JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Pu-erh Tea Using Chemical and Metabolic Profiling Approaches

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In this study, the chemical constituents of pu-erh tea, black tea, and green tea, as well as those of pu-erh tea products of different ages, were analyzed and compared using a chemical profiling approach. Differences in tea processing resulted in differences in the chemical constituents and the color of tea infusions. Human biological responses to pu-erh tea ingestion were also studied by using ultraperformance liquid chromatography−quadrupole time-of-flight mass spectrometry (UPLC−QTOFMS) in conjunction with multivariate statistical techniques. Metabolic alterations during and after pu-erh tea ingestion were characterized by increased urinary excretion of 5-hydroxytryptophan, inositol, and 4-methoxyphenylacetic acid, along with reduced excretion of 3-chlorotyrosine and creatinine. This study highlights the potential for metabonomic technology to assess nutritional interventions and is an important step toward a full understanding of pu-erh tea and its influence on human metabolism.

KEYWORDS: UPLC−QTOFMS; pu-erh tea; metabonomics; multivariate statistical analysis

INTRODUCTION

Tea (*Camellia sinensis* L.) has been considered a natural medicine for 4,000 years, and it is now one of the most popular beverages in the world. Its popularity is due to its attractive aroma, taste, and putative health effects, making it an ideal after-dinner drink.

Teas can generally be classified into three major categories according to the degree of fermentation: unfermented green teas, partially fermented oolong and paochong teas, and fully fermented black and pu-erh teas. Green and oolong teas are consumed mainly in Asia and Northern Africa, whereas black tea is consumed worldwide (*1*). Pu-erh tea, produced mainly in the Yunnan province of China, is consumed in large quantities in Asia, especially in southwestern China. Both black tea and pu-erh tea are fermented teas; however microorganisms like *Aspergillus niger* are found only in pu-erh tea during its fermentation (*2*). The microorganisms in pu-erh tea oxidize tea polyphenols more completely than the enzymatic oxidation process which occurs in black tea, resulting in lower concentrations of tea polyphenols and tea catechins.

The dried pu-erh tea product is often pressed into cakes or bricks, making it suitable for long storage periods. Further, it is believed that the quality of pu-erh tea increases with age (*3*), in contrast to green tea, which is unfermented and consumed as fresh as possible (*3*). A number of studies have shown that pu-erh tea has a wide range of biological and pharmaceutical properties, including hypocholesterolemic effects in rats (*4*); free radical scavenging effect (*5*); microbicidal activity against *Mycoplasma pneumoniae* and *Mycoplasma orale* (*6*); bactericidal activity against *Bordetella pertussis* (*7*); antiobesity, antimutagenic, and antimicrobial activity (*8*); plasma triglyceride, overall cholesterol, and LDL-cholesterol lowering action in rats (*9*); and protection against LDL oxidation (*10*).

Teas produced by various techniques, as well as pu-erh teas of various ages, are reported to differ in their chemical constituents and pharmaceutical effects (*11*). Here, we have attempted to distinguish between the chemical constituents of the various teas, to determine the quality of the pu-erh teas of various ages, and to determine the global metabolic response to pu-erh tea ingestion in humans. To this end, we applied a UPLC−QTOFMS-based metabonomic approach. Extracts of pu-erh tea and biofluid MS spectra are extremely rich in composition information which allows a rapid assessment of a broad range of metabolites. To analyze the generated mega data sets and assess any treatment-related effects on the complex

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10.1021/jf804000y CCC: $40.75
Published on Web 03/25/2009

biofluid MS profiles, multivariate statistical analysis is required (*12*) which comprises a state-of-the-art tool for probing network integrity, systems diversity, and the complexity of human individuals (*13*). Quality assessment of green tea (*14*), and the metabolic impact of black tea, green tea (*15*), and chamomile (*16*) in humans has been successfully performed with NMR and GC−MS-based metabonomics. In this study, we investigated the differences among green tea, black tea, and pu-erh tea; the quality of pu-erh teas aged for various periods; and the human metabolic response to pu-erh tea ingestion over a 6-week period using a metabonomics strategy combining UPLC−QTOFMS and multivariate statistical analysis.

MATERIALS AND METHODS

Chemicals and Materials. Leucine-enkephalin, formic acid, theanine, arginine, and epicatechin were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and methanol of HPLC grade were obtained from Merck (Germany). Analytical-grade methanol was obtained from the Shanghai Lin Feng Chemical Reagent Co, Ltd. (China). All aqueous solutions were prepared with ultrapure water produced by a Milli-Q system (18.2 MΩ, Milipore, Bedford, MA). Myricetin and theaflavine were purchased from the J & K Chemical Ltd. (Shanghai, China). Catechin, epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), gallocatechin gallate (GCG), and inositol were purchased from the Shanghai Shunbo Bioengineering Co, Ltd. (Shanghai, China). Quercetin, kaempferol, chlorogenic acid, and gallic acid were purchased from the National Institute for the Pharmaceutical and Biological Products (Beijing, China).

Information on the collected pu-erh teas, black teas, and green teas can be seen in the Supporting Information. Genuine pu-erh tea has the following four characteristics. It must have been cultivated in Yunnan province, particularly in the Simao district or Xishuangbanna prefecture. It must use fresh leaves of a large-leaved variety of *Camellia sinensis* as the raw material and must undergo postfermentation processes to produce its unique shape and inherent characteristics (*17*). The quality of the pu-erh tea material collected in this study was assessed to ensure that it met the requirements of the local standard DB53/T103-2006 (*17*) of Yunnan Province, China.

Tea Sample Preparation for UPLC−QTOFMS Analysis. Dried tea leaves were ground into a fine powder and filtered through a 20 mesh screen. The resulting fine powder (∼0.1 g) was weighed and standardized, and 70% (v/v) methanol (3 mL) was added (*18*). The powder was then extracted in an ultrasonic water bath at 60 °C for 60 min. The extraction was repeated two times. After cooling, the solution was centrifuged at 13,000 rpm for 15 min and the resulting supernatants were immediately stored at −80 °C pending UPLC−QTOFMS analysis. Ultrapure water (500 μL) was added to tea extraction (500 μL) and vortexed for 1 min, and then the supernatant was filtered through a syringe filter (0.22 μm) for UPLC−QTOFMS analysis.

Analysis of Tea Pigments. Tea pigments are complex, group compounds which are difficult to separate into single compounds. So the analysis of main tea pigments including theabrownin (TB), theaflavin (TF) and thearubigin (TR) was performed with a system approach (*19*) and determined by ultraviolet−visible spectrophotometer (UNIC UV-2102 PCS, UNIC, USA).

Participants and Study Design. Approximately 5 kg of 5-year-old pu-erh tea was mixed to produce a homogeneous sample, and then ground into a fine powder and filtered through a 20 mesh screen. The pu-erh tea was prepared by infusing 10 g of powder in 200 mL of boiling water for 10 min, followed by straining. Participants received a daily dose equivalent to 5 cups of a commercial preparation of tea. Twenty healthy men ($n = 10$) and women ($n = 10$) whose mean age was 25 ± 2 years (range 22−32) were enrolled in this study, and written consent was obtained from each participant.

Spot urine samples were collected from participants daily between 11:00 and 11:30 a.m. during a 6-week period that included a 2-week baseline phase, a 2-week daily pu-erh tea ingestion phase, and a 2-week "postdosing" phase. During the pu-erh tea ingestion phase, participants were given 200 mL of pu-erh tea each day at approximately 10:00 a.m. Participants reported no adverse effects after the ingestion of pu-erh tea. A standard food regimen was supplied during the experiment in order to avoid the potential influence of the diet on the metabolic effects of pu-erh tea. Food intake and body weight were recorded.

Urine Sample Preparation. The collected urine samples were centrifuged at 13,000 rpm for 10 min at 4 °C, and the resulting supernatants were immediately stored at −80 °C pending UPLC−QTOFMS analysis. Ultrapure water (500 μL) was added to urine (500 μL) and vortexed for 1 min, and then filtered through a syringe filter (0.22 μm) for UPLC−QTOFMS analysis.

UPLC−QTOFMS Analysis. Tea and urine metabolite profiling was performed using a Waters ACQUITY UPLC system equipped with a binary solvent delivery manager and a sample manager (Waters Corporation, Milford, MA), coupled to a Micromass Q-TOF Premier mass spectrometer equipped with an electrospray interface (Waters Corporation, Milford, MA). Chromatographic separations were performed on a 2.1 × 100 mm 1.7 μm ACQUITY BEH C18 chromatography column. The column was maintained at 45 °C and eluted with a 1−99% acetonitrile (0.1% (v/v) formic acid)−aqueous formic acid (0.1% (v/v) formic acid) gradient over 10 min at a flow rate of 0.40 mL/min. A 5 μL aliquot sample was injected onto the column. The mass accuracy analysis and detailed MS parameters were optimized according to our previous work (*20*). During metabolite profiling experiments, centroid data were acquired for each sample from 50 to 1000 Da with a 0.10 s scan time and a 0.01 s interscan delay over a 10 min analysis time.

Data Processing and Statistical Analysis. The UPLC−QTOFMS data of the urine samples was analyzed to identify potential discriminant variables. The ES+ raw data was analyzed by the MarkerLynx applications manager version 4.1 (Waters, Manchester, U.K.) using parameters reported in our previous work (*20*). A list of the intensities of the detected peaks was generated for the first sample, using retention time (RT) and the *m*/*z* data pairs as the identifier for each peak. The resulting three-dimensional matrix containing arbitrarily assigned peak index (retention time−*m*/*z* pairs), sample names (observations), and peak intensity information (variables) was exported to SIMCA-P software 11.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis. The multivariate statistics for UPLC−QTOFMS-based metabolic profiling was performed with the method previously reported (*13*, *20*).

RESULTS AND DISCUSSION

Quality Assessment of Tea Samples. Using the method for metabolite profiling reported in our previous study, pu-erh teas, black teas, and green teas were analyzed with UPLC−QTOFMS coupled with a multivariate statistical analysis method. **Figure 1A** shows the UPLC−QTOFMS base peak intensity (BPI) chromatograms of pu-erh tea, black tea, and green tea. Differences among the samples were not apparent by visual examination of the UPLC−QTOFMS chromatograms (**Figure 1A**). However, by applying multivariate statistical analysis, a clear separation of the three types of teas was observed in the OPLS-DA plot (**Figure 1B**). The orthagonal partial least squares-discriminant analysis (OPLS-DA) plot in **Figure 1B** is readily divided into three large clusters, confirming that the pu-erh tea, black tea, and green tea are chemically distinct.

The patterns of chemical constituents varied among the three teas (see Supporting Information Table S2). The variations in the concentrations of these chemical constituents, including tea pigments (e.g., theaflavin) and catechins (eg, epigallocatechin gallate), are summarized in **Tables 1** and **2**. From **Figures 2** and **3** we can see that the contents of the main tea pigments, including TB, TF and TR, were significantly different and the contents of some catechins were changed significantly. Furthermore, each pair of teas—pu-erh tea and black tea, pu-erh tea and green tea, and black tea and green tea—was compared in detail and their relative chemical differences such as caffeine, kaempferol, and catechins are shown (Supporting Information

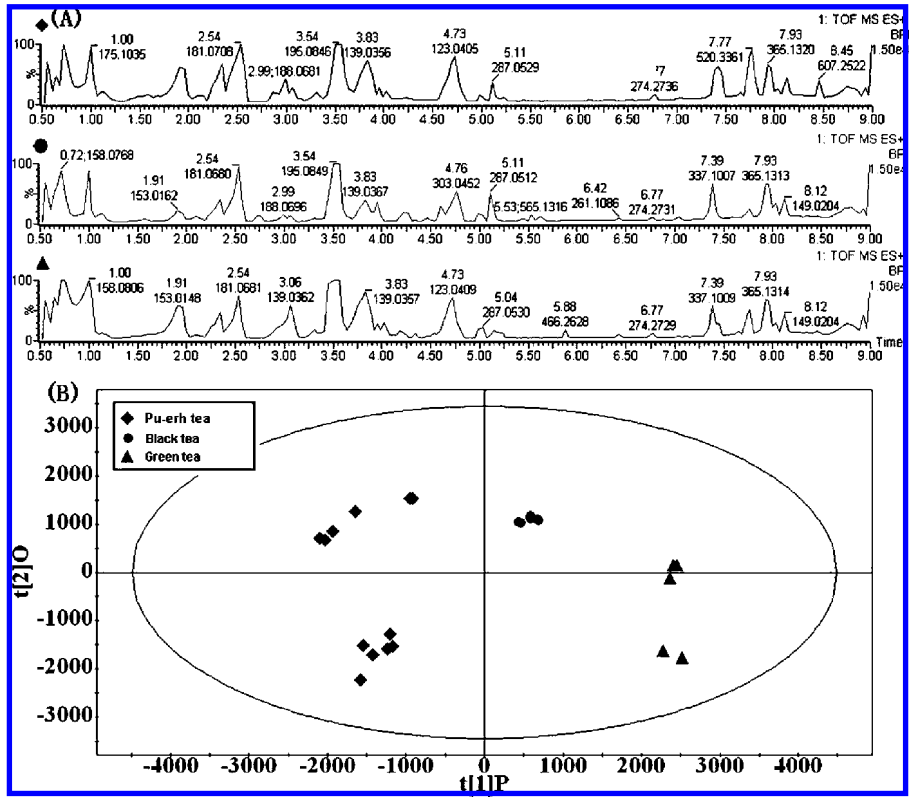

Figure 1. Comparison of UPLC−QTOFMS base peak intensity (BPI) chromatograms (**A**) and scores plot (*t*[1] vs *t*[2]) generated from OPLS-DA of UPLC−QTOFMS spectra of pu-erh teas (◆), black teas (●), and green teas (▲) (**B**).

Table 1. The Contents of Tea Pigments in Different Teas

sample no.	type	preservation period (yr)	TF (%)	TR (%)	TB (%)
1	pu-erh tea	1	0.29	0.36	8.33
2	pu-erh tea	3	0.23	0.99	8.75
3	pu-erh tea	5	0.22	0.76	8.95
4	pu-erh tea	8	0.21	0.75	9.29
5	pu-erh tea	10	0.25	0.73	10.72
6	pu-erh tea	1	0.20	0.76	11.92
7	pu-erh tea	5	0.19	0.47	9.82
8	pu-erh tea	1	0.21	0.83	8.91
9	pu-erh tea	2	0.21	0.43	11.25
10	pu-erh tea	5	0.23	0.27	8.88
11	pu-erh tea	8	0.19	—[a]	13.65
12	pu-erh tea	10	0.16	—[a]	11.30
13	black tea	1	0.37	8.84	6.66
14	black tea	1	1.11	10.00	8.56
15	black tea	1	0.47	8.33	7.19
16	black tea	1	0.31	8.20	7.29
17	green tea	0.5	0.14	4.90	3.26
18	green tea	1	0.16	4.36	2.78
19	green tea	1	0.23	5.49	5.49
20	green tea	1	0.22	5.03	2.67
21	green tea	1	0.21	4.67	2.87
22	green tea	1	0.19	5.32	3.50
23	green tea	0.5	0.18	3.28	2.17
24	green tea	0.5	0.12	3.13	2.12

[a] Not detected.

Figures S1−S3 and Tables S3−S5). The characteristic constituents of the teas were found to be polyphenols and theanine in green tea, theaflavin (TF) and thearubigin (TR) in black tea, and theabrownin (TB) and gallic acid (GA) in pu-erh tea (*18*) (**Figure 2**). During pu-erh tea fermentation, the catechin content was reduced compared to green tea and black tea (*21*) and ECG, EGCG, and EC were almost undetectable (*11*) (**Figure 3**). As fermentation progressed, tea polyphenols, catechins, TF, TR, amino acids, and soluble sugar greatly decreased, while the TB content increased (*22*). In contrast to black tea (*23*), pu-erh tea contained almost no TF or theaflavic acid (TFA), compounds that contribute to the astringency and bitterness of tea (*22*).

Tea pigments have certain pharmaceutical-like properties, including anticarcinogenic activity and the ability to modulate blood lipids and reduce cholesterol (*24*). The increase in the tea pigment TB gives pu-erh its characteristic brown color and plays an important role in the quality of the final product, but its pharmaceutical-like properties are not well understood. GA, one of the main products of EGCG degradation during fermentation, is another active component of pu-erh tea. GA is reported to reduce blood sugar and lipids in humans (*25*) and inhibit the synthesis of cholesterol by HepG2 cells (*26*). Our finding of decreased EGCG and greatly increased GA during pu-erh tea ingestion agrees with previous studies (*27*). The increased GA may explain the putative cholesterol-reducing effect of pu-erh tea.

As mentioned above, it is reported that longer aging improves the quality of pu-erh tea. For that reason, we collected pu-erh tea of different ages and analyzed them using UPLC−QTOFMS coupled with a multivariate statistical analysis method, and pretreatment methods from our previous work (*20*). A two component PLS scores plot of UPLC−QTOFMS data depicts the general chemical variation of pu-erh teas (**Figure 4**). The substances accounting for this separation were identified (**Table 3**), and a detailed comparison of 1- and 10-year-old pu-erh tea is shown in **Figure 5**. Differences between UPLC−TOFMS chromatograms of the various samples were not apparent by visual examination (chromatograms not shown). The PLS scores plot (**Figure 4**) could be readily separated by PC1, and the samples were associated with age. From **Figure 4**, we can see that the 1-year-old and 3-year-old pu-erh tea samples differed in their concentrations of certain chemical constituents and they both differed from the 5-, 8-, and 10-year-old pu-erh tea samples. The separation of 5-, 8-, and 10-year-old pu-erh tea was less

Table 2. The Contents of Determined Constituents in Different Teas (μg/mL)

sample no.	type	preservation period (yr)	TFA	C	CG	GCG	EGC	EC	ECG	quercetin
1	pu-erh tea	1	0.75	–[a]	–	8.29	1.82	9.06	9.89	1.14
2	pu-erh tea	3	1.11	0.25	–	10.80	3.79	21.13	10.66	1.22
3	pu-erh tea	5	4.34	1.35	–	8.29	4.98	44.36	18.21	1.30
4	pu-erh tea	8	4.16	1.32	–	8.85	5.17	40.26	11.35	1.18
5	pu-erh tea	10	4.05	1.31	–	8.29	5.62	35.88	8.19	1.16
6	pu-erh tea	1	35.65	10.86	207.48	163.00	70.24	114.41	537.14	3.93
7	pu-erh tea	5	28.34	4.82	180.96	90.50	34.01	75.04	471.65	5.94
8	pu-erh tea	1	0.39	–	–	8.29	1.26	–	8.19	0.94
9	pu-erh tea	2	0.25	–	–	8.29	1.26	2.51	8.19	0.99
10	pu-erh tea	5	0.70	–	–	8.29	1.56	6.30	8.19	0.98
11	pu-erh tea	8	0.23	–	–	8.16	1.33	6.02	8.02	0.91
12	pu-erh tea	10	0.23	–	–	8.23	1.26	5.68	7.87	0.89
13	black tea	1	21.94	0.70	37.06	57.60	10.57	36.50	116.20	5.82
14	black tea	1	33.12	0.36	19.02	23.10	6.86	15.85	71.64	0.84
15	black tea	1	34.73	–	–	13.00	2.10	0.16	11.32	0.84
16	black tea	1	30.50	–	1.60	19.30	3.18	6.62	28.63	0.84
17	green tea	0.5	43.58	3.32	174.16	315.00	47.23	85.65	454.84	2.73
18	green tea	1	29.19	1.61	81.55	238.00	97.10	72.33	226.11	1.93
19	green tea	1	24.71	2.62	154.41	338.00	95.44	55.21	406.06	4.72
20	green tea	1	39.25	5.58	236.62	298.00	91.86	114.78	609.12	4.81
21	green tea	1	37.87	6.40	255.32	350.00	122.19	97.21	655.30	7.65
22	green tea	1	44.31	3.60	116.64	225.00	127.12	87.10	312.77	4.70
23	green tea	0.5	44.24	3.10	123.62	337.00	157.21	95.55	330.01	2.36
24	green tea	0.5	47.80	3.89	168.57	269.00	58.14	74.69	441.03	2.72

[a] Not detected.

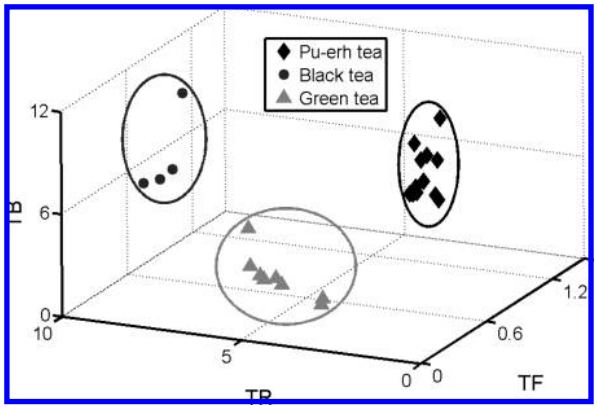

Figure 2. The contents of tea pigments in pu-erh teas (◆), black teas (●), and green teas (▲).

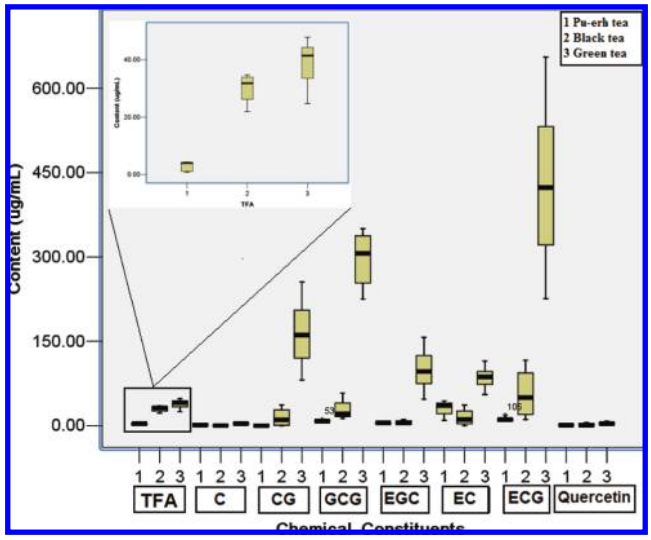

Figure 3. The contents of TFA, C, CG, GCG, EGC, EC, ECG, and quercetin in pu-erh teas, black teas, and green teas.

clear, which may indicate that, although the chemical constituents (and quality) of pu-erh tea change over time, the quality stabilizes after a given period. For that reason, 5-year-old pu-erh tea was selected for this study of human biological responses to pu-erh tea ingestion.

Optimization of Urine Sample Preparation. Before UPLC−QTOFMS analysis, urine samples were diluted with ultrapure water or water with 0.1% formic acid (v/v), according to the method of Plumb et al. (*28*). The detailed experiment parameters are listed in the Supporting Information. The influence of different dilution volumes on the determination of metabolites was studied, and the subsequent UPLC−QTOFMS BPI chromatograms are provided in the Supporting Information. Examination of the chromatograms reveals that the peak retention time of 3.79 min in S1 was saturated, and the peaks located between 7 to 9 min appear until the condition S3. The effect of diluting with water and 0.1% formic acid solution was also studied, but the separation was not improved by this dilution. Therefore, a 2-fold volume of water was added to the urine throughout this study.

Analysis of Urine UPLC−QTOFMS Profiles. UPLC−QTOFMS spectra obtained from urine of a male participant before, during, and after pu-erh tea intake are shown in **Figure 6**. Visual examination of these chromatograms revealed little difference among the urines obtained before, during, and after pu-erh tea intake, although some peak intensities varied among the samples. To detect more subtle treatment-related metabolic differences, pattern recognition techniques were applied.

PCA was performed on all urinary metabolite profiles (**Figure 7**). Urine samples from day 14 were classified as outliers and were removed from the analysis because the participants did not eat the standard diet on that day. The PCA (scores) plot shows a clustering of the urine samples obtained before, during, and after pu-erh tea ingestion. The corresponding loadings plot and S-plot showed that increased urine UPLC−QTOFMS peaks from inositol, myristic acid, 5-hydroxytryptophan, and 4-methoxyphenylacetic acid and decreased urine UPLC−QTOFMS peaks from creatinine, 3-chlorotyrosine, and tyramine contributed most to the separate clustering of the groups.

Effects of Pu-erh Tea Intake. In this study, the urine samples obtained from 10 men and 10 women before pu-erh tea intake

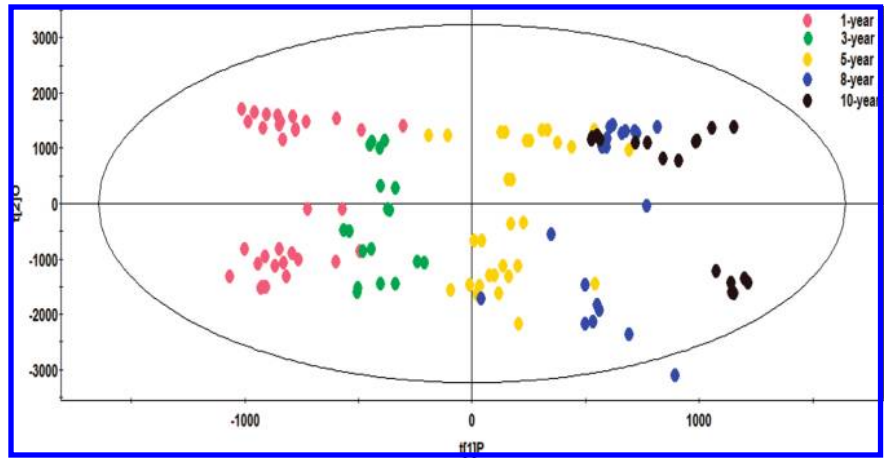

Figure 4. PLS-DA scores plot of pu-erh tea of different ages: 1-, 3, 5-, 8-, and 10-year-old.

Table 3. Summary of the Differential Metabolites from VIP Values of Two Component PLS-DA Model (R2Y = 0.982, Q2Y = 0.946) of Pu-erh Tea (10 Year)[a]

metabolite identification	VIP
glucose	(5)↑
caffeine	(1)↓
kaempferol	(2)↓
gallocatechin	(4)↓
catechin	(3)↓
epicatechin	(7)↑
arginine	(6)↓
theanine	(8)↓

[a] Compared to the pu-erh tea (1 year), ↑ represents significantly elevated concentration, whereas ↓ represents significantly lowered concentration.

(days 1, 7 and 14), during pu-erh tea intake (days 16, 21 and 28), and after pu-erh tea intake (days 30, 36, and 42) were analyzed. Typical BPI chromatograms are shown in **Figure 6**. PCA, an unsupervised pattern recognition method, was performed to increase accuracy. The trajectory of the PCA (scores) plot reveals the separation of urine samples obtained before, during, and after pu-erh tea ingestion (**Figure 7**). The three samples were clearly separated by the principal component 1 (PC1); the plot shows that the metabolic patterns of objects deviate from baseline during the consumption of pu-erh tea, and approaches the baseline during the postdosing phase, although the postdose metabolic pattern is still distinct from the predose pattern.

A three-component PLS-DA model (R2X = 0.606, R2Y = 0.927, Q2Y = 0.821) was subsequently constructed to identify the metabolites accountable for the separation of the metabolic patterns of the control group and pu-erh tea ingestion group. The set of differential urine metabolites most responsible for the altered metabolic profiles is summarized in **Table 4**. Human urinary metabolites were affected by gender as we can see from **Figure 8**. Metabolites from male and female during pu-erh tea intake can be separated clearly by PC2 (*t*[1]), which has been discussed by Wang et al. (*16*). It is also reported that urine samples can be separated by diet and time of collection (*29*). All of these influences on the metabolic profile can obscure the effect of specific dietary interventions. To establish metabolic changes due specifically to pu-erh tea, we established a standard diet and a fixed urine collection time.

An S-plot model was used to visualize the metabolites that were differentially produced before and during pu-erh tea intake (**Figure 9**). The metabolites most strongly influencing the differentiation are listed in **Table 4** along with the variable importance parameter (VIP), a measure of their relative influence on the model. The most influential factors were increased urinary

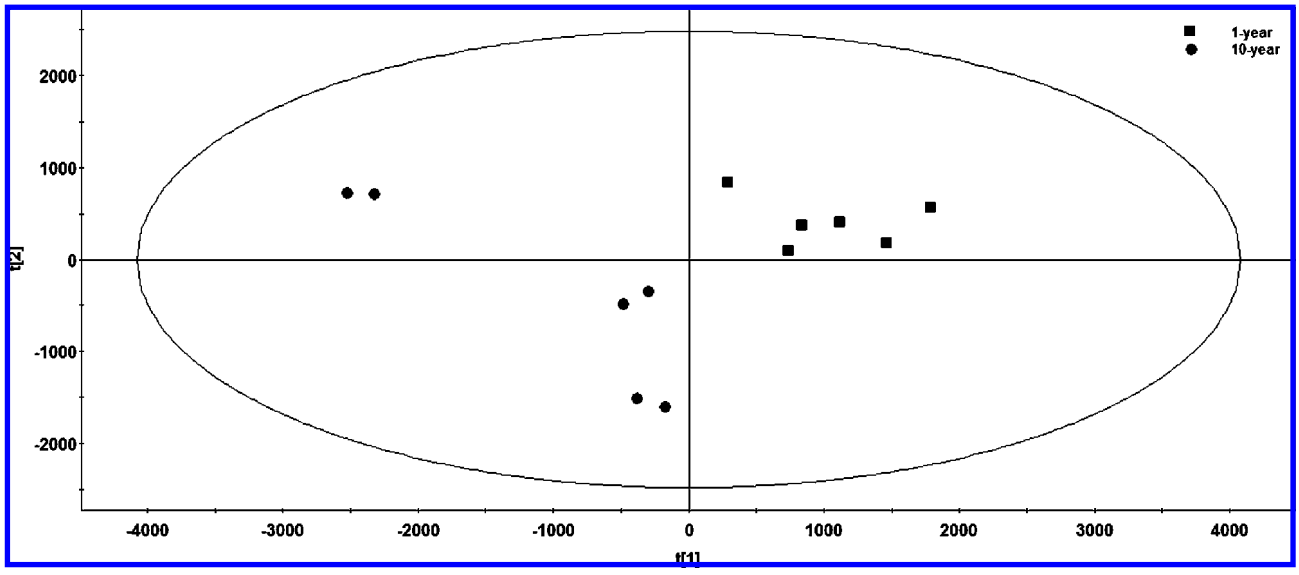

Figure 5. Metabolic profiles depicted by PLS-DA scores plot of UPLC−QTOFMS spectra between samples of pu-erh tea (1 year) and pu-erh tea (10 year).

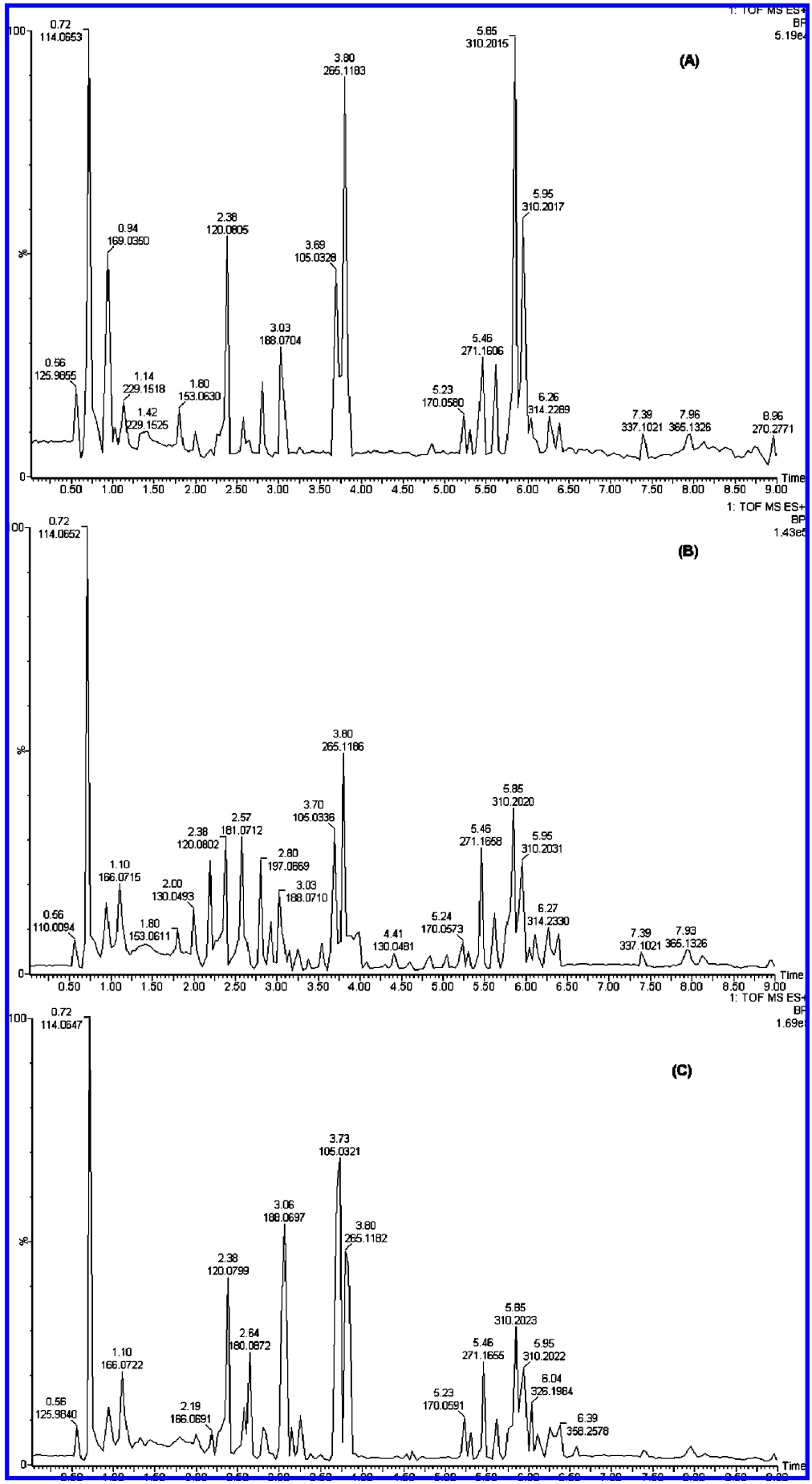

Figure 6. Comparison of UPLC−QTOFMS base peak intensity (BPI) chromatograms of urine collected during the predose phase (**A**), pu-erh tea intake phase (**B**), and postdose phase (**C**).

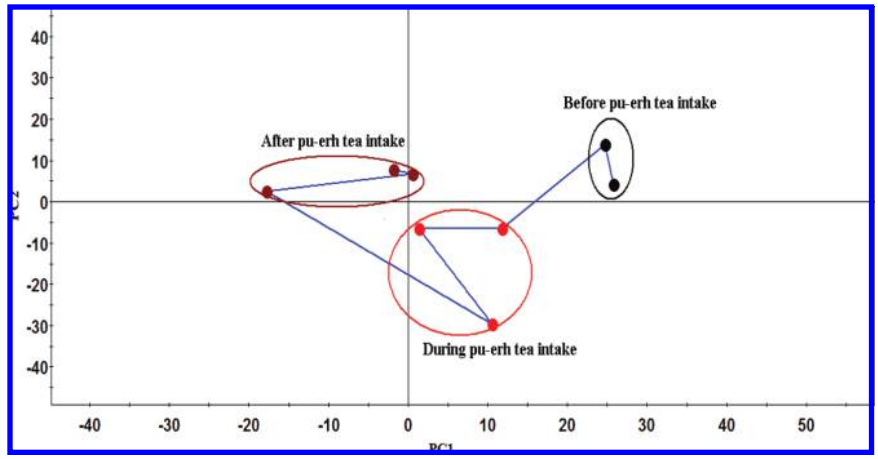

Figure 7. Trajectory of PC1 vs PC2 scores for UPLC−QTOFMS data of urine samples collected during the predose phase, pu-erh tea intake phase, and postdose phase. Each dot denotes mean score at different time points (e.g., predose period (day 1, day 7), "dosing" period (day 16, day 21, day 28), and postdose period (day 30, day 36, day 42).

Table 4. Changes of Metabolites Observed in Human Urine Obtained after Pu-erh Tea Intake and the Contribution of Each Metabolite

retention time (min)	metabolite	VIP rank
0.97	inositol	3(↑)
1.45	myristic acid	4(↑)
2.01	5-hydroxytryptophan	5(↑)
2.19	4-methoxyphenylacetic acid	1(↑)
0.71	creatinine	8(↓)
1.03	3-chlorotyrosine	7(↓)
1.15	tyramine	6(↓)
2.01	pyroglutamic acid	2(↑)

excretion of 4-methoxyphenylacetic acid, inositol, myristic acid, and 5-hydroxytryptophan, and decreased urinary excretion of 3-chlorotyrosine, tyramine and creatinine (**Figure 9** and **Table 4**). Inositol has been reported to lower cholesterol levels (*30*), thus the increased inositol levels in the urine during pu-erh tea intake may be at least partially responsible for the cholesterol-reducing effects of pu-erh tea reported in the literature (*9*). 3-Chlorotyrosine, a specific marker for myeloperoxidase-catalyzed oxidation, is markedly elevated in low-density lipoprotein (LDL) isolated from human atherosclerotic blood vessels. Reduced 3-chlorotyrosine levels result in a lower LDL concentration, which may be the mechanism of reducing plasma triglyceride (*9, 31*). 5-Hydroxytryptophan, as an intermediate in 5-hydroxytryptamine biosynthesis from the essential amino acid tryptophan, increases 5-hydroxytryptamine levels, thereby helping to prevent headaches, to lose weight, and to alleviate stress and insomnia. 5-Hydroxytryptophan is also a clinically important nerve medicament (*32*). The reason for the observed change in creatinine is unclear. However, it is known that oxidative stress promotes urinary excretion of creatinine (*33*) and antioxidants reduce urinary creatinine in rabbits (*34*). It is therefore possible that the antioxidative activity of pu-erh tea caused the reduced level of urinary creatinine following pu-erh tea intake.

The metabolic profiles of urine samples obtained during the 2 week post-treatment phase were distinct from those obtained during the pretreatment phases (**Figure 8** and Supporting Information Figure S6), suggesting an incomplete recovery during the washout phase. Disruption of the gut microbial populations by pu-erh tea could account for these results. The concentration of creatinine and other metabolic species in urine has been shown to be modulated by microbes. 3-Chlorotyrosine, reported to promote microbiological activity (*35*), was decreased in the metabolic profiles during the pu-erh tea ingestion phase.

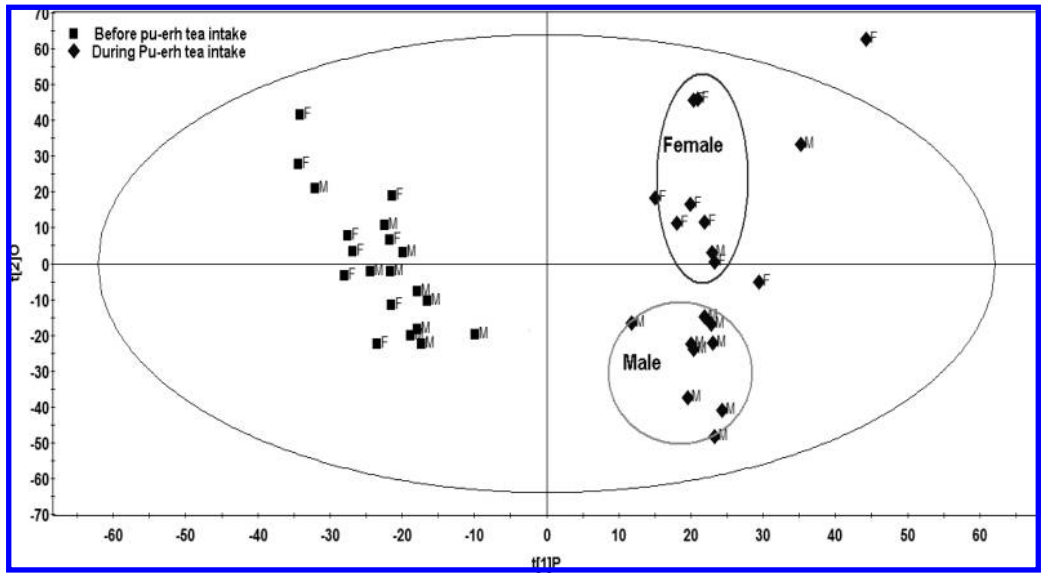

Figure 8. Scores plot (*t*[1] vs *t*[2]) generated from OPLS-DA of UPLC−QTOFMS spectra from control group and pu-erh tea ingestion group.

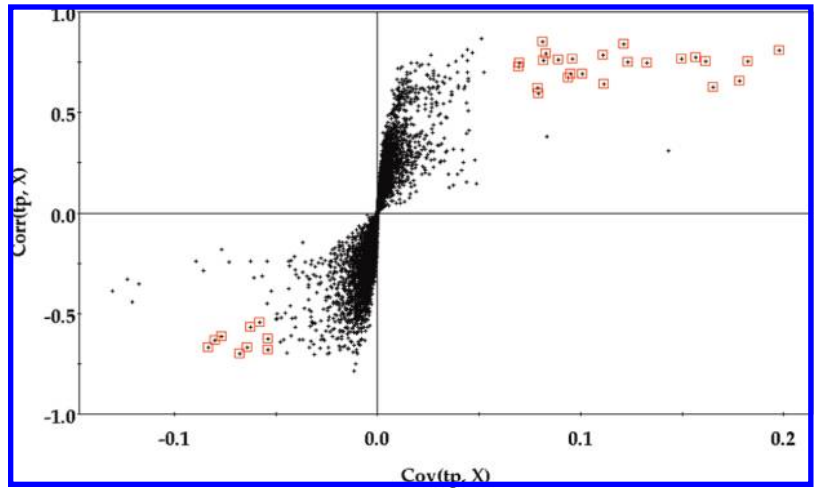

Figure 9. S-plot of the differentiate metabolites.

Pu-erh tea has been shown to possess antimicrobial activity (*6*, *7*), and more than 2 weeks may be required to reestablish gut microbial populations after pu-erh tea ingestion. Gut microbes exert a profound impact on the development and structure of the intestinal epithelium, the digestive and absorptive capabilities of the intestine, and host immune system function (*36*); therefore disturbances of the gut microbial populations by pu-erh tea ingestion would be expected to affect health. Identifying specific changes in the microbial community would help in understanding the metabolic implications of pu-erh tea ingestion.

In conclusion, we applied an MS-based metabonomics approach to evaluate the quality of pu-erh tea, black tea, and green tea, as well as the effects of dietary intervention with pu-erh tea. We found that differences in tea processing resulted in differences in the chemical constituents and the color of tea infusions. The characteristic components were polyphenols and theanine in green tea, TFA and TR in black tea, and TB and GA in pu-erh tea. We also found that the urinary metabolic profile was affected by the tea intervention, deviating from the baseline during the period of pu-erh tea ingestion and regressing to a pattern approaching baseline during the 2-week washout phase. It was found that the depletion of creatinine and the elevation of 4-methoxyphenylacetic acid, inositol, myristic acid, 5-hydroxytryptophan were strongly associated specifically with pu-erh tea intake. In addition, the metabolic consequences of pu-erh tea ingestion persisted during a 2-week postdosing period, implying an ongoing disruption of the resident gut microflora. The current study is an important step toward a full understanding of pu-erh tea and its influence on human metabolism. Further pharmaceutical research is warranted to elucidate the metabolic effects of pu-erh tea.

ABBREVIATIONS USED

UPLC−QTOFMS, ultraperformance liquid chromatography−quadrupole time-of-flight mass spectrometry; PCA, principal component analysis; PLS-DA, partial least-squares-discriminant analysis; ANN, artificial neural networks; BPI, base peak intensity; VIP, variable importance parameter; EGC, epigallocatechin; EGCG, epigatechin gallate; ECG, epicatechin gallate; GCG, gallocatechin gallate; TR, thearubigin; TF, theaflavin; TFA, theaflavic acid; TB, theabrownin; GA, gallic acid.

ACKNOWLEDGMENT

We thank the volunteers for their help in making this study possible.

Supporting Information Available: Sources of the tea samples (Table S1); comparison of chemical constituents of pu-erh tea, black tea, and green tea (Table S2); summary of the differential metabolites from VIP values of two component OPLS model (R2Y = 0.994, Q2Y=0.991) in the green tea (compared to the black tea, ↑ represents significantly elevated concentration, whereas ↓ represents significantly lowered concentration) (Table S3); summary of the differential metabolites from VIP values of two component PLS-DA model (R2Y = 0.999, Q2Y = 0.995) in the black tea (compared to the pu-erh tea, ↑ represents significantly elevated concentration, whereas ↓ represents significantly lowered concentration) (Table S4); summary of the differential metabolites from VIP values of two component PLS-DA model (R2Y = 0.999, Q2Y = 0.995) in the green tea (compared to the pu-erh tea, ↑ represents significantly elevated concentration, whereas ↓ represents significantly lowered concentration) (Table S5); dilution parameters of urine (Table S6); metabolic profiles depicted by PLS-DA scores plot of UPLC−QTOFMS spectra between samples of green tea and black tea (Figure S1); metabolic profiles depicted by PLS-DA scores plot of UPLC−QTOFMS spectra between samples of pu-erh tea and black tea (Figure S2); metabolic profiles depicted by PLS-DA scores plot of UPLC−QTOFMS spectra between samples of pu-erh tea and green tea (Figure S3); UPLC−QTOFMS base peak intensity (BPI) chromatograms of urine samples diluted by different volumes of ultrapure water (Figure S4); UPLC−QTOFMS base peak intensity (BPI) chromatograms of urine samples diluted by 2-fold volumes of ultrapure water and 0.1% formic acid solution (Figure S5); scores plot (t[1] vs t[2]) generated from OPLS-DA of UPLC−QTOFMS spectra from pu-erh tea ingestion group and after pu-erh tea intake (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review December 23, 2008. Revised manuscript received February 18, 2009. Accepted February 18, 2009. This work was financially supported by the National Basic Research Program of China (2007CB914700), the International Collaborative Project, Chinese Ministry of Science and Technology (2006DFA02700) and Xishuangbanna prefecture of Yunnan province.

JF804000Y