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High-performance isotope-labeling liquid chromatography mass spectrometry for investigating the effect of drinking Goji tea on urine metabolome profiling

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Urine is a human biofluid that is widely used for metabolomics research on disease biomarker discovery. Ideally, the metabolome profiles generated from comparative groups of individuals should mainly consist of the endogenous human metabolites that reflect the healthy states of the individuals. However, external factors, such as diet, may alter the urine metabolome profile by either introducing a significant amount or variety of exogenous metabolites to urine or inducing changes of the metabolome profile. Thus, strict control of the external factors during the sample collection process is critical for urine metabolomics aimed at discovery of disease biomarkers. In this work, we describe a study to determine the effect of drinking Goji tea, which is considered a nutritional supplement drink in some regions of the world, on urine metabolome profile. The purpose of this work is not to determine the nutritional values of Goji tea, but to investigate whether drinking a moderate amount of Goji tea 1–3 h (short-term effect) or 12 h (longer-term effect) before urine collection can cause significant variations of urine metabolome profiles. A highly sensitive dansylation isotope labeling liquid chromatography mass spectrometry (LC-MS) method was used to determine the urine metabolomes before and after drinking Goji tea. From the studies of the short term (< 3 h) and longer term (12 h) effects of drinking Goji tea, it is clear that the consumption of a moderate amount of Goji tea does not affect the urine metabolome significantly. Fasting for 12 h should be sufficient to remove any potential interference of Goji metabolites from the human urine metabolome profile.

metabolomics, urine metabolites, mass spectrometry, liquid chromatography, isotope labeling, Goji, diet

1 Introduction

Urine metabolomics has been widely used to search for potential metabolite biomarkers for disease diagnosis or prognosis [1–7]. However, urine metabolome can be very complex because, along with human endogenous metabolites, exogenous metabolites (such as those from food, drinks, nutritional supplements, and pharmaceuticals) can also be present in urine. The presence of these exogenous metabolites in significant amounts or varieties in urine can complicate biomarker discovery, as any chemical residues from food, drinks, etc. can affect the overall urine metabolome composition [8]. Another potential complication in urine metabolomics is related to the effect of diet or other factors on metabolome profiles [9–13]. For example, diet-induced metabolome changes may mask the differences of the metabolomes between a diseased group and a control group. Thus, it is critical to minimize the influence of these external factors in the profiling of endogenous metabolites in urine samples used for discovering specific disease biomarkers.

The effect of external factors on urine metabolome may not be detectable if an analytical technique is not sufficiently sensitive or quantitative. Technological advances in recent years have made possible to detect and quantify many

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metabolites present at low concentrations in urine [14, 15]. Although sensitive techniques are very useful for detecting low-abundance metabolites as potential biomarkers, they also increase the likelihood of detecting exogenous metabolites in an untargeted metabolome profiling work, as well as of revealing subtle changes due to external factors such as diet. Thus, it is necessary to pay special attention to the potential interferences of external factors for urine metabolomics. We have recently developed a highly sensitive isotope labeling liquid chromatography mass spectrometry (LC-MS) for quantitative metabolomics based on rational design of chemical labeling reagents for simultaneous improvement in metabolite separation, detection, quantification and identification [16, 17]. For example, urine metabolites that contain primary amine, secondary amine, or phenolic hydroxyl groups can be labeled with ^{12}C- or ^{13}C-dansyl chloride prior to LC-MS analysis [16]. This labeling strategy allows the separation of polar or ionic metabolites on a reversed phase (RP) column, as well as signal enhancement of tenfold to a thousandfold, compared to the unlabeled counterparts. In a human urine sample, we can detect more than 1000 putative metabolites from more than 10000 features typically found in a 30-min LC-MS run.

In this work, we report our investigation of using the dansylation isotope labeling LC-MS method to study the effect of drinking Goji tea on urine metabolome profile. The objectives of this study were: (1) to present the metabolome profile of Goji, which has never been reported; and (2) to examine how the intake of Goji tea affects the urine metabolome of a small number of healthy individuals. The ultimate goals were to determine the external factors that can cause significant alternation of the urine metabolome, as well as to make recommendations to avoid or properly control these external factors during sample collection, in order to eliminate or reduce their influence in profiling the human endogenous metabolites in urine. This work was not intended to study the effect of drinking Goji tea on human health, which would require a much larger sample size from diverse populations.

Goji tea is considered a nutritional supplement in some regions of the world. Goji, the red and sweet fruit of *Lycium barbarum*, is native to southeastern Europe and Asia [18], and commonly used in meals, drinks, and traditional Chinese medicine (TCM) [19]. Like many fruits, Goji contains many nutrients including vitamins, trace minerals, amino acids, and essential oils [20–22], as well as active ingredients such as *Lycium Barbarum* polysaccharides (LBP), scopoletin, zeaxanthin dipalmitate, and β-carotene [18, 22–25]. Dried Goji berries and Goji tea made from the berries are being sold as nutritional supplements or health food products. In this work, we applied the very sensitive dansylation LC-MS method of targeting amine- and phenol-containing metabolites to analyze the urine metabolomes of healthy individuals with and without drinking Goji tea to determine whether drinking Goji tea would have any effects on their urine metabolome profiles.

2 Experimental

2.1 Chemicals and reagents

^{12}C-dansyl chloride was purchased from Sigma-Aldrich Canada (Markham, ON, Canada). ^{13}C-dansyl chloride was synthesized in our lab [16] and the chemicals used to synthesize this isotope reagent were also purchased from Sigma-Aldrich. LC/MS-grade water, acetonitrile (ACN), and formic acid were purchased from Thermo Fisher Scientific (Edmonton, AB, Canada).

2.2 Goji tea

Goji fruits purchased from a local Chinese herbal medicine store were imported from Qinghai, China. Goji tea was made by mixing 15.075 ± 0.007 g Goji in 500 mL water, heating the water to its boilong point, and then leaving it to stand for 1 h at room temperature. For Goji metabolome analysis, two aliquots of 50 μL of Goji tea were labeled with ^{12}C- and ^{13}C-dansyl chloride, respectively. A dansylated Goji tea sample was prepared by mixing equal volumes of ^{12}C- and ^{13}C-dansyl labeled solutions. The mixture sample was centrifuged for 10 min at 14000 rpm, after which it was ready for analysis.

2.3 Urine sample collection

Urine samples were collected from seven healthy individuals, with ethics approval from the University of Alberta. The samples were stored at 4 °C immediately after collection. The urine samples were centrifuged at 4000 rpm for 10 min, after which the supernatants were filtered twice through an 0.2 μm filter. The individually filtered urine was aliquoted and stored at −80 °C until further use.

2.4 Dansylation labeling reaction

The frozen urine samples were thawed in an ice bath and then diluted twofold, followed by dansylation labeling [16, 26]. The 50 μL sample was mixed with 25 μL sodium carbonate/sodium bicarbonate buffer (500 mM) and 25 μL ACN in a reaction vial. The solutions were vortexed, spun down, and mixed with either 50 μL of freshly prepared ^{12}C-dansyl chloride solution (18 mg/mL) for light labeling or ^{13}C-dansyl chloride (18 mg/mL) for heavy labeling. The dansylation reaction was allowed to proceed for 60 min at 40 °C. After 60 min, the mixtures were vortexed and spun down, and then 10 μL of sodium hydroxide (250 mM) was added to the reaction mixture to consume the excess dansyl

chloride and quench the dansylation reaction. After an additional 10 min of 40 °C incubation, a 50 μL formic acid (425 mM in 50% acetonitrile) was added to consume the excess sodium hydroxide and to adjudge pH value. The ^{12}C-labeled or ^{13}C-labeled mixtures were centrifuged for 10 min at 14000 rpm, after which they were ready to be injected onto an ultrahigh performance liquid chromatography (UPLC) column for UV quantification [27]. According to the UV quantification results, an appropriate volume of ^{13}C-labeled sample was combined with a ^{12}C-labeled sample in a fixed amount ratio. The mixtures were centrifuged for 10 min at 14000 rpm and were then ready to be injected into LC-MS for analysis.

2.5 LC-UV quantification

A Waters ACQUITY UPLC system (Milford, MA, USA) with a binary solvent manager, a sample manager, and a photodiode array (PDA) detector was used for the quantification step. First, 2 μL of the labeled urine solution were injected onto a Waters ACQUITY BEH C18 column (2.1 mm × 5 cm, 1.7 μm particle size, 130 Å pore size) for a fast step-gradient run. Solvent A was 0.1% (*v*/*v*) formic acid in 5% (*v*/*v*) acetonitrile; Solvent B was 0.1% (*v*/*v*) formic acid in acetonitrile. The gradient started with 0% B for 1 min and was stepwise increased to 95% within 0.01 min and held at 95% B for 1 min to ensure complete elution of all labeled metabolites. The gradient was restored to 0% B in 0.5 min and held in this condition for 3.5 min to re-equilibrate the column. The flow rate was 0.45 mL/min. The detection wavelength was set at 338 nm.

2.6 LC-MS analysis

The HPLC system consisted of an Agilent 1100 liquid chromatograph (Palo Alto, CA, USA) equipped with a binary pump system. The labeled urine samples were separated with a reversed-phase Eclipse plus C18 column purchased from Agilent (2.1 mm × 100 mm, 1.8 μm particle size, 95 Å pore size) in an optimal injection amount. Solvent A was 0.1% (*v*/*v*) formic acid in 5% (*v*/*v*) ACN; Solvent B was 0.1% (*v*/*v*) formic acid in ACN. The gradient started at 20% B and stepwise increased to 35% B within 3.5 min, followed by 14.5-min ramp to 65% B, then raised to 99% B within 6 min and finally held at 99% B for 4 min to ensure complete elution of all labeled metabolites. The flow rate was 180 μL/min. The flow from HPLC was loaded to the electrospray ionization (ESI) source of a Bruker maXis Impact Ultra-High Resolution Quadrupole Time-of-Flight (QTOF) mass spectrometer (Bruker, Billerica, MA, USA). The MS conditions were as follows: nebulizer gas pressure 1.8 bar; drying gas temperature 230°C; drying gas flow 8 l/min; capillary voltage 4500 V; and scan range 150–800. All MS spectra were obtained in the positive-ion mode.

2.7 Data processing and statistical analysis

The resulting MS spectral peaks were picked by using the Bruker DataAnalysis software 4.0 and an in-house visual basic (VB) script, and then processed by using an in-house peak-pair-picking software called IsoMS, written in R language, for finding ^{12}C/^{13}C-peak-pairs with their accurate mass difference of 2.0067 Da. The IsoMS software also removed the redundant peaks of the same metabolite, such as natural isotopic peaks, common adduct ion peaks, dimer peaks, and multiply charged peaks. Only the protonated ion pair from a metabolite was exported for further analysis. The extracted peak-pair data, including peak-ratio values, were aligned by retention time and accurate mass, and only those peak-pair features shared by no less than 50% of the samples were retained for multivariate analysis. The multivariate analysis was performed by Metaboanalyst (www.metaboanalyst.ca) [28], and the data were mean-centered and autoscaled (unit variance) prior to analysis.

2.8 Metabolite database search

For putative metabolite identification based on accurate mass matches with the metabolites in a database, the MyCompoundID program (www.mycompoundid.org) [29] was used to search against the Human Metabolome Database (www.hmdb.ca) of 8021 known human endogenous metabolites and the Evidence-Based Metabolome Library (EML) of about 375809 predicted human metabolites. The mass-accuracy window used for database search was set at 10 ppm.

3 Results and discussion

3.1 Experimental design

Urine typically contains many endogenous metabolites as well as exogenous metabolites; the latter are metabolic breakdown compounds from a wide range of foods, drinks, nutritional supplements, and other sources. The presence of exogenous metabolites in high abundance may complicate the discovery of potential biomarker metabolites from urine. To study the effect of taking nutritional supplements (in this case, Goji tea) on urine metabolome, we designed two experiments: one to study the short-term effect (< 3 h) and the other to study a longer-term effect (12 h). Figure 1 shows the workflows of urine sample collections for both experiments. To study the short-term effect (Figure 1(a)), after overnight fasting, five urine samples were collected from one volunteer each day for three continuous days. The first and second urine samples for each day were used as the control group. After the second urine sample was collected, the volunteer drank 500 mL of Goji tea. Three urine samples were then collected at 1-h intervals and treated as an experimental group. This workflow yielded a total of 15

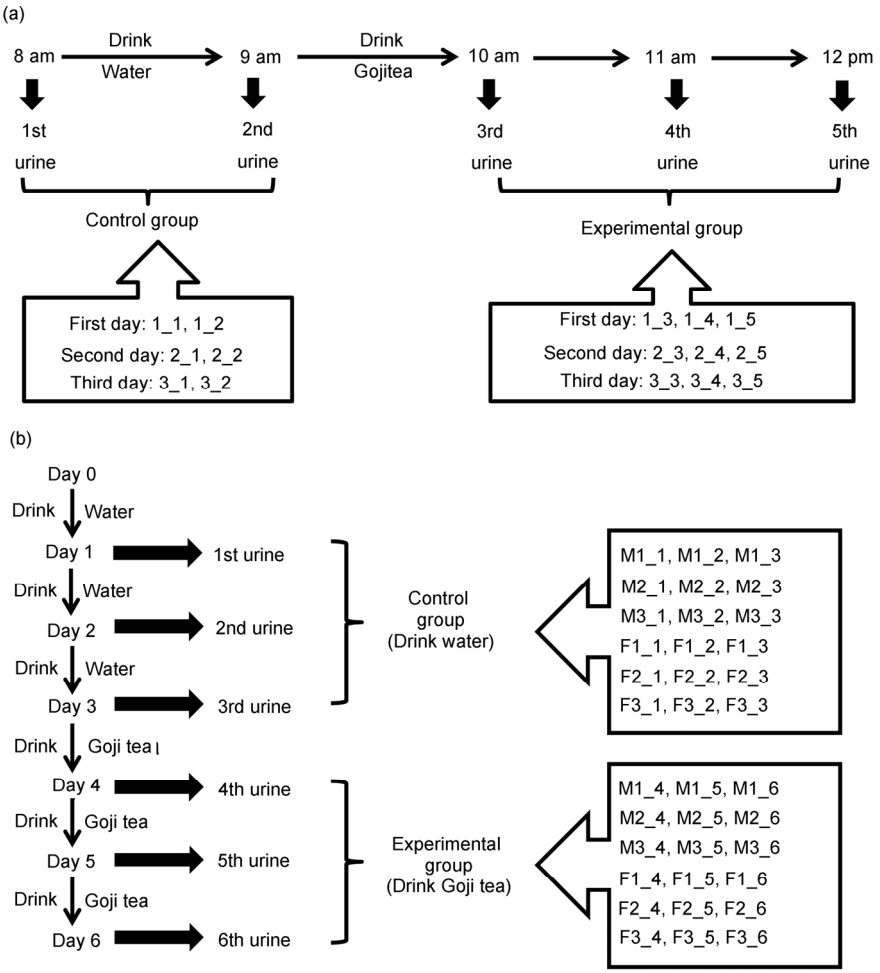


Figure 1 Experimental design for urine sample collection: (a) studying the short term effect of drinking Goji tea on urine metabolome and (b) studying the longer term effect of drinking Goji tea on urine metabolome (drinking water was used as a control).

urine samples from one individual. Each urine sample was analyzed in experimental triplicates.

In studying the longer-term effect (Figure 1(b)), urine samples were collected from six volunteers (three males and three females) over six continuous days. For the first three days, volunteers were asked to drink 500 mL of water in the evenings, 12 h before their urine samples were to be collected. These urine samples were used as the control group. For the following three days, they were asked to drink 500 mL of Goji tea in the evenings, 12 h before their urine samples were to be collected. These urine samples were treated as an experimental group. This workflow generated 36 urine samples in total. For each sample, experimental duplicates were performed.

3.2 Goji metabolome profiling

Before analyzing the human urine samples, we profiled the Goji metabolome to determine the detectability of the dansyl-labeling LC-MS method on the Goji metabolites. Figure 2 shows the base-peak ion chromatograms of two separate LC-MS runs from the same injection amount of plain Goji tea and dansylated Goji tea. Many peaks with high intensities were detectable in the dansylated Goji tea, whereas only a few low-intensity peaks were detected in the plain Goji tea at the same injection amount.

Because the dansylated Goji tea sample was prepared by mixing equal volumes of ^{12}C- and ^{13}C-dansyl-labeled aliquots of the same tea sample, the expected peak intensity ratio for each labeled metabolite should have been 1.0. The IsoMS software was used to process the LC-MS data and calculate the peak ratio for each peak pair picked by the software. We excluded < 10% of the overall number of peak pairs detected that had ratios of < 0.67 and > 1.5 (i.e., the outliers due to experimental variations). The final results show that the average number of peak pairs or putative metabolites detected in the Goji tea was 1629 ± 15 (n = 3) and that 1475 (91%) metabolites were commonly found in the triplicate experiments.

To putatively identify these peak pairs, the accurate mass of each metabolite from an individual peak pair was fed into a search using MyCompoundID against the HMBD and

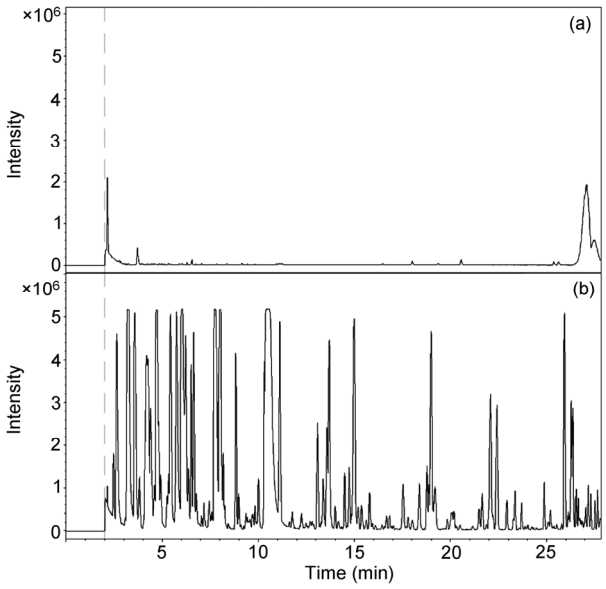


Figure 2 Base-peak ion chromatograms obtained by LC-MS from (a) plain Goji tea and (b) dansylated Goji tea.

EML databases. Of the 1475 commonly found metabolites, 361 peak pairs matched the HMDB metabolites and 575 matched the predicted metabolites in EML, for a total of 936 matches (63%) (see Supporting Information Tables S1 and S2 online). Thus, many of the detected peak pairs could be putatively identified based on this accurate mass match. Of course, authentic standards are needed for positive identification, which is beyond the scope of this work. It should be noted that we used a database of human metabolites for matches because an equivalent resource is not available for plant metabolites. One would expect that plants and humans share many similar metabolic processes and that they should share many common metabolites. We reasoned that the unique metabolites found in Goji tea, which are different from humans either in identity or quantity, could serve as the tracers to examine the effect of drinking Goji tea on the human urine metabolome profile.

3.3 Short-term effect on urine metabolome

To generate a reference sample for comparison with the individual samples, aliquots of individual urine samples were taken and mixed to produce a pooled sample, which was then labeled by ^{13}C-dansyl chloride. An equal amount of ^{12}C-dansyl-labeled individual sample was mixed with the ^{13}C-dansyl-labeled pooled sample, followed by running the mixture in LC-MS. The peak-intensity ratios of the isotope-labeled peak pairs detected in the mass spectra were calculated. Because the same pooled sample was used, the peak ratios of each individual peak pair from different samples represented the different concentrations of the same putative metabolite in those samples. The peak ratio values found in different samples were then exported into a statistics tool for multivariate analysis. The results of the analysis are shown in Figure 3; each point represents an average of triplicate runs. The unsupervised principal component analysis (PCA) was applied to generate an overview on how the data were scattered (see Figure 3(a)). The PCA plot shows that there is no apparent separation between the control group and the experimental group. The data points appear to be clustered by different days.

In order to determine if the control group and the experimental group could be separated, the supervised partial least squares discriminant analysis (PLS-DA) was applied; the resulting score plot is shown in Figure 3(b). This plot shows that the two groups are separated from each other. Next, we applied the permutation test (n = 100) to the PLS-DA model to determine whether this separation is valid. Figure 3(c) shows that the observed statistic p-value is 0.83 which is much greater than the validation threshold of 0.05. Thus, the PLS-DA model is not valid, which indicates that the two groups are not really separate from each other.

We also analyzed the data using a Volcano plot, which is another statistical tool that combines the fold-change and t-test information to determine the significant metabolites that can be used to separate the two groups. Using the twofold change and a p-value of 0.05 as the threshold cutoff values, the Volcano plot is shown in Figure 3(d). Only six peak pairs are found to be significant; they appear in the upper right. Positive fold changes of these six peak pairs indicate that the concentrations of these putative metabolites are higher in the control group. Three out of the six peak pairs could match with some metabolites in HMDB [30] or METLIN [31] (see Table S3). The p-value of these metabolites for group separation ranges from 0.015 to 0.049. If a cutoff value of 0.05 is used, these metabolites would be considered significant.

Results from the PLS-DA and Volcano plot analyses indicate that there is little or no separation between the control and experimental groups. In other words, drinking a moderate amount of Goji tea (500 mL) did not have a significant effect on urine metabolome even after the urine samples were collected within 1 to 3 h. It should be noted that in our work, a moderate amount of Goji tea from a typical-sized drink was consumed by an individual, which would be better representative of the participants of a biomarker discovery study in which some participants may ingest nutritional supplements (such as Goji tea) in moderate amounts.

To compare the urine metabolomes at the individual metabolite level, we examined the metabolite distributions in the Goji tea and urine samples collected before and after subjects drank the Goji tea. Figure 4(a) shows a comparison of the number of peak pairs found in urine before drinking tea (control group) and in the Goji tea itself. The total number of peak pairs detected in the six control-urine samples is 2168. Of these, 622 (18%) peak pairs match the total number of peak pairs found in the Goji tea (i.e., 1917 from three runs). Figure 4(b) shows a comparison of the peak pair

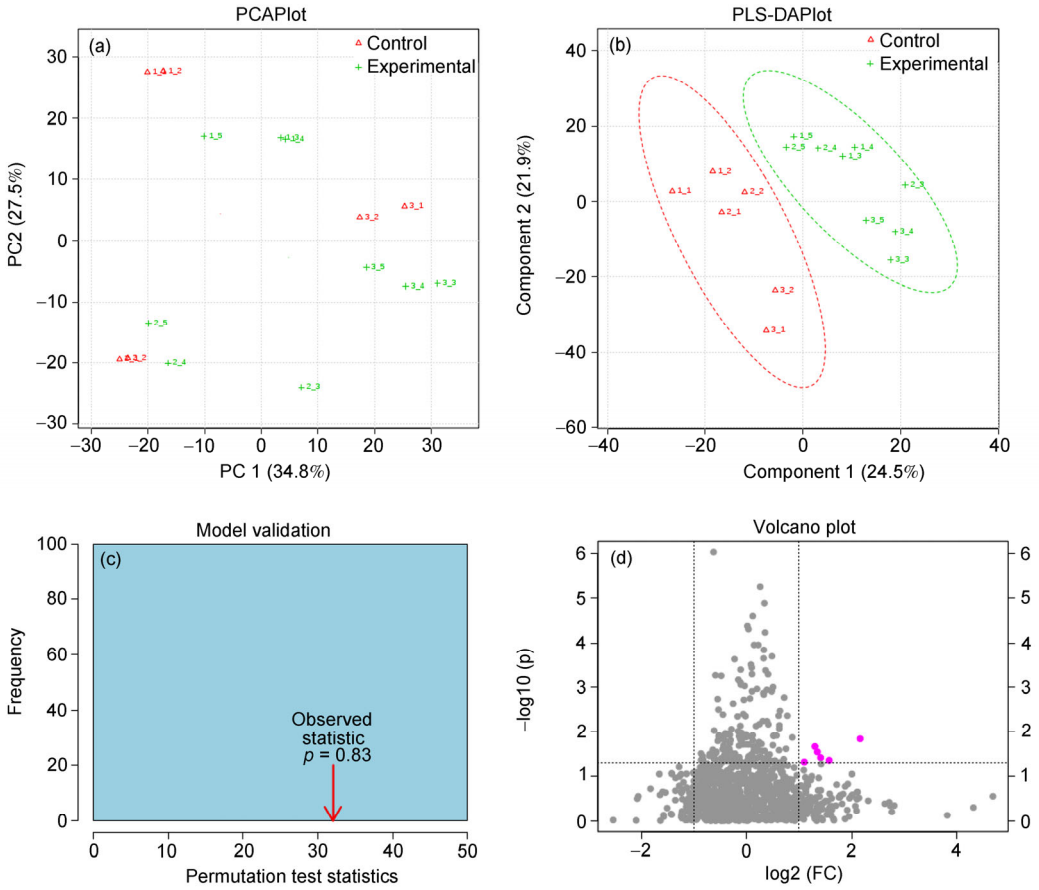


Figure 3 Multivariate analysis of the metabolome data obtained from the urine samples collected in the short-term effect study: (a) PCA score plot, (b) PLS-DA score plot, (c) permutation test of the PLS-DA model, and (d) Volcano plot. Each point in PCA and PLS-DA score plots represents the average of three replicate experiments.

numbers found in urine before and after subjects drank the Goji tea (i.e., control group vs. experimental group). The total number of peak pairs detected in the experimental group is 2187. Of these, 2143 (98%) peak pairs are found in both groups. Although there are 25 unique peak pairs found in the control group and 44 unique peak pairs in the experimental group, most of these peak pairs are only found in an individual sample or were found in one day. After removing these pairs, only two unique peak pairs are consistently found in the experimental group. Comparing these two pairs with those found in the Goji tea, one with *m/z* 285.0644 and a retention time of 1283 s was also observed in the Goji tea. As shown in Figure 4(a), the number of the unique peak pairs found in the Goji tea itself is 1295; however, only 1 could be observed in the urine samples after subjects drank the Goji tea. It is clear that the concentrations of these compounds from the Goji tea were so diluted that they could not be detected in the urine samples. Alternatively, these compounds might have been absorbed or converted into other metabolites by the human body. In any case, these results are consistent with the global classification results generated by the PLS-DA and Volcano plot models: the urine metabolome was not significantly affected by drinking a moderate amount of the Goji tea.

3.4 Longer-term effect on urine metabolome

Although the results described above illustrate that there is no short-term effect on urine metabolome, we cannot rule out the possibility of some metabolism occurring over an extended period, after the intake of food or drink, that would result in the late appearance of these metabolites in urine. Fasting for 12 h is a common practice before urine-sample collection. We examined the effect on urine metabolome of drinking Goji tea 12 h before sample collection. To compare the urine metabolomes from six individuals and six days, a pooled sample was produced by mixing aliquots of individual urine samples and labeling them with ^{13}C-dansyl chloride. An equal amount of ^{12}C-dansyl-labeled individual sample was mixed with ^{13}C-dansyl-labeled pooled sample, followed by running the mixture in LC-MS. The whole dataset of 36 samples was analyzed by statistical tools; results are shown in Figure 5. Each point represents an average of duplicate experiments.

Figure 5(a) depicts the PCA score plot, which does not show any clear separation between drinking water or Goji

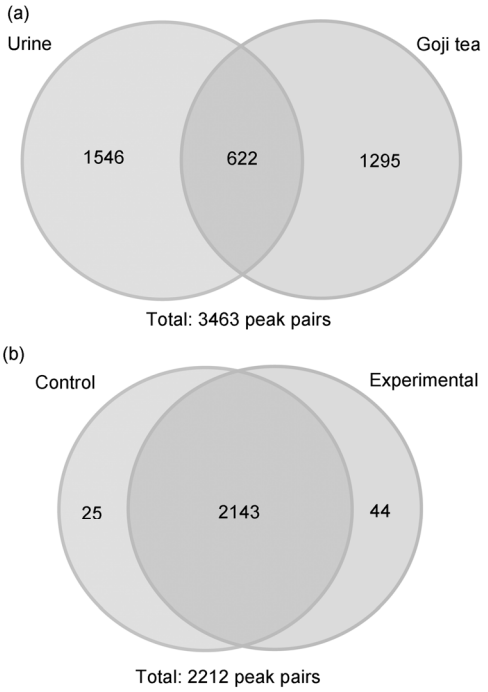


Figure 4 Venn diagrams of the distributions of the numbers of peak pairs or putative metabolites detected: (a) comparison between the number detected in urine before drinking Goji tea (control group) and the number detected in the Goji tea itself, and (b) comparison between the number detected in urine before drinjing Goji tea (control group) and the number detected in urine after drinking Goji tea (experimental group).

tea 12 h before urine samples were collected; moreover, most of the data points are clustered within an individual. The PLS-DA score plot is shown in Figure 5(b) and the permutation test (n = 100) result of the PLS-DA model is shown in Figure 5(c). The p-value from the test is 0.98 which indicates that the model is not valid (i.e., there is no separation between the control and experimental groups). This conclusion is supported by the Volcano plot shown in Figure 5(d), where there is no significant metabolite found that would differentiate the two groups. These results indicate that 12 h after taking the Goji tea, there was no effect on the urine metabolome profiles.

4 Conclusions

In this work, a dansylation-isotope-labeling LC-MS method was used to study the effect of drinking Goji tea on human urine metabolome profiles. This sensitive method allows the detection of an average of 1629 ± 15 (n = 3) peak pairs or putative metabolites in the Goji tea itself, compared to very few metabolites detectable without labeling using the same amount of injection into LC-MS. From the studies of the short-term (< 3 h) and longer-term (12 h) effects of drinking Goji tea, it is clear that the consumption of a moderate amount of Goji tea does not affect the urine metabolome significantly. Fasting for 12 h should be sufficient to

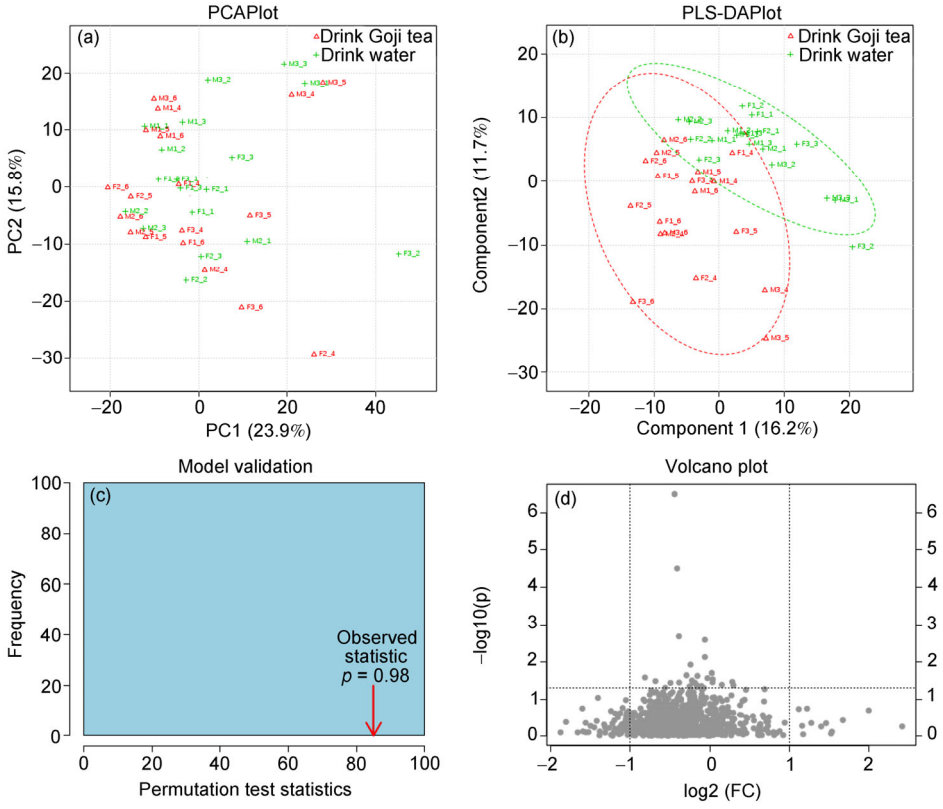


Figure 5 Multivariate analysis of the metabolome data obtained from the urine samples collected in the longer-term effect study: (a) PCA score plot, (b) PLS-DA score plot, (c) permutation test of the PLS-DA model, and (d) Volcano plot. Each point in PCA and PLS-DA score plots represents the average of two replicate experiments.

remove any potential interference of the Goji metabolites in human urine metabolome profiling. Although this work focused only on the profiling of amine- and phenol-containing metabolites in urine, the high sensitivity of the technique allows us the examination of any small changes in urine metabolome. We have shown that a conventional LC-MS method, without dansylation labeling of the metabolites, could detect only very few metabolites in Goji tea that was prepared at a typical concentration used for consumption. Therefore, we did not expect that a conventional LC-MS could detect any Goji metabolites in urine after consumption. In the future, we will apply this sensitive analytical approach and the same study design to examine the effects of other types of food or drinks on urine metabolome, with the goal of developing guidelines about avoiding certain types of commonly consumed food and drinks for urine metabolomics-based biomarker discovery studies.

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