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RESEARCH ARTICLE

Advantage of LC-MS Metabolomics to Identify Marker Compounds in Two Types of Chinese Dark Tea after Different Post-fermentation Processes

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Abstract Chinese dark tea (CDT) was investigated for chemical characteristics using liquid chromatography coupled with tandem mass spectrometry (LC-MS) metabolomics analysis. The LC-MS^n method was used for detection of secondary metabolites in 10 CDT varieties. Pattern recognition methods, including principal component analysis (PCA) and orthogonal projection on latent structure-discriminant analysis (OPLS-DA), revealed clear differences and classified the CDT varieties into 2 categories. One category is fermented with *Aspergillus niger* while the other (fu teas) is fermented with *Eurotium cristatum*. Chemical analyses identified 25 marker compounds that contribute to the classification of the 2 CDT types. Flavonoid triglycosides and catechin derivatives were the distinctive compounds of fu teas, based on MS/MS fragmentation analysis. Although both fu teas and dark teas are commonly called CDTs, differences between these two CDT types are significant in the chemical classification.

Keywords: Chinese dark tea, liquid chromatography-mass spectrometry, metabolomics, post-fermentation

Introduction

Chinese dark tea (CDT) is a post-fermented tea product that is produced using microbial fermentation of mature tea leaves. In China and Southeast Asia, CDTs are popular for their flavors, which are different from green tea. The post-fermentation process, including a strong and fast process to ripen crude tea leaves, has proved to be critical in the formation of CDT characteristics. CDTs are mainly produced in southwestern China in Yunnan, Hunan, Hubei, Sichuan, and Guangxi provinces. The CDT variety known as pu-erh tea has been widely reported to promote health (1,2) and to contain many beneficial chemical constituents (3).

The varieties of CDT are correlated with regional characters. Storing of tea leaves in piles under controlled conditions is an important process in preparation of CDT varieties. The 2 popular CDTs known as pu-erh tea and fuzhuan brick tea are, respectively, produced in Yunnan and Hunan provinces (4,5). Their qualities are highly dependent on local environmental and climatic features. The fungi that are involved in post-fermentation probably play an important role in the different features of these 2 varieties. *Aspergillus* has been reported as the prevalent microorganism in ripened pu-erh tea, while *Eurotium cristatum* is the main microorganism in fuzhuan brick tea (6).

Chemical analysis of CDTs showed that amounts of the major catechins in raw tea materials are decreased after post-fermentation (7). Some catechin derivatives are supposedly derived from microbial metabolites of tea polyphenols during post-fermentation (8,9). These compounds are important for development of the characteristics of some CDTs, but they are not sufficient to characterize the final CDT product (10). Metabolomics has been an important tool used to solve system biology problems (11). Plant metabolomics has been widely used in the chemical analysis of plant samples with differences involving geographical factors, species, and processing treatments (12). Gas chromatography-time of flight mass spectrometry (GC-TOF-MS) analysis is preferred by researchers for study of volatile plant ingredients. Liquid chromatography coupled

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with tandem mass spectrometry (LC-MS) is a powerful metabolomics tool that provides identification information and also detects a broad group of metabolites (13). Recently, metabolomics combined with multivariate analysis was used in chemical research of pu-erh teas (14).

This study focused on the chemical characteristics of CDT varieties using metabolomics to classify traditional CDTs which, up to now, have only been distinguished based on the place of production.

Materials and Methods

Chemicals and materials Gallic acid (GA, >98%), caffeine (>98%), (+)-catechin (C, >98%), (-)-epicatechin (EC, >98%), (-)-gallocatechin (GC, >98%), (-)-epigallocatechin (EGC, >98%), (-)-gallocatechin gallate (GCG, >98%), (-)-epigallocatechin gallate (EGCG, >98%), and (-)-epicatechin gallate (ECG, >98%) standards were purchased from Shanghai Tongtian Biotechnology Co. (Shanghai, China) for quantitative analysis.

A total of 10 traditional CDT types were purchased from different locations in China. Kangzhuan (1), zangzhuan (2) and jinjian (6) teas were purchased from Ya'an in Sichuan province. Qinzhuan (4) tea was produced in Chibi of Hubei province. Liubao tea (6) was purchased from Wuzhou in the Guangxi autonomous region. Heizhuan (5), qianliang (7) and fuzhuan (8) tea were purchased from Yiyang in Hunan province. Pu-erh tea was produced in Xishuangbanna in Yunnan province. Jingwei fu tea was provided by Cangshan Tea Company of Shaanxi province.

Extraction of tea samples A 50 mg sample of each tea was weighed and extracted using ultrasonic extraction (KQ-100DV; Shumei, Kunshan, China) with 2 mL of methanol in a water bath at 45°C for 30 min. Samples were analyzed in triplicate. The extract was centrifuged (TGL-16C; Anting, Shanghai, China) at 5,300×*g* for 10 min. Supernatants were filtered through a 0.22 μm filter (P36010003; Jingteng, Tianjin, China) and preserved at 4°C for HPLC-MS^n analysis and HPLC determination.

Determination of the major compounds of CDT extracts Chromatography was performed on a Breeze HPLC system (Milford, MA, USA) consisting of a Waters 2695 series HPLC pump, a Waters 2498 dual-wavelength absorbance detector, and Waters Breeze software (Milford) for instrument control and data handling. The elution conditions followed a previous report (7). A total of 9 major compounds were identified and linear regression curves were calculated for each compound. A total of 5 μL of CDT extract was used for HPLC analysis.

LC-DAD-MS analysis of CDTs extracts An Agilent G6300 series HPLC-MS system (Santa Clara, CA, USA) consisted of a Surveyor MS pump, an auto sampler, a diode array detector and a LC/MSD ion trap mass spectrometer with Xcalibur software (ThermoFinnigan, San Jose, CA, USA) for data acquisition and analysis. Separations were carried out using an Agilent SB-Aq C18 reverse phase column (250×4.6 mm i.d., 5 μm) protected with a Gemini C 18 Guard Cartridge (2 mm×4 mm; Phenomenex, Torrance, CA, USA). Elution consisted of a linear gradient program from 5 to 30% acetonitrile in a 0.4% formic acid aqueous solution over 60 min. The flow rate was 0.8 mL/min and 10 μL of sample was injected. A 15 min re-equilibration time was used between HPLC runs. The diode array detection (DAD) acquisition wavelength was set in the range of 200-400 nm. After passing through the flow cell of the DAD, the column eluate was split and 0.2 mL/min was directed to a trap mass spectrometer with an electrospray interface (ESI) (Santa Clara, CA, USA), operating in full scan MS mode from m/z=100 to 2,000. Mass spectra were acquired in both negative and positive modes with an ion spray voltage of 3.5 kV, a capillary temperature of 350°C, a capillary voltage of 35 V, a sheath gas pressure of 25 psi, and an auxiliary gas pressure of 12 psi. A total of 7 traditional CDT varieties and 3 fu teas produced in 2012 were subjected to LC-MS metabolomics analysis.

Data analysis CDT sample LC-MS data were analyzed to identify potential discriminant variables. +MS/-MS raw data were analyzed using Xcalibur software (ThermoFinnigan) for data acquisition and analysis. Intensities of the detected peaks were determined for each sample using retention times (t_R) and m/z data pairs as identifiers for each peak. The resulting 3 dimensional matrix containing arbitrarily assigned peak indices (retention time-m/z pairs), sample names (observations), and peak intensity information (variables) was exported to SIMCA-P software 12.0 (Umea, Sweden) for multivariate statistical analysis, which was performed using LC-DAD-MS based metabolic profiling statistical data following a method previously reported (10,11). Statistical significance was defined as $p<0.05$.

Results and Discussion

The levels of tea polyphenols in 2 CDT types The major polyphenol and caffeine contents of 2 Chinese dark teas did not show any significant differences between the 2 teas ($p>0.05$) (Fig. 1).

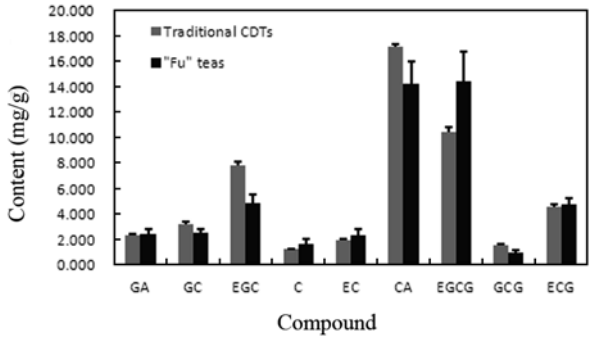

Fig. 1. The amounts of major compounds in different Chinese dark teas (mg/g) (mean±SEM). GA, Gallic acid; GC, (-)-gallocatechin;EGC, (-)-epigallocatechin; C, (+)-catechin;EC, (-)-epicatechin; CA, caffeine; EGCG, (-)-epigallocatechin gallate; GCG, (-)-gallocatechin gallate; ECG, (-)-epicatechin gallate

Metabolite profiling and CDT multivariate statistical analysis using LC-MS Using a published method for metabolite profiling (7), 10 CDT samples were analyzed using HPLC-DAD-MS^n coupled with a multivariate statistical analytical method. HPLC-MS peaks from CDT chromatograms were similar (Fig. 2A), but the automatic search program with intensity threshold more than 20,000 for compounds showed differences. From the 10 CDT samples, a total 367 major peaks were indentified using Xcalibur software with the LC-MS data set. The amounts of 9 major CDT compounds were also used in multivariate statistical analysis. Detailed MS information, including retention time (min) and the +MS/-MS (*m*/*z*) ion and peak intensities were subjected to principal components analysis (PCA) and Orthogonal partial least-squares discriminant

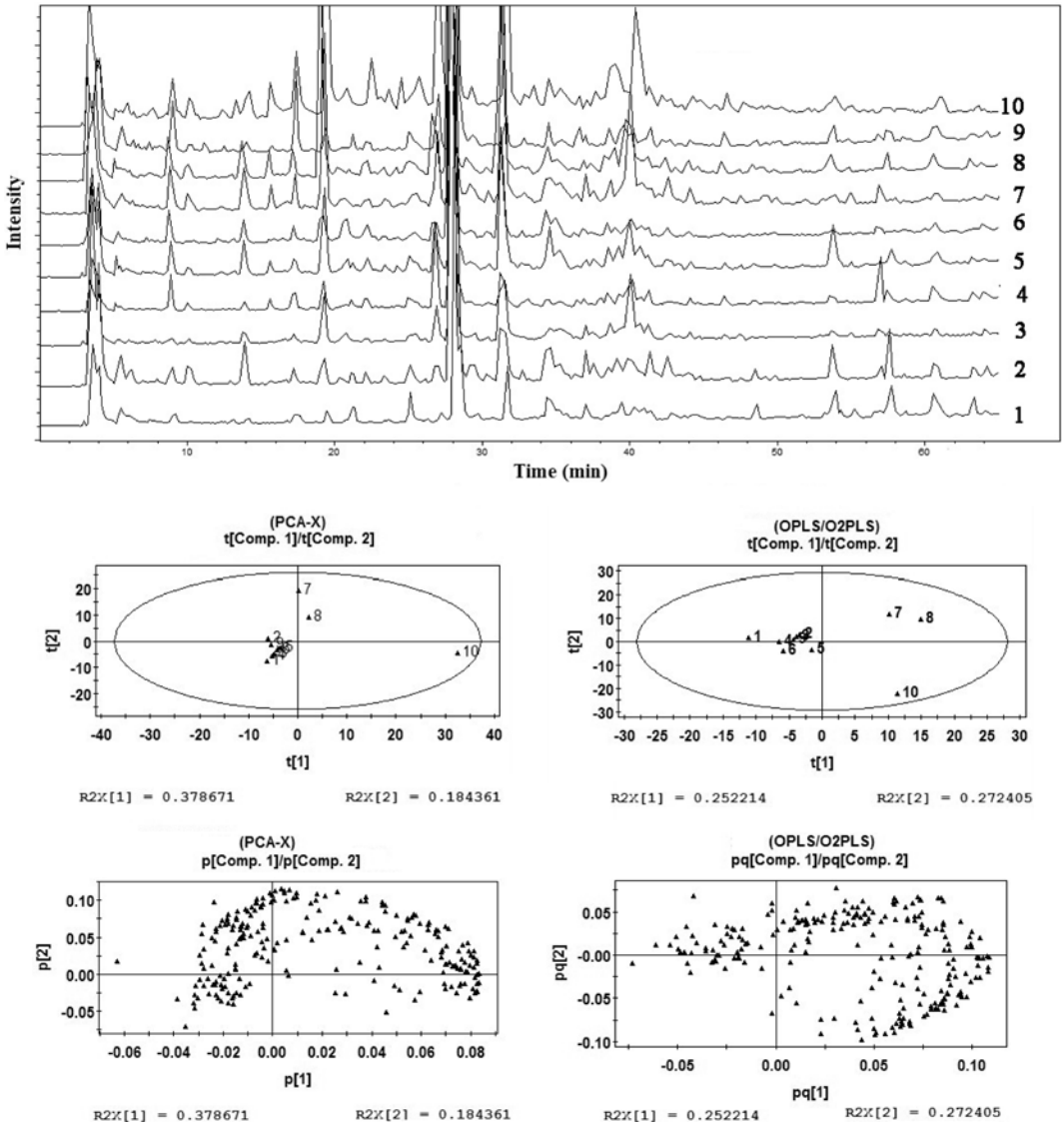

Fig. 2. Classification of various Chinese dark teas (CDTs) using principal component analysis (PCA) and orthogonal projection on latent structure-discriminant analysis (OPLS-DA) with LC-MS profiles. (A) the TIC (total ions chromatography) of various CDT varieties; (B) PCA score plot of CDT varieties; (C) PCA loading plots of CDT varieties; (D) OPLS-DA score plot of CDT varieties; (E) OPLS-DA loading plots of CDT varieties

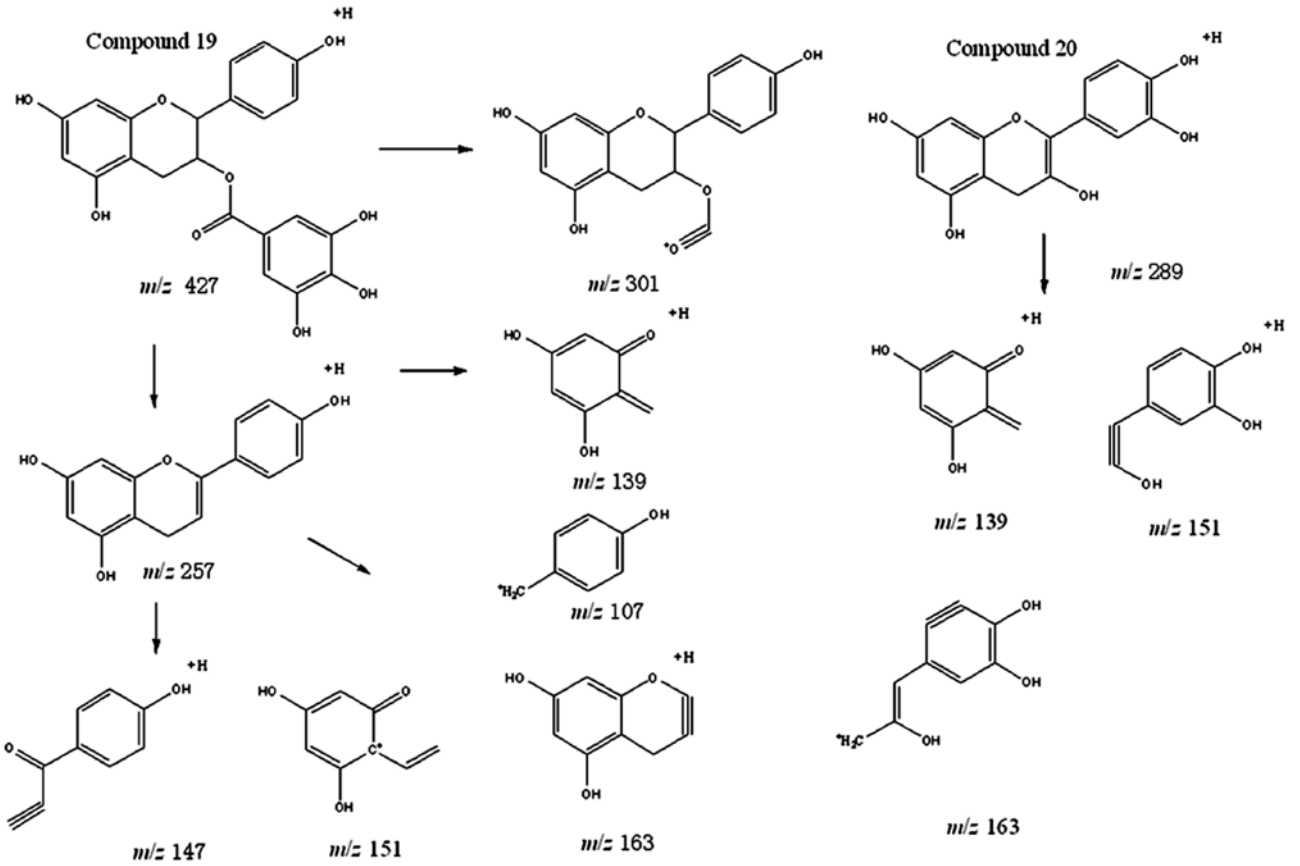

Fig. 3. The mass fragmentation of catechin derivatives under the positive mode of MS/MS.

analysis (OPLS-DA).

Preliminary PCA with mean centering was performed on all samples. The PCA scores plot derived from the LC-MS data set explained 97.6% of the total variance (R^2) with a prediction goodness parameter (Q^2)=84.6%. As a result, CDT varieties were clearly discriminated by both PC1 (37.9%) and by PC2 (18.4%) based on post-fermentation features (Fig. 2B). The loading plots of PCA were shown in Fig. 2C.

To identify the compounds correlated with CDT types, OPLS-DA was applied to LC-MS data sets. The ion intensities of 367 ions were configured as X variables, while the amounts of compounds in tea were used as Y variables in OPLS-DA modeling. A 6-component model explained 97.0% of the variance, with the first three components explaining 66.7%. Using PC1 and PC2, the 10 CDT samples used in this study were clustered into 2 groups (Fig. 2D). The loading plots of OPLS-DA were shown in Fig. 2E. Samples 1, 2, 3, 4, 5, 6, and 9 were classified as one type of CDT, while samples 7, 8, and 10 were classified as another type of CDT. The 2 types were clearly separated using only one predictive component that derived from the more sophisticated OPLS-DA (orthogonal partial least squares discriminate analysis) model because information noise irrelevant to major tea polyphenols was removed. The PCA and OPLS-DA classifications were similar, but OPLS-DA showed better separation of tea samples.

Analysis of marker compounds resulting in classification of CDTs Differences among sample HPLC-MS chromatograms were not apparent (Fig. 2A). OPLS-DA score plots readily divided all of the CDT samples into 2 types. The R^2X, R^2Y, and Q^2 values of this model were 0.970, 0.994, and 0.646, respectively. The VIP (variable importance in the projection) value of the OPLS-DA model was greater than 1.3. The marker compound variables were identified using co-chromatography, MS/MS spectral data, and UV spectral data of authentic compounds. A total of 25 marker compounds are listed in Table 1. Based on these compounds, 2 CDT types were distinguished. These critical compounds were used as precursor ions for fragmentation analysis using collision-induced dissociation (CID).

A total of 6 flavonoid glycosides were identified based on MS/MS fragmentation. Under the negative mass mode (-MS), the compound precursor ions were gradually fragmented to eliminate the glucose (−162 Da) and/or the rhamnose (−146 Da) moieties to produce the flavonoids aglycone such as kaempferol (m/z=285 $[M\text{-}H]^-$) and

Table 1. Retention time, mass spectral characteristics, and identification of VIP compounds of metabolomics analysis of dark teas and fu teas[1)]

No.	Peak No.	t_R (min)	Mw	Precursor ion (*m/z*)	MS^n (*m/z*)	UV λ_{max} (nm)	Compound
1[b]	175[f]	34.1	626	625.4 $[M-H]^-$	625.2-479.8-316.8-178,6/190.7	-	Myricetin-glucose-rhamonse
2[b]	270[fd]	44.1	594	593.3 $[M-H]^-$	593.3-447.2-284.8-150.7/162.6	265,350	Kaempferol-glucose-rhamnose
3[b]	247[f]	42.1	594	593.4 $[M-H]^-$	593.4-440.9-284.8-150.7/162.6	-	Kaempferol-rhamnose-glucose
4[b]	244[f]	41.5	756	755.4 $[M-H]^-$	755.4-592.8-431.0-284.8-150.7/184.8	265,350	Kaempferol-glucose-glucose-rhamnose
5[b]	262[f]	43.1	738	739.5 $[M+H]^+$	739.4-593.1-446.8-284.8-150.7/162.8	-	Kaempferol-glucose-rhamnose-rhamnose
6[a]	284[fd]	46.7	448	447.1 $[M-H]^-$	447.2-284.8-151.0/162.9	-	Kaempferol-3-*O*-β-D-glucopyranoside
7[a]	178[fd]	34.6	458	457.2 $[M-H]^-$	457.2-304.9-136.9/168.7/192.7	230,274	GCG
8[a]	125[fd]	22.1	290	289.1 $[M-H]^-$	289.1-136.8/150.7/160.8	230,280	Catechin
9[a]	44[fd]	13.8	306	305.1 $[M-H]^-$	305.1-136.8/160.7	230,280	GC
10[a]	234[fd]	40.1	442	441.2 $[M-H]^-$	441.2-288.9-136.9/150.7/160.9	230,278	ECG
11[a]	71[fd]	19.3	306	307.3 $[M+H]^+$	307.3-139.1/150.9/163.0	230,270	EGC
12[a]	154[fd]	31.8	458	459.2 $[M+H]^+$	459.2-289.0-139.0/150.9/162.9	230,275	EGCG
13[b]	82[f]	21.5	454[n]	453.4 $[M-H]^-$	453.4-304.9-134.9/178.6/204.6	230,270	Gallocatechin derivative
14[b]	107[f]	23.9	294[n]	295.1 $[M+H]^+$	295.1-139.0/162.9	-	Catechin derivative
15[b]	295[fd]	49.2	553[n]	552.0 $[M-H]^-$	552.0-400.0-148.8/161.6/178.7/204.7	-	Catechin gallate derivative
16[b]	246[f]	41.8	569[n]	568.4 $[M-H]^-$	568.4-416.0-138.7/168.7/192.6	-	Gallocatechin gallate derivative
17[b]	89[f]	22.2	358[n]	357.1 $[M-H]^-$	357.1-288.8-136.7/150.7	230,280	Catechin derivative
18[b]	141[fd]	29.6	417[n]	418.1 $[M+H]^+$	418.1-205.0/139.1/170.9/177.0	280	Catechin derivative
19[b]	287[fd]	47.5	426	427.2 $[M+H]^+$	427.2-257.0/301.0-107.0/139.0/147.0/151.0/163.0	280	5,7, 4'-Flavanol-3-gallate
20[b]	151[f]	31.4	288	289.3 $[M+H]^+$	289.3-139.0/150.9/163.0/205.0	230,275	2-(3,4-Dihydroxyphenyl)-4H-chromene-3,5,7-triol
21[b]	63[fd]	18.3	338	337.2 $[M-H]^-$	337.2-162.7/190.7/202.5	-	Catechin derivative
22[b]	81[f]	21.0	594	595.5 $[M+H]^+$	595.5-443.1-289.0-139.0/162.9/194.9	280	Catechin galloyl-gallate derivative
23[b]	84[f]	21.7	484	483.1 $[M-H]^-$	483.1-330.9-168.7	-	Digalloyl-glucose
24	214[fd]	38.3	512	510.9 $[M-H]^-$	310.8,340.9,341.9,374.8,394.9, 412.8,430.9, 431.9,444.5; 162.5,248.7,282.3,310.8,311.7,340.9,341.8,359.8	-	Unknown
25	51[fd]	15.7	266	267.3 $[M+H]^+$	114.1,204.0,249.0,267.1	-	Unknown

[1)][f]Only detected in fu teas; [fd]detected in both fu teas and dark teas; [n]new compounds formed after post-fermentation; [a]identified with reference to chemical standards; [b]identified using MS/MS ions and UV absorbance of HPLC-DAD-MS^n

myricetin (*m/z*=317 $[M-H]^-$). These flavonoid triglycosides were exclusively detected in the second CDT type. The polyphenols catechin and catechin esters were identified in both CDT types. The fragmentation features of catechins under both positive and negative mass modes were established using standard compounds. Catechins were fragmented to 139, 151, 161, and 205 under the positive mass mode. Based on these fragments, some catechin derivatives were identified as marker compounds of the second CDT type. For example, the precursor ion of compound 14 (*m/z*=295.1) $[M+H]^+$ produced 2 MS^2 ions (*m/z*=139 and 163). These fragments were also detected after MS/MS fragmentation of compound 14-22, which contained fragments typical of catechins (*m/z*=139, 151, 161, and 163). Compounds 19 and 20 were probably 5,7,4'-flavanol-3-gallate and 2-(3,4-dihydroxyphenyl)-4H-chromene-3,5,7-triol, based on mass fragmentation data. Speculative identifications of compounds 19 and 20 are shown in Fig. 3.

Fresh tea leaves were studied to identify new compounds after post-fermentation. A total of 6 marker compounds were identified as catechin derivatives (Table 1). Compounds 15 and 16 contained the gallate moiety, deduced based on a loss of gallic acid (170 Da) from ions of *m/z*=554 and 570 to ions of *m/z*=384 and 400. MS/MS data for ions of *m/z*=384 and 400 continued to produce critical catechin mass fragmentation ions of *m/z*=139, 151, 161, and 163. These compounds consisted of catechins, gallocatechins, and gallocatechin gallate.

According to classification criteria of manufacturing technology, Chinese dark tea has a unique post-fermentation process that is different from green, black, oolong, white, and yellow teas. Although post-fermentation is common among all CDT varieties, the fungi used for individual CDTs varieties vary. Fungal fermentation is indispensible to develop the distinctive CDT flavor and taste. In this

study, 10 commercial CDT varieties from different areas of China were analyzed. The metabolomics analytical method was applied for CDT classification. In addition, major CDT compounds were also determined based on qualitative analysis.

Results of an analysis of CDT microorganisms were in conformity with the metabolomics classification results. PCA and OPLS-DA analyses of LC-MS data showed that 7 CDT varieties were classified as a distinct CDT type. The prevailing fungus of this group of 7 teas was identified as *Aspergillus niger*. The other CDT type consisted of jingweifu, fuzhuan, and the fu teas containing the golden ascocarps of *Eurotium cristatum.* These results show that the chemical characteristics of CDT varieties are highly correlated with the particular post-fermentation fungus used. Representative compounds affecting the metabolomics classification of CDT varieties were identified using LC-MS^n fragment information.

Fresh tea leaves contain 3 types of polyphenol, including flavonoid glycosides, catechins, and tannins. Using metabolomics analysis, the distributions of these 3 polyphenols were investigated between the 2 typical CDT types. Fu brick tea contained 7 kinds of gallotannin while pu-erh tea did not (data not shown). The flavonoid triglycosides were also presented in fu teas but not in other dark teas. The major catechins were identified in both CDT types, but fu teas had a more diverse population of catechin derivatives than other dark teas. The post-fermentation process of fu tea is a less strongly enzymatic process that does not affect the glycosidic bonds of flavonoid triglycosides. The levels of EGCG in fu teas were also higher than in dark teas. The number of different catechin derivatives were greater in fu teas than in dark teas. Fu teas not only contained all of the characteristic compounds of dark teas, but also possessed some unique compounds. In conclusion, fu teas share the common constituents of other dark teas, but also contain other unique compounds.

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