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A comparative analysis for the volatile compounds of various Chinese dark teas using combinatory metabolomics and fungal solid-state fermentation

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ABSTRACT

A total of 98 compounds including 20 aldehydes, eight arenes, six acids, 17 alcohols, 13 ketones, nine esters, nine methoxyphenolics, three alkenes, seven alkanes, and six other components were tentatively identified in six Chinese dark teas (CDTs) using gas chromatography–mass spectrometry. Multivariate statistical analysis revealed that dark teas from Yunnan and Guangxi provinces could be classified into one group, and other CDTs belonged to the other cluster. The diagnostic volatile compounds being responsible for CDTs' discrimination were observed as (*E*,*E*)-2,4-decadienal, methoxyphenolics, geraniol, α-terpineol, 2,4-heptadienal, *cis*-jasmone, linalool oxides, and 2-nonenal. Furthermore, mature tea leaves were separately fermented using *Eurotium cristatum* and *Aspergillus niger*. The results showed that *E. cristatum* increased the contents of *cis*-jasmone, α-terpineol, ß-ionone, nonanal, and 2-pentylfuran, whereas *A. niger* advanced the levels of geraniol, linalool oxides, 9,12-octadecadienoic acid, and ß-ionone after short-term fermentation. Fungus species may contribute to forming the flavor of Chinese dark teas by affecting the volatile compounds during postfermentation.

1. Introduction

Chinese dark tea (CDT) is a type of postfermented tea product from Southwestern China. CDTs usually possess significant geographical features. For example, ripened pu-erh tea is the typical CDT in Yunnan province. Furthermore, there are other famous CDTs in different regions, such as Ya'an Tibet tea (Sichuan dark tea; SCDT), Liubao tea (Guangxi dark tea; GXDT), Jingweifu tea (Shaanxi dark tea; SXDT), Fu-brick tea (Hunan dark tea; HNDT), and Qingzhuan tea (Hubei dark tea, HBDT)

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[1]. CDTs have been favored by the consumers because of their special flavors. They also contain some specific chemical constituents different from other unfermented or semi-fermented teas, such as green tea and oolong tea [2]. It has been reported that postfermentation produced the special secondary metabolites of dark teas [3]. Fungal fermentation could transfer the flavan-3-ols and L-theanine into 8-C-N-ethyl-2-pyrrolidinone substituted flavan-3-ols [4]. Also, the primary fungus is able to play an important role in the transformation of catechins during postfermentation [5]. Furthermore, some other B-ring oxidized flavan-3-ols have been identified in fu-brick tea [6]. These unique metabolites of catechins of CDTs are highly depended on the fermentation process and predominant fungi.

Traditionally, tea was classified according to the critical manufacture process, to be exact, the fermentation technology. For example, it could be classified as unfermented, semifermented, fully-fermented, and postfermented teas [7]. Fully-fermented tea (black tea) is oxidized by polyphenol oxidase(s) of tea leaves. The postfermentation process of CDTs could be depicted as a piling solid-state fermentation of deactivated tea leaves under natural or controlled conditions [8]. Some studies have demonstrated that there are multiple fungi species involved in postfermentation [9]. Different from other teas, the dark teas are more complicated in terms of flavor compounds because the tea ingredients can be highly affected by environmental microorganisms, temperature, and humidity.

To chemically distinguish CDTs, liquid chromatography coupled mass spectrometry combining metabolomics analysis has been applied in the reclassification of various CDTs [10]. The volatile compounds of some CDTs have been studied by comparing the teas' flavor compounds before and after postfermentation. It has been reported that methoxyphenolic compounds were the main volatile compounds of ripened pu-erh tea [11,12]. It was suggested that methoxyphenolic compounds were the metabolites of gallic acid and tannins after postfermentation. Furthermore, other types of CDTs, such as fu-brick tea, contained a high content of limonene. The unsaturated hydrocarbons in fu-brick tea usually gave a woody and fruity fragrance [13].

Although there are some studies regarding the volatile compounds of pu-erh tea and fu-brick tea, the systematic comparative study on various CDTs is still lacking. Furthermore, the effects of predominant microorganisms on the formation of the unique flavor of CDTs have not been studied in depth. Metabolomics has become a robust tool of non-targeted analysis of plant and bio-samples [14]. Integrative gas chromatography–mass spectrometry (GC-MS) and liquid chromatography coupled mass spectrometry analysis has been successfully applied in the research of primary and secondary metabolites in tea plants. Through the multivariate analysis, some marker volatile compounds were identified in different types of CDTs [10]. During the postfermentation, the fungi are very important for the transformation of compounds of CDTs. For example, *Aspergillus niger* is an important and main microorganism in the long-term fermentation of many CDTs rather fu-brick tea. Although the fungal fermentation caused a significant change of flavor in dark tea, very little is known about the effects of fungus on the transformation of raw volatile compounds of CDTs. In addition, the distinct aroma of CDT is a reflection of hundreds of chemicals, rather than a single flavor-active compound.

To explore the volatile compound profiling of different CDTs, a simultaneous distillation extraction (SDE) was used to extract the volatile compounds of tea samples, and subsequently subject to GC-MS analysis. The marker volatile compounds being responsible for the classification of various CDTs were characterized. Solid-state fermentation of deactivated tea leaves was also conducted by single fungus to study the trajectory of volatile compounds.

2. Methods

2.1. *Tea samples*

Yunnan dark tea (YNDT; pu-erh tea) was purchased from the Xishuangbanna, Yunnan province. HNDT (fu-brick tea) was purchased from the Anhua, Hunan province. HBDT (Qingzhuan tea) was produced in Chibi, Hubei province. GXDT (Liubao tea) was produced in Wuzhou, Guangxi province. SCDT (Tibet tea) was produced in Ya'an, Sichuan province. SXDT (Jingweifu tea) was produced in Xianyang, Shaanxi province. The green tea sample was used as control in the present study. Furthermore, the mature tea leaves of *Camellia sinensis* were used as the raw material for solid-state fermentation of single fungus.

2.2. *Sample preparation*

Six kinds of CDTs were extracted using SDE method. Briefly, for each kind of CDT, 15 g of tea leaves were placed in a 1-L flat-bottom flask containing 300 mL of boiling distilled water and immediately attached to the SDE apparatus. The flat-bottom flask containing tea was on one side of the Likens-Nickerson apparatus, and the flask containing solvents was on the other side. The solvent and tea infusions were heated via hot plates. Volatile compounds were extracted using diethyl ether for 90 minutes with three replications. After extraction, the extracts were dried using a small amount of anhydrous sodium sulfate and subsequently filtered. Then, the extract was concentrated to 1 mL. This concentrate was used for GC-MS.

During the SDE, 50 μL of ethyl decanoate was added into the tea sample as an internal standard, with the final concentration of 2.78 μg/g. The relative contents of volatile compounds were calculated with reference to the internal standard. For each tea sample, analysis was conducted in triplicate.

2.3. *The solid-state fermentation by* A. niger *and* E. cristatum

The solid-state fermentation of tea leaves single fungus referenced our previous study [5]. In brief, 5 mL of *A. niger* and *E. cristatum* spore suspension was incubated in potato dextrose agar plates with concentrations of 3.0–4.0 × 10^5/mL and 9.0–10.0 × 10^3/mL, respectively. The incubation condition was set at 30°C and 95% relative humidity. After 48 hours of

enrichment culture, 15 g of mature tea leaves were added to the culture plate for fermentation test. At 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, and 144 hours post-fermentation, the whole samples in one plate were collected and extracted using SDE method. The volatile compounds were analyzed using GC-MS. Before this experiment, the mature tea leaves were deactivated by heating. Each sample was prepared in duplicate.

2.4. GC-MS analysis

To analyze volatile compounds of CDTs, an Agilent 7890A gas chromatograph coupled with an Agilent 5975C mass spectrometer was used to perform the volatile analysis (Agilent Technologies, Santa Clara, CA, USA). An HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies) was equipped, with purified helium as the carrier gas, at a constant flow rate of 1 mL/min. The oven temperature was held at 60°C for 3 minutes and then increased to 210°C at a rate of 1°C/min, and then held at 210°C for 2 minutes, and then increased to 270°C at a rate of 5°C/min and held at 270°C for 7 minutes. Ion source temperature was at 250°C and spectra was produced in the electron impact mode at 70 eV. Mass ranged from *m/z* 40 *m/z* to 600 *m/z*.

The fermented tea samples by *A. niger* and *E. cristatum* were also extracted as above-mentioned. The extracted volatile compounds of fermented tea were also injected in to GC-MS for analysis.

The volatile compounds were identified by using the deconvolution reporting software in combination with National Institute of Standards and Technology 98Las well as by comparison of their retention indexes with literature data or National Institute of Standards and Technology database. The relative proportions of the constituents were obtained by flame ionization detector peak area normalization. To calculate the Kovats retention index (RI) for each peak, 1 mL *n*-alkane mixture (C_7–C_{40}; Sigma-Aldrich, St. Louis, Mo, USA) was injected under the same GC-MS conditions. RI was calculated using the following equation:

$$RI = 100n + 100(tx - tn)/(tn + 1 - tn) \quad (1)$$

where t_x is the retention time, n and n + 1 are respectively the number of carbon atoms in the alkanes eluting before and after the compound X.

2.5. Multivariate data analysis

The GC-MS data of CDTs samples was analyzed to identify potential discriminant variables. A list of the intensities of the detected peaks was generated for each sample, using retention time (t_R) and the *m/z* data pairs as the identifier for each peak. The resulting three-dimensional matrix containing arbitrarily assigned peak index (retention time-*m/z* pairs), sample names (observations), and peak intensity information (variables) was exported to SIMCA-P software 12.0 (Umetrics, Umea, Sweden) for principle components analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA). The multivariate statistics for GC-MS-based metabolic profiling was performed with the method previously reported [10].

3. Results

3.1. The volatile compounds of various CDTs

The GC–MS chromatograms of the flavor profiles of various CDTs are shown in Figure 1. In total, major volatile compounds were identified in six types of Chinese dark tea as shown in Table 1.

In the YNDT, similar to the published results, methoxyphenolics were the major volatile compounds, with a total content of 5.623 ± 0.351 μg/g [15]. Among these methoxyphenolic compounds, 1,2,3-trimethoxybenzene was identified as one of the major volatile compounds of YNDT, which had highest relative content (3.159 ± 0.114 μg/g), followed by 1,2,4-trimethoxybenzene (1.368 ± 0.132 μg/g). Furthermore, alcohols were also the other type of volatile compound in YNDT, the main alcohols were the linalool oxides, which contributed to the total content of 3.479 ± 0.137 μg/g. Compared with green tea, the contents of linalool in post-fermented teas (CDTs) were significantly decreased.

In SCDT, the major volatile compounds were aldehydes and ketones, with the total contents of 14.022 ± 1.551 μg/g and 11.350 ± 2.005 μg/g, respectively. (*E,E*)-2,4-heptadienal was the main aldehyde in HNDT, followed by (*E,E*)-2,4-decadienal and nonanal. These C_6aldehydes were usually described as having a "grassy" flavor. Furthermore, α-ionone, ß-ionone, geranylacetone, and hexahydrofarnesyl acetone had the content of 1.506 ± 0.193 μg/g, 2.701 ± 0.176 μg/g, 2.243 ± 0.186 μg/g, and 1.878 ± 0.306 μg/g, respectively. The HNDT and SXDT belong to the fu-brick teas which are mainly postfermented by *E. cristatum*. In HNDT, the volatile compounds were ß-ionone, geranylacetone, hexahydrofarnesyl acetone, and damascenone. In SXDT, ketones and esters were the main volatile compounds, with the contents of 2.528 ± 0.209 μg/g and 0.954 ± 0.104 μg/g, respectively, and the main ketone was hexahydrofarnesyl acetone.

In the GXDT, alcohols were the main volatile compounds. Among these alcohols, cedrol were the most abundant alcohols in GXDT (2.598 ± 0.068 μg/g). The integral contents of volatile compounds were much less than other CDTs. The contents of aldehydes, acids, ketones, and esters were the lowest in all six types of CDTs. In the HBDT, the contents of hexadecanoic acid and nonanoic acid were the highest in all CDTs. Furthermore, it also contained high contents of ketones, mainly composed of ß-ionone, geranylacetone, and hexahydrofarnesyl acetone.

3.2. Metabolomics analysis of GC-MS data of various CDTs

Chemo-metric pattern recognition techniques, such as PCA, are valuable tools for reducing the complexity of GC-MS data sets. The scores plot for the entire data set is shown in Figure 2. PCA was performed on all data from GC-MS in all types of CDTs sample (Figure 2). The results showed that YNDT and GXDT samples were classified as one type.

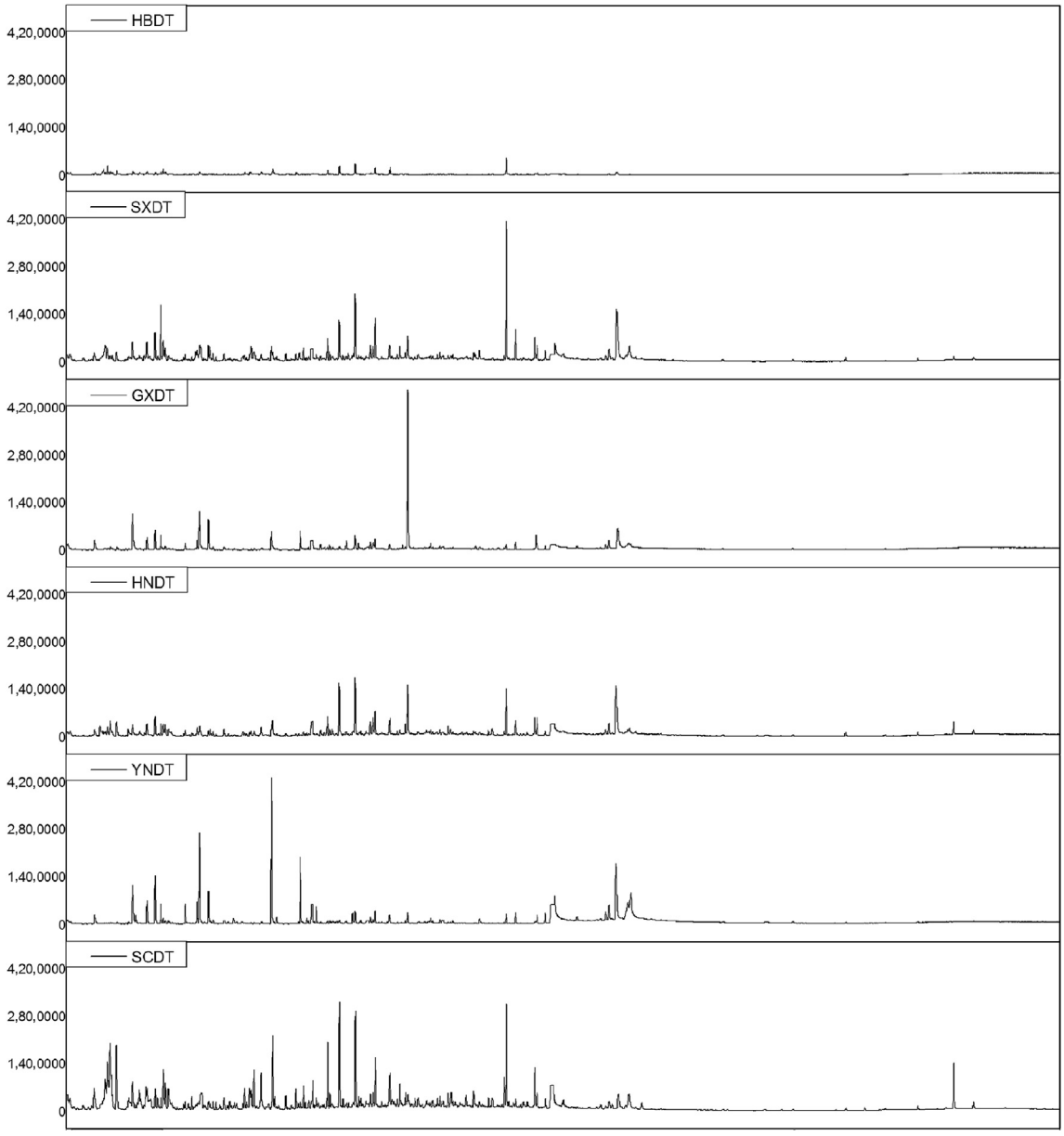

Figure 1 – The representative total ion current chromatograms of various Chinese dark teas using gas chromatography–mass spectrometry. GXDT = Guangxi dark tea; HBDT = Hubei dark tea; HNDT = Hunan dark tea; SCDT = Sichuan dark tea; SXDT = Shanxi dark tea; YNDT = Yunnan dark tea.

The PLS model provided a similar classification results for all kinds of CDTs. The summary of the fit of the model is displayed with R2X (cum) [R2X shows the percentage of variance in the data that is explained by a particular component, R2X (cum) sums up the R2X as they accumulate with an increase in the number of components] and Q2 (cum) [indicates the predictive ability of the model]. In total, PLS describes 75.5% of the variable X (R2X), 94.9% of the variable Y in the data with a Q2 of 86.8% for the six main components indicating good prediction properties of the model. The score plots also indicated that six types of CDTs were obviously divided into two types, one type included YNDT and GXDT, and the other type had SCDT, HNDT, HBDT, and SXDT.

OPLS-DA score plots readily divided all of the CDT samples into two types. The variable importance in the projection value of the OPLS-DA model was greater than 1.2. The marker compound variables were identified as shown in Table 2. A total of 17 marker compounds are listed in Table 2.

3.3. *Change of marker volatile compounds during solid-state fermentation by* Aspergillus niger

The mature tea leaves were fermented by *A. niger*. As shown in Figure 3, five marker volatile compounds including geraniol, linalool oxides, 9,12-octadecadienoic acid, and ß-ionone were monitored during the solid-state fermentation. Samples at

Table 1 – Gas chromatography–mass spectrometry analysis results of volatile compounds in six types of Chinese dark teas samples.

No.	RT. (min)	RI[a]	RI[b]	Compounds	Relative content[c] (μg/g, mean ± S.D; $n = 3$)						
					SCDT	HBDT	YNDT	HNDT	GXDT	SXDT	GT
1	4.259	n.d.	n.d.	(*E*)-2-hexenal	0.488 ± 0.127	0.274 ± 0.026	0.048 ± 0.008	0.192 ± 0.040	0.096 ± 0.056	0.077 ± 0.027	0.137 ± 0.033
2	5.283	n.d.	n.d.	Heptanal	0.538 ± 0.118	0.353 ± 0.055	0.050 ± 0.007	0.232 ± 0.051	n.d.	0.119 ± 0.004	0.383 ± 0.070
3	6.714	953	960	2-heptenal	0.160 ± 0.052	0.160 ± 0.192	n.d.[e]	0.126 ± 0.127	n.d.	0.054 ± 0.064	n.d.
4	6.867	958	963	Benzaldehyde	0.930 ± 0.007	0.304 ± 0.064	0.350 ± 0.037	0.294 ± 0.019	0.284 ± 0.016	0.138 ± 0.019	0.141 ± 0.023
5	7.879	996	982	(*E*,*E*)-2,4-heptadienal	2.845 ± 0.302	0.552 ± 0.325	0.037 ± 0.010	0.383 ± 0.009	0.048 ± 0.001	0.070 ± 0.004	0.063 ± 0.019
6	8.311	1009	1012	2,4-heptadienal	2.130 ± 0.073	0.616 ± 0.113	0.071 ± 0.007	0.485 ± 0.032	0.080 ± 0.009	0.106 ± 0.006	0.130 ± 0.023
7	9.380	1042	1048	Benzene acetaldehyde	0.830 ± 0.174	0.484 ± 0.078	1.132 ± 0.059	0.360 ± 0.051	0.804 ± 0.046	0.229 ± 0.017	0.195 ± 0.039
8	9.819	1056	1060	2-octenal	0.371 ± 0.276	0.318 ± 0.139	0.027	0.216 ± 0.028	n.d.	0.036 ± 0.007	0.099 ± 0.036
9	11.371	1104	1104	Nonanal	1.266 ± 0.015	0.698 ± 0.118	0.109 ± 0.005	0.364 ± 0.030	0.017 ± 0.007	0.258 ± 0.017	0.440 ± 0.056
10	13.242	1158	1151	2-nonenal	0.284 ± 0.013	0.170 ± 0.027	n.d.	0.077 ± 0.012	n.d.	0.036 ± 0.003	0.030 ± 0.005
11	14.444	1193	n.d.	3-hydroxy-6-methyl-benzaldehyde	0.132 ± 0.058	0.063 ± 0.009	0.076 ± 0.034	0.198 ± 0.014	0.016 ± 0.003	0.139 ± 0.009	0.044 ± 0.038
12	14.641	1199	1178	2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde	0.202 ± 0.026	0.136 ± 0.023	0.101 ± 0.014	0.151 ± 0.018	0.051 ± 0.005	0.072 ± 0.004	0.086 ± 0.018
13	14.813	1204	1185	Decanal	0.168 ± 0.002	0.104 ± 0.017	0.035 ± 0.004	0.056 ± 0.002	n.d.	0.032 ± 0.001	0.026 ± 0.005
14	15.354	1221	1123	2,6,6-trimethyl-2-cyclohexene-1-carboxaldehyde	0.310 ± 0.014	0.148 ± 0.020	0.124 ± 0.009	0.209 ± 0.025	0.070 ± 0.016	0.062 ± 0.003	0.186 ± 0.040
15	16.582	1257	1261	2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde	0.105 ± 0.095	0.153 ± 0.159	n.d.	0.154 ± 0.011	0.031 ± 0.004	0.059 ± 0.009	0.326 ± 0.293
16	16.703	1261	1265	*E*-2-decenal	0.424 ± 0.002	0.270 ± 0.063	n.d.	0.068 ± 0.008	0.010 ± 0.003	0.038 ± 0.001	n.d.
17	18.529	1315	1318	(*E*,*E*)-2,4-Decadienal	1.825 ± 0.066	0.912 ± 0.157	3.159 ± 0.114	0.491 ± 0.051	n.d.	0.081 ± 0.004	0.139 ± 0.028
18	20.055	1362	1262	2-decenal	0.438 ± 0.040	0.394 ± 0.040	n.d.	0.048 ± 0.043	n.d.	0.058 ± 0.007	0.020 ± 0.002
19	33.683	1837	1850	5,9,13-trimethyl-4,8,12-tetradecatrienal	0.576 ± 0.091	n.d.	n.d.	0.231 ± 0.037	n.d.	n.d.	n.d.
20	38.442	2025	n.d.	3-(4-methyl-3-pentenyl)-3-cyclohexene-1-carboxaldehyde	n.d.	0.083 ± 0.021	n.d.	n.d.	n.d.	n.d.	0.223 ± 0.086
	Aldehydes			Total contents	14.022 ± 1.551	6.056 ± 1.646	5.139 ± 1.308	4.383 ± 0.616	1.507 ± 0.166	1.718 ± 0.206	2.670 ± 0.795
21	4.462	n.d.	868	Ethylbenzene	0.181 ± 0.070	0.127 ± 0.033	0.097 ± 0.004	0.123 ± 0.024	0.102 ± 0.028	0.053 ± 0.014	0.193 ± 0.061
22	4.602	n.d.	870	p-xylene	0.353 ± 0.171	0.148 ± 0.034	0.105 ± 0.014	0.125 ± 0.014	0.152 ± 0.009	0.045 ± 0.010	0.299 ± 0.084
23	5.092	n.d.	890	Styrene	0.558 ± 0.306	0.382 ± 0.080	0.138 ± 0.007	0.214 ± 0.080	0.221 ± 0.054	0.119 ± 0.006	0.253 ± 0.065
24	29.484	1679	n.d.	1,2,3-trimethyl-4-[(1*E*)-prop-1-en-1-yl]naphthalene	0.270 ± 0.016	0.159 ± 0.053	0.097 ± 0.006	0.126 ± 0.095	0.048 ± 0.003	0.068 ± 0.002	0.058 ± 0.009
25	29.681	1686	n.d.	(4-acetylphenyl)-phenylmethane	0.225 ± 0.054	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
26	30.311	1708	1668	2,2′,5,5′-tetramethyl-1,1'-biphenyl	0.127 ± 0.027	0.054 ± 0.036	0.066 ± 0.008	0.188 ± 0.106	0.030 ± 0.003	0.039 ± 0.001	0.026 ± 0.024
27	31.806	1765	1541	1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	n.d.	0.008 ± 0.015	n.d.	0.021 ± 0.001	0.067 ± 0.001	0.034 ± 0.034	n.d.
28	32.041	1774	1793	Anthracene	0.160 ± 0.052	0.049 ± 0.043	0.174 ± 0.010	0.091 ± 0.013	n.d.	0.091 ± 0.001	0.101 ± 0.016
	Arenes			Total contents	1.874 ± 0.696	0.927 ± 0.294	0.677 ± 0.049	0.888 ± 0.333	0.620 ± 0.098	0.449 ± 0.068	0.930 ± 0.259

29	7.497	981	981	Hexanoic acid	n.d.	1.845 ± 0.745	0.021 ± 0.003	0.191 ± 0.146	0.026 ± 0.005	0.246 ± 0.170	0.077 ± 0.025
30	13.980	1180	1191	Octanoic acid	1.272 ± 0.103	0.463 ± 0.412	n.d.	0.041 ± 0.005	0.016 ± 0.008	n.d.	0.064 ± 0.018
31	17.269	1277	1297	Nonanoic acid	0.600 ± 0.046	0.397 ± 0.242	0.046 ± 0.005	0.153 ± 0.049	0.029 ± 0.005	0.140 ± 0.019	0.056 ± 0.049
32	20.265	1369	1387	n-decanoic acid	n.d.	n.d.	1.358 ± 0.201	0.069	n.d.	0.073 ± 0.016	n.d.
33	31.685	1760	1765	Tetradecanoic acid	n.d.	0.113 ± 0.065	n.d.	0.088 ± 0.016	n.d.	0.062 ± 0.004	n.d.
34	41.947	2138	2134	9,12-octadecadienoic acid	0.569 ± 0.329	0.272 ± 0.195	1.514 ± 0.369	0.376 ± 0.075	0.206 ± 0.293	0.260 ± 0.091	n.d.
	Acids			Total contents	2.441 ± 0.375	3.090 ± 1.659	2.933 ± 0.578	0.918 ± 0.36	0.277 ± 0.311	0.781 ± 0.300	0.197 ± 0.092
				Alcohols							
35	9.087	1033	1024	Benzyl alcohol	0.919 ± 0.090	n.d.	0.058 ± 0.002	0.299 ± 0.097	0.028 ± 0.003	0.078 ± 0.008	0.220 ± 0.121
36	10.322	1071	1077	Linalool oxide	0.579 ± 0.026	0.339 ± 0.131	0.549 ± 0.032	0.401 ± 0.030	0.211 ± 0.010	0.211 ± 0.035	0.248 ± 0.072
37	10.850	1088	1094	trans-linalool oxide (furanoid)	0.526 ± 0.036	0.199 ± 0.038	1.100 ± 0.055	0.540 ± 0.033	0.329 ± 0.021	0.274 ± 0.032	0.122 ± 0.033
38	11.225	1099	1100	Linalool	0.271 ± 0.007	0.270 ± 0.043	0.420 ± 0.024	0.284 ± 0.024	0.233 ± 0.011	0.477 ± 0.038	0.793 ± 0.143
39	11.518	1108	1114	2,6-dimethylcyclohexanol	0.708 ± 0.031	0.272 ± 0.064	0.092 ± 0.008	0.315 ± 0.018	0.069 ± 0.007	0.131 ± 0.006	0.153 ± 0.044
40	11.785	1116	1115	Phenylethyl alcohol	0.697 ± 0.057	0.163 ± 0.088	0.120 ± 0.015	0.219 ± 0.009	0.046 ± 0.062	0.066 ± 0.010	0.151 ± 0.056
41	13.496	1166	1168	4-ethylphenol	0.026 ± 0.013	n.d.	n.d.	n.d.	0.017 ± 0.004	0.112 ± 0.005	n.d.
42	13.757	1173	1173	Linalool oxide II (pyranoid)	0.055 ± 0.007	0.436 ± 0.071	1.830 ± 0.050	0.442 ± 0.003	0.664 ± 0.070	0.358 ± 0.009	0.186 ± 0.030
43	14.336	1190	1190	α-terpineol	0.197 ± 0.042	0.119 ± 0.024	0.643 ± 0.067	0.134 ± 0.008	0.466 ± 0.021	0.147 ± 0.007	0.158 ± 0.041
44	16.506	1255	1267	Geraniol	0.157 ± 0.043.	0.044 ± 0.019	0.083 ± 0.023	n.d.	n.d.	n.d.	0.083 ± 0.105
45	22.257	1432	n.d.	1-(4-tert-butylphenyl) propan-2-one	n.d.	0.162 ± 0.012	n.d.	n.d.	n.d.	n.d.	n.d.
46	25.094	1525	n.d.	5-pentyl-1,3-benzenediol	0.466 ± 0.036	0.250 ± 0.149	0.110 ± 0.026	0.503 ± 0.042	0.180 ± 0.010	0.149 ± 0.001	0.193 ± 0.043
47	26.214	1563	1531	Nerolidol	0.711 ± 0.397	1.123 ± 0.253	0.267 ± 0.010	0.608 ± 0.035	0.137 ± 0.003	0.220 ± 0.022	0.596 ± 0.108
48	27.372	1603	1608	Cedrol	0.472 ± 0.053	0.116 ± 0.014	0.211 ± 0.026	1.104 ± 0.093	2.598 ± 0.068	0.271 ± 0.035	0.060 ± 0.019
49	28.854	1656	1650	α-cadinol	0.144 ± 0.075	0.061 ± 0.024	0.114 ± 0.004	0.128 ± 0.021	0.083 ± 0.003		0.057 ± 0.002
50	36.361	1945	1948	Isophytol	0.267 ± 0.072	0.177 ± 0.087	0.276 ± 0.025	0.135 ± 0.020	0.082 ± 0.006	0.105 ± 0.002	0.219 ± 0.053
51	41.005	2111	2122	Phytol	0.852 ± 0.255	1.107 ± 0.574	n.d.	n.d.	0.991 ± 0.127	1.187 ± 0.110	n.d.
	Alcohols			Total contents	7.057 ± 1.237	4.834 ± 1.592	5.874 ± 0.430	5.112 ± 0.635	6.125 ± 0.451	3.776 ± 0.390	3.236 ± 0.969
52	10.112	1065	1065	Acetophenone	0.526 ± 0.050	n.d.	0.037 ± 0.003	0.082 ± 0.014	0.027 ± 0.004	0.058 ± 0.010	0.074 ± 0.015
53	10.996	1092	1100	3,5-octadien-2-one	0.364 ± 0.019	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
54	12.701	1143	1142	2,6,6-trimethyl-2-cyclohexene-1,4-dione	0.124 ± 0.037	0.056 ± 0.002	0.025 ± 0.012	0.058 ± 0.013	n.d.	0.034 ± 0.004	n.d.
55	19.394	1342	n.d.	2-pentylcyclopentanone	n.d.	0.256 ± 0.045	n.d.	n.d.	n.d.	n.d.	n.d.
56	21.232	1399	1396	*cis*-jasmone	0.491 ± 0.779	0.214 ± 0.260	n.d.	0.024 ± 0.003	n.d.	0.008 ± 0.001	0.185 ± 0.050
57	21.385	1404	1408	Pseudoionone	0.240 ± 0.106	0.135 ± 0.055	0.362 ± 0.014	0.020 ± 0.003	0.013 ± 0.010	0.035 ± 0.001	n.d.
58	22.123	1428	1427	α-ionone	1.506 ± 0.193	0.520 ± 0.004	0.083 ± 0.018	0.475 ± 0.037	0.043 ± 0.002	0.183 ± 0.010	0.075 ± 0.003
59	22.880	1452	1458	Geranylacetone	2.701 ± 0.176	1.056 ± 0.094	0.104 ± 0.031	1.281 ± 0.082	0.056 ± 0.033	0.319 ± 0.034	0.125 ± 0.051
60	23.834	1483	1388	Damascenone	0.055 ± 0.028	0.393 ± 0.631	0.329 ± 0.022	0.870 ± 0.681	n.d.	0.049 ± 0.003	0.233 ± 0.404
61	23.930	1486	1490	ß-Ionone	2.243 ± 0.186	1.036 ± 0.070	0.295 ± 0.025	1.272 ± 0.065	0.237 ± 0.005	0.511 ± 0.023	0.673 ± 0.117
62	28.052	1627	1621	Benzophenone	0.282 ± 0.010	0.054 ± 0.031	0.068 ± 0.009	0.062 ± 0.026	0.010 ± 0.001	0.062 ± 0.004	0.050 ± 0.008
63	33.823	1843	1838	Hexahydrofarnesyl acetone	1.878 ± 0.306	1.801 ± 0.128	0.248 ± 0.030	1.130 ± 0.244	0.093 ± 0.002	1.044 ± 0.109	0.218 ± 0.086
64	35.674	1917	1921	Farnesyl acetone	0.940 ± 0.115	0.345 ± 0.119	n.d.	0.458 ± 0.043	0.330 ± 0.120	0.225 ± 0.010	0.155 ± 0.186
	Ketones			Total contents	11.35 ± 2.005	5.866 ± 1.439	1.551 ± 0.164	5.732 ± 1.211	0.809 ± 0.177	2.528 ± 0.209	1.788 ± 0.92
65	12.949	1150	1159	2-ethylhexyl acetate	0.188 ± 0.133	n.d.	n.d.	n.d.	n.d.	0.010 ± 0.005	0.099 ± 0.021
66	14.451	1194	1187	Methyl salicylate	n.d.	n.d.	n.d.	0.221 ± 0.032	n.d.	0.138 ± 0.010	n.d.
67	18.210	1306	1320	2-hydroxybenzoic acid, 1-methylethyl ester	n.d.	0.097 ± 0.021	n.d.	n.d.	0.021 ± 0.006	0.038 ± 0.005	0.360 ± 0.130

(*continued on next page*)

Table 1 – (*continued*)

No.	RT. (min)	RI[a]	RI[b]	Compounds	Relative content[c] (μg/g, mean ± S.D; $n = 3$)						
					SCDT	HBDT	YNDT	HNDT	GXDT	SXDT	GT
68	20.641	1380	1382	(3Z)-3-hexenyl hexanoate	0.296 ± 0.303	0.042 ± 0.005	0.115 ± 0.007	0.039 ± 0.009	0.014 ± 0.001	0.048 ± 0.056	0.078 ± 0.037
69	24.152	1493	n.d.	Tetrahydroactinidiolide	0.220 ± 0.018	0.086 ± 0.010		0.057 ± 0.001	0.125 ± 0.008	0.032 ± 0.003	0.095 ± 0.004
70	25.240	1530	1525	Dihydroactindiolide	1.147 ± 0.184	0.810 ± 0.010	0.275 ± 0.014	0.613 ± 0.065	0.170 ± 0.032	0.360 ± 0.008	0.143 ± 0.086
71	35.840	1923	1928	Hexadecanoic acid, methyl ester	0.335 ± 0.004	0.198 ± 0.041	0.229 ± 0.053	0.392 ± 0.065	0.115 ± 0.005	0.129 ± 0.009	0.119 ± 0.047
72	40.325	2090	2092	(Z,Z)- 9,12-octadecadienoic acid, methyl ester	0.090 ± 0.004	0.060 ± 0.015	0.205 ± 0.017	0.197 ± 0.021	0.087 ± 0.003	0.056 ± 0.003	0.121 ± 0.114
73	40.535	2097	2105	(Z,Z,Z)-9,12,15-octadecatrienoic acid, methyl ester	0.261 ± 0.018	0.260 ± 0.114	0.402 ± 0.027	0.393 ± 0.049	0.188 ± 0.011	0.143 ± 0.005	0.319 ± 0.092
	Esters			Total contents	2.537 ± 0.664	1.553 ± 0.216	1.226 ± 0.118	1.912 ± 0.242	0.72 ± 0.066	0.954 ± 0.104	1.333 ± 0.531
74	12.803	1146	1149	1,2-dimethoxybenzene	0.185 ± 0.037	0.109 ± 0.024	0.449 ± 0.028	0.121 ± 0.017	0.125 ± 0.007	0.063 ± 0.011	n.d.
75	15.958	1239	1230	3,4-dimethoxytoluene	0.223 ± 0.118	0.027 ± 0.003	0.121 ± 0.009	0.052 ± 0.007	0.025 ± 0.002	0.025 ± 0.004	n.d.
76	18.459	1313	1309	1,2,3-trimethoxybenzene	n.d.	0.211 ± 0.019	3.159 ± 0.114	0.220 ± 0.016	0.289 ± 0.100	0.116 ± 0.004	n.d.
77	18.783	1323	n.d.	4-ethyl-1,2-dimethoxybenzene	0.228 ± 0.071	0.075 ± 0.051	0.123 ± 0.008	n.d.	0.024 ± 0.001	0.028 ± 0.003	n.d.
78	20.202	1367	n.d.	4-ethyl-1,2-dimethoxybenzene	0.290 ± 0.306	0.040 ± 0.028	0.042 ± 0.002	0.085 ± 0.013	n.d.	0.083 ± 0.028	0.013 ± 0.003
79	20.329	1371	n.d.	1,2,4-trimethoxybenzene	0.161 ± 0.024	0.062 ± 0.022	1.368 ± 0.132	n.d.	0.337 ± 0.044	n.d.	0.022 ± 0.006
80	22.721	1447	n.d.	1,2,3,4-tetramethoxybenzene	n.d.	0.961 ± 0.083	0.055 ± 0.013	0.483 ± 0.695	0.022 ± 0.002	n.d.	0.043 ± 0.006
81	23.758	1481	n.d.	3,5-dimethoxy-4-hydroxyacetophenone	0.112 ± 0.074	0.027 ± 0.003	0.248 ± 0.020	0.067 ± 0.017	n.d.	n.d.	n.d.
82	24.273	1497	1408	1,2-dimethoxy-4-propenylbenzene	0.245 ± 0.022	0.032 ± 0.006	0.058 ± 0.025	0.091 ± 0.025	n.d.	0.013 ± 0.005	n.d.
	26.010	1558	1559	1,2,3-trimethoxy-5-(2-propenyl)-benzene	0.065 ± 0.020	n.d.	n.d.	n.d.	n.d.		n.d.
	Methoxyphenolics			Total contents	1.509 ± 0.672	1.544 ± 0.239	5.623 ± 0.351	1.119 ± 0.79	0.815 ± 0.156	0.328 ± 0.055	0.078 ± 0.015
83	21.665	1413	1409	α-cedrene	0.057 ± 0.022	0.018 ± 0.008	n.d.	0.106 ± 0.030	n.d.	0.032 ± 0.010	0.053 ± 0.001
84	24.592	1508	1507	α-farnesenea	0.153 ± 0.073	n.d.	n.d.	n.d.	n.d.	0.013 ± 0.011	0.134 ± 0.017
85	63.069	2820	2660	Squalene	0.881 ± 0.126	0.126 ± 0.024	n.d.	0.315 ± 0.028	n.d.	n.d.	n.d.
	Alkenes			Total contents	1.091 ± 0.221	0.144 ± 0.032		0.421 ± 0.058		0.045 ± 0.021	0.187 ± 0.018
86	29.999	1697	n.d.	Hexadecane	0.349 ± 0.035	0.081 ± 0.012	0.043 ± 0.007	0.231 ± 0.052	0.026 ± 0.003	0.042 ± 0.005	0.055 ± 0.011
87	32.652	1797	n.d.	Octadecane	0.169 ± 0.038	0.102 ± 0.059	n.d.	0.110 ± 0.021	n.d.	n.d.	0.046 ± 0.020
88	35.171	1896	n.d.	Nonadecane	0.127 ± 0.003	0.097 ± 0.016	n.d.	0.073 ± 0.020	n.d.	n.d.	n.d.
89	37.614	1996	n.d.	Eicosane	n.d.	0.254 ± 0.034	n.d.	n.d.	n.d.	n.d.	n.d.
90	47.985	2295	n.d.	Octacosane	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.306 ± 0.073
91	56.020	2496	n.d.	Tetracosane	n.d.	0.087 ± 0.002	n.d.	0.141 ± 0.019	n.d.	0.071 ± 0.028	0.494 ± 0.167
92	60.734	2693	n.d.	Eicosane	n.d.	0.088 ± 0.010	n.d.	0.124 ± 0.026	n.d.	0.100 ± 0.052	0.210 ± 0.088
	Alkanes			Total contents	0.645 ± 00.073	0.709 ± 00.133	0.040 ± 00.007	0.679 ± 00.138	0.026 ± 00.003	0.213 ± 00.085	1.111 ± 00.359
93	9.564	1048	1016	1-methyl-1H-pyrrole-2-carboxaldehyde	n.d.	n.d.	0.258 ± 0.014	n.d.	n.d.	0.013 ± 0.003	0.243 ± 0.279
94	7.732	990	996	2-pentylfuran	2.185 ± 0.229	1.306 ± 0.417	0.042 ± 0.007	0.255 ± 0.018	0.034 ± 0.003	0.184 ± 0.012	0.159 ± 0.046

95	17.791	1293	1292	Indole	0.765 ± 0.141	0.410 ± 0.075	n.d.	0.223 ± 0.024	0.076 ± 0.021	0.060 ± 0.005	0.324 ± 0.083
96	26.665	1579	1583	Fluorene	0.212 ± 0.037	0.066 ± 0.008	0.044 ± 0.001	n.d.	0.028 ± 0.006	0.054 ± 0.004	0.079 ± 0.016
97	28.599	1647	1649	2,6,10-trimethyl-pentadecane	0.219 ± 0.124	n.d.	n.d.	n.d.	n.d.	0.040 ± 0.001	n.d.
98	50.618	2353	1375	(Z)-9-octadecenamide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.322 ± 0.175
	Others			Total contents	3.381 ± 0.531	1.782 ± 0.500	0.344 ± 0.022	0.478 ± 0.042	0.138 ± 0.030	0.351 ± 0.025	1.127 ± 0.599

[a] RI = retention indices calculated in the experiment.

[b] RI = retention indices in the literature or National Institute of Standards and Technology website; volatiles were identified according to the following: MS = mass spectrum comparison using National Institute of Standards and Technology libraries; RI = retention index in agreement with literature value.

[c] μg/g = concentration was expressed in microgram per gram of tea samples, ethyl decanoate as internal standard, and data listed were the mean of three assays ± standard deviation. GT = Green tea sample; GXDT = Guangxi dark tea; HBDT = Hubei dark tea; HNDT = Hunan dark tea; n.d. = not detected or RI was not provided in literature; RI = retention index; SCDT = Sichuan dark tea; SXDT = Shanxi dark tea; YNDT = Yunnan dark tea.

different times were detected by GC-MS to analyze the dynamic changes of aroma of tea samples in the process of fermentation. According to the GC-MS results, we discovered the regularity of flavor compounds in the process of fermentation.

Geraniol is the aroma of rose and geranium. It was also considered as the typical flavor compound of green tea. During the solid-state fermentation, the content of geraniol is rapidly increased in 24 hours, but after 48 hours this flavor compound was not detected in the fermented tea leaves (Figure 3). This result suggested that the green tea aroma may disappear after long-term postfermentation, but short-term fermentation may assist in enhancing the flavor of green tea. In the industrial production, the postfermentation could last for months, even years, so the flavor of CDTs is highly different from green tea.

3.4. *Change of marker volatile compounds during solid-state fermentation by* E. cristatum

E. cristatum is usually called "golden flower" in the fu-brick tea (HNDT and SXDT). *Cis*-jasmone, α-terpineol, ß-ionone, nonanal, and 2-pentylfuran were detected in the tea leaves fermented by *E. cristatum* (Figure 4). *Cis*-jasmone showed a strong floral element and makes a highly significant contribution to the profile of oolong tea [16]. In the tea samples fermented by *E. cristatum*, this compound may give the HNDT the special aroma. The changes of ß-ionone and *cis*-jasmone were similar to the results obtained from the fermentation by *A. niger*.

2-Pentylfuran with a burnt and sweet odor was increased during the fermentation. In comparison with YNDT, the content of 2-pentylfuran in HNDT was significantly higher. Its formation may be related to the oxidation of linoleic acid. Furthermore, the change of α-terpineol was similar to that of geraniol, which could also be produced by the hydrolysis of glycosidic aroma precursors [17,18].

4. Discussion

Comparing with the volatile compounds of green tea, the CDTs were highly different. It was suggested that some typical green and fresh odorants were degraded and transformed into typical volatile compounds of CDTs during postfermentation. For example, CDTs contained higher contents of ketones which were mainly derived from the oxidation and degradation of fatty acids and carotenoids. It is well-known that these ketones are very important flavor compounds in various teas, providing a special floral and woody odor. Furthermore, the total acids (long chain fatty acids) of CDTs were significantly increased. It was suggested that the esters were hydrolyzed into fatty acids by the extracellular lipase produced by microorganism.

Through the multivariate analysis of GC-MS, YNDT and GXDT were distinguished from SCDT, SXDT, HBDT, and HNDT. Class prediction analysis has proven to be a valuable technique in characterization and authentication of traditional medicinal plants [19]. The class prediction model allows assigning categories into previously determined groups in an unbiased analysis. The OPLS-DA analysis gave some critical volatile compounds responsible for the classification of various CDTs.

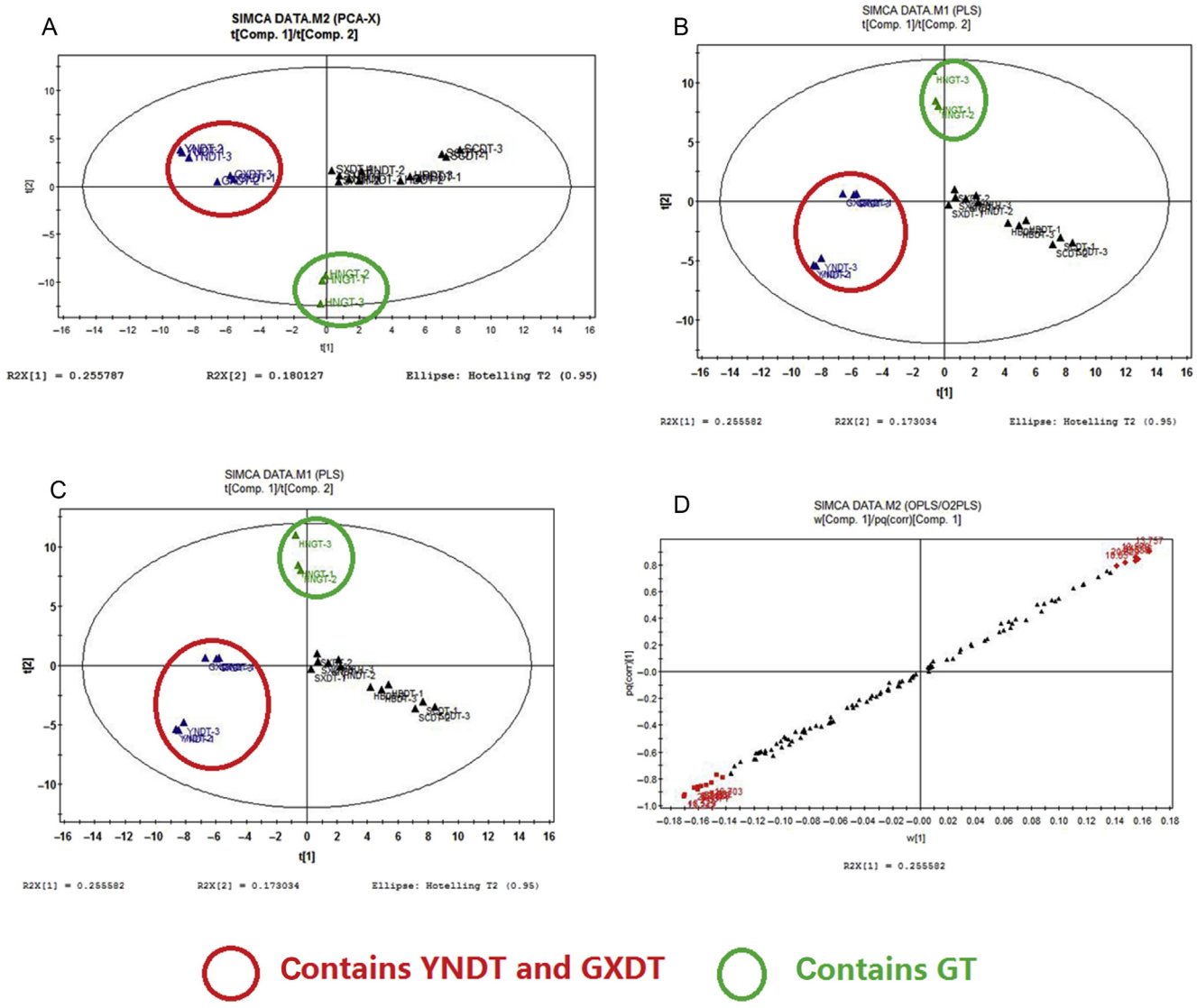

Figure 2 – Classification of various Chinese dark teas using principal component analysis, partial least squares (PLS), and orthogonal projection on latent structure-discriminant analysis (OPLS-DA) with gas chromatography–mass spectrometry profiles. (A) Score plot of principal component analysis; (B) score plot of PLS; (C) score plot of OPLS-DA; (D) S-plot of various Chinese dark teas under the OPLS-DA model. GT = green tea; GXDT = Guangxi dark tea; HBDT = Hubei dark tea; HNDT = Hunan dark tea; SCDT = Sichuan dark tea; SXDT = Shanxi dark tea; YNDT = Yunnan dark tea.

For example, eight compounds including (*E*,*E*)-2,4-decadienal, 1,2-dimethoxybenzene, 1,2,4-trimethoxybenzene, geraniol, linalool oxide II (pyranoid), 9,12-octadecadienoic acid, trans-linalool oxide (furanoid), α-terpineol, and 3,5-dimethoxy-4-hydroxyacetophenon showed the highest contents in YNDT. Among these marker compounds, methoxyphenolic compounds have been reported as the main typical compounds in YNDT (pu-erh tea). Other marker compounds also contributed to the classification of various CDTs. They are geraniol, 2,4-heptadienal, *cis*-jasmone, 2-nonenal, geranylacetone, 2-pentylfuran, (*E*,*E*)-2,4-heptadienal, ß-ionone, and nonanal. *Cis*-jasmone and ß-Ionone are the potent flavor compounds of flora. Geranylacetone was also the abundant compound in fu-brick tea (HNDT).

The differences of volatile compounds of CDTs may be related to its own manufacturing process and the predominate fungi involved in fermentation. During the fermentation of tea leaves by *A. niger*, The geraniol derived from the glycosidic aroma precursors, which could be hydrolyzed to release the free form of geraniol by hydrolase secreted by *A. niger* [20,21]. But after long-term fermentation, this compound may be degraded and oxidized. ß-ionone is known for violet aroma and described as a complex woody and fruity scent [22]. It is known that ß-ionone is synthesized from carotenoids in the tea by oxidative degradation or enzymatic oxidation. It is also the typical flavor of green tea and oolong tea with low odor threshold [23]. In the CDTs, ß-ionone also decreased to a very low content after long-term fermentation.

Table 2 – The VIP variables responsible for the classification of six types of Chinese dark teas (CDTs).

No.	Retention time (min)	Compound identification	Highest relative content in CDTs	Lowest relative content in CDTs	VIP index
1	18.529	(E,E)-2,4-decadienal	YNDT	GXDT	1.288
2	12.803	1,2-dimethoxybenzene	YNDT	SXDT	1.285
3	20.329	1,2,4-trimethoxybenzene	YNDT	HNDT/SXDT	1.283
4	16.506	Geraniol	SCDT	HNDT/GXDT/SXDT	1.273
5	8.311	2,4-heptadienal	SCDT	YNDT	1.273
6	13.757	Linalool oxide II(pyranoid)	YNDT	SXDT	1.268
7	21.232	cis-jasmone	SCDT	YNDT/GXDT	1.260
8	13.242	2-nonenal	SCDT	YNDT/GXDT	1.256
9	22.880	Geranylacetone	SCDT	YNDT/GXDT	1.255
10	7.732	2-pentylfuran	SCDT	YNDT/GXDT	1.238
11	41.947	9,12-octadecadienoic acid	YNDT	GXDT	1.237
12	7.879	(E,E)-2,4-heptadienal	SCDT	YNDT	1.229
13	10.850	Trans-linalool oxide (furanoid)	YNDT	HBDT	1.229
14	23.758	3,5-dimethoxy-4-hydroxyacetophenon	YNDT	GXDT/SXDT	1.218
16	14.336	α-terpineol	YNDT	HBDT	1.217
16	23.930	ß-ionone	SCDT	GXDT	1.213
17	11.371	Nonanal	SCDT	GXDT	1.208

GT = Green tea sample; GXDT = Guangxi dark tea; HBDT = Hubei dark tea; HNDT = Hunan dark tea; SCDT = Sichuan dark tea; SXDT = Shanxi dark tea; VIP = variable importance in the projection; YNDT = Yunnan dark tea.

These compounds are the critical volatile compounds responsible for the classification of various dark teas. The volatile components of tea could be affected by diverse factors such as maturity of tea leaves, variation of tea plants and processing conditions. To explore the flavor compounds of tea, GC-MS were the regular tools, but the unbiased metabolomics analysis of GC-MS dataset could provide more chemical classification for various teas, or tea samples during different process. In the present study, various CDTs were analyzed using GC-MS, the datasets of which were

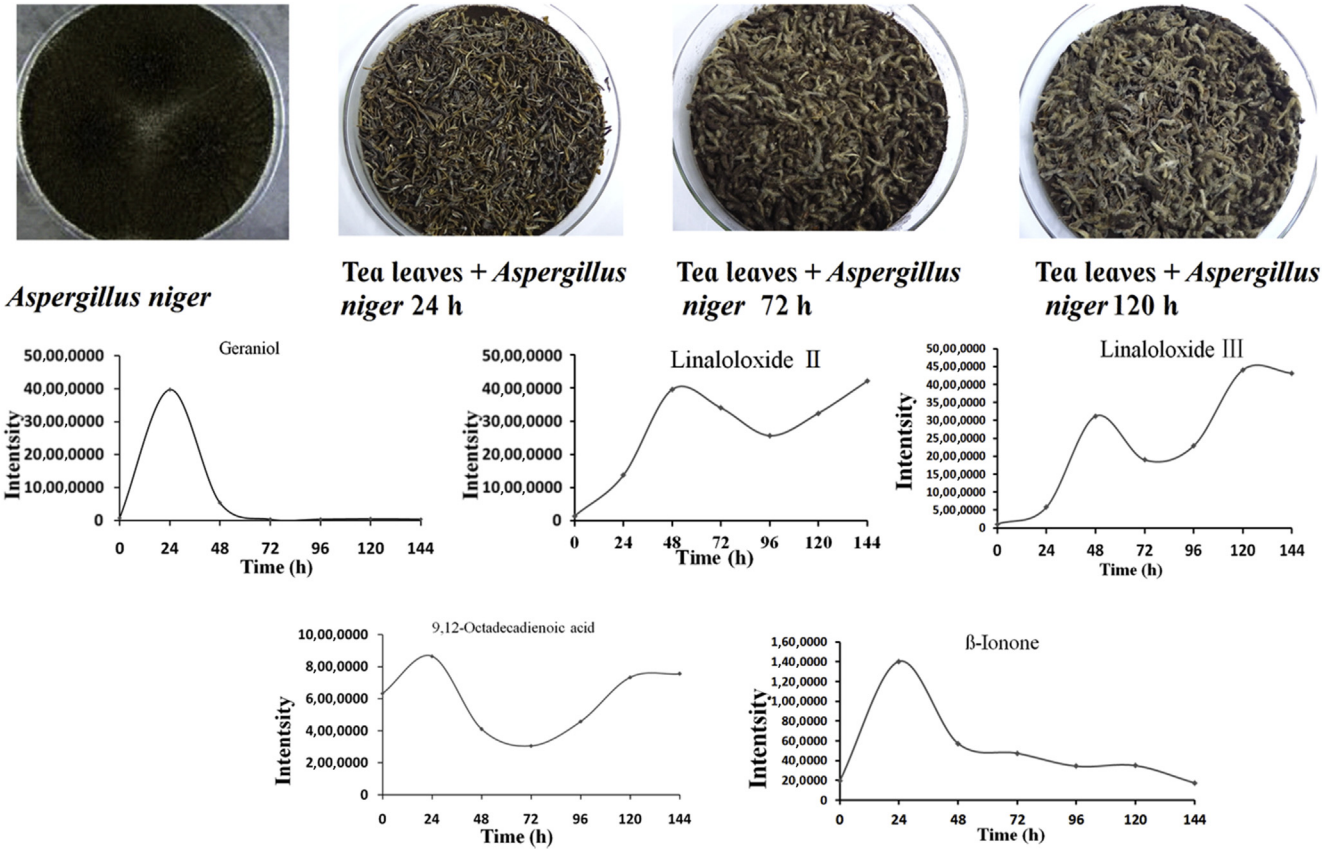

Figure 3 – The trajectories of marker volatile compounds in mature tea leaves fermented with *Aspergillus niger* from 0 hours to 120 hours.

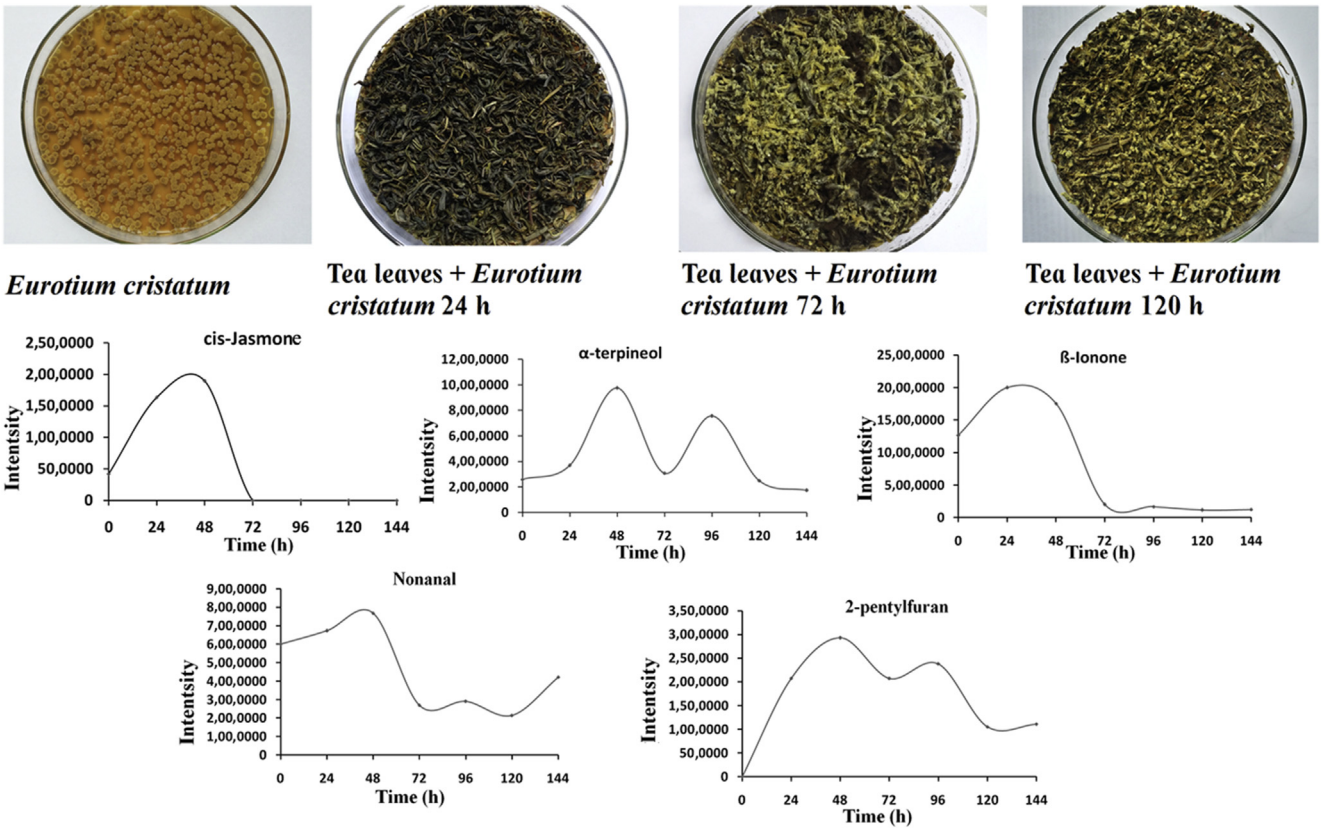

Figure 4 – The trajectories of marker volatile compounds in mature tea leaves fermented with *Eurotium cristatum* from 0 hours to 120 hours.

subsequently analyzed using multivariate analysis models. The results successfully reclassified and obtained some critical marker compounds being responsible for the properties of each cluster.

To clarify the relationship between these marker compounds and the effects of dominant fungi in post-fermentation, an artificial solid-state fermentation model was established. The contents of volatile compounds of mature tea samples during fermentation were continuously analyzed. It showed that the post-fermentation promoted the production and transformation of some volatile compounds. The hydrolysis of glycosidic aroma precursors produced α-terpineol and geraniol by the glycosidase(s) of *A. niger* and *E. cristatum*. However, these typical alcohols were almost undetectable after long-term fermentation. Although it was supposed that the methoxyphenolic compounds were degraded from gallic acid by the methylation of microorganisms, the apparent increment did not occur for methoxyphenolic compounds following the solid-state fermentation with *A. niger*. It was suggested that the generation of methoxyphenolic compounds may need a long-term fermentation or the participation of other microorganism in the postfermentation.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfda.2016.11.020.

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