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Comparison of volatile profiles and bioactive components of sun-dried Pu-erh tea leaves from ancient tea plants on Bulang Mountain measured by GC-MS and HPLC#

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Abstract: To explore the volatile profiles and the contents of ten bioactive components (polyphenols and caffeine) of sun-dried Pu-erh tea leaves from ancient tea plants on Bulang Mountain, 17 samples of three tea varieties were analyzed by headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) and high-performance liquid chromatography (HPLC). A total of 75 volatile components were tentatively identified. Laomaner (LME), Laobanzhang (LBZ), and other teas on Bulang Mountain (BL) contained 70, 53, and 71 volatile compounds, respectively. Among the volatile compounds, alcohols (30.2%–45.8%), hydrocarbons (13.7%–17.5%), and ketones (12.4%–23.4%) were qualitatively the most dominant volatile compounds in the different tea varieties. The average content of polyphenol was highest in LME (102.1 mg/g), followed by BL (98.7 mg/g) and LBZ (88.0 mg/g), while caffeine showed the opposite trend, 27.3 mg/g in LME, 33.5 mg/g in BL, and 38.1 mg/g in LBZ. Principal component analysis applied to both the volatile compounds and ten bioactive components showed a poor separation of samples according to varieties, while partial least squares-discriminant analysis (PLS-DA) showed satisfactory discrimination. Thirty-four volatile components and five bioactive compounds were selected as major discriminators (variable importance in projection (VIP) >1) among the tea varieties. These results suggest that chromatographic data combined with multivariate analysis could provide a useful technique to characterize and distinguish the sun-dried Pu-erh tea leaves from ancient tea varieties on Bulang Mountain.

Key words: Sun-dried Pu-erh tea; Ancient tea plant; Bulang Mountain; Volatile compound; Bioactive component
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1 Introduction

Bulang Mountain is located in Menghai County, Xishuangbanna Dai Autonomous Prefecture, Yunnan Province, China, near the Chinese–Burmese border. Bulang Mountain, with an area of 1016.34 km^2, is a famous Pu-erh tea-producing area and is one of the best preserved areas of ancient tea plants. There are 600 ha of gardens containing the Laomaner (LME), Laobanzhang (LBZ), and other ancient tea plants (BL) of the area. The tea plants are distributed mainly at altitudes of 1300–1900 m (Tao et al., 2013). The teas in Bulang Mountain are characterized by their unique aroma, similar to the sweet smell of malt sugar. They are yellow or green and have an immediate sweet taste followed by mellow and bitter tastes. They are very

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astringent (Xia et al., 2012). The plant stems are strong and bear numerous hairs. As a valuable resource, the ancient teas in Bulang Mountain have been consistently pursued by tea traders and Pu-erh tea enthusiasts worldwide.

Food flavor mainly includes taste and aroma. Non-volatile compounds usually contribute to taste, while volatile components contribute to aroma (Lin et al., 2012; Du et al., 2014). The volatile components of sun-dried Pu-erh tea leaves from ancient tea plants are similar to those of common Pu-erh green or Pu-erh raw tea leaves and include mainly alcohols, hydrocarbons, aldehydes, ketones, and esters (Lv et al., 2015). However, the relative volatile composition, concentrations, and perception thresholds of individual components give sun-dried Pu-erh tea leaves of these ancient tea plants their distinctive aroma. The analysis of the volatile components of Pu-erh green teas has been used for various purposes including distinguishing between teas from different mountains (Wu et al., 2016) and differentiating between varieties and quality grades (Lv et al., 2015). The non-volatile components of the tea leaves include polyphenols, alkaloids, polysaccharides, free amino acids, and proteins. Among these, polyphenol and alkaloid bioactive components have been well studied, including gallic acid, (+)-catechin, (−)-gallocatechin, (−)-catechin gallate, (−)-epigallocatechin, (−)-epicatechin gallate, (−)-epicatechin, (−)-epigallocatechin gallate, caffeine, and (−)-gallocatechin gallate (Fan et al., 2017). Up to date, scientific information on volatile constituents, polyphenols, and alkaloids of different varieties of Pu-erh tea from ancient tea plants on Bulang Mountain is rather scarce (Cai et al., 2014). Therefore, an in-depth study is necessary to explore their volatile profiles and bioactive component characteristics.

The aim of this work was to chemically characterize the volatile components and ten bioactive components of Pu-erh tea leaves from ancient tea plants on Bulang Mountain using headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) and high-performance liquid chromatography (HPLC). Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were used to investigate the relationships between the samples. To our knowledge, no similar publications are available in the literature.

2 Materials and methods

2.1 Samples and chemicals

Seventeen sun-dried Pu-erh tea samples belong to three tea varieties, including five LME, six LBZ, and six BL samples (Table 1), which were identified by the Pu'er Institute of Pu-erh tea (Pu'er, Yunnan, China). Those samples were harvested in 2017 and kept in a dry environment. The samples were ground to pass through a 30–60 mesh and sealed before use.

Sodium chloride (99.5%, chromatographic grade, the same below), linalool (98.0%), α-terpineol (98.0%), geraniol (99.0%), nerol (99.0%), α-ionone (92.0%), β-ionone (97.0%), (*E*)-nerolidol (≥85.0%), methyl salicylate (98.0%), and phytol (95.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A homologous series of *n*-alkanes solutions (C_8–C_{32}) was purchased from AccuStandard (USA) and used to calculate the retention index (RI) of each compound. Gallic acid, (+)-catechin, (−)-gallocatechin, (−)-catechin gallate, (−)-epigallocatechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate, (−)-gallocatechin gallate, and caffeine were purchased from the Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of all these standards was above 95%. Acetonitrile, methanol, and phosphoric acid (HPLC grade) were purchased from Macklin (Shanghai, China). Ultra-pure water was obtained from a Milli-Q purification system (Millipore, Bedford, USA).

2.2 HS-SPME of samples

SPME is an equilibrium technique. Many experimental parameters affect this equilibrium. To ensure good-quality SPME extraction, optimization was needed (He et al., 2016). In this study, the effects of major factors, such as extraction fiber, extraction

Table 1 Information about sun-dried Pu-erh tea leaves from ancient tea plants

Sample ID	Name	Region	Harvest time
LME1–LME5	Laomaner tea	Bulang Mountain	Apr. 2017
LBZ1–LBZ6	Laobanzhang tea	Bulang Mountain	Apr. 2017
BL1–BL6	Other sun-dried Pu-erh tea of ancient tea plants	Bulang Mountain	Apr. 2017

temperature, extraction time, and the amount of water, on extraction efficiency were investigated comprehensively before the experiment. Through comparison of the total peak area, the optimal extraction process was as follows: 4.0 g of tea sample was transferred into a 100-mL SPME headspace vial, following the addition of 4.0 g of sodium chloride and 16 mL of water, and then the vial was sealed using a polytetrafluoroethylene (PTFA) septum. The vial was then placed on a magnetic stirring apparatus at 300 r/min and equilibrated at 60 °C for 5 min. A 65-μm polydimethylsiloxane (PDMS)/divinylbenzene (DVB) fiber (Supelco Inc., Bellefonte, USA) was exposed to the headspace over the sample for 60 min. After extraction, the fiber was exposed in the GC-MS injector at 250 °C for 4 min and then the headspace was immediately analyzed by GC-MS.

2.3 Volatile component analysis by GC-MS

Volatile components were analyzed according to the method described by Lv et al. (2015) with some modifications. After the extraction procedure, the SPME device was removed from the SPME vial and sent for GC analysis. An Agilent 7890A GC directly interfaced with an Agilent 5975C mass selective detector quadrupole MS instrument (Agilent Technologies, Palo Alto, CA, USA) was used. An HP-5MS capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness) from Agilent Technologies was used. Experimental conditions of the GC procedure were as follows: the initial temperature was 50 °C, increased to 150 °C at a rate of 2 °C/min, and then ramped at 10 °C/min to 250 °C, maintained for 5 min. The total GC runtime was 65 min. The temperature of the sample injection port was 250 °C and the injection mode was splitless. The carrier gas (He, percentage purity >99.9%) was in constant-flow mode, with a flow velocity of 0.8 mL/min. The MS was operated in an electron-impact mode at 70 eV. The temperatures of the interface (280 °C), ion source (230 °C) and quadrupole (150 °C) were all at default settings for GC-MS. The acquisition mode was full-scan (from 35 to 350 atomic mass units (aum)), and the solvent delay time was 3.0 min. The volatile compounds were identified as previously described (Wang et al., 2017), and peaks were deconvoluted using AMDIS software (NIST, USA). Identification was based on mass matching with the NIST 11.L and WILEY 07 mass spectra databases and/or comparison against standards where available. The RIs of target components were obtained using *n*-alkanes (C_8–C_{32}) under the same GC-MS procedure. The calculated RI was compared with the reported RI. The percentage amount of target compound was calculated by the percentage of peak area of target compound relative to the total peak area of all compounds.

2.4 Polyphenols and alkaloids analyzed by HPLC

The HPLC analysis was based on a slightly modified method described by Wang et al. (2016a). Each sample (about 0.2 g) was placed into a 10-mL centrifuge tube, and 4 mL of preheated 70% methanol was added. The sample was extracted for 10 min in a 70 °C water bath, and then the extract was centrifuged at 3500 r/min for 10 min. The supernatant was transferred into a 50-mL volumetric flask, and the sample was extracted again using the above process. Finally, the extracts were combined and made up to 50 mL with ultrapure water. The solution was filtered through a 0.45-μm membrane prior to injection into the HPLC system. The polyphenols and alkaloids were analyzed using an Agilent 1260 series HPLC system, including a G1311C quaternionic pump, a G1329B autosampler, a G1316A thermostatted column compartment, and a G1315D DAD detector (Agilent Technologies) to acquire quantitative chromatograms. A Phenomenex Synergi Hydro-RP 80A-C18 column (4 μm, 4.6 mm× 250 mm) was used. Acetonitrile (solvent A) and 0.05% phosphoric acid aqueous solution (solvent B) were used as the mobile phase at a flow rate of 1 mL/min. The gradient program was set as follows: 0–50 min, linear gradient 2%–25% A; 50–55 min, linear gradient 25%–2% A. The column temperature was 30 °C and the injection volume was 10 μL. Nine polyphenol compounds and caffeine were simultaneously detected at 210 nm. Each target compound was identified by comparing its retention time with that of a standard substance. All samples were tested in triplicate.

2.5 Statistical analysis

PCA and PLS-DA were performed using XLSTAT v. 2016 (Addinsoft, New York, NY, USA) and SIMCA-P 12 software (Umetrics, Umea, Sweden), respectively, to investigate the relationships among the 17 tea samples. Duncan's multiple range tests were used for the significance of differences ($P \leq 0.05$) among the tea samples using SPSS statistics 17.0 software (SPSS Inc., Chicago, IL, USA).

3 Results and discussion

3.1 Recovery and repeatability of the HS-SPME method

We investigated the intra-day (five replicates within one day) and inter-day repeatability (one replicate on each of five consecutive days) of nine major compounds selected, to examine the repeatability and accuracy of the established HS-SPME-GC-MS method. To investigate recovery rates, two levels (2 and 8 μg) of mixed standard were added before and after HS-SPME. The repeatability and recovery rates of the HS-SPME method are presented in Table 2. The HS-SPME method showed satisfactory repeatability. The intra-day relative standard deviation (RSD) ranged from 3.36% to 6.71%. As an example, detailed total ion chromatograms and peak areas of the nine compounds are shown in Fig. S1 and Table S1, respectively. The inter-day RSD ranged from 4.19% to 7.04%. Satisfactory accuracy was achieved, with recoveries of low-level compounds ranging from 72.7% to 93.6% and recoveries of high-level compounds ranging from 70.6% to 106.4%. These results indicate that this technique is suitable for the analysis of volatile compounds of Pu-erh tea leaves.

3.2 Volatile component analysis

To investigate the aroma characteristics, HS-SPME-GC-MS was used for detection and identification of the volatile components of the tested tea samples. The representative total ion chromatograms of Pu-erh tea leaves from the three varieties of ancient trees are shown in Fig. 1. The relative contents of identified compounds are shown in Table S2. The average contents of identified compounds of the same kind of tea samples as well as their standard deviation are summarized in Table 3.

Among 75 volatile components tentatively identified according to their mass spectra and RI, 70, 53, and 71 volatile compounds were identified in LME, LBZ, and BL, respectively. These compounds included

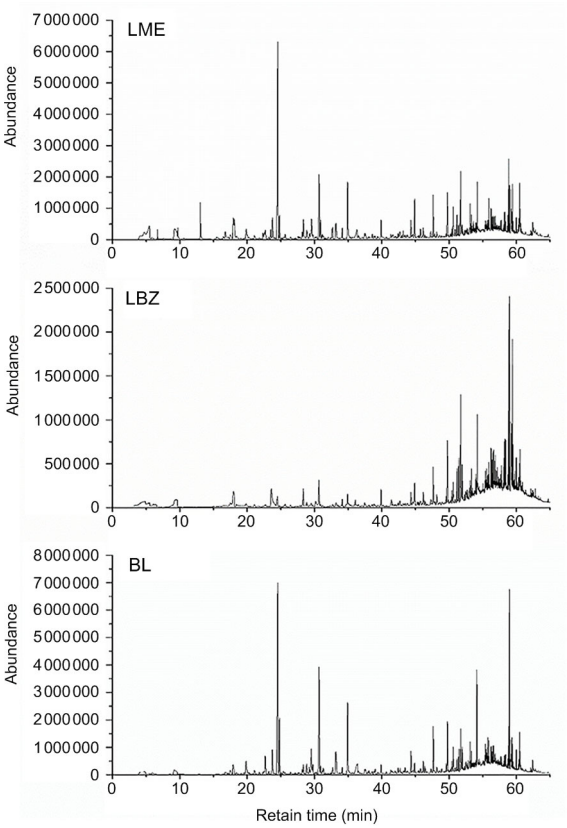

Fig. 1 Representative total ion chromatograms of three tea varieties

Table 2 Repeatability and recovery rates using the HS-SPME method

Compound	RI	Intra-day repeatability (%) (n=5)	Inter-day repeatability (%) (n=5)	Recovery (%)	
				Spiked 2 μg (n=3)	Spiked 8 μg (n=3)
Linalool	1101	4.81	5.63	85.34	88.79
α-Terpineol	1192	4.07	4.19	79.37	77.15
Methyl salicylate	1195	6.47	6.68	93.62	89.54
Nerol	1230	3.36	5.35	87.38	90.12
Geraniol	1256	5.57	6.18	92.16	106.39
α-Ionone	1429	4.12	5.31	90.29	88.74
β-Ionone	1487	4.75	4.29	79.36	83.55
(*E*)-Nerolidol	1569	6.71	7.04	72.69	70.57
Phytol	2116	6.04	6.84	88.42	84.69

RI: retention index

Table 3 Volatile compounds identified in tea leaf samples

Compound	ID	RI[1]	RI[2]	Average relative percentage content (%)[3]			Odor note[4]	Reference
				LME	LBZ	BL		
1-Octen-3-ol	MS, RI	982	986	0.782±0.371[a]	0.476±0.532[a]	0.880±0.849[a]	Mushroom	1, 2
β-Myrcene	MS, RI	991	992	0.658±0.084[a]	0.315±0.489[a]	0.299±0.329[a]	Sweet, floral	2
D-Limonene	MS, RI	1028	1030	1.807±0.495[a]	0.630±0.525[b]	1.052±0.568[b]	Lemon-like	3, 4
(*Z*)-Ocimene	MS, RI	1037	1039	0.526±0.089[a]	0.510±0.399[a]	0.329±0.267[a]	Herbaceous	5
(*Z*)-2-Octen-1-ol	MS, RI	1062	1066	0.455±0.324[a]	0.000±0.000[a]	0.296±0.508[a]	Fatty	5
1-Octanol	MS, RI	1064	1068	0.198±0.275[ab]	0.000±0.000[b]	0.584±0.557[ac]	Orange, rose	5
Linaloloxide I	MS, RI	1068	1062	0.699±0.962[a]	0.256±0.292[a]	0.954±1.082[a]	Floral, woody	1, 4
Linaloloxide II	MS, RI	1072	1074	1.660±1.435[ab]	0.766±0.945[b]	2.561±1.351[ac]	Floral, woody	1, 4
Linalool	MS, RI, STD	1101	1106	16.749±3.678[a]	12.500±4.511[a]	17.014±4.681[a]	Floral, woody, fruity	1, 4
Hotrienol	MS, RI	1106	1109	2.041±0.377[a]	0.510±0.576[b]	1.937±1.277[a]	Musty	2, 6
Phenylethyl alcohol	MS, RI	1118	1114	0.071±0.158[a]	0.000±0.000[a]	0.091±0.143[a]	Honey, spice, rose, lilac	1, 4
L-Menthone	MS, RI	1160	1166	0.592±0.223[a]	0.000±0.000[b]	0.460±0.275[a]	Mint	5
Benzyl acetate	MS, RI	1165	1165	1.488±0.588[a]	0.617±0.833[b]	1.638±0.536[a]	Jasmine-like	7
Linaloloxide III	MS, RI	1169	1171	2.857±0.447[a]	2.280±1.746[a]	3.329±1.106[a]	Floral, woody	2, 8
(−)-4-Terpineol	MS, RI	1174	1175	0.572±0.433[a]	0.000±0.000[b]	0.325±0.310[ab]	Fruity, sweet	3
α-Terpineol	MS, RI, STD	1192	1190	7.018±1.686[a]	5.903±1.414[a]	6.430±1.140[a]	Lilac-like	1, 3
Methyl salicylate	MS, RI, STD	1195	1193	0.806±0.404[a]	0.217±0.531[a]	0.895±0.696[a]	Mint, wintergreen-like	4, 7
Estragole	MS, RI	1199	1196	0.469±0.291[a]	0.000±0.000[b]	0.305±0.197[a]	Anise-like	1
Safranal	MS, RI	1207	1205	0.273±0.261[a]	0.000±0.000[b]	0.257±0.216[a]	Herbaceous, sweet	3, 4
β-Cyclocitral	MS, RI	1213	1216	0.268±0.406[a]	0.452±0.518[a]	0.279±0.488[a]	Floral, fruity	1, 4
Nerol	MS, RI, STD	1230	1232	2.068±0.411[a]	0.303±0.340[b]	1.825±1.009[a]	Rose-like, sweet	1, 3, 4
5-Methyl-isothiazole	MS, RI	1247		0.456±0.481[a]	0.451±0.659[a]	0.822±0.311[a]	Unpleasant odor	5
Geraniol	MS, RI, STD	1256	1258	4.879±1.012[a]	2.973±1.199[a]	4.249±2.247[a]	Rose, geranium	1, 7
Nonanoic acid	MS, RI	1278	1272	1.329±0.373[a]	0.919±0.566[a]	1.359±0.685[a]	Fatty	5
2-Methyl-naphthalene	MS, RI	1298	1299	0.477±0.342[a]	0.359±0.565[a]	0.083±0.204[a]	Herbal-like	2
Indole	MS, RI	1303	1300	0.315±0.373[a]	0.805±1.033[a]	0.489±0.506[a]	Unpleasant flavor	1, 3
1-Methyl-naphthalene	MS, RI	1312	1315	0.162±0.268[a]	0.000±0.000[a]	0.343±0.386[a]	Unpleasant flavor	3
Geranic acid	MS, RI	1354	1355	0.180±0.246[a]	0.000±0.000[a]	0.083±0.203[a]	Floral, sweet	5

To be continued

Table 3

Compound	ID	RI[1]	RI[2]	Average relative percentage content (%)[3]			Odor note[4]	Reference
				LME	LBZ	BL		
3-Methyl-tridecane	MS, RI	1372	1369	0.190±0.268[a]	0.259±0.315[a]	0.000±0.000[a]		
3,5-Dimethyldodecane	MS, RI	1373		0.216±0.484[a]	0.612±0.650[a]	0.846±1.578[a]		
(*Z*)-3-Hexenyl hexanoate	MS, RI	1381	1381	0.306±0.183[a]	0.566±0.558[a]	0.339±0.314[a]	Fruity, green	3, 6
β-Damascenone	MS, RI	1385	1388	0.202±0.312[a]	0.000±0.000[a]	0.065±0.160[a]	Apple, rose, honey	6, 9
Hexyl hexanoate	MS, RI	1386	1385	0.000±0.000[a]	0.000±0.000[a]	0.143±0.222[a]	Herbaceous-like	5
(*E*)-2-Hexenyl hexanoate	MS, RI	1392	1391	0.000±0.000[a]	0.000±0.000[a]	0.072±0.115[a]	Fruity, green	5
(*Z*)-Jasmone	MS, RI	1397	1396	0.000±0.000[a]	1.744±2.126[b]	0.000±0.000[a]	Fruity, jasmine	10
Cyclododecanone	MS, RI	1407		0.000±0.000[a]	0.000±0.000[a]	0.312±0.353[b]		
β-Caryophyllene	MS, RI	1418	1417	0.475±0.318[a]	0.724±0.679[a]	0.558±0.327[a]	Clove-like	3, 4
α-Ionone	MS, RI, STD	1429	1427	0.739±0.189[a]	2.056±0.313[ab]	3.326±3.035[b]	Wood, violet	1, 4
Geranyl acetone	MS, RI	1454	1457	2.065±1.174[a]	4.074±1.810[b]	3.288±1.160[ab]	Magnolia, green, fruity	1, 4
(*Z*)-β-Farnesene	MS, RI	1458	1457	0.158±0.220[a]	0.145±0.355[a]	0.330±0.590[a]	Floral, green	5
Dehydro-β-ionone	MS, RI	1484	1485	1.460±2.359[a]	0.658±0.561[a]	0.241±0.209[a]	Woody, fruity	5
β-Ionone	MS, RI, STD	1487	1485	2.628±1.265[a]	7.373±1.004[b]	1.709±1.880[a]	Woody, fruity	1, 9
α-Farnesene	MS, RI	1507	1510	0.489±0.365[a]	0.963±0.897[a]	0.690±0.404[a]	Fruity	3, 4
Butylated hydroxytoluene	MS, RI	1516	1518	0.695±0.673[a]	1.791±2.401[a]	1.229±0.767[a]	Faint musty	1
α-Amorphene	MS, RI	1517	1516	0.038±0.085[a]	0.000±0.000[a]	0.048±0.119[a]		
5-Pentylresorcinol	MS, RI	1531		2.667±1.786[ab]	1.133±2.776[a]	4.519±2.555[b]		
Dihydroactinidiolide	MS, RI	1539	1535	1.213±0.618[a]	3.180±0.988[b]	1.306±0.360[a]	Coumarin, musky	3, 8
α-Calacorene	MS, RI	1543	1542	0.502±0.403[a]	0.382±0.603[a]	0.361±0.572[a]		
Cyclopentadecane	MS, RI	1549		0.279±0.385[a]	0.242±0.594[a]	0.103±0.253[a]		
4-Methyl-pentadecane	MS, RI	1559		0.113±0.253[a]	0.283±0.467[a]	0.741±0.197[b]		
(*E*)-Nerolidol	MS, RI, STD	1569	1562	1.519±1.226[a]	1.591±1.824[a]	2.221±1.364[a]	Rose-like	3, 8
3-Methyl-pentadecane	MS, RI	1571	1570	0.293±0.656[a]	1.348±1.151[a]	0.402±0.632[a]		
Farnesyl acetaldehyde	MS, RI	1580		0.318±0.712[a]	0.000±0.000[a]	0.598±0.964[a]		
Diethyl phthalate	MS, RI	1602	1603	0.040±0.089[a]	0.000±0.000[a]	0.000±0.000[a]		
Cedrol	MS, RI	1605	1607	0.208±0.466[a]	0.000±0.000[a]	0.639±0.992[a]	Woody	1
Benzophenone	MS, RI	1621	1625	0.616±0.858[a]	0.248±0.385[a]	0.079±0.195[a]	Rose-like	2

To be continued

Table 3

Compound	ID	RI[1]	RI[2]	Average relative percentage content (%)[3]			Odor note[4]	Reference
				LME	LBZ	BL		
τ-Muurolol	MS, RI	1643	1648	0.000±0.000[a]	0.000±0.000[a]	0.255±0.625[a]		
α-Cadinol	MS, RI	1658	1663	0.585±0.448[a]	0.000±0.000[b]	0.216±0.529[ab]	Spicy	5
2,2',5,5'-Tetramethyl-1,1'-biphenyl	MS, RI	1665	1668	2.900±1.532[a]	3.727±1.345[a]	2.006±1.823[a]		
4'-Benzylacetophenone	MS, RI	1697		1.549±0.717[a]	1.911±1.078[a]	0.986±0.838[a]		
2,6,10,14-Tetramethyl-pentadecane	MS, RI	1705	1703	2.443±1.545[a]	2.569±1.327[a]	1.573±1.396[a]		
3-Methyl-heptadecane	MS, RI	1771	1771	0.696±0.559[a]	0.161±0.396[a]	0.176±0.277[a]		
2,6,10,14-Tetramethyl-hexadecane	MS, RI	1808	1810	1.973±0.693[a]	1.553±1.366[a]	1.132±0.629[a]		
3-Octadecyne	MS, RI	1833		2.158±1.482[a]	2.734±3.472[a]	2.595±2.478[a]		
6,10,14-Trimethyl-2-pentadecanone	MS, RI	1839	1838	2.515±1.094[a]	5.295±2.900[a]	3.307±1.684[a]	Floral, jasmine	1
Caffeine	MS, RI	1842	1842	2.349±1.564[ab]	4.832±1.736[a]	2.117±2.371[b]		
Diisobutyl phthalate	MS, RI	1869	1871	1.363±1.326[a]	1.145±0.974[a]	0.319±0.501[a]		
Methyl palmitate	MS, RI	1927	1928	0.901±0.600[a]	1.066±0.830[a]	0.704±0.372[a]	Ester-like	5
Isophytol	MS, RI	1947	1948	1.049±1.442[a]	0.000±0.000[b]	0.359±0.166[ab]	Floral, herbal, green	1
n-Hexadecanoic acid	MS, RI	1965	1964	1.997±0.987[a]	1.661±0.969[a]	1.326±0.890[a]		
Dibutyl phthalate	MS, RI	1969	1965	0.790±0.280[a]	2.165±0.960[a]	1.326±1.696[a]		
Methyl linoleate	MS, RI	2095	2092	0.091±0.127[a]	0.245±0.267[a]	0.147±0.132[a]		
Methyl linolenate	MS, RI	2098	2101	0.030±0.068[a]	0.000±0.000[a]	0.156±0.116[b]		
Phytol	MS, RI, STD	2116	2119	1.449±0.529[a]	2.614±3.154[a]	1.585±0.993[a]	Floral, balsam	1, 4
Oleic acid	MS, RI	2143	2140	0.109±0.244[a]	0.000±0.000[a]	0.000±0.000[a]	Fresh cut grass like	5
Alcohols				44.866±6.566[a]	30.177±14.142[b]	45.757±11.229[a]		
Hydrocarbons				16.560±2.444[a]	17.523±6.163[a]	13.672±4.707[a]		
Ketones				12.369±1.426[a]	23.363±6.380[b]	13.777±3.705[a]		
Esters				7.032±1.351[a]	9.204±1.768[b]	7.048±1.302[a]		
Acids				3.616±1.367[a]	2.580±0.976[a]	2.768±0.373[a]		
Nitrogen compounds				3.121±2.045[a]	6.089±2.766[a]	3.429±2.377[a]		
Aldehydes				0.860±0.665[a]	0.452±0.518[a]	1.135±1.364[a]		
Phenols				3.362±2.419[a]	2.924±3.172[a]	5.748±2.934[a]		
Methoxy-phenolic compounds				0.469±0.291[a]	0.000±0.000[b]	0.305±0.197[a]		

[1] The calculated retention index (RI) on HP-5MS. [2] The RI values found in the literature (on HP-5MS), which were provided by the NIST Chemistry WebBook (http://webbook.nist.gov/chemistry). [3] The content of volatile compounds is represented as mean value±standard deviation (SD), and different letters indicate significant differences (P<0.05, ANOVA, Duncan's multiple range test). [4] Odor descriptions of odor-active compounds identified in the literature. LME: Laomaner; LBZ: Laobanzhang; BL: other ancient tea plants on Bulang Mountain; ID: identification; MS: mass spectrometry; STD: standard. References: 1, Du et al., 2014; 2, Xu et al., 2015; 3, He et al., 2016; 4, Wang et al., 2017; 5, Burdock, 2010; 6, Zhu et al., 2008; 7, Wang et al., 2008; 8, Lv et al., 2012; 9, Schuh and Schieberle, 2006; 10, Kumazawa and Masuda, 2002

alcohols, hydrocarbons, ketones, esters, acids, nitrogen compounds, aldehydes, phenols, and methoxyphenolic compounds. Nineteen alcohols were identified in all tea samples. The relative content of total alcohols ranged from 30.2% to 45.8%, the lowest in LBZ and the highest in BL. Linalool, α-terpineol, geraniol, linalool oxide, hotrienol, phytol, (*E*)-nerolidol, and nerol were the most abundant alcohols. These principal volatile compounds found in green teas contribute greatly to the flavor of the tea. Linalool has been identified as one of the key aroma compounds in Pu-erh raw tea, green tea, black tea, and tea infusions, and contributes citrus and floral aroma notes. α-Terpineol provides "lilac-like" and "sweet" aroma notes. Nerol, geraniol, and (*E*)-nerolidol provide "rose-like" aroma notes. Geraniol is an extremely potent volatile with a threshold of 10 μg/kg, and is present at trace levels. Geraniol has been identified as the key component of black tea, greatly contributing to the tea flavor (Shi et al., 2014). The results were consistent with those of Dianhong tea (Wang et al., 2017). (*E*)-Nerolidol is a sesquiterpene, which exists in many teas as a main aroma compound, especially oolong tea, at relatively high content, and can be regarded as one of the key aroma components for the high-quality flavor of oolong tea (Wang et al., 2016b). Linalool oxides have a "floral" and "woody" odor (Alasalvar et al., 2012). Hotrienol adds a "musty" aroma note and is one of the major compounds in oolong tea (Pripdeevech and Machan, 2011). Phytol has a "floral" and "balsam" odor and is a volatile compound common in many plants (Byju et al., 2013).

Twenty hydrocarbon compounds, including saturated and unsaturated hydrocarbons, were identified in tea samples. Saturated hydrocarbons usually have low odor notes, whereas unsaturated hydrocarbons have high odor notes and contribute more to the overall flavor of tea (Wang et al., 2016b). Unsaturated hydrocarbons, such as D-limonene, are regarded as the prominent components of green tea volatile and are described as having "lemon-like" aroma notes (He et al., 2016). Moreover, another important aroma compound (*Z*)-ocimene offers a strong sense of "warm herbaceous" aroma. β-Myrcene has "sweet" and "floral" aroma notes. β-Caryophyllene has a "woody-spicy" and "clove-like" aroma. α-Farnesene provides "fruity" aroma and has been reported as the main flavor component of oolong tea (Lin et al., 2013).

Eleven ketones were detected in all tea samples, with β-ionone, 6,10,14-trimethyl-2-pentadecanone, geranyl acetone, dehydro-β-ionone, and α-ionone predominant. β-Ionone has pleasant violet fragrance and complex woody and fruity aroma notes. It has been reported as the dominant aroma compound in various teas (Gulati and Ravindranath, 1996). 6,10,14-Trimethyl-2-pentadecanone was described as having a "floral" and "jasmine" odor in Kangra tea and Pu-erh tea, and was found in all tea leaf samples. Geranyl acetone plays an important role in the aroma of Pu-erh and Dianhong teas (Xu et al., 2015; Wang et al., 2017). Dehydro-β-ionone has "woody" and "fruity" aroma notes. α-Ionone was detected in all tea samples and has been regarded as an important aroma compound in infusions of Dianhong tea (Wang et al., 2017).

Twelve esters were identified in tea samples, of which dihydroactinidiolide, benzyl acetate, methyl salicylate, (*Z*)-3-hexenyl hexanoate, diisobutyl phthalate, methyl palmitate, and dibutyl phthalate were predominant. Dihydroactinidiolide in Pu-erh tea was described as "smelling like coumarin" and "musky", and from *Eleocharis coloradoensis* is known to be a potent growth inhibitor (Stevens and Merrill, 1981). Benzyl acetate provides "jasmine-like" aroma notes. Methyl salicylate has been reported in various teas and is recognized as an important compound for the formation of overall tea aroma (Lv et al., 2014). (*Z*)-3-Hexenyl hexanoate was previously reported in oolong tea (Kawakami et al., 1995). Diisobutyl phthalate and dibutyl phthalate were reported in Pu-erh tea (Du et al., 2016). Methyl palmitate is produced from the esterification reaction of hexadecanoic acid and was detected in rooibos tea (Kawakami et al., 1993).

Acids, namely nonanoic acid, *n*-hexadecanoic acid, geranic acid, and oleic acid, were detected in all tea samples. Branched-chain acids are usually generated from the degradation of branched-chain amino acids, whereas straight-chain acids are generated from the degradation of fatty acids. Nonanoic acid provides "fatty" aroma notes, and was once identified in Pu-erh tea (He et al., 2016), whereas oleic acid provides an aroma like fresh cut grass. Geranic acid gives "floral" and "sweet" aroma notes. The nitrogen compounds in the tea samples were 5-methylisothiazole, indole, and caffeine. 5-Methylisothiazole and indole have an unpleasant odor, and caffeine affects mainly the taste

characteristics of the tea. Aldehydes, including safranal, β-cyclocitral, and farnesyl acetaldehyde, were identified in the tea samples. Safranal provides a "herbaceous" and "sweet" aroma note and has been reported as a character aroma in infusions of Zijuan tea (He et al., 2016). β-Cyclocitral has "floral" and "fruity" aroma notes, which is identified as an major odorant contributing to the aroma of Zijuan and Pu-erh green teas (He et al., 2016). Farnesyl acetaldehyde was first reported in Pu-erh green tea. Aldehydes usually have lower odor thresholds than their homologous alcohols. Two phenols, namely 5-pentylresorcinol and butylated hydroxytoluene, were detected in all samples. These compounds were previously identified in various tea samples (Kumazawa and Masuda, 2002; Yao et al., 2005). Estragole is a methoxyphenolic compound found in tea samples and was previously reported in ripened Pu-erh tea leaves (Du et al., 2014).

3.3 Polyphenols and alkaloids

Comparisons of the polyphenols and alkaloids identified in the tea leaf samples are reported in Tables 4 and S3. The total polyphenol content ranged from 84.5 to 111.4 mg/g in LME, from 79.5 to 98.4 mg/g in LBZ, and from 93.2 to 104.8 mg/g in other sun-dried Pu-erh tea leaves (BL) from the ancient tea plants. The average content of caffeine was higher in LBZ teas (range, 28.9–43.6 mg/g; mean, 38.1 mg/g) than in BL (range, 24.8–42.2 mg/g; mean, 33.5 mg/g) and LME (range, 23.6–35.2 mg/g; mean, 27.3 mg/g) teas. The possible reason is differences among the varieties of the ancient tea plants.

3.4 Relationships among sun-dried Pu-erh teas

To identify the relationships among sun-dried Pu-erh teas from ancient tea plants, PCA and PLS-DA were performed using the detection data from GC-MS and HPLC, respectively. The results showed that the first two principal components of the PCA applied to the volatile and bioactive component data accounted for 36.2% and 57.4% of the total variance, respectively. The varieties were poorly separated (Fig. 2), especially for the PCA score plot of the volatile data in which good discrimination was observed only between LME and LBZ teas. The overlaps observed between LME and BL teas, and between LBZ and BL teas suggest that they have some similar chemical characteristics. As for the PCA score plot of the bioactive component data, a trend showing discrimination among samples along PC1 is apparent (score plot, Fig. 2b). LME teas were located on the right hand side of the plot and were well separated from LBZ, and BL teas were located between LME and LBZ teas.

Table 4 Comparisons of the polyphenol and alkaloid content of sun-dried Pu-erh tea leaves of ancient tea plants from Bulang Mountain detected by HPLC

Compound	Polyphenol or alkaloid content (mg/g)					
	LME		LBZ		BL	
	Mean	Range	Mean	Range	Mean	Range
Polyphenols						
GA	8.604[a]	8.074–9.140	7.405[b]	6.735–7.921	8.546[a]	7.218–9.243
GC	3.858[a]	3.151–4.368	2.670[b]	1.863–3.523	2.785[b]	2.305–3.054
EGC	7.221[a]	6.058–9.271	4.088[b]	2.257–5.483	5.930[a]	4.807–7.344
C	5.630[a]	3.864–7.940	7.049[a]	5.401–10.064	5.720[a]	4.494–8.339
EC	16.063[a]	11.467–22.540	16.464[a]	12.546–21.420	16.141[a]	11.843–19.060
EGCG	25.899[a]	22.878–30.573	16.462[b]	12.870–21.231	23.912[a]	20.645–26.749
GCG	5.417[a]	4.410–7.243	4.539[a]	3.842–6.086	4.314[a]	2.269–5.821
ECG	25.775[a]	18.560–30.069	24.325[a]	20.676–29.881	27.998[a]	24.373–31.039
CG	3.642[a]	2.545–5.603	4.978[a]	3.374–7.930	3.377[a]	1.422–5.536
Total content	102.109[a]	84.463–111.424	87.980[b]	79.499–98.393	98.723[a]	93.155–104.757
Alkaloids						
CAF	27.300[a]	23.597–35.210	38.100[b]	28.908–43.566	33.516[ab]	24.831–42.222

GA: gallic acid; GC: (−)-gallocatechin; EGC: (−)-epigallocatechin; C: (+)-catechin; EC: (−)-epicatechin; EGCG: (−)-epigallocatechin gallate; GCG: (−)-gallocatechin gallate; ECG: (−)-epicatechin gallate; CG: (−)-catechin gallate; CAF: caffeine. Different letters indicate significant differences (P<0.05, ANOVA, Duncan's multiple range test)

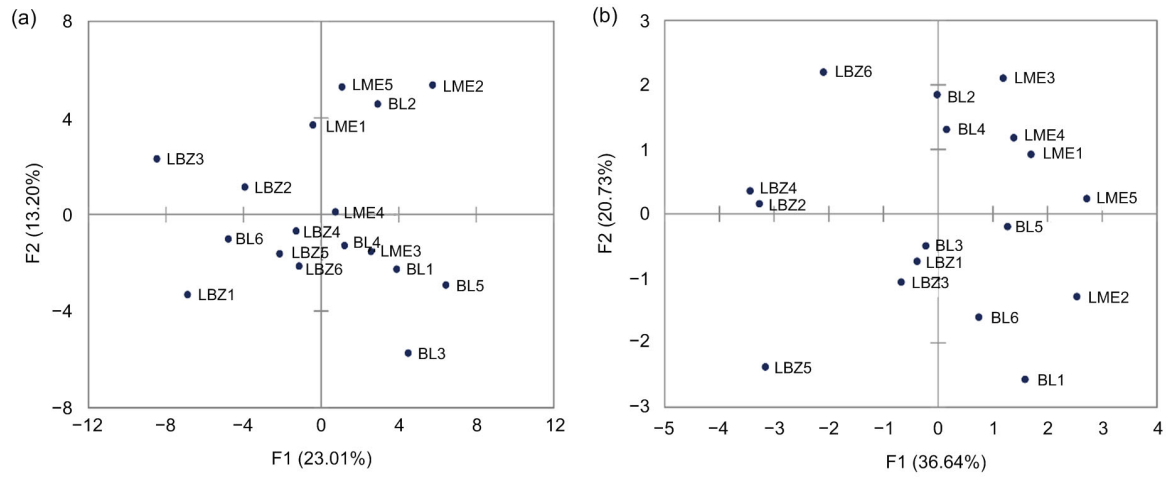

Fig. 2 PCA score plots of the volatile and bioactive component data
(a) PCA score plot of volatile data; (b) PCA score plot of data for ten bioactive compounds

PLS-DA was carried out using 75 volatile and 10 bioactive components. Different teas were well separated in the two scatter plots (Figs. 3a and 3b). Cross-validation results (R^2=0.389 and Q^2=−0.187 using volatile components; R^2=0.225 and Q^2=−0.269 using bioactive components) showed that there was no over-fitting in the models. The 17 tea samples were well distributed and showed the possibility of outliers, groups, similarities, and other patterns in the data. To explain the relationships between variables and tea samples, loading scatter plots were performed (Figs. 3c and 3d). The volatile components, D-limonene (V3), L-menthone (V12), estragole (V18), nerol (V21), cyclododecanone (V36), α-ionone (V38), β-ionone (V42), dihydroactinidiolide (V47), 4-methyl-pentadecane (V50), and methyl linolenate (V73) had high-loading values. Among the bioactive components, (−)-gallocatechin, (−)-epigallocatechin gallate, and (−)-epigallocatechin had higher-loading values. These components contributed most to the discrimination between the varieties of sun-dried Pu-erh tea leaves. To weigh the importance of the effect of each variable on discrimination, variable importance in projection (VIP) plots (Figs. S2a and S2b), which summarize the importance of each variable in explaining X and correlate each variable to Y, were constructed. According to the VIP plots, 34 volatile compounds and 5 bioactive components had VIP values greater than 1.0 (Table S4), which means that these variables were primarily responsible for the discrimination of the three tea cultivar samples. Among them, 4-methyl-pentadecane (VIP value=1.590), β-ionone (1.495), D-limonene (1.425), L-menthone (1.423), methyl linolenate (1.411), and (−)-gallocatechin (1.410) had the largest VIP values, consistent with the analytical results of Duncan's multiple range tests (Tables 3 and 4).

4 Conclusions

This study presents the characterization of aroma profiles by HS-SPME-GC-MS and analysis of the contents of ten bioactive components (polyphenols and alkaloids) by HPLC of sun-dried Pu-erh leaves from ancient tea plants from Bulang Mountain. Chemometric methods, including unsupervised PCA and supervised PLS-DA, were used to distinguish the tea samples according to their varieties. PLS-DA was proven to be satisfactory in distinguishing the different varieties of the tea samples according to their volatile compounds and bioactive components. Major contributors to odor and taste were selected to distinguish the different tea varieties based on VIP values. This study suggested that HS-SPME-GC-MS and HPLC combined with multivariate data analysis are sensitive and ideal methods for characterizing and distinguishing the different varieties of Pu-erh tea from Bulang Mountain. Further investigations on Pu-erh tea leaves from these ancient plants are recommended.

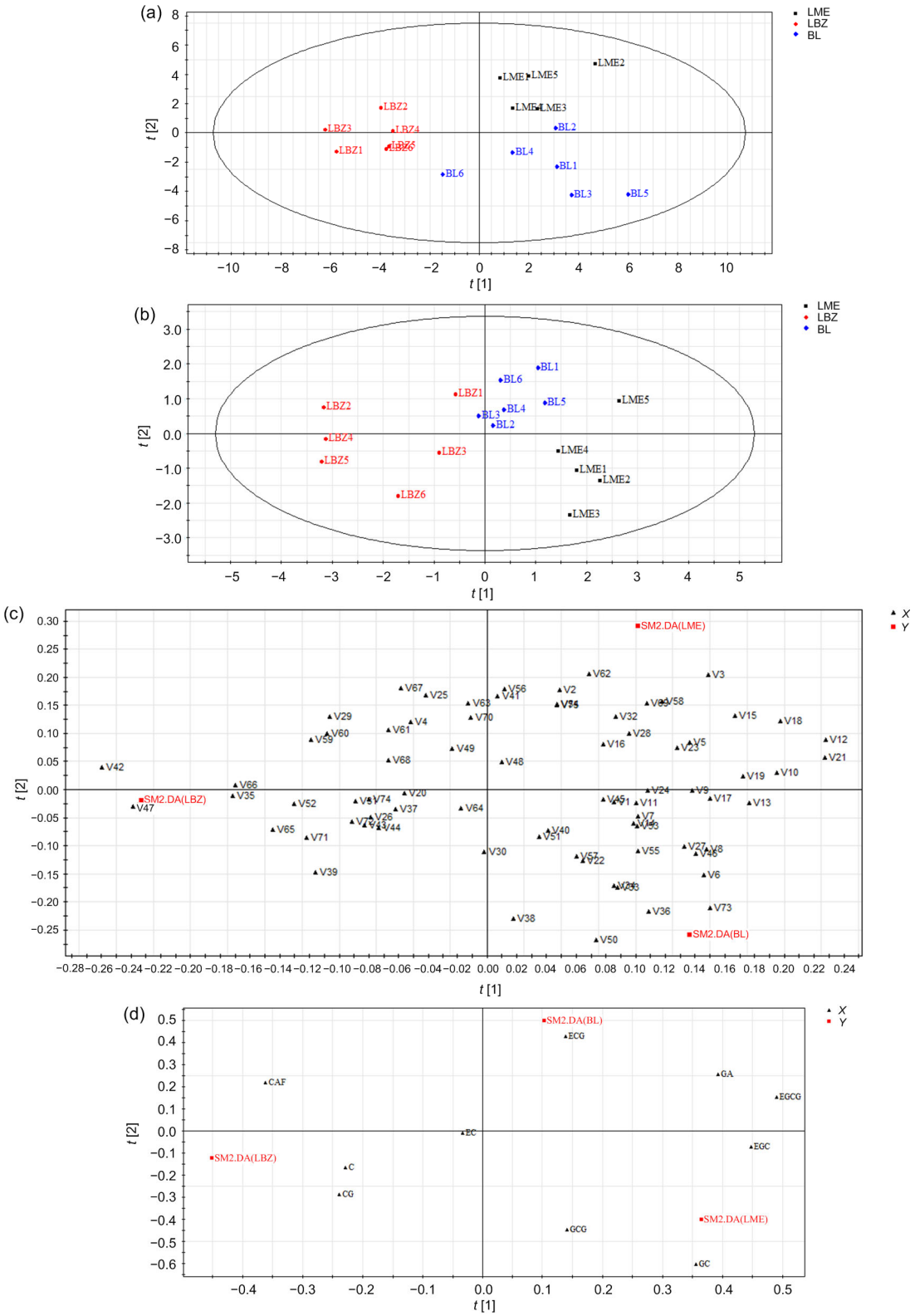

Fig. 3 Plots of PLS-DA scores and loading scatter of volatile and compound data

(a) PLS-DA score plot of volatile data; (b) PLS-DA score plot of bioactive compound data; (c) PLS-DA loading scatter plot of volatile data; (d) PLS-DA loading scatter plot of bioactive compound data. GA: gallic acid; GC: (−)-gallocatechin; EGC: (−)-epigallocatechin; C: (+)-catechin; EC: (−)-epicatechin; EGCG: (−)-epigallocatechin gallate; GCG: (−)-gallocatechin gallate; ECG: (−)-epicatechin gallate; CG: (−)-catechin gallate; CAF: caffeine. V1–V75 are shown in Table S2

Contributors

Rui-juan YANG and Ting-ting ZHENG collected data of the GC-MS. Miao-miao ZHAO and Li MA collected data of the HPLC. Wen-jie ZHANG analyzed the data and wrote the manuscript. Cong LIU revised the paper. Liang YAN designed the study. All authors read and approved the final manuscript. Therefore, all authors have full access to all the data in the study and take responsibility for the integrity and security of the data.

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Compliance with ethics guidelines

Wen-jie ZHANG, Cong LIU, Rui-juan YANG, Ting-ting ZHENG, Miao-miao ZHAO, Li MA, and Liang YAN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 Total ion chromatograms of the intra-day repeatability experiments

Fig. S2 VIP plots of PLS-DA based on volatiles data and bioactive compounds data

Table S1 Detail results of intra-day repeatability of the HS-SPME method

Table S2 Volatile compounds in seventeen tea samples

Table S3 Contents of ten bioactive compounds in seventeen tea samples

Table S4 Compound list with VIP value larger than 1.0

中文概要

题　目：采用气质联用技术和高效液相色谱对布朗山古树普洱晒青茶挥发性成分和生物活性成分进行比较分析

目　的：为布朗山不同品种古树普洱晒青茶香气物质和活性成分（多酚类和咖啡碱）深入研究提供理论依据。

创新点：首次采用气质联用技术（GC-MS）和高效液相色谱（HPLC）结合化学计量学对布朗山不同品种的古树普洱晒青茶进行研究，探究不同品种古树晒青茶成分之间的差异性。

方　法：采用顶空固相微萃取法（HS-SPME）结合 GC-MS 对香气成分进行鉴定；采用 70%甲醇溶液对活性成分进行富集，使用 HPLC 进行分析；结合主成分分析（PCA）和偏最小二乘判别分析（PLS-DA）对不同品种的古树普洱晒青茶进行区分并对区分起主要作用的物质进行筛选。

结　论：三个品种的古树普洱晒青茶（老曼峨、老班章和布朗）香气成分组成及含量差异较大，但主要成分均为醇类、碳氢类和酮类化合物。老曼峨中多酚类化合物含量较高，其次为布朗和老班章，相比于多酚类，咖啡碱含量表现出了相反的趋势。相比于 PCA 分析，PLS-DA 分析对三个品种的古树普洱晒青茶均显示出了较好的区分效果。变量投影重要性准则（VIP）分析结果显示 34 种香气和 5 种生物活性成分对不同品种的古树普洱晒青茶区分贡献较大。

关键词：普洱晒青茶；古茶树；布朗山；挥发性成分；生物活性成分