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Application of Metabolomics in the Analysis of Manufacturing Type of Pu-erh Tea and Composition Changes with Different Postfermentation Year

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Liquid chromatography−mass and multivariate analyses were employed to measure the composition of pu-erh teas and to determine the general changes in the compositional patterns of pu-erh teas during postfermentation. Principle component analysis of pu-erh teas indicated two large distinct clusters in the score plot: ripened pu-erh teas and raw pu-erh teas. The raw pu-erh teas contained more antioxidant compounds compared to ripened pu-erh teas. As a result, the raw pu-erh teas showed significantly higher antioxidant activities than the ripened pu-erh teas in the 1,1-diphenyl-2-picrylhydrazyl, Trolox equivalent antioxidant capacity, and ferric reducing antioxidant power assays. In addition, raw pu-erh teas showed significantly higher NO inhibitory and cell protective activities than the ripened pu-erh teas. Significant correlations between compounds and postfermentation year were observed in raw pu-erh teas; epigallocatechin-3-gallate, epigallocatechin, epicatechin-3-gallate, and quinic acid were decreased and gallic acid was increased in a year-dependent manner. The antioxidant activity was shown to decrease as the number of antioxidant compounds in raw pu-erh tea decreased. These findings indicate that a metabolomic approach is a useful tool for analyzing manufacturing type, postfermentation year, and antioxidant activity of pu-erh tea.

KEYWORDS: Pu-erh; tea; antioxidant; metabolomics; LC-MS

INTRODUCTION

Pu-erh tea is traditionally made with leaves from old, wild tea trees of a variety known as "broad leaf tea", *Camellia sinensis* (L.) O. Kuntze var. assamica Kitamura, which is found in southwestern China. Pu-erh tea is a postfermented tea as opposed to fermented tea, such as black tea. Investigations of pu-erh teas are relatively rare, although green tea (*C. sinensis* L., Theaceae), which is consumed worldwide, has been extensively studied and shown to have many health-promoting benefits (*1*, *2*). Recent studies have revealed that pu-erh tea has unique biological effects: inhibitory effect on nitric oxide production (*3*, *4*), antiobesity effect (*5*−*7*), hypolipidemic effect (*8*, *9*), hypocholesterolemic effect (*10*), and free radical scavenging effect (*11*).

Pu-erh teas are typically manufactured via two methods. Raw pu-erh tea is traditionally produced by pressing large and unoxidized tea leaves that are then fermented for several years at room temperature. Ripened pu-erh teas are "ripened" for several months using microbes under optimum conditions prior to being pressed (*12*). A previous study reported that *Aspergillus niger*, *Aspergillus gloucu*, and several species of *Penicillium*, *Rhizopus*, *Saccharomyces*, and *Bacterium* were found in pu-erh tea. Among them, *A. niger* is the most predominant followed by *Saccharomyces* spp. (*13*). It is generally believed that pu-erh teas, with their longer postfermentation period, have better quality and taste. Thus, tea connoisseurs and speculators are willing to pay higher prices, upward of thousands of dollars per cake, for older pu-erh tea.

Demand for older pu-erh tea has recently increased as people have shifted to more health-centered diets. In 2003, the consumption of authentically aged pu-erh tea in Guangdong, Hong Kong, France, Japan, Korea, Taiwan, and Malaysia reached 20000 tons (*12*). However, because authentically aged pu-erh is difficult to find and identify, many counterfeit varieties exist in the marketplace. Thus, accurate and systemic methods of predicting the age of pu-erh and identifying the type of manufacturing process used for its production are required not only for the protection of consumers but also for the producers in terms of quality control.

Metabolomics can help us gain a broader insight into the biochemical composition of living organisms and how this composition changes with time and processing (*14*). Recent developments in plant metabolomics now allow for the simultaneous detection of several hundred metabolites. Thus, reliable

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comparisons between nonspecific samples are also possible in an untargeted manner (*15*). Metabolomics is a powerful tool that can be used to differentiate phytochemical composition among different origins (*16*), varieties (*17*), or products (*18*, *19*). Moreover, metabolomics can also be utilized for quality assessment (*20*−*25*).

This study aims to identify biomarkers for distinguishing manufacturing types used for pu-erh tea production and analyze how the chemical composition changes during postfermentation by using liquid chromatography−mass spectrometry (LC-MS) and multivariate analysis. These analyses should help us to pinpoint composition changes of pu-erh teas during the postfermentation process. Correlations between the antioxidant activity of pu-erh tea and both the composition of its antioxidant compounds and postfermentation years were determined.

MATERIALS AND METHODS

Preparation of Samples. Pu-erh teas at different postfermentation years were purchased from markets in the Republic of Korea (**Table 1**). All samples were cultivated in Yunnan province in China. The samples were infused with 60% aqueous acetone for 2 h at 70 °C in a water bath in triplicate. The mixtures were filtered through a 0.2 μm PTFE filter for LC-MS analysis.

Chemicals and Reagents. (+)-Catechin, (−)-epicatechin-3-gallate, NaOH, $AlCl_3$, sodium carbonate, gallic acid, Folin−Ciocalteu's phenol reagent, potassium persulfate, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-*s*-triazine (TPTZ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), $FeCl_3 \cdot 6H_2O$, $C_2H_3NaO_2 \cdot 3H_2O$, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, lipopolysaccharide (*Escherichia coli*, serotype 0.55:B5), phosphoric acid, pyrrolidine dithiocarbamate, dimethyl sulfoxide (DMSO), and formic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and water used in LC-MS were of the optima grade from Fisher Scientific (Pittsburgh, PA).

Liquid Chromatography−Potodiode Array−Electrospray Ionization−Mass Spectrometry (LC-PDA-ESI/MS) Conditions. We used a 212-LC binary solvent delivery system, a MetaTherm HPLC column heater, a Prostar 410 autosampler, a Prostar 335 photodiode array detector, and a 500-ion trap mass spectrometer from Varian Technologies (Palo Alto, CA). The system was operated using the MS workstation software (version 6.9.2, Varian, Inc., Palo Alto, CA). The LC-MS conditions were set on the basis of previous papers (*26*−*28*). The chromatographic separation was performed on a ChromSep HPLC column SS 100 × 2.0 mm i.d., 3 μm particle size (Varian Inc., Lake Forest, CA), with a ChromSep guard column PurSuit XRs 3-C18 (Varian Inc., Lake Forest, CA) at a column oven temperature of 45 °C. A flow rate of 0.2 mL/min was used during separation. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient was increased linearly from 15 to 30% B (v/v) at 15 min, to 100% B at 30 min, and held at 100% B until 35 min, after which it was decreased linearly to 15% at 35.01 min and held at 15% for 40 min until injection of the next analytical sample. The UV spectrum was set at 280 nm for real-time monitoring of the peak intensities. A photodiode array was used to continuously record the absorbance from 200 to 600 nm for tea component identification. Mass spectra were simultaneously acquired using electrospray ionization in negative ionization (NI) mode at 70 V over the range of m/z 50−1000. A drying gas pressure and temperature of 30 psi and 350 °C, respectively, a nebulizer pressure of 50 psi, and a capillary voltage of 70 V for NI were used.

Trolox Equivalent Antioxidant Capacity (TEAC). The TEAC assay was conducted as described by Re et al. (*29*). Following this, 7 mM ABTS ammonium was dissolved in a potassium phosphate buffer (pH 7.4) and treated with 2.45 mM potassium persulfate. The mixture was then allowed to stand at room temperature for 12−16 h until it turned dark blue. The solution was then diluted with the potassium phosphate buffer until the absorbance reached 0.7 ± 0.02 at 734 nm. The latter was measured using a BioTek EL 808 microplate reader (Power Wave XS, Biotek Instruments Inc., Winooski, VT). Subsequently, 190 μL of the solution was mixed with 10 μL of the sample or blanks (60% aqueous acetone). The absorbance was recorded at room temperature after 6 min. Results were expressed in millimols of Trolox equivalent concentration per gram of pu-erh tea. The concentrations of the standard solutions ranged from 0.25 to 4 mM. Experiments were carried out in triplicate.

Table 1. Sample List of Pu-erh Teas from Yunnan Province, China

raw pu-erh teas			ripened pu-erh teas		
no.	production year	sample name	no.	production year	sample name
1	2008	rp0Y1	1	2007	p1Y1
2	2008	rp0Y2	2	2007	p1Y2
3	2008	rp0Y3	3	2007	p1Y3
4	2007	rp1Y1	4	2005	p3Y1
5	2007	rp1Y2	5	2005	p3Y2
6	2007	rp1Y3	6	2005	p3Y3
7	2005	rp3Y1	7	2003	p5Y1
8	2005	rp3Y2	8	2003	p5Y2
9	2005	rp3Y3	9	1998	p10Y1
10	2004	rp4Y1	10	1998	p10Y2
11	2004	rp4Y2	11	1995	p13Y1
12	2004	rp4Y3	12	1995	p13Y2
13	2003	rp5Y1			
14	2003	rp5Y2			
15	2000	rp8Y			
16	1997	rp11Y			
17	1995	rp13Y			
18	1993	rp15Y			

Determination of the Antioxidant Activity by the DPPH Free Radical Scavenging Assay. The DPPH assay was conducted as described, with minor modifications, by Dietz et al. (*30*). Reaction mixtures containing test samples or blanks (60% aqueous acetone) (10 μL) and 190 μL of a 200 μM DPPH ethanol solution were incubated at room temperature for 30 min in 96-well plates. The absorbance of the DPPH free radical was measured at 515 nm with a microplate reader. Results were expressed in millimols of Trolox equivalent concentration per gram of pu-erh tea. The concentration of the standard solutions ranged from 0.156 to 2.5 mM. Experiments were carried out in triplicate.

Ferric Reducing/Antioxidant Power (FRAP) Assay. The antioxidant capacity of each standard and sample was estimated according to the procedure described by, with minor modifications, Benzie and Strain (*31*). Briefly, 300 μL of FRAP reagent, prepared freshly and warmed at 37 °C, was mixed with 10 μL test sample of solutions or blanks (60% aqueous acetone). The absorbance was measured at 593 nm after 6 min using a microplate reader. The FRAP reagent contained 2.5 mL of a 10 mM/L TPTZ solution in 40 mM/L HCl in distilled water, 2.5 mL of 20 mM/L $FeCl_3 \cdot 6H_2O$ in distilled water, and 25 mL of 0.3 M/L acetate buffer of pH 3.6. Results were expressed in millimols of Trolox equivalent concentration per gram of pu-erh tea. The concentration of standard solutions ranged from 0.25 to 2 mM. Experiments were carried out in triplicate.

Determination of Total Phenolics and Flavonids. Total phenol content was determined using the method of Singleton et al. (*32*). The assay conditions were as follows: a 10 μL sample was added to 0.2 N Folin−Ciocalteu's phenol reagent (160 μL) in 96 wells. After 3 min, 30 μL of a saturated sodium carbonate solution was added to the mixture and subsequently incubated at room temperature for 1 h. The resulting absorbance of the mixture was measured at 750 nm using a microplate reader. The total phenol content was calculated on the basis of a standard curve with gallic acid. The standard solution concentrations ranged from 12.5 to 400 μg/mL. Results were expressed in milligrams of gallic acid equivalent (GAE) per gram of pu-erh tea. Experiments were carried out in triplicate.

Total flavonoid content was analyzed using the method described by Yoo et al. (*33*), with minor modifications. Twenty microliters of the samples or standard solutions of (+)-catechin was added to each well in the 96-well plates. The standard solution concentrations ranged from 100 to 800 μg/mL. Distilled water (40 μL) and 6 μL of 5% (w/v) sodium nitrite were added to each well. After 5 min, 12 μL of 10% (w/v) $AlCl_3$ was added. After 6 min, 40 μL of 1 M NaOH was added to the mixture followed by 42 μL of distilled water. Absorbance was measured at 515 nm using a microplate reader, and the flavonoid content was expressed as milligrams

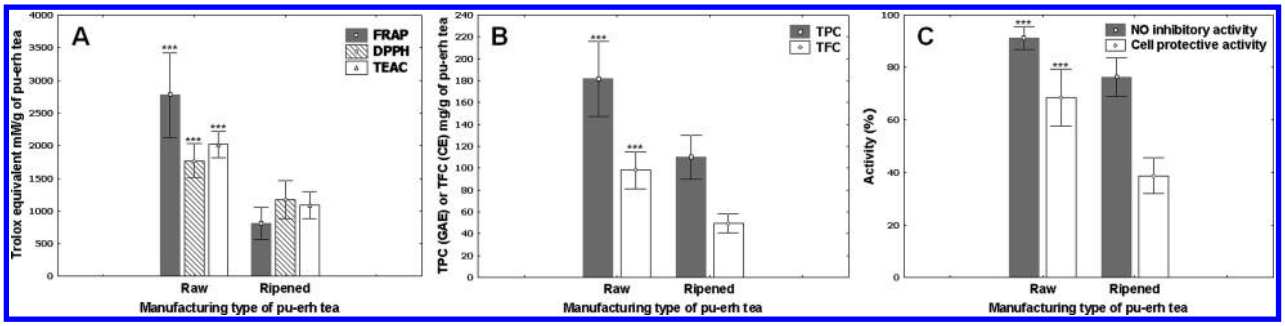

Figure 1. (**A**) Antioxidant activity of two types of pu-erh tea; (**B**) total phenol content (TPC) (GAE, gallic acid equivalent) and total flavonoid content (TFC) [CE, (+)-catechin equivalent]; (**C**) NO inhibitory activity and cell protective activity. ***, significant difference between two groups by Student *t* test at $p < 0.001$.

of (+)-catechin equivalent per gram of pu-erh tea. Experiments were carried out in triplicate.

Cell Culture. The mouse macrophage cell line (RAW 264.7, KCLB, Seoul, South Korea) was cultured in a high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 4 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco). Cells were grown in a humidified incubator at 37 °C under 5% CO_2.

Inhibitory Effect on Nitric Oxide (NO) Production Activated by LPS. Macrophages were cultured in 96-well (1×10^5/mL) plates for 2 days and incubated overnight (16 h) with or without pu-erh tea samples. They were then reconstituted in the presence of 1 μg/mL LPS (*E. coli*, serotype 0.55:B5) in high-glucose, phenol red free DMEM (Gibco) containing 0.5% FBS, 4 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultured cells with or without LPS served as a positive or negative control, respectively. The production of NO was determined by measuring nitrite in the culture medium using the Griess reaction (*34*). Briefly, 100 μL aliquots of medium were incubated with an equal volume of modified Griess reagent (50 μL of 1% sulfanilamide in 5% H_3PO_4 and 50 μL of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in H_2O). After 10 min, the absorbance was measured at 570 nm using a microplate reader. Pyrrolidine dithiocarbamate was used as a positive control (final concentration was 5 μM).

Cell Protective Activity Using Raw 264.7. Cell protective activity was analyzed using the method of Hu et al. (*35*). Macrophages were cultured in 96-well (1×10^5/mL) plates for a day. The cell cultures were preincubated with the pu-erh tea extracts for 1 h, and then hydrogen peroxide was added to a final concentration of 100 μM in medium for 24 h. Cell protective activity was determined using the MTT assay, which yields a blue formazan product in living cells, but not in dead cells or their lytic debris. MTT dissolved in phosphate-buffered saline (10 μL) was added to a final concentration of 5 mg/mL at the end of incubation and then incubated further at 37 °C for 4 h. The resultant formazan product was dissolved with 200 μL of DMSO and detected using a microplate reader at 570 nm.

Data Processing. Data preprocessing was performed with Varian MS Workstation 6.9 software (Palo Alto, CA). LC-MS spectra were converted into NetCDF formats using Vx Capture (version 2.1, Laporte, MN). After conversion, LC-MS spectra were processed by XCMS. The XCMS parameters for the R language were performed by simple commands as XCMS's default settings with the following (http://masspec.scripps.edu/xcms/documentation.php). The resulting data were exported in Excel (Microsoft, Redmond, WA), and then statistical analysis was performed using other statistical programs.

Statistical and Multivariate Analysis. Statistical analyses were performed on all continuous variables using the SIMCA-P+ (version 11.5, Umetrics, Umeå, Sweden). Univariate statistics for multiple classes was performed by breakdown and one-way ANOVA using Statistica (version 7.0, StatSoft Inc., Tulsa, OK). Multivariate statistics was performed by unsupervised principal component analysis (PCA) to obtain a general overview of the variance of metabolites in the study. In addition, supervised partial least-squares (PLS) statistics were performed, which requires information about the assigned variable classes (*X* variable; LC-MS peak and *Y* variable; postfermentation year). All variables were pareto scaled with mean-centering in a columnwise manner before PCA and PLS analyses.

RESULTS AND DISCUSSION

Comparison of Antioxidant Activity of Two Types of Pu-erh Teas. To compare the antioxidant activity of pu-erh teas produced using the two manufacturing methods, TEAC, DPPH, and FARP assays were performed. These antioxidant assays are based on electron-transfer reaction, but each specified mechanism is different (*36*). Thus, these three different antioxidant assays will give us credible and comprehensive antioxidant data for samples. As displayed in **Figure 1A**, the antioxidant activity of raw pu-erh teas was significantly higher than that of ripened pu-erh teas in the three different antioxidant assays. Whereas ripened pu-erh teas showed 1170 (DPPH), 810 (FRAP), and 1090 (TEAC) mM of Trolox equivalent (per gram of pu-erh tea) antioxidant activity, raw pu-erh tea showed 1770 (DPPH), 2780 (FRAP), and 2020 (TEAC) mM of Trolox equivalent (per gram of pu-erh tea) antioxidant activity (further detailed in Table S1 of the Supporting Information). In summary, raw pu-erh teas exhibited higher antioxidant activity than ripened pu-erh teas.

Comparison of Total Phenol and Total Flavonoid Contents. Raw pu-erh teas were found to have a higher content of phenolic and flavonoid compounds than ripened pu-erh teas. As shown in **Figure 1B**, the total phenol content of raw pu-erh teas (182 mg of gallic acid equiv/g of pu-erh tea) was 1.65 times higher than that of ripened pu-erh teas (110 mg of gallic acid equiv/g of pu-erh tea). The total flavonoid content of raw pu-erh teas (98 mg of gallic acid equiv/g of Pu-erh tea) was 2 times higher than that of ripened pu-erh teas (49 mg of gallic acid equiv/g of Pu-erh tea) (further detailed in Table S1 of the Supporting Information). Previous research has suggested that total phenol content is closely related to antioxidant activity (*37*−*40*). Phenolic compounds and flavonoid compounds are well-known antioxidants and cancer-preventing agents (*41*, *42*). In this aspect, raw pu-erh teas appear to be more suitable teas for antioxidant and cancer-preventing activities than ripened pu-erh teas.

NO Production Inhibitory Activity. As shown in **Figure 1C**, raw pu-erh teas more strongly inhibited NO production than ripened pu-erh teas (further detailed in Table S1 of the Supporting Information). Previous studies reported that the water extract of pu-erh tea showed stronger NO production inhibitory activity than other types of tea extracts (*4*). However, this study indicates that the NO inhibitory activity of pu-erh teas was dependent on the method of manufacturing.

Cell Protective Activity Using Raw 264.7. Raw pu-erh teas exhibited significantly stronger cell protective activity against H_2O_2-induced toxicity than ripened pu-erh teas. Whereas ripened pu-erh teas displayed a 38.8% cell protective activity, raw pu-erh

Table 2. Correlation Coefficients between Bioactivities and Postfermentation Year[a]

	postfermentation year	FRAP	TPC	DPPH	TEAC	TFC	NO inhibitory
postfermentation year	1.00						
FRAP	**-0.60**	1.00					
	(0.12)						
TPC	**-0.65**	0.16	1.00				
	(0.20)	(0.50)					
DPPH	0.15	−0.10	0.13	1.00			
	(0.32)	(0.40)	(**0.87**)				
TEAC	−0.25	0.37	−0.02	−0.10	1.00		
	(0.08)	(**0.64**)	(**0.91**)	(**0.75**)			
TFC	−0.44	**0.75**	0.16	−0.24	**0.71**	1.00	
	(0.38)	(0.22)	(**0.80**)	(0.56)	(**0.63**)		
NO inhibitory	0.18	−0.07	−0.11	0.07	0.23	−0.02	1.00
	(−0.05)	(**0.77**)	(0.45)	(0.29)	(**0.59**)	(0.18)	
cell protective	0.20	0.16	0.02	−0.16	0.39	0.42	0.29
	(−0.06)	(0.24)	(0.07)	(0.04)	(0.14)	(−0.15)	(−0.07)

[a] Upper values are correlation coefficient of raw pu-erh tea, and lower values are correlation coefficient of ripened pu-erh tea. Significant correlation coefficients are given in bold ($p < 0.05$).

teas showed a 68.6% cell protective activity (**Figure 1C**) (further detailed in Table S1 of the Supporting Information). The high contents of antioxidant compounds such as polyphenol and flavonoid are probably responsible for the cell protective effect against H_2O_2-induced toxicity or the scavenging effect against hydroxyl radical.

Correlation Coefficients between Bioactivities and Total Compounds Assay and Postfermentation Year. To determine the correlation between bioactivities and both total compound compositions and postfermentation year of pu-erh teas, correlation coefficients were calculated using Statistica (**Table 2**). Bioactivities of raw pu-erh teas were significantly correlated with their postfermentation year, although there was no significant correlation coefficient between the bioactivities of ripened pu-erh teas and their postfermentation year. More specifically, the postfermentation year of raw pu-erh teas was negatively correlated with FRAP ($r = -0.60$, $p < 0.05$) and total phenol content ($r = -0.65$, $p < 0.05$). This finding is in agreement with a previous study, which showed that total phenol content decreased during postfermentation of pu-erh tea (*43*). The total flavonoid content of raw pu-erh teas was positively correlated with FRAP ($r = 0.75$, $p < 0.05$) and TEAC ($r = 0.71$, $p < 0.05$), whereas the total phenol content of ripened pu-erh tea was positively correlated with DPPH ($r = 0.87$, $p < 0.05$) and TEAC ($r = 0.91$, $p < 0.05$). Although all three antioxidant assays are based on electron-transfer reaction (*36*), the results obtained from them were not same. Considering the sensitivity of FRAP assay against postfermentation year and total phenol content of raw pu-erh teas, it is speculated that the FRAP assay is useful to analyze raw pu-erh teas (*38*).

Multivariate Analysis of Different Manufacturing Types of Pu-erh Teas. To identify biomarkers that are significantly different between raw and ripened pu-erh teas, multivariate statistical analyses were performed. Multivariate statistics using the unsupervised principal component analysis (PCA) indicated that the dominant factor separating metabolic profiles was the manufacturing type and not the postfermentation year. The PCA score plot showed clear separation of ripened pu-erh teas and raw pu-erh teas based on 377 principle peaks by PC1 ($R^2X = 75.6\%$) (**Figure 2A**). The relatively large variation observed in raw pu-erh teas was the result of the different postfermentation years. On the other hand, the compositions of the ripened pu-erh teas, of which the raw materials are diverse, might become similar to each other during rapid fermentation. This indicates that postfermentation of raw pu-erh teas has a significant effect on their composition,

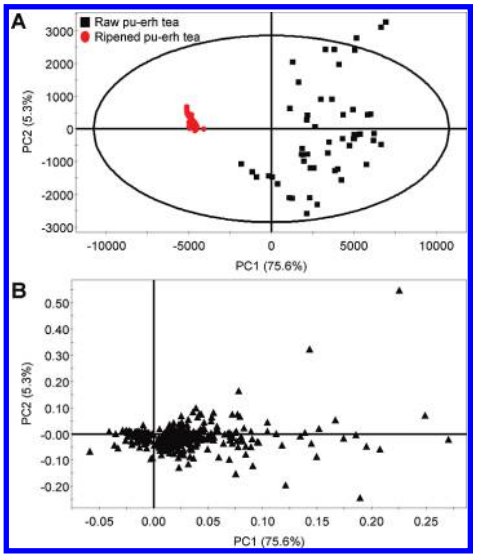

Figure 2. (**A**) PCA score plot of pu-erh teas (black squares, raw pu-erh teas; red circles, ripened pu-erh teas); (**B**) PCA loading plot obtained using pareto scaling with mean centering.

although this effect does take many years. However, the compositional patterns of ripened pu-erh teas were not heavily influenced by their postfermentation period.

The PCA loading plot (**Figure 2B**) was obtained using pareto scaling with mean-centering from the LC- MS peak intensity of each peak. Some outstanding peaks were observed in the PCA loading plot. Peak identification was performed using an in-house database (*44*) and cochromatography, the MS/MS spectral data, and the UV spectral data of the authentic compounds available and/or by comparison with published literature (*27*, *28*, *45*−*47*). Significantly different peaks were identified using the above techniques (**Table 3**). Some peaks were detected as a dimer and an isotope form along with their monomer *m*/*z* 883 (441), 884 (441), 916 (457), 915 (457), and 634 (633). As displayed in **Figure 3A**, raw pu-erh teas contained stricitinin (*m*/*z* 633), trigalloylglucose (*m*/*z* 635), caffeoylquinic acid (*m*/*z* 353), epigallocatechin (*m*/*z* 305), epicatechin-3-gallate (*m*/*z* 441), epigallocatechin-3-gallate (*m*/*z* 457), and galloylquinic acid (*m*/*z* 343), at levels significantly higher than those in ripened pu-erh tea (*t* test, $p < 0.001$). However, gallic acid was found at significantly lower levels in raw pu-erh teas (*t* test, $p < 0.001$). According to previous

Table 3. Biomarker Identification from Pu-erh Teas

no.	t_R (min)	UV λ_{max} (nm)	$[M-H]^-$ (m/z)	MS^n fragment ions (m/z)	identification	p value[b] (t test)	correl coeff (p value)[c]	ref
1	2.853	nd[a]	191	173, 127, 111, 85	quinic acid	<0.001	**−0.688** ($p < 0.001$)	*44*[d]
2	3.688	223, 271	169	125	gallic acid	<0.001	**0.546** ($p < 0.001$)	*44*, standard[e]
3	3.737	225, 273	343	191 (quinic acid; $[M-H]^-$ − galloyl), 169	galloylquinic acid	<0.001	−0.004 (0.997)	*27*, *28*, *45*, *47*
4	7.138	234, 271	305	221, 219, 179, 165	epigallocatechin	<0.001	**−0.505** ($p < 0.001$)	*44*, *45*, *47*
5	8.335	232, 276	353	191 (quinic acid; $[M-H]^-$ − caffeoyl), 179	caffeoylquinic acid	<0.001	**−0.565** ($p < 0.001$)	*27*, *45*, *47*
6	8.412	244, 272	633	463, 301, 275	strictinin	<0.001	**−0.495** ($p < 0.001$)	*28*
7	10.515	241, 272	457	331, 305, 227, 193, 169	epigallocatechin gallate	<0.001	**−0.567** ($p < 0.001$)	*27*, *28*, *44*, *45*, *47*
8	12.125	233, 275	635	483 ($[M-H]^-$ − Glc), 465, 313, 295, 169 (gallic acid; $[M-H]^-$ − Glc-Glc-Glc)	trigalloylglucose	<0.001	−0.214 (0.120)	*27*
9	14.249	240, 272	441	331, 289, 245, 203, 161	epicatechin gallate	<0.001	**−0.435** ($p < 0.01$)	*27*, *28*, *46*, standard

[a] nd, not determined. [b] Indicates significant difference between raw and ripened pu-erh teas by t test. [c] Indicates correlation coefficient between compound and postfermentation year of raw pu-erh teas. Significant values are in bold ($p < 0.05$). [d] Identified using an in-house library. [e] Positively identified by direct comparison with standard. Glc, glucosyl.

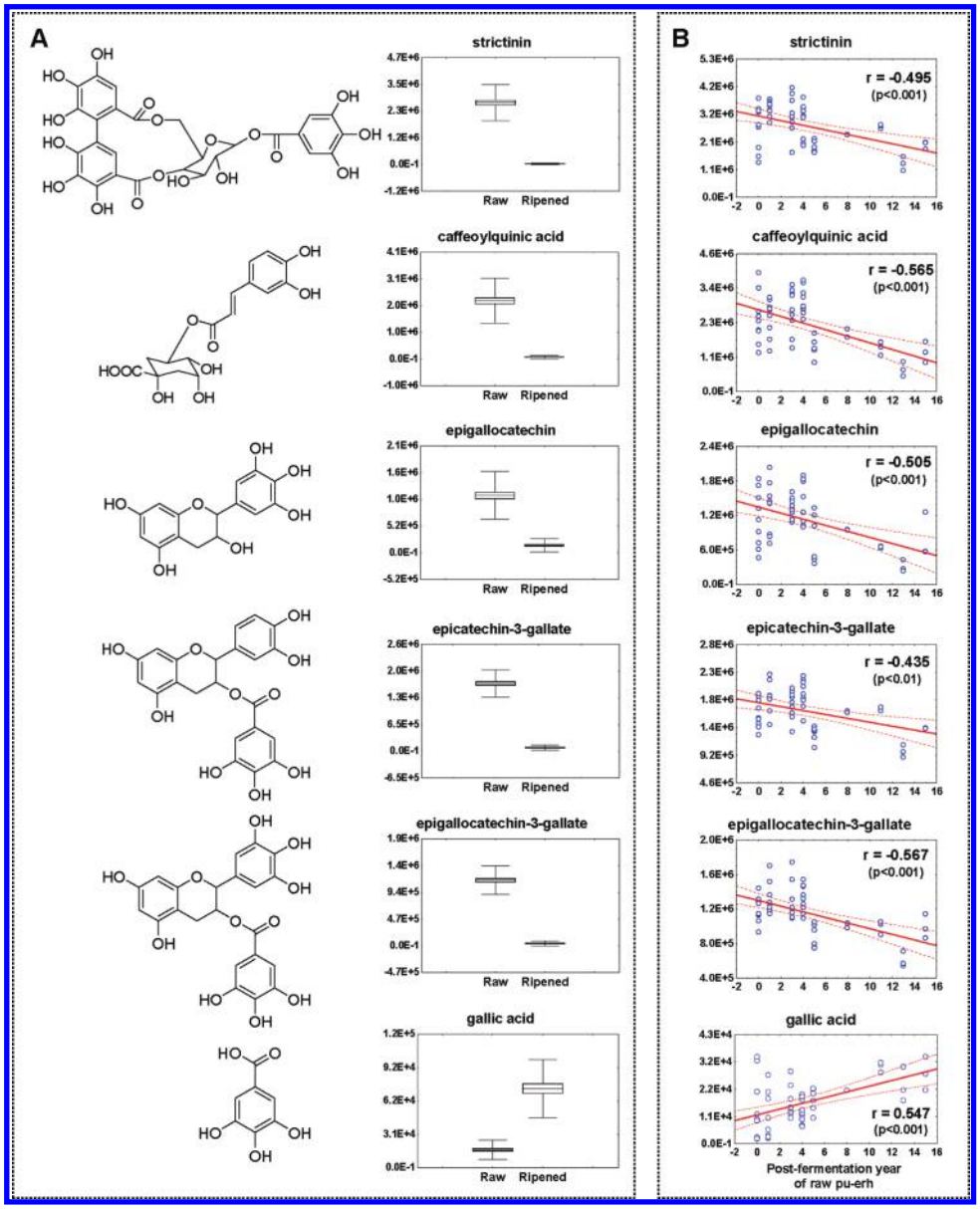

Figure 3. (**A**) Box and whisker plot of significantly different compounds between raw pu-erh tea and ripened pu-erh tea; (**B**) significant compounds associated with postfermentation. Y axis of plot in both (**A**) and (**B**) indicates mass peak intensity.

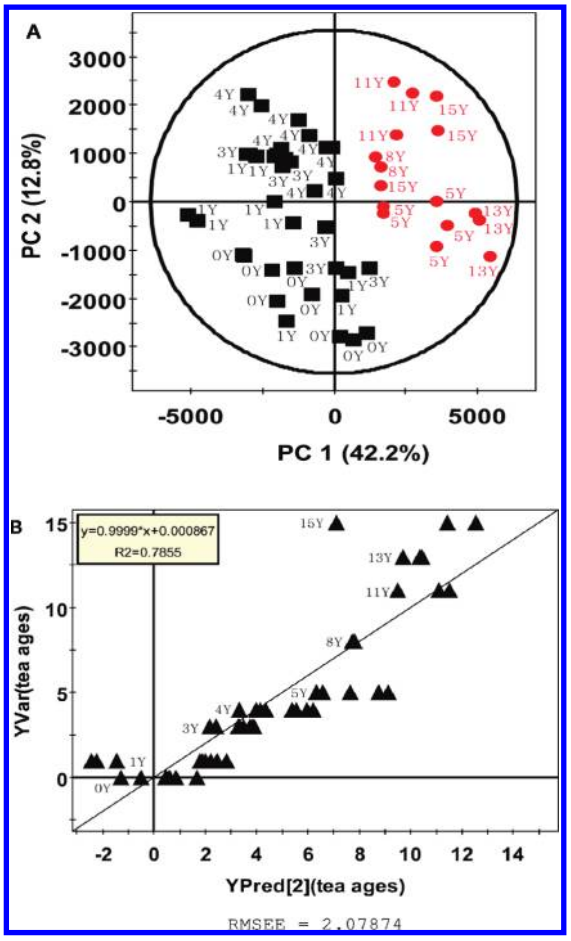

Figure 4. (**A**) PLS score plot of raw pu-erh teas (red circles, ≥5 years old; black squares, ≤4 years old); (**B**) PLS regression model for prediction of postfermentation year of raw pu-erh tea.

studies, epicatechin-3-gallate, epigallocatechin-3-gallate, epigallocatechin, galloylquinic acid, and caffeoylquinic acid were identified as the main antioxidant compounds in teas on the basis of ABTS online HPLC (*47*, *48*). These antioxidant compounds are responsible for the high antioxidant activity of raw pu-erh teas. Overall, ripened Pu-erh teas have a low level of catechins but a high level of gallic acid (**Figure 3A**). Hence, antioxidant activity, such as DPPH and TEAC, of ripened pu-erh teas showed a high correlation with total phenol content rather than with total flavonoid content. Also, a previous study reported that epicatechin-3-gallate and epigallocatechin-3-gallate inhibit inducible NOS (iNOS) proteins in activated macrophages (*49*). Raw pu-erh teas have significantly more of these two compounds than ripened pu-erh teas. Hence, we beleive that epicatechin-3-gallate and epigallocatechin-3-gallate are responsible for the higher NO inhibition activity in raw pu-erh teas.

From 90 samples of various pu-erh teas, 377 peaks were extracted by XCMS. Among them, 343 peaks showed significantly different mass intensities between raw and ripened pu-erh teas ($p < 0.001$).

Ripened pu-erh teas have a mild taste due to the reduced catechins, which contribute to astringency during rapid fermentation. Also, a previous study reported that only ripened pu-erh tea exhibited a remarkably reduced weight gain compared to raw pu-erh tea (*50*). However, the present study shows that raw pu-erh teas have a higher bioactivity than ripened pu-erh teas in terms of antioxidant, NO inhibitory, and cell protective activity.

PLS Analysis of Various Postfermentaiton Years of Raw Pu-erh Teas. To determine the correlation between compound composition and postfermentation year of raw pu-erh teas, PLS analysis was performed using SIMCA-P. As shown in **Figure 4A**, aged raw pu-erh teas (≥5 years old) and young raw pu-erh teas (≤4 years old) were discriminated by PLS component 1 as well as its PCA score plots (Figure S2-A in the Supporting Information). PLS analysis might be used as an algorithm to create a quality prediction model for tea. Previous study successfully created a quality-predictive model for green tea (*23*). As shown in **Figure 4B**, the postfermentation year-predictive model created successively from PLS regression showed the root-mean-square error of the fit for observations in the workset (RMSEE) of 2.08 years. Using this analysis, we found that the composition of raw pu-erh teas gradually changed depending on their postfermentation year ($R^2 = 78.5$, $Q^2 = 73.8$). This postfermentation-predictive model could be used to predict the postfermentation year of raw pu-erh tea.

To identify certain compounds that were specifically associated with fermentation, correlation coefficients were calculated using Statistica. Significant compounds were identified in the same manner described above (**Table 3**). Certain compounds such as quinic acid ($r = -0.688$) (not shown as a figure), epigallocatechin-3-gallate ($r = -0.567$), caffeoylquinic acid ($r = -0.565$), epigallocatechin ($r = -0.505$), strictinin ($r = -0.495$), epicatechin-3-gallate ($r = -0.435$), and gallic acid ($r = 0.546$), which were significantly correlated with postfermentation year ($p < 0.05$), can serve as biomarkers for determining the age of raw pu-erh teas (**Figure 3B**). As mentioned above, TPC and FRAP of raw pu-erh teas were also significantly correlated with their postfermentation year. These biomarkers can explain why aged raw pu-erh teas have less total phenol content and less antioxidant activity. According to previous studies, microbes in pu-erh teas grow at the expense of several benzene compounds (*51*, *52*). Therefore, the polyphenol content gradually decreased during postfermentation (*43*). Gallic acid is most likely produced from the degradation of epigallocatechin-3-gallate during fermentation (*53*). Due to postfermentation of raw pu-erh tea, gallic acid was gradually produced in raw pu-erh teas at the expense of epigallocatechin-3-gallate degradation. Gallic acid has been reported to inhibit fatty acid synthase (*54*) and cholesterol biosynthesis in a Hep G2 cell line (*55*). There is a general belief that the more aged the pu-erh tea is, the better it is. The product produced from tea fermentation such as gallic acid supports this general belief.

PLS Analysis of Various Postfermentaiton Years of Ripened Pu-erh Teas. As shown in **Figure 5A**, aged and young ripened pu-erh teas were not clearly discriminated by neither PLS nor PCA analysis (Figure S2B in the Supporting Information). The relatively large variation observed in ripened pu-erh teas may be a result of the genetic factors of the tea (*56*) or postfermentation conditions. As mentioned before, the compositions of the ripened pu-erh teas were significantly changed by rapid fermentation. This indicates that secondary metabolites of ripened pu-erh teas were not heavily influenced by their postfermentation period. Contrary to raw pu-erh teas, ripened pu-erh teas did not show a high coefficient of determination ($R^2 = 60.9$, $Q^2 = 40.1$) (**Figure 5B**). Through correlation analysis between compounds and postfermentation year, we identified only a few peaks that were significantly correlated with postfermentation year; however, they were hard to identify due to the low intensity.

In conclusion, this study revealed details on chemical composition differences of various pu-erh teas in terms of manufacturing type and postfermentation year. As a result of the PCA, two different types of pu-erh teas were clearly separated by PC1. Raw pu-erh teas, which have high levels of phenols and flavonoids,

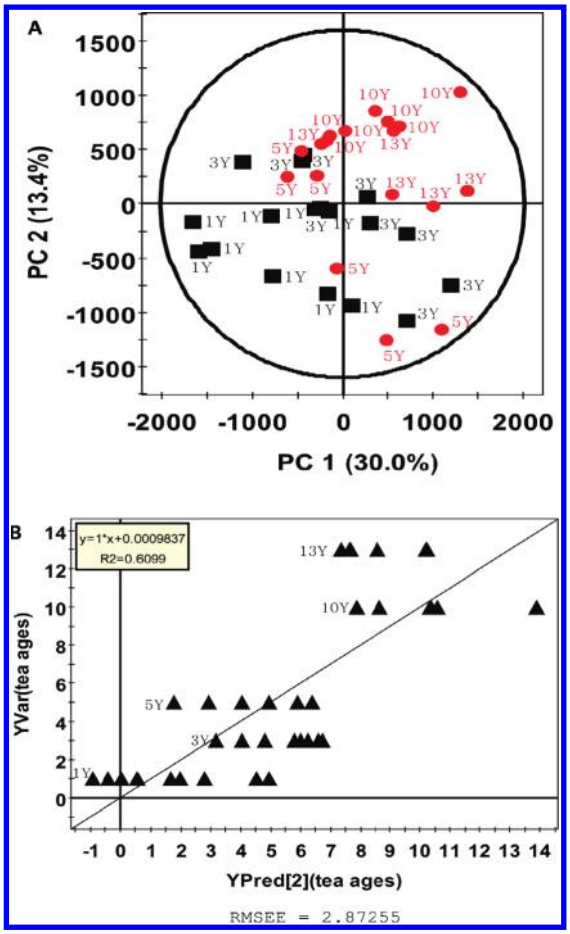

Figure 5. (**A**) PLS score plot of ripened pu-erh teas (red circles, ≥5 years old; black squares, ≤4 years old); (**B**) PLS regression model for prediction of postfermentation year of ripened pu-erh tea.

showed higher bioactivities than ripened pu-erh teas. Using LC-MS and multivariate analyses, we also obtained a broad overview of the composition changes that occur in raw pu-erh teas during postfermentation. Some compounds such as epigallocatechin, epicatechin-3-gallate, strictinin, caffeoylauinic acid, and epigallocatechin-3-gallate significantly decreased with increasing postfermentation year. These compounds were identified as free radical scavengers in previous studies (*47*, *48*). Thus, these compounds can explain why naturally long-term fermented pu-erh teas showed a low antioxidant activity. A postfermentation-predictive model was established through PLS analysis based on metabolites of raw pu-erh teas. As a result of PLS regression analysis, various commercially available raw pu-erh teas in the Republic of Korea showed a particular pattern of composition change in a postfermentation year-dependent manner. Furthermore, we demonstrated that metabolomics is useful for composition pattern analysis and can also help to highlight correlations between the bioactivity of pu-erh teas and their compositional changes.

Supporting Information Available: Figures S1 and S2 and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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