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Sample Preparation and Stability of Human Serum and Urine Based on HPLC-DAD for Metabonomics Studies

Yun Liu,[†,‡] Xiaoming Sun,[†,‡] Duolong Di,[†,*] Yuxiang Feng,[§] and Fengling Jin[#]

[†]*Key Laboratory of Chemistry of Northwestern Plant Resources and Key Laboratory for Natural Medicine of Gansu Province, Chinese Academy of Sciences, Lanzhou Institute of Chemical Physics, Lanzhou 730000, China*
[*]*E-mail: didl@licp.cas.cn*
[‡]*Graduate University of the Chinese Academy of Sciences, Beijing 100049, China*
[§]*Gansu Province Hospital of Traditional Chinese Medicine, Lanzhou 730000, China*
[#]*The First Affiliated Hospital of Lanzhou University, Lanzhou 730000, China*
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Many literatures focus on the biological relevance and the identification of biomarkers for disease activity assessment while less attention has been paid to the development of standard procedures for sample preparation and storage based on liquid chromatography technique. The influencing factors including protein precipitation, storage temperature, storage time, and reconstitution by ultra pure water were analyzed employing HPLC-DAD. The effects were investigated from five participants over three months by principal components analysis (PCA) and the values of percent changes (PC). The samples with protein precipitation might slow the rate of bacterial enzymatic conversion. After protein precipitation, the average PC of urine samples (0.136 ± 0.013, $n = 5$) is relatively less than that of the serum samples (0.173 ± 0.026, $n = 5$) for three months. Minimal effects on metabolic profiles of serum and urine ($PC < 0.15$) are reasonable for metabolomic studies after protein precipitation and storage at −20 ºC for two months.

Key Words : Stability, HPLC/UPLC, Serum, Urine, Metabonomics

Introduction

Metabolomics, attempting to provide the dynamic profiling changes in metabolites, has recently demonstrated enormous potentials in many fields such as the pharmaceutical industry, toxicology and clinical diagnosis.[1-3] The analytical techniques of nuclear magnetic resonance (NMR),[4,5] high performance liquid chromatography (HPLC)[6,7] and gas chromatography-mass spectrometry (GC-MS)[8,9] have all been employed for metabolomic study. However, no single technique can be expected to analyze all the metabolites, though each of them has its own merits and demerits. Compared with the other techniques for analyzing biological samples, HPLC is characterized by the advantage of avoiding the troubled pre-treatment, analyzing a much wider range of metabolites and controlling critical elements in experimental design.[10] Accordingly, a number of literatures reporting HPLC-based biomarker discoveries in liver cancer, breast cancer, myocardial ischemia, bladder tumor and ovarian cancer have now appeared.[11-15] Biomarkers that have emerged from these studies can be very useful in diagnosis, prognosis, choice of therapy, and assessment of disease activity as well as exploration of the metabolic pathways and biological alterations.[16,17] Therefore, it is necessary to ensure the reliability and accuracy of biomarkers for clinic diagnosis.

The organisms or tissues that were used in metabolomic analysis include urine, serum, epithelium, adipose tissue, liver and cerebral spinal fluid and so on.[18-21] Due to non-invasive collection and easy acquisition of multiple samples in a short time, urine is considered as a common biofluid for metabolomic investigations. Urine gives a "time-averaged" pattern for polar metabolites that are excreted in variable amounts according to the variations in whole-body homeostatic control.[22] Serum is also related to most of tissues that may contain molecules and biological information about many physiological processes and their pathological counterparts.[23] Therefore, serum and urine samples are both analyzed in this paper for metabolomics study.

The accurately and timely evaluation of the stability for serum and urine samples related to the validation of diagnosis is urgently desirable due to the unknown significant metabolites prior to analysis when using a non-targeted approach for the discovery of biomarkers and the long time needed for the collection of samples in clinic. Some factors such as experimental bias, instrumental noise and data acquisition methods may influence the experimental results. It should be also noticed that most of biofluids have some levels of intrinsic biochemical activity, *e.g.* esterase, transaminase, and peptidase, which may be markedly altered in disease states.[24] The autologous biochemical changes also complicate the sample handling problem in the clinic analysis. Furthermore, the discrepancies between the methods of sample preparation and stability based on HPLC technique perhaps contribute significantly to the production of extra, unwanted, false biomarkers. In order to keep the samples in the original state, procedures of eliminating the individual deviations and optimizing sample treatment and stability are still of interest. Alternatively, if degenerative samples were

detected, it would be appropriate to remove them from the analytes in case of confusing the biomarker's discovery.

In most reports, NMR-based metabolomic study on the effectiveness of sample storage condition *e.g.* storage temperature, the freeze-thaw process, preservatives for human urine has been systematically addressed.[25-27] However, such conclusions are indefinite when applied to LC due to the differences in the detection mechanism of the two techniques. When carrying out HPLC-based metabolomic investigations for human serum and urine, it is crucial to precipitate protein that may cause the blockage of chromatography column. The urine of healthy human contains little protein, but urinary protein might appear in persons with impaired renal function and the decrease of creatinine clearance rate. In 2008, Gika et al. studied the handling and storage conditions of human urine that was not pretreated for protein precipitation based on LC-MS patterns.[28] According to the results of Elizabeth J. Want, 100% methanol was proved to the best solvent system for protein precipitation that could offer low protein interference, a comprehensive metabolite profile with the most straightforward sample preparation, and reproducible results.[29] In this paper, methanol is also used to precipitate protein for human urine and serum samples. The effects of handling procedures, storage temperature and storage time were systematically studied based on the metabolic profiles measured by HPLC and analyzed by chemometric methods.

Experimental Section

Chemicals. Methanol (HPLC grade) and acetic acid (analytical reagent) were provided from YuWang Group Co., Ltd (Shandong, China) and Bodi Chemical Holding Co., Ltd (Tianjin, China), respectively. Ammonium acetate (analytical reagent) was purchased by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Ultra-pure water was used for the preparation of all the samples and solutions

Sample Collection and Preparation. Serum and urine samples were obtained from 18 healthy volunteers (9 females and 9 males) receiving a normal diet and were processed immediately after collection in order to analyze the effect of gender difference. Samples from five of the 18 donors (one female and four males) were selected for the experiment of samples preparation and stability. Each of the samples from five donors was divided into 17 portions of 200 μL serum (50 μL, urine). The freshly each sample of five donors was immediately analyzed as raw data by high performance liquid chromatography-diode array detector (HPLC-DAD) technique after the collection of 4 hours. In order to precipitate protein, 200 μL serum and methanol (600 μL) were vortex mixed for 10 s followed by centrifugation at 10 000 g for 10 min before HPLC analysis. The supernatant was evaporated under a steam of nitrogen gas atmosphere and the residues were reconstituted by 50 μL ultra pure water, and then filtered through a millipore filter (0.45 μm). For the preparation of urine samples, 150 μL of methanol was added to 50 μL of urine to precipitate protein. After the evaporation of supernatant, urine samples were reconstituted by ultra pure water ($V_{original\ urine}$: V_{H_2O}, 1:3). All the other procedures were the same as serum samples.

Two major sections are divided in the experiment of sample preparation and storage. In the first part, samples without any treatment was directly stored at −20 °C, naming the method A. As the treatment of method B, samples were for protein precipitation according to the above-described procedure and then stored at −20 °C after evaporation step. Each sample from five participants was split into 8 aliquots. All aliquots was treated by the method A or B and analyzed after 0.5, 1, 2 and 3 month, respectively. In the second part, all samples were pretreatment for protein precipitation as the above-described. After that, samples were directly stored at 4 °C after evaporation step (method C) and −20 °C after reconstituted by ultra pure water (method D) for 3 months. In this part, the stability of human serum and urine based on three different preparing ways (the method B, C and D) were simultaneously compared.

Chromatography Analysis. An Agilent 1200 series LC system equipped with a G1312A binary pump, a G1315B DAD detector, a G1328B manual injector and Agilent Chemstation software was used in our experiment. Chromatography separation was performed on a SinoChrom ODS-BP C18 column (250 × 4.6 mm, 5 μm, Elite, Dalian, China) equipped with a C18 guard column (12.4 × 4.6 mm, 5 μm, Elite, Dalian, China), the temperature of which was maintained at 25 °C during the analysis. A 15 μL sample during each injection was eluted with different combination of 30 mM ammonium acetate aqueous solution (A) at pH = 4.65 adjusted by acetic acid and methanol (B). The elution followed a gradient at a constant flow rate of 1.0 mL/min.

For the separation of serum samples, the gradient elution of mobile phase was set as 0.5-9.7% (B) in 0-11 min, 9.7-16.6% (B) in 11-24 min, 16.6-21.8% (B) in 24-30 min, 21.8-35.7% (B) in 30-40 min, 35.7-61.4% (B) in 40-43 min, 61.4-100% (B) in 43-55 min. Detection was carried out at the wavelength of 254 nm with the reference wavelength of 360 nm. Re-equilibration duration was 10 min between each individual run.

The gradient program for urine samples was as follows: 0.5% (B) in 0-5 min, 0.5-9.7% (B) in 5-18 min, 9.7-16.6% (B) in 18-35 min, 16.6-21.8% (B) in 35-41 min, 21.8-64.3% (B) in 41-50 min, 64.3-100% (B) in 50-60 min. Detection was carried out at the wavelength of 254 nm with the reference wavelength of 400 nm. Re-equilibration duration was 10 min between each individual run.

Data Handling and Analysis. After analysis with HPLC-DAD technique, the chromatographic peaks were integrated automatically by Agilent Chemstation software. Since the metabolites in serum were at relatively low levels, these integral parameters were set as follows: slope sensitivity: 1.5, peak width: 0.01, area reject: 1, height reject: 0.5. The peak information of these samples was incorporated in a matrix and exported in CSV format. The row of dataset represented peak areas at one retention time, and the column was corresponding to the samples. During the collection of

chromatography signals, the changes of retention time were apparent from day-to-day, In order to match the chromatograms accurately, the peak algorithm was carried out based on adjusted retention factor (arf).[11] Uric acid was selected as the reference peak in the algorithm. At last, there are 27 peaks in serum and 35 in urine as the common peaks for the further investigation.

A visualization tool for the discovery of relationships between these complex data sets, is necessary. Based on this purpose, multivariate statistical analysis was accomplished with SIMCA-P 12.0 software (Umetrics AB, Umeå, Sweden). Prior to chemometric analysis, the raw data was processed with *pareto* scaling, which provide a reasonable balance of contributions from high and low amplitude signals by the square root of standard deviation. Principle component analysis (PCA) was exploited to construct initial scores plots and the validation of models was carried out with leave-one-out procedure.

The degree of variation on the basis of each preparation and storage method was determined by the PC values and the peak intensity for all the tested peaks. The formula of PC was expressed as follows: $PC = |A_R - A| / A_R \times 100\%$ where A_R represents the raw peak area of each samples, A is the corresponding peak area at one time after preparation. To compare and characterize significant differences in samples handling, the distribution of PC value and average PC values were also used.

Results and Discussion

Establishment of Metabolic Profiles. For healthy persons, the larger molecular proteins in urine cannot permeate into the glomerulus of kidney and the lower ones are reabsorbed by renal tubular. However, when the investigated objects are infected the glomerular disease or some systematic diseases concerning kidney, albuminuria will always exist before recovery. Therefore, the influence of precipitate protein by methanol was also investigated. Considering both the content of peak information and peak resolution, chromatograms were measured at 254 nm for metabolic profiles. Water was selected as the reconstituted solvent since the chromatographic information of serum and urine was consistent with the original ones. In addition, the precision of the method, which are responsible for the stability and accuracy of metabolic analysis, were also researched. The intraday precision of method was determined by the consecutive analysis of 6 injections for the same sample. The relative standard deviation of common peaks intensity and retention time were < 3.3% and 0.28% (n = 6), respectively. The results suggested that the method is reliable and accurate for metabolic analysis based on the optimized conditions.

Analysis of Possible Gender Difference. During the collection of serum and urine, there were many uncontrollable factors such as ages, diet, body mass index and gender that might cause subtle distinctions at HPLC-DAD chromatograms. If there were significant differences in the concentrations of the metabolites between male and female, the stability of samples between them should be respectively investigated and the classification result of the sample stability based on the PCA model might be also interfered. Therefore, we have firstly evaluated the possible gender difference based on the HPLC-DAD chromatograms from fresh samples. The typical metabolic profiles were presented in Figure 1 based on serum and urine. 42 metabolites were chosen as common chromatography peaks from serum samples (39 metabolites from urine). The PCA score model was calculated by the first two principal components (R^2: 0.701). The result indicated that no significant differences between males and females were found based on the above described procedures of sample pretreatment (data not shown). At the same time, the gender difference between urine samples was also not discovered using the same method (data not shown). Therefore, the gender element was excluded from the influencing factors that may affect the preparation and stability of serum and urine samples.

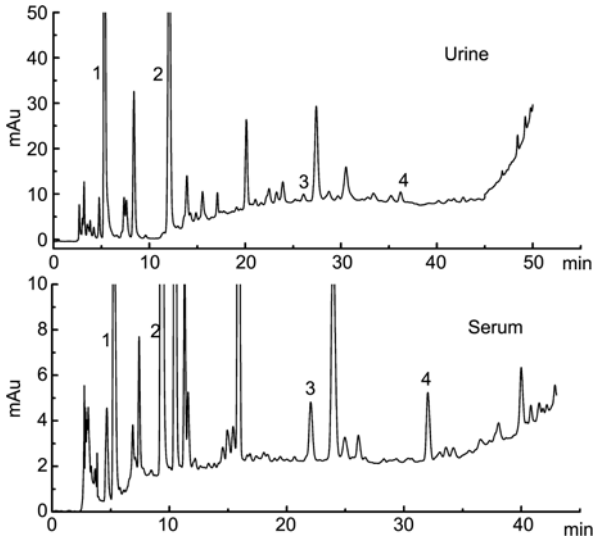


Figure 1. Typical chromatogram of RP-HPLC from fresh serum and urine samples. 1. Creatinine; 2. Uric acid; 3. Adenine; 4. Guanine.

Comparison of the Preparation Method A and B. It sometimes takes several months or even longer time for the collection of human serum and urine during the clinic study. For this reason, it is important to develop appropriate procedures for sample preparation and storage to ensure the accuracy of metabolite analysis. Two different preparation methods (A and B) were compared for maintaining the composition of metabolites. The effects of both two methods were evaluated according to the PC values of each peak over the period of three months stored at –20 °C. The type of analysis is designed to highlight systematic variation according to the chromatography data analyzed by the PCA score plot when the peak areas of total 40 samples. It might be expected that some dramatic changes would appear based on visual comparisons between the preparation methods A and B. The PCA score model was calculated by the first two principal components (R^2: 0.773). The discrimination results (Fig. 2) indicated that the major discrepancy of chromatography

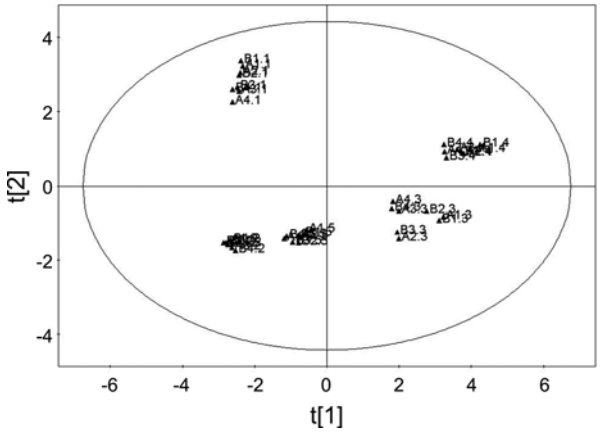
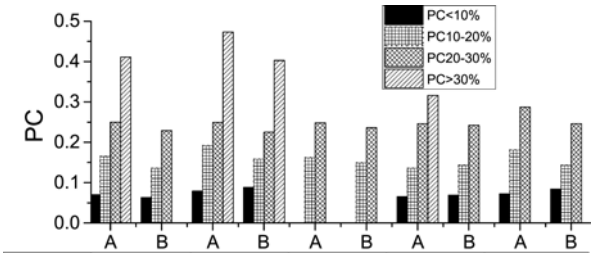
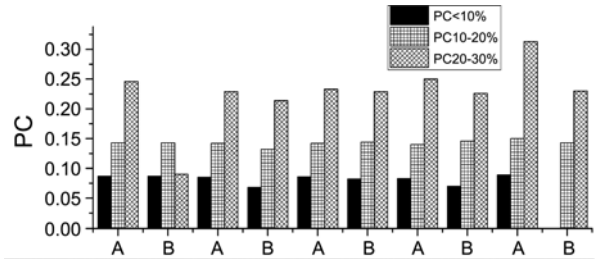


Figure 2. PCA score plot showing typical effects of preparation method A and B from serum sample. Codes: the first numeral designates preparation method (A and B), the middle letter designates storage time 0.5, 1, 2 and 3 month, respectively (1, 2, 3 and 4) and the third numeral designates donor (1-5).



	P1		P2		P3		P4		P5	
	A	B	A	B	A	B	A	B	A	B
PC<10%	0.41	0.41	0.21	0.07	0	0	0.35	0.35	0.22	0.27
PC 10-20%	0.24	0.41	0.53	0.40	0.70	0. 70	0.55	0.55	0.64	0.50
PC 20-30%	0.18	0.18	0.13	0.33	0.30	0.30	0.05	0.10	0.14	0.23
PC>30%	0.17	0	0.13	0.20	0	0	0.05	0	0	0
Average PC	0.216	0.206	0.248	0.218	0.205	0.193	0.191	0.151	0.180	0.158

Figure 3. The effects of method A and B for serum samples from five participants. The distribution of PC and the percentage of peak numbers in each PC intervals were used to evaluate the two methods.



	P1		P2		P3		P4		P5	
	A	B	A	B	A	B	A	B	A	B
PC<10%	0.14	0.14	0.29	0.33	0.13	0.29	0.30	0.40	0.05	0
PC 10-20%	0.77	0.77	0.52	0.62	0.75	0.63	0.60	0.40	0.65	0.65
PC 20-30%	0.09	0.09	0.19	0.05	0.12	0.08	0.10	0.20	0.30	0.35
Average PC	0.159	0.146	0.147	0.143	0.154	0.152	0.158	0.147	0.199	0.187

Figure 4. Comparison method A with B for urine samples from five participants. The distribution of PC and the percentage of peak numbers in each PC intervals were used to evaluate the two methods.

variation was at the metabolite levels of the participants (individual variation) and there might be no significant difference for the treatment method. However, this does not necessarily mean that the chromatography information of directly frozen samples is same with that of the samples frozen after precipitating protein. Many literatures[24,26] have proved that the position of samples in the score plot may be unchanged although the relative levels of some metabolites may change due to the different ways of treatment and storage. Therefore, another quantitative measures defined as distribution of various PC intervals (> 30%, 20-30%, 20-10%, < 10%) and the average PC for evaluation of the sample stability was took. Figure 3 and Figure 4 respectively reported the distribution of PC values in each serum and urine samples over three months' storage based on two different preparation methods. As shown in Figure 3, we obtained 41% of peaks with PC < 10%, 24% with PC 10-20%, 18% with PC 20-30%, 17% with PC >30% and average PC 21.6% for participant 1 based on the method A. In contrast, using method B, we obtained 41% of peaks with PC< 10%, 41% with PC 10-20%, 18% with PC 20-30%, 0% with PC > 30%, and the average PC 20.6%. It thus can be seen that the distribution of PC is relatively concentrated and the value of average PC is a bit small after preparation by method B. For serum samples, the result of participant 1 was consistent with that of the other participant. Therefore, we consider the method B slightly better than the method A. The conclusion is in general agreement with whole distribution of all serum and urine samples. The likely explanation for the above changes is the elimination of bacterial enzymatic conversion by way of precipitating protein, reducing inorganic ions and evaporating water, under which conditions the bacteria are hard to survive.

Comparison of the Preparation and Storage Method B, C and D. For the preservation of biological samples, the default method is using the lowest temperature of −80 °C, the apparatus of which is far more expensive than that of −20 °C. In addition, Gika and Theodoridis[28] have reported that there were no remarkable changes in the profile of urine samples stored at −20 and −80 °C through LC analysis. Therefore, in our study, we only compared both temperatures (−20 °C and 4 °C) with different preparation methods.

In this part, all samples were pretreated to precipitate protein according to the described method prior to analysis. At the beginning, we evaluated the differences qualitatively among three processing methods (B, C and D) based on the PCA score plot (Fig. 5). The serum samples from two random participants were used to establish the PCA model. The model parameters were calculated by the first two principal components. R^2 provides an estimate of how well the model fits the Y data whereas Q^2 is an estimate of how well the model predicts the Y. The cumulative values of R^2 (0.981) and Q^2 (0.953) close to 1 indicate a reliable model. The score plot visually shows that the cluster originated from the method B is comparatively centralized and nearer to the initial state. The change of serum creatinine concentration in each random participant over three months was shown in

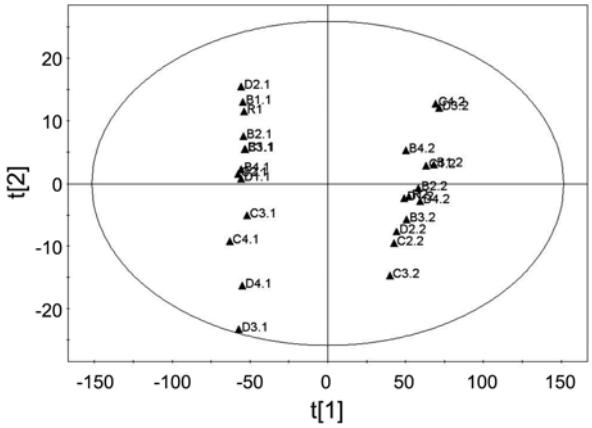


Figure 5. The effects of method B, C and D for serum samples from two participants based on PCA score plot. Codes: the first numeral designates preparation method (B, C and D), the middle letter designates storage time 0.5, 1, 2 and 3 month, respectively (1, 2, 3 and 4) and the third numeral designates donor (1and 2), R1and R2 designates the raw chromatography information from participant 1 and 2.

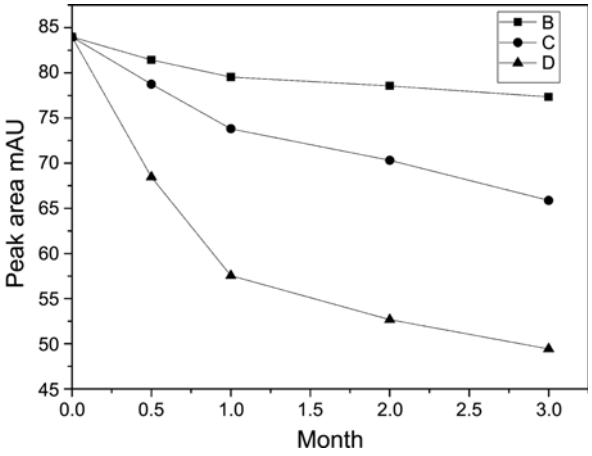


Figure 6. The change of serum creatinine concentration in random one participant over three months based on three preparation methods (B, C and D).

Figure 6. As indicated in Table 1, the peak areas of four compounds identified were listed out including creatinine, uric acid, adenine and guanine in serum from each random participant over three months. Based on the result of Figure 6 and Table 1, the variable rate of creatinine peak areas from high to low is respectively the method D, C and B. The quantitative results (data not shown) also show that the average PC values of method B are the smallest among the three methods, while the average PC values of the method D are smaller than those of the method C. This is in agreement with the results from urine samples. Studies have shown that the creatinine in urine can be degraded by bacteria and will be converted to creatine by the enzyme of creatininase.[29] Therefore, the reduction of creatinine is suggestive of bacterial contamination in human serum and urine samples. There was some a possible reason that samples reconstituted by ultra pure water (stored at −20 °C) changed significantly after three months. The bacterial growth can be affected by some factors such as temperature, acidity, energy sources and the presence of oxygen, nitrogen, minerals and water. Water, for most of bacteria, is a neccessity for spontaneous metabolic events. Though stored at lower temperature, endogenous compounds in serum and urine can still be degraded by bacteria. Our study suggests that the presence of bacteria can affect the metabolic levels in serum and urine samples and such as protein precipitation, water evaporation under a steam of nitrogen gas atmosphere, and storage conditions at −20 °C, must be taken to keep the samples at their original state.

Comparison the Stability of Method B for Serum and Urine. From the above discussion, we may conclude method B is a suitable procedure for preparation samples during the metabolomics study. In this part, we compared the stability of serum and urine over three months' storage based on the method B.

The changes during the storage of serum and urine were easy to discern from Table 2, which exhibits a series of PC values from five participants over 0.5, 1, 2, 3 months' storage. It is evident that the storage time is responsible for the largest changes in the chromatography data. The changes of metabolites over the period of 3 months from the origin serum and urine samples are highly suggestive of bacterial contamination and those metabolites do decompose with the prolonging of time.[30,31] Comparatively, serum samples are strongly affected based on method B after three months' storage. The distribution of PC was shown in Figure 7. The PC values of serum and urine started to increase dramatically

Table 1. The average peak areas of four serum metabolites from fiver participants (n=5) after three months' storage by different preparation methods (B, C and D)

	Creatinine	RSD%	Uric acid	RSD%	Adenine	RSD%	Guanine	RSD%
Raw	83.98	0.78	1232.20	1.45	18.52	0.38	5.30	0.40
0.5 months								
B	81.43	3.81	1192.10	5.30	17.86	1.22	5.18	0.57
C	78.75	2.56	1141.95	7.31	17.04	0.98	4.92	0.34
D	68.45	4.17	1089.66	4.67	15.94	1.43	5.06	0.49
3 months								
B	76.33	11.98	985.01	15.68	15.25	5.67	4.56	1.48
C	65.87	13.26	906.65	20.45	13.31	5.91	4.31	1.59
D	49.43	18.72	849.69	16.44	11.02	4.73	3.07	1.93

Table 2. The PC values and the average PC from fiver participants over a period of 0.5, 1, 2 and 3 months based on serum and urine samples

	PC(0.5M)	PC(1M)	PC(2M)	PC(3M)	Average PC
Serum					
P1	0.136	0.148	0.157	0.237	0.169
P2	0.118	0.179	0.242	0.328	0.216
P3	0.095	0.117	0.238	0.252	0.175
P4	0.092	0.159	0.181	0.196	0.157
P5	0.059	0.164	0.173	0.199	0.148
Urine					
P1	0.053	0.121	0.169	0.236	0.145
P2	0.040	0.089	0.127	0.205	0.116
P3	0.058	0.105	0.144	0.224	0.133
P4	0.079	0.097	0.139	0.228	0.136
P5	0.070	0.139	0.165	0.230	0.151

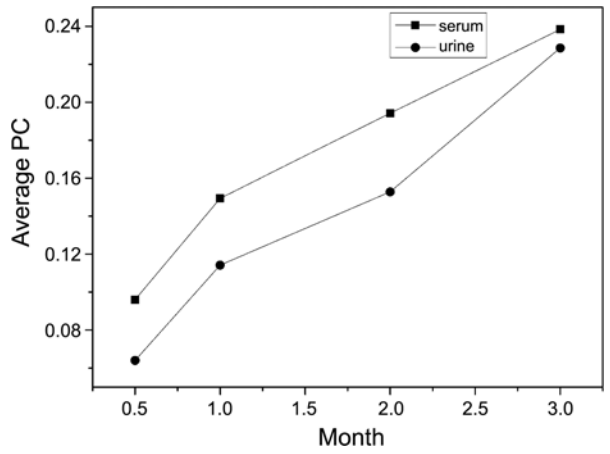


Figure 7. The distribution of average PC values from serum and urine over three months after protein precipitation.

between 0.5 and 1 month's storage and then increased slowly between 1 and 2 month's storage. However, there was a noticeable increase for urine samples after 2 months. It was noted that the average PC value of the serum samples (average PC = 0.173 ± 0.026, n = 5) and the degree of scatter are all higher than those of the urine samples (average PC = 0.136 ± 0.013, n = 5). It is thus recommended that the time for storing urine samples may be longer than that for serum samples based on metabolomics study. Storage at –20 °C for 2 months limited the loss of metabolites to some extent. However, no storage method was able to inhibit the significant reduction of metabolites in serum and urine samples. As a result, the use of –20 °C for up to two months' storage when the average PC values for serum and urine samples is less than 15%, seems to be effective for maintaining the stability of samples and may provide meaningful results.

Conclusions

Careful optimization conditions are necessary to ensure accurately report of biological characteristics or disease states. Investigators must take appropriate measures to avoid or inhibit bacterial influence that plays a leading role in the loss of metabolites. The analysis of the metabolic profile for the serum and urine samples was employed based on HPLC-DAD and PCA. Although many disadvantages are present in the HPLC-DAD technique for biological studies such as low sensitivity, incapability of many metabolites without UV absorption, the results based on all metabolite we detected can be used to explain the preparation and stability for samples. It is all acceptable and reasonable within a certain error. The average PC values for serum and urine samples with protein precipitation and the storage of samples at –20 °C for two months are less than 15%, which provides a suitable procedure in clinic. Compared with the serum samples, the urine sample has less metabolite change and thus can be stored for a longer time.

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