compared to day 2. (C) 1D STOCSY plot driven from δ3.23 showing statistically correlated resonances appearing at approximately δ3.42. (D) O-PLS coefficients plot from model where urine from day 4 from participant 2 was compared to urine collected on all other days (inset showing zoomed region between δ3.65 and δ3.95).

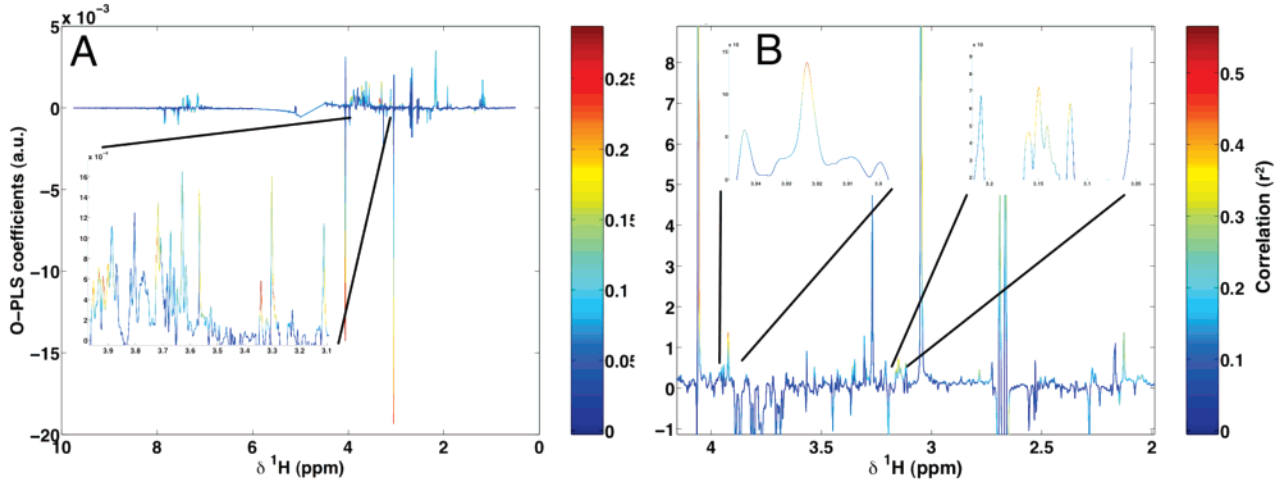

Figure 4. O-PLS-DA comparing urine collected at different times of the day. (A) Data from first morning's urine grouped against both on arrival (T_{AM}) and afternoon (T_{FM}) urine. (B) T_{AM} urine (with positive O-PLS coefficients) compared to T_{PM} urine (negative O-PLS coefficients).

The PCA shown in Figure 1 suggested systematic changes in the data for each day of collection, which were dependent on the time of day of collection. T_{FM} urines were distinguished by a singlet at δ3.34, statistically correlated with singlets at δ3.30 and δ3.90, a singlet at δ3.91, and a singlet at δ2.71, when compared to the rest of the day's urines ($Q^2\hat{\mathbf{Y}} = 0.48$). Figure 4A shows that urine collected later in the day was distinguished by the presence of creatinine. This is due to higher relative levels of creatinine, due to dilution of the urine, rather than higher amounts excreted. Figure 4B shows that when T_{AM} and T_{PM} urine were compared ($Q^2\hat{\mathbf{Y}} = 0.76$), they were primarily distinguished by higher creatinine relative to other metabolites in "on arrival" urine,

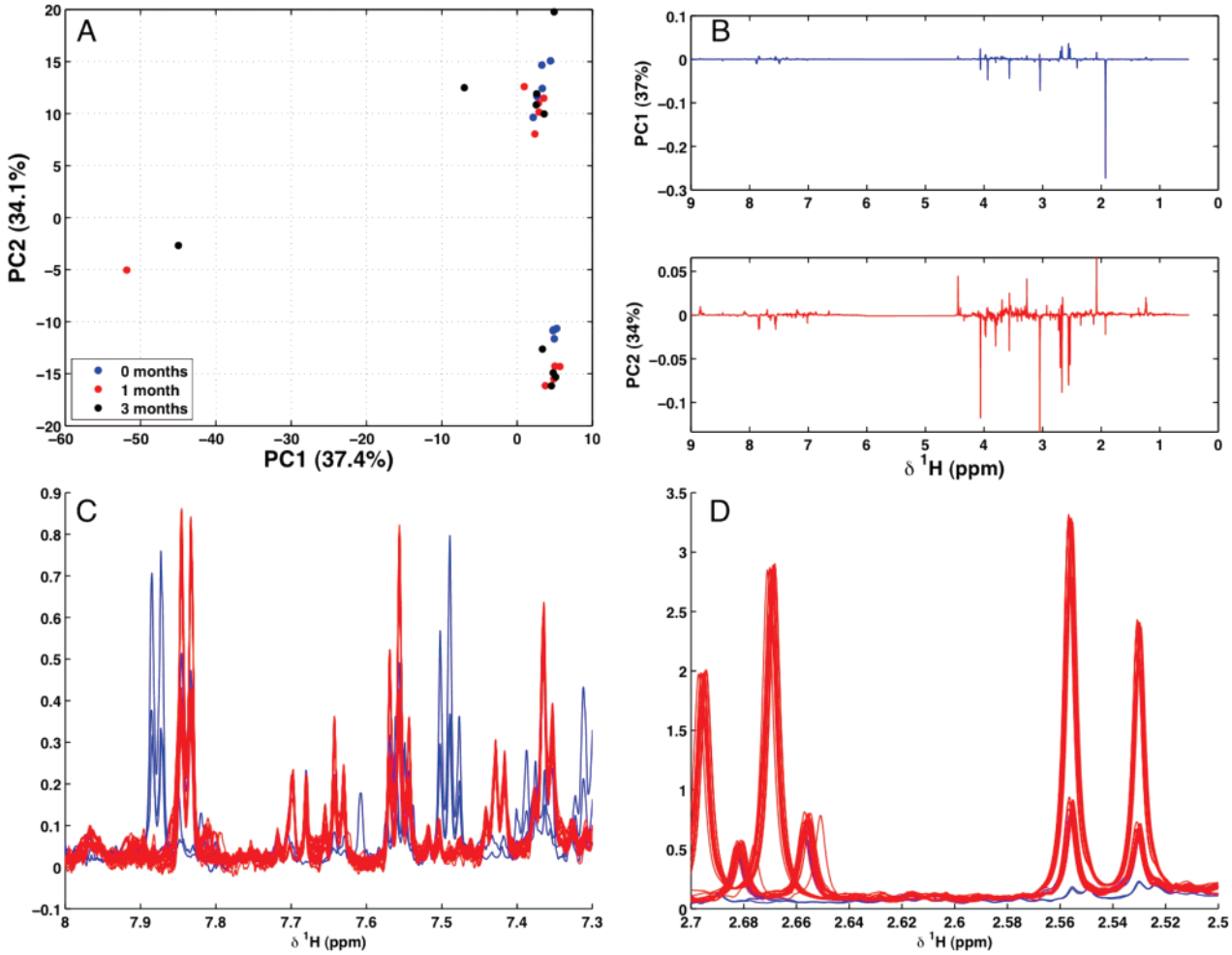

Figure 5. PCA and O-PLS-DA analysis of urinary ^{1}H NMR data stored for up to 3 months at different temperatures. (A) Scores plot for the first two PCs, colored according to storage time: blue = 0 months; red = 1 month; green = 3 months. (B) PCA loadings plotted as a function of chemical shift. (C, D) ^{1}H NMR spectra showing regions of the spectra that differed considerably across stored samples. Blue traces = samples stored at room temperature, red traces = samples frozen or refrigerated.

plus resonances at multiplet δ3.15 (statistically correlated with a multiplet at δ2.06) and singlet at δ3.92 (not statistically correlated).

Effects of Long-Term Storage on Observed Urinary Metabolite Signatures. The second part of this study aimed to determine the effect of long-term storage conditions on the ^{1}H NMR metabolic profile of human urine. From each participant, one sample was split into 15 aliquots. Each aliquot was stored at one of five temperatures; −80, −40, −20, and 4 °C and room temperature, for 3 months, 1 month, or overnight (0 time). From PCA, it was evident that the major source of variation was time-dependent changes associated with 1-month and 3-month room-temperature storage (Figure 5 A). Samples stored at room temperature had very negative PC1 scores, as can be seen in Figure 5A. The remainder of the data were clustered together and showed little systematic variation. To determine the resonances responsible for this variation, O-PLS-DA was used to compare urine stored at room temperature with all other temperatures. Figure 5B plots the corresponding PC loadings as a function of chemical shift and shows that long-term storage gave rise to increased resonances at δ1.92 (acetate), along with decreased hippurate, and new resonances at δ7.49 and δ7.88, respectively. Panels C and D in Figure 5 show expanded regions of the ^{1}H NMR spectra showing the appearance of the new resonances at δ7.49 and δ7.88 (benzoic acid) and the depletion of citrate, respectively. Increased signals were also observed at δ3.64 (glycine), δ1.48 (alanine), δ1.33 (lactate), and δ8.46 (formate). Analysis of the same data set by O-PLS-DA yielded similar results (not shown). The likely explanation for the observed changes is bacterial enzymatic conversion of hippurate to benzoic acid and glycine[28] and conversion of citrate to acetate and oxaloacetate. Oxaloacetate is then converted into pyruvate and then lactate.[29] When observed by eye, no apparent decrease in the intensity of the urea signal was observed (data not shown). These findings are consistent with a recently published study on the effects of storage time and temperature of human urine.[30]

DISCUSSION

The complex metabolic profile of urine as determined by ^{1}H NMR spectroscopy can be readily visualized using multivariate statistical approaches such as PCA and O-PLS-DA, which allow classification and discrimination of sample classes. The STOCSY approach, when combined with recently published enhanced

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visualization methods,[24] has also proven valuable in identification of biomarker metabolites responsible for observed classes variations. The discrimination identifiable by PCA and O-PLS-DA analysis of the entire data set (Figure 1) indicated that the major source of metabolite spectral variation was at the level of the participant, followed by day of collection, and then time of day of collection. The clustering seen in the PCA plot associated with time of day of collection is interesting in that it shows that these two collections are not equivalent, despite the period of fasting prior to the collection of urine. In previous studies, we have shown that marked diurnal variations in rat urine metabolic profiles occur[11] and that such variations would also be expected in humans because of variations in eating patterns. Of course this is well-known clinically as most patient samples are collected for chemical analysis in the morning (preprandial). Furthermore, other aspects of physiological state such as hydration level, fasting, and exercise also affect urine metabolic profiles,[16,31] and these might also reasonably be expected to vary diurnally.

The combination of O-PLS-DA[23] with STOCSY greatly facilitated the assignment of metabolites responsible for the variations seen between the samples. In Figure 3, it was observed that TMAO was a major discriminator between the two participants. This is consistent with dietary influences[19] and suggests that overnight fasting on its own is insufficient to eliminate differences related to food intake. The discovery of acetaminophen metabolites as a major discriminator between urines collected on different days highlights the need to monitor drug and medication intake for clinical studies in metabonomics. In this study, both participants reported discomfort in fasting for the 6 h prior to the afternoon urine collection, increasing the likely need for analgesia. The detection of mannitol, a common food sweetener, in all urine samples from 1 day (Figure 3D), shows again that dietary influences can exert their effects on the results. Changes in urine collected at different times of the day were mostly due to differences in creatinine content, as shown in Figure 4. Creatinine is excreted in uniform amounts throughout the day in humans,[32] and since NMR measures the relative amounts of metabolites, the higher creatinine levels found in on arrival urine, compared to afternoon urine, reflect the dilution of the urine with water. This is likely the result of alleviation of the discomfort associated with daytime fasting by drinking water.

In general, the variations associated with handling and storage of the urine sample following collection were minimal. This indicates that urine is stable, for the purposes of ^{1}H NMR-based metabonomics, over 24 h even at room temperature. The changes we have identified over long-term room-temperature storage, as shown in Figure 5, offer a means of identifying samples that have degraded over time, for example, due to freezer failure, etc. This is most likely due to laboratory contamination with airborne bacteria, and characterization of the bacteria responsible and identification of the source of such infection of samples will be an important development in understanding the critical steps in minimizing the risk of contamination. However, it is also possible that some enzyme activity is present due to normal renal cell turnover and lysis into the urine and it has been shown that raised enzymatic activity in abnormal samples can result in selective deuteration of metabolites from the ^2H_2O used as field-frequency lock.[34] In this context, it is important to recall that this study was on normal urines and that the effects of storage and handling may not be the same if urine from diseased patients were analyzed.

The systematic differences observed in the metabolic profiles of the urines collected in this study underscore the importance of instructions being given to clinical participants. It is clear that "fasting overnight and no alcohol the night before" is insufficient to reduce observations to the level of natural biological variability in controls. When designing such studies, it is essential to ensure participants' medication and dietary intake is recorded for up to 24 h prior to sampling. A comprehensive understanding of the relationship between the urinary metabolic profile and the previous day's meal, e.g., high or low in fats/carbohydrates, etc., will be an important development in this field. This would also help distinguish between diet- and medication-related effects. In light of the differences observed between T_{AM} and T_{PM} urine, we recommend that participants should be seen at the same time of the day for research projects of this nature. To circumvent problems associated with variations in hydration levels, alternative normalization procedures can be explored.[33]

It is intended that this work is used to aid researchers analyzing data from epidemiological studies to facilitate identification of nonbiological variation in samples. In specific circumstances, samples containing confounding metabolites (e.g., acetaminophen conjugates), it is possible to remove the spectral regions corresponding to these resonances prior to pattern recognition. This would still permit optimal information recovery, especially if the sample size is small. Alternatively, if degraded samples were detected, it would be preferable to remove them from the analysis to avoid confounding biomarker discovery.

In conclusion, we have detected patterns of metabolic variation across urine samples collected from healthy participants and accounted for this by identification of the metabolites responsible. While normal urine has been shown to be metabolically stable over 24 h at room temperature, postcollection, it is advisable to control and monitor the circumstances under which is has been collected. This work highlights the need for vigilance in metabonomic and metabolomic studies, particularly for human disease biomarker detection as spurious statistical association could easily arise in poorly randomized sample collections.

ACKNOWLEDGMENT

Funding for this work was provided by the EU Framework 6 MolPAGE project (A.D.M. and S.F.M.Z.). Drs. Dorrit Baunsgaard, Marc Dumas, and Magnus Aberg are thanked for helpful discussion. Dr. Olivier Cloarec for chemometric software and for statistical advice.

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Received for review February 1, 2007. Accepted May 2, 2007.

AC070212F