Beverage Plant Research

ARTICLE

Open Access

https://doi.org/10.48130/bpr-0024-0001

Beverage Plant Research **2024**, 4: e009

Revealing the flavor profile of citrus Pu-erh tea through GC-MS-O and untargeted metabolomics

Jiaqing Sun[1#], Weitong Cai[1#], Tao Feng[1*], Da Chen[2], Jun Lu[3], Shiqing Song[1], Lingyun Yao[1], Min Sun[1], Huatian Wang[1], Qian Liu[1] and Chuang Yu[1]

[1] *School of Perfume & Aroma Technology, University of Shanghai Institute of Technology, Shanghai 201418, China*
[2] *Department of Animal, Veterinary and Food Sciences, University of Idaho, 875 Perimeter Drive, Moscow, ID83844, USA*
[3] *Auckland Bioengineering Institute, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand*
[#] These authors contributed equally: Jiaqing Sun, Weitong Cai
* Corresponding author, E-mail: fengtao@sit.edu.cn

Abstract

Chinese citrus Pu-erh tea is recognized for its unique flavor, which is composed of key aroma-active compounds and affected by taste-impact metabolites. In this study, the whole citrus Pu-erh tea (CP), its out-layer fruit (OF) container and inside tea (IT) powder, were analyzed by solvent-assisted flavor evaporation (SAFE) coupled with GC-MS-O and UHPLC-MS/MS. As a result, 47 important volatiles were identified, including 27 (IT), 30 (OF) and 27 (CP) volatiles that were screened out based on their odor activity value (OAV) and aroma character impact value (ACI), and further validated by aroma omission/recombination experiment. Combined with the sensory evaluation and PLSR model, the aroma profile of CP was characterized with the following ten flavor attributes: sweet (vanillin); floral (β-ionone); fruity (methyl anthranilate, methyl methanthranilate, citronellal); roasted (thymol); musty (p-cymene), woody (perillaldehyde); herbal (linalool, α-terpineol); phenolic (2,4-di-tert-butylphenol, p-cresol); minty (dihydrocarvone); and fatty (octanoic acid) volatiles. As for the non-volatile taste-impact chemicals, the most prominent metabolites were identified as flavonoids that mainly contributed to the taste of bitter (catechin, epicatechin, gallocatechin), astringency (leucopelargonidin) and sweet (neohesperidin). This novel finding has provided an insight and better understanding of the aroma profile of citrus Pu-erh tea and some guidance for flavor pairing and taste improvement.

Citation: Sun J, Cai W, Feng T, Chen D, Lu J, et al. 2024. Revealing the flavor profile of citrus Pu-erh tea through GC-MS-O and untargeted metabolomics. *Beverage Plant Research* 4: e009 https://doi.org/10.48130/bpr-0024-0001

Introduction

Citrus Pu-erh tea (CPT), also known as Ganpu tea, is a novel type of citrus blend-tea that is co-fermented with citrus peel (*Citrus reticulata* Blanco cv. Chachiensis) and Pu-erh tea (*Camellia sinensis* var. *assamica*)[1]. The earliest record of tea processed with citrus peel appeared 1,400 years ago in the Tang Dynasty[2]. Nowadays, thousands of enterprises have been involved in the production of citrus tea which was tailored to satisfy consumers' flavor desires along with the emergence of food-pairing hypothesis[3]. The citrus Pu-erh tea which was made by peel produced between August and September in Xinhui District of Guangdong, China, and the ripened Pu-erh tea produced in Yunnan, China so-called 'Xiao Qing Gan' is the most popular, and is familiar with most consumers[4,5]. However, few systematic studies have been conducted on its flavor.

The mellow taste and hedonistic aroma of citrus Pu-erh tea were generated during stuffing fermented tea to citrus pericarps and then redrying them together[2,6]. During the sun-drying and fermentation process, numerous enzymes and compounds were catalyzed to transform in the citrus peels, producing specific compounds with a fruity aroma[4]. For example, flavonoid glycosides such as hesperidin and phenolic acids were usually considered to be the critical flavor contributors[2,7]. Meanwhile, the degradation, oxidation, glycosylation and other reactions that occur in Pu-erh tea under conditions of high humidity and temperature with microorganisms also lead to the generation of volatile compounds[7]. The previous results have investigated the impact of different citrus species on CPT and showed that the fundamental odorants associated with the aroma of citrus blend black teas were mainly the outstanding combination of heptanal, limonene, linalool, and trans-β-ionone[8,9]. Wang et al. also demonstrated that an interaction of various volatiles originating from white tea and citrus occurred and significantly changed the properties of their olfactory properties[10]. Thus, the tangy aroma of the citrus peel is a perfect match for the mellow earthiness of Pu-erh tea, resulting in its attractive aroma.

Flavor wheel had a strong advantage for revealing the flavor characteristics of samples. Flavor wheel is a quantitative analysis tool that standardizes the quality of a sample, and flavor descriptions are organized according to categories and arranged in a disc-shaped frame that is systematic yet concise and clear[11,12]. Flavor is affected by both aroma (volatiles) and taste (non-volatiles)[13]. In 1987, Suffet et al. created the first drinking water flavor wheel containing both olfactory and gustatory descriptions to represent the diversity of odor and aroma qualities[14], while the Specialty Coffee Association of America (SCAA) developed the first flavor wheel in 1995, which has now been updated to a more detailed and comprehensive

flavor wheel with three levels and nine categories[15]. The establishment and updating of flavor wheel can enable professional sensory evaluation groups or amateur consumers to have more standards and bases for judging when conducting sensory evaluations and favoritism tests[16,17], which shows that flavor wheel is instructive for the industry in new product research and development, while for academic research, it has a great advantage in revealing the composition of aroma and odor characteristics. In addition, with the flavor wheel providing the basic framework for the formation of the aroma and odor of that sample, and a rough grasp of the compounds corresponding to each kind of flavor, it can shed some light on food cooking and even flavor pairing[18,19].

Based on this, in this paper, the solvent-assisted flavor evaporation (SAFE) with high recovery rate was used to extract the three different parts of the CPT (CP, OF, IT), which was combined with the GC-MS-O method to analyze the volatile chemical components. And then the untargeted metabolomics by ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS/MS) is to study its non-volatile compounds, in order to identify the different metabolic pathways and metabolites affecting the flavor of our CPT. After obtaining the key volatile compounds and non-volatile compounds, a more complete flavor wheel was drawn by combining both of them with the sensory evaluation results for co-analysis, so as to reveal the crucial compounds affecting the flavor of CPT brewing and the differences among the three parts of CPT. This study will significantly increase the knowledge of CPT infusion flavors and provide insights into manufacturing techniques for CPT qualities that will enable companies to improve the quality of blended tea in the future to meet the marketing standards of the products in question. It will also provide data for future flavor matching practices.

Materials and methods

Sample preparation of citrus Pu-erh tea

Citrus Pu-erh tea samples which were processed by full-sunlight withering and have been stored for five years were purchased from local tea plantations in Jiangmen City (Guangdong, China) in October 2021. In these samples, the citrus peel of Xiao Qing Gan refers to the citrus peel produced in Xinhui, Guangdong province, while Pu-erh tea refers to the sun dried green raw tea of Yunnan large leaf tea. The purchased samples were vacuum-packed and stored at room temperature until usage. Before the chemical analyses, the sample was divided into three parts: out-layer fruit (OF) peel, inside tea (IT) powder, and whole citrus Pu-erh tea (CP). Samples were ground into small particles and passed through 30 mesh filter. The infusion was prepared by the methods reported earlier[20]. Each sample was taken by 5 g and mixed with 100 mL of boiling water for 5 min to take the 1st round of extract, the residues were repeated by mixing with 75 mL of boiling water at two time intervals (3 min and 2 min respectively). Then, the three rounds of each infusion (extract) were combined after filtration and cooled in an ice bath for subsequent instrumental analysis and sensory evaluation.

The preparation of metabolites was as follows: Each powdered samples (i.e., CP, IT, OF) in 0.05 g constant weight was respectively mixed with 400 μL of methanol-acetonitrile (1:1) (v:v) solvent in a 2 mL centrifuge tube. After the mixture was ground for 6 min (−10 °C, 50 kHz) and extracted using low-temperature ultrasonic extraction for 30 min (5 °C, 40 kHz), the extract was placed at −20 °C for 30 min and then centrifuged for 15 min (13,000 g, 4 °C) to precipitate the dissolvable residues. Finally, the supernatant was taken for analysis.

Chemicals

The following authentic standards were commercially purchased, including (+)-limonene (≥ 99%), γ-terpinene (≥ 95%), (+)-dihydrocarvone (98%), (E)-p-mentha-2,8-dien-1-ol (98%), (−)-pinocarveol (98%), (+)-carvone (≥ 98%), perillaldehyde (98%) from Sigma-Aldrich (Shanghai, China). 2,3-dimethyl pyrazine (98%), nonanal (95%), phenol (≥ 99%), 2- pyrrolaldehyde (98%), carvacrol (99%), methyl anthranilate (99%), 2,4-di-tert-butylphenol (97%) from TCI (Shanghai, China). 2,4-dimethyl styrene (98%), piperitone (> 94%), p-cymene (99%), prenol (≥ 99.5%), decanal (97%) and limonene glycol (98%) from Aladdin (Shanghai, China). (Z)-carveol (97%), p-cymenol (99%), linalool (≥ 98%), perillalcohol (≥ 98%) from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 2-ethylhexanol (99%), β-pinene (95%), methyl methanthranilate (98%), palmitic acid (99%), benzophenone (99.5%), dimethyl sulfone (99%) and 1,2-dimethoxybenzene (98%) from Merck (Darmstadt, Germany). β-ionone (98%), citronellal (≥ 98%) and vanillin (99.5%) from Yuanye Bio-Technology Co., Ltd (Shanghai, China). 4-methoxyacetophenone (99%), styrene (> 99.5%), o-Cresol (≥ 99.7%), p-Cresol (≥ 99.7%), terpinen-4-ol (≥ 98%), (−)-carveol (97%) and α-terpineol (≥ 96%) from Macklin (Shanghai, China). Octanoic acid (≥ 99.9%), thymol (> 99%), lauric acid (98%), benzaldehyde (> 99%), benzyl alcohol (≥ 99%) and phenylethyl alcohol (≥ 99%) from Boer (Shanghai, China).

C_7–C_{30} (n-alkanes) and 1,2-dichlorobenzene (internal standard) were purchased from Sigma-Aldrich (Shanghai, China). Dichloromethane, anhydrous sodium sulfate, acetone and sodium chloride were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All reagents were of analytical grade.

Solvent-Assisted Flavor Evaporation (SAFE)

Isolation of the volatiles was performed according to a typically adopted method[21]. For each sample, 100 mL of infusion and 100 μL of 1,2-dichlorobenzene (100 mg/kg, solved in acetone) were mixed and extracted with dichloromethane (3 × 100 mL) at 200 rpm using a magnetic stirrer (85-1, Shanghai Meiyingpu Instrument Manufacturing Co., Ltd., Shanghai, China) at room temperature for 3 h (3 × 1 h). Afterwards, all of the organic phase extract was combined, filtered, and concentrated to 150 mL using a rotary evaporator (RE 52-86A, Shanghai Yarong Biochemical Instrument Factory, Shanghai, China), then poured into a 500 mL distillation flask of SAFE apparatus (Glasbläserei Bahr, Manching, Germany) under a 40 °C water bath and 5 × 10^{-5} mbar vacuum to concentrate the organic phase, and separate the volatiles from the nonvolatile fraction. Each extract of SAFE was further concentrated to 5 mL by a rotary evaporator under 40 °C and finally concentrated to 1 mL by a nitrogen stream. The concentrate was immediately analyzed in Dr Feng's lab.

Gas Chromatography–Mass Spectrometry–Olfactometry (GC–MS–O) analysis

A gas chromatograph 6890A equipped with a 5975C mass selective detector (Agilent Technologies, Santa Clara, CA, USA) and an ODP-3 olfactory detection port (Gerstel, Mühlheim an

der Ruhr, Germany) was used for flavor analyses. Separation of the volatiles was performed using two fused silica capillaries: HP-INNOWAX and HP-5 (both 60 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies, USA) at a flow rate of 1.0 mL/min. The flow split ratio at the end of the column was 1:1 between the detector and olfactory port. The SAFE extract (2 μL) was injected into the injection port at 250 °C in a splitless mode. The oven temperature was programmed at 40 °C (held for 3 min), ramped at 5 °C /min to 100 °C (held for 1 min), then ramped to 180 °C at a rate of 3 °C /min, and ramped to 230 °C at a rate of 4 °C /min and held at 230 °C for 5 min. Mass spectrometer condition was set at electron ionization (EI) mode with the ionization energy of 70 eV and the ion source temperature of 230 °C. The scan range was 30–450 m/z in full-scan mode.

Five trained panelists (two males and three females, aged 23–42 years, nonsmokers) were selected for GC-O analysis. The retention times (RTs), odor attributes smelled from the sniffing port, and aroma intensities (AIs) were recorded. The AIs were evaluated on a five-point intensity scale: 0 (none), 3 (moderate) and 5 (strong). Each sniff was performed in triplicate and consisted of two evaluation sessions for the compounds that were eluted between 0–30 min and 31–60 min to minimize nasal discomfort and fatigue[22].

Metabolite analysis based on UHPLC–MS/MS

An UHPLC-Q Exactive HF-X system was used to separate and analyze the metabolites. An ACQUITY HSS T3 (100 mm × 2.1 mm i.d., × 1.8 μm; Waters, Milford, USA) column was used for chromatographic separation of the metabolites. Mobile phase A was composed of 95% water and 5% acetonitrile (containing 0.1% formic acid), and mobile phase B was composed of 47.5% acetonitrile, 47.5% isopropanol and 5% water (containing 0.1% formic acid). The injection volume was 3 μL, and the column temperature was set at 40 °C. The details of the gradient elution procedure and the experimental parameters were the same as a previous study[23].

Identification and quantification of the volatile compounds

Identification of the volatiles was based on mass spectra compared with NIST Mass Spectral Library 11 Vision; standard chemicals; odor descriptions of authentic, and retention indices (RI) with reference values (https://webbook.nist.gov/chemistry/). The retention indices (RI_x) of detected chemicals was calculated as below: $RI_x = 100Z + 100[(\lg t_x - \lg t_z)/(\lg t_{z+1} - \lg t_z)]$, where Z is the number of carbon atoms of n-alkane which appears in front of the identified compound in the same GC condition; t_x, t_z and t_{z+1} are the retention time of the identified compounds, the lower alkane, and upper alkanes, respectively[24].

Quantification of the volatiles was calculated according to the standard curves. Firstly, three infusion samples were extracted by dichloromethane until achieving each odorless matrix. The standard chemicals were then dissolved and diluted with the artificial odorless matrix at a concentration ranging from 50 to 30,000 mg/L for six levels (1:5, 1:25, 1:100, 1:250, 1:500 and 1:1,000). Each standard chemical matrix (100 mL) with 100 μL of 1,2-dichlorobenzene was extracted by the SAFE procedure and finally analyzed by GC-MS (As described above). Calibration curves were constructed by the following formula[24]: $A_x/A_i = a(C_x/C_i) + b$. A and C represent the peak area and the concentration, while x and i represent the authentic compound and internal standard, respectively. The concentration of each volatile compound was calculated based on the calibration equation above. The result was an average of three replicates. The limit of detection (LOD) and the limit of quantitation (LOQ) was defined as the concentration of a standard compound whose signal-to-noise (S/N) ratio was 3 and 10, respectively[25].

Aroma Extract Dilution Analysis (AEDA)

Each concentrated original solution of SAFE was stepwise diluted with dichloromethane for proportions of 1:2; 1:4; 1:8 … 1:2n and submitted to GC-O analysis under the same GC conditions as described above using an HP-INNOWAX column. The maximum dilution factor of a sample (2n) in which no odorant could be detected by GC-O was defined as the flavor dilution factor (FD)[21]. The larger FD values indicate the greater contribution of the aroma compound to the overall aroma.

Calculation of aroma contribution index

Odor activity value (OAV) was calculated as the ratio of the concentration to the odor detection threshold in water. Aroma character impact value (ACI) is the fraction of the sum of OAV for individual compounds in a mixture, which can further estimate the aroma contribution of individual components[26]. It is calculated as the following formula: $ACI\% = (P_i/T_i)/(\sum P_k/T_k)$, where ∑P is the sum of concentration percentage of all compounds, T is the odor threshold of the compounds in the water[27].

Sensory evaluation

The sensory evaluation of the three infusion samples was performed using quantitative descriptive analysis. The sensory evaluation procedures were carried out according to Wang et al. with slight modification[28]. Thirty-five healthy and non-smoking assessors were recruited from the students and staff members of the School of Perfume and Aroma Technology (Shanghai Institute of Technology, Shanghai, China). A panel of 10 well-trained panelists (five males and five females with age ranging from 20–42 years) were selected for their familiarity with three infusion samples based on the enforced triangle test. Before sensory evaluation, all panelists were trained about the characteristics of infusion samples and the sensory evaluation requirements (such as the definition of quality attributes and the method of scoring) for more than 2 h per day and lasted a week to familiarize them with the descriptive terms of the infusion. Thereafter, the vocabulary of CPT infusion samples' sensory attributes was generated. In addition, the panelists were trained to reach consensus on rating the intensity of the ten defined aroma attributes, including 'sweet', 'minty', 'fruity', 'woody', 'fatty', 'phenolic', 'roasted', 'floral', 'herbal', and 'musty' which were identified using reference compounds of vanillin, dihydrocarvone, prenol, perillaldehyde, octanoic acid, 2,4-dimethyl styrene, 2-pyrrolaldehyde, benzophenone, 2-ethylhexanol, and p-cymene, respectively. Each sensory attribute was taken on a 10-point intensity scale (0–3, weak; 4–6, middle; 7–9, strong). To validate the reliability of the intensity scale, the recorded data of repeated panel performances were compared using different means of the analysis of variance (ANOVA).

The sensory analysis was performed at room temperature under daylight with individual booths. Before sensory evaluation, the infusion samples were presented in plastic cups

labeled with randomly selected three-digit numbers. The assessors were asked to take three short sniffs to sense the aroma of the samples first and to rinse their mouths with pure water to minimize any residual effect. Each sample was evaluated in triplicate and carefully scored after sensory judgment.

E-tongue measurements for taste evaluation

The E-tongue (TS-5000Z, Insent Inc., Japan) comprises lipid membrane sensors of basic tastes (umami, sourness, sweetness, saltiness, bitterness, astringency) and corresponding aftertaste (aftertaste-astringency, aftertaste-bitterness and richness) was used. The sensors were conditioned by a conditioning, calibration and diagnostic process before the analysis. Reference solution (30 mM KCl and 0.3 mM tartaric acid aqueous) and washing solution (30% ethanol adding 100 mM hydrochloric acid for the negatively charged sensors; 100 mM potassium chloride and 10 mM potassium hydroxide for the positively charged sensors.) were prepared[29]. Three measurement phases were performed as follow: sample detection (120 s), aftertaste detection (40 s), and washing (10 s). The average taste strength value from 110 to 120 s during sample detection was calculated to be the final result. Each sample was measured in triplicate, and each tea infusion was measured four times[30].

Statistical and data processing

The statistical data from GC-MS was analyzed by Microsoft Excel 2019 (Microsoft, Redmond, WA, USA) and expressed as mean ± standard deviation (SD). The descriptive analysis data was evaluated by SPSS version 26.0 (SPSS Inc., Chicago, IL, USA) and performed one-way analysis of variance (ANOVA). The significant differences ($p \leq 0.05$) among individual samples for each aroma attribute were identified by the SNK test. Other figures were made by Origin Pro 2021 (OriginLab Corporation, Northampton, MA, USA). The correlations between sensory attributes and volatile compounds were analyzed by PLSR using the Simca 14.1 (Umetrics, Sweden). The identification of metabolites was based on biochemical databases, such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg). The data were analyzed on the online platform of Majorbio Cloud Platform. Heatmap and bubble diagram were employed using the scipy (Version 1.0.0) Python on the Majorbio Cloud Platform (https://cloud.majorbio.com).

Results and discussion

Sensory evaluation and E-tongue analysis of CPT infusion

The sensory evaluation of the three samples (CP, IT, OF) showed significantly different results ($p \leq 0.01$ or $p \leq 0.001$) (Supplemental Table S1). As shown in Fig. 1a, the CP sample had more prominent aromas in characteristics of phenolic (7.75), fatty (6.00) and minty (4.75) flavors, the IT sample had more roasted (7.25), musty (8.00) and herbal (7.25) aromas, and the OF sample outperformed the other two samples in woody (3.00), fruity (3.25), and floral (7.00) aroma attributes. However, these three samples had similar scores in the aroma of fruity attribute (IT = 2.75, OF = 3.25, CP = 2.25, $p \leq 0.01$).

Figure 1b showed the significant difference ($p \leq 0.01$) in the taste attributes of three CPT infusion samples according to the data of Supplemental Table S2 analyzed by E-tongue. Except for the sweetness and sourness, all the other seven attributes showed the highest scores in the IT sample and the lowest scores in the OF sample. This indicates that the taste attributes' intensity of CP sample was moderated by the IT and OF. For the score of sweetness attribute, it seems there is a synergistic effect between the OF and IT, making the CP samples with the highest score in sweetness. Overall, the differences in the scores of the aromas and taste attributes among the three CPT samples were obvious, which could be clearly distinguished even after years of co-fermentation. Thus, the differences in their specific substances deserve further study.

Identification and quantitation of volatiles by SAFE-GC-MS-O

Based on the four identification methods (Table 1), 47 volatile compounds were identified, including six alkenes, six phenols, 14 alcohols, seven aldehydes, six ketones, two esters, three acids, and three others. The aroma descriptions and FD factors of aroma-active compounds determined by AEDA were also listed (Table 1). Compounds with low FD factors are assumed to be less or not important for odor impressions[22]. The results showed that the FD factor of one compound varied greatly from sample to sample. For example, 2,4-dimethyl styrene (FD = 2,048), α-terpineol (FD = 1,024), p-cymenol (FD = 128), 2-ethylhexanol (FD = 2,048), and phenylethyl alcohol (FD

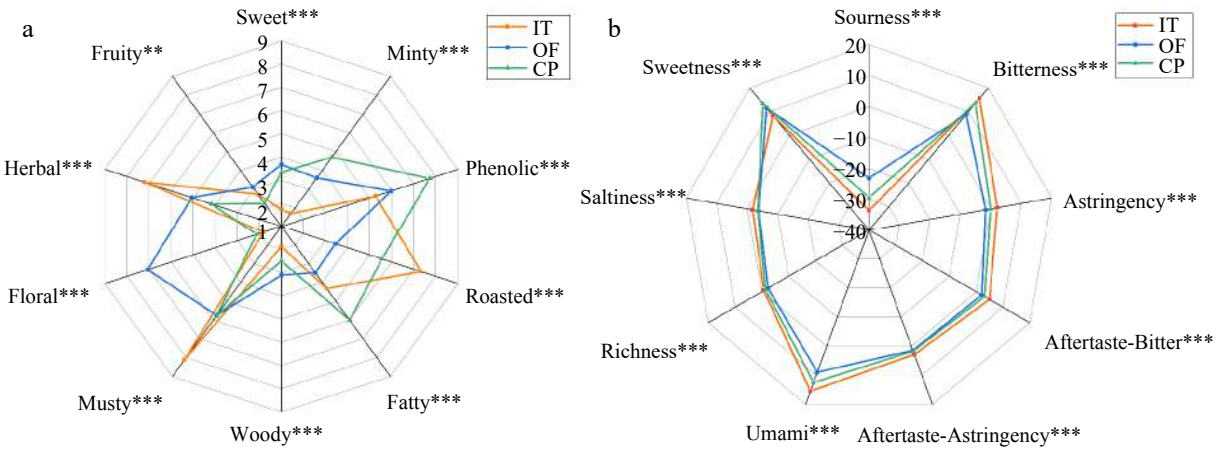

Fig. 1 Sensory spider plot of three CPT infusion samples, (a) sensory evaluation of three CPT samples based on ten aroma attributes, (b) taste profiles of three CPT samples by E-tongue. Note: *, ** and *** significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

Table 1. Identification analysis of volatile compounds in citrus Pu-erh tea samples.

No.	Compounds	RI[a]				Aroma description[b]	FD[c]			IM[d]
		HP-INNOWAX		HP-5MS						
		cal.	ref.	cal.	ref.		IT	OF	CP	
Alkenes										
A1	styrene	1295	1272	–	890	sweet, balsam, floral(acacia)	2	1	–	MS, RI, S, O
A2	β-pinene	1135	1115	–	978	woody, pine, hay, green	2	–	–	MS, RI, S, O
A3	(+)-limonene	1232	–	1001	–	citrus, herbal, sweet	4	4	4	MS, RI, S, O
A4	γ-terpinene	1277	1255	–	1064	oily, woody, lime, herbal	–	1	–	MS, RI, S, O
A5	p-cymene	1305	1280	1103	1026	musty, woody, spice	1024	1024	1024	MS, RI, S, O
A6	2,4-dimethyl styrene	1459	1433	1076	1078	phenolic, spicy, soil, plastic	2048	64	2	MS, RI, S, O
Phenols										
B1	phenol	2037	2028	–	981	phenolic, plastic, rubber	2	–	4	MS, RI, S, O
B2	o-cresol	1998	2010		1060	musty, phenolic, herbal, leathery	1	–	1	MS, RI, S, O
B3	p-cresol	2950	2079	1097	1098	phenolic, floral(narcissus)	–	–	8	MS, RI, S, O
B4	carvacrol	2226	2225	1296	1307	spice, woody, phenolic	2048	1024	1024	MS, RI, S, O
B5	thymol	2239	2172	1292	1297	herbal, phenolic, roasted	1024	1024	1024	MS, RI, S, O
B6	2,4-di-tert-butylphenol	2323	2330	1512	1513	phenolic	–	2048	2048	MS, RI, S, O
Alcohols										
C1	prenol	1340	1323	–	778	fruity, green, floral(lavender)	2	4	–	MS, RI, S, O
C2	linalool	1553	1549	1101	1104	herbal, green, floral(rose),	16	8	8	MS, RI, S, O
C3	α-terpineol	1714	1170	1190	1191	pine, citrus, woody, floral(lilac)	1024	512	32	MS, RI, S, O
C4	Z-carveol	1842	1869	1220	1220	vegetable,green, caraway	16	8	4	MS, RI, S, O
C5	p-cymenol	1868	1851	1185	1188	sweet, fruity(cherry), camphor	128	8	4	MS, RI, S, O
C6	(-)-carveol	1884	1846	1231	1225	minty, green, herbal, spicy	–	4	4	MS, RI, S, O
C7	limonene glycol	2298	2325	1345	1342	minty, roasted,	2	1	1	MS, RI, S, O
C8	perillalcohol	2018	2021	–	1300	spicy(cardamom), floral(violet)	1	2	1	MS, RI, S, O
C9	benzyl alcohol	1903	1885	–	1034	floral(rose), phenolic	–	2048	2	MS, RI, S, O
C10	2-ethylhexanol	1496	1490	–	1026	citrus, fresh, floral, oil, sweet	2048	4	–	MS, RI, S, O
C11	terpinen-4-ol	1602	1636		1177	herbal, woody, earthy, musty	4	32	64	MS, RI, S, O
C12	(-)-pinocarveol	1679	1666	1137	1140	warm, woody, fennel, cereal	–	4	2	MS, RI, S, O
C13	phenylethyl alcohol	1940	1923	–	1121	floral(rose)	16	–	–	MS, RI, S, O
C14	(E)-p-mentha-2,8-dien-1-ol	1645	1641	1120	1121	fatty, popcorn, minty	4	4	1024	MS, RI, S, O
Aldehydes										
D1	vanillin	2615	2550	1397	1394	sweet(chocolate), creamy	–	1	4	MS, RI, S, O
D2	nonanal	1410	1396	–	1102	waxy, fatty, orange	–	64	–	MS, RI, S, O
D3	decanal	1513	1504	–	1195	sweet, waxy, citrus(orange), floral	4	–	–	MS, RI, S, O
D4	citronellal	2023	1488	–	1158	sweet, floral, herbal, waxy, citrus	–	4	–	MS, RI, S, O
D5	benzaldehyde	1556	1529	–	961	almond, fruity(cherry)	16	8	–	MS, RI, S, O
D6	perillaldehyde	1814	1807	1276	1279	woody, pine, sweet(balsam), minty	–	32	16	MS, RI, S, O
D7	2-pyrrolaldehyde	2065	2048	1013	1015	musty, beefy, burnt, roasted, smoky	1024	–	1024	MS, RI, S, O
Ketones										
E1	dihydrocarvone	1636	1645	1198	1200	herbal, minty, rubber, rice	–	16	8	MS, RI, S, O
E2	β-ionone	1964	1953	1485	1490	powdery, floral(orris), woody	–	8	–	MS, RI, S, O
E3	piperitone	1758	1743	1266	1268	woody, minty, camphor	–	8	4	MS, RI, S, O
E4	(+)-carvone	1764	1744	1244	1245	minty, fruity, spice	1024	2048	1024	MS, RI, S, O
E5	benzophenone	2533	2505	–	1625	floral (rose, geranium)	4	4	2	MS, RI, S, O
E6	4-methoxyacetophenone	2106	2120	1341	1345	fatty, sweet, anisic	2	–	2	MS, RI, S, O
Esters										
F1	methyl methanthranilate	2100	2068	1408	1402	fruity, musty, sweet	2048	2048	2048	MS, RI, S, O
F2	methyl anthranilate	2283	2257	–	1338	floral (orange flower), fruity(grape)	1024	2048	1024	MS, RI, S, O
Acids										
G1	octanoic acid	2033	2070		1191	fatty, waxy, rancid, oily, green, cheesy	2	1	4	MS, RI, S, O
G2	lauric acid	2489	2502		1570	fatty, fruity(coconut), oily	2	–	2	MS, RI, S, O
G3	palmitic acid	2512	2890	1968	1964	phenolic, waxy, fatty	–	2	4	MS, RI, S, O
Others										
H1	dimethyl sulfone	1944	1912	–	915	roasted, sulfurous, burnt	2	2	2	MS, RI, S, O
H2	2,3-dimethyl pyrazine	1373	1352	–	911	nutty, butter, coffee, caramel, roasted	4	–	–	MS, RI, S, O
H3	1,2-dimethoxybenzene	1743	1740	1145	1143	musty, creamy, phenolic, sweet	2	–	1	MS, RI, S, O

[a] Retention index of compounds on an HP-INNOWAX column and HP-5MS column. Cal means the RI value calculated by the formula. Ref means the RI value confirmed by comparison retention index to reference standards in the same condition (https://webbook.nist.gov/). [b] Aroma description. The aroma description vocabulary was generated by the GC-O evaluation team by comparing the aroma characteristics at actual concentrations with the literature and spectral library descriptions. [c] FD factor, flavor dilution factor determined on a HP-INNOWAX column. '–' means not being detected. [d] Identification method: MS means identified by comparison with the NIST mass spectral library 11 Vision database; RI means confirmed by comparison retention index; S means confirmed by authentic standard chemicals; O means confirmed by aroma descriptor.

= 16) were determined in the IT sample, but those compounds had no or much lower FD factors in the CP and OF samples. This indicates that these compounds contributed more to the aroma profile of the IT samples. Similarly, benzyl alcohol (FD = 2,048), nonanal (FD = 64), and dihydrocarvone (FD = 16) showed higher FD values in the OF sample than in IT and CP samples. Nevertheless, some compounds with larger FD values in CP, such as p-cymene (1,024), carvacrol (1,024), thymol (1,024), linalool (8), (+)-carvone (1,024), methyl methanthranilate (2,048), and methyl anthranilate (1,024), also showed higher values both in IT and OF samples. The above results were initially obtained by the AEDA sniffing method to screen the key aroma-active compounds of each sample, which are needed for validation.

The concentration of each individual compound was determined by its standard curve (Supplemental Table S3) and listed

Table 2. Quantitative analysis of volatile compounds in citrus Pu-erh tea samples.

No.	Compounds	OT (mg/kg)[A]	Concentration (mg/kg)[B]			OAV[C]			ACI%[D]		
			IT	OF	CP	IT	OF	CP	IT	OF	CP
A1	styrene	0.065	26.54^{a}	8.29^{b}	–	408.26	127.49	–	0.0526	0.0041	–
A2	β-pinene	0.14	4.09^{a}	–	–	29.24	–	–	0.0038	–	–
A3	(+)-limonene	0.034	233.62^{a}	159.49^{b}	120.07^{c}	6871.15	4691.00	3531.55	0.8852	0.1513	0.9724
A4	γ-terpinene	1	–	37.35^{a}	–	–	37.35	–	–	0.0012	–
A5	p-cymene	7.2	94.59^{a}	80.77^{b}	76.74^{c}	13.14	11.22	10.66	0.0017	0.0004	0.0029
A6	2,4-dimethyl styrene	0.085	16.71^{c}	30.69^{b}	56.52^{a}	196.62	361.01	664.97	0.0253	0.0116	0.1831
B1	phenol	5	179.14^{a}	–	150.89^{b}	35.83	–	30.18	0.0046	–	0.0083
B2	o-cresol	1.4	19.59^{b}	–	20.07^{a}	14.00	–	14.34	0.0018	–	0.0039
B3	p-cresol	0.0039	–	–	83.12^{a}	–	–	21311.63	–	–	5.7137
B4	carvacrol	2.29	1070.68^{c}	1413.74^{b}	2900.15^{a}	467.54	617.35	1266.44	0.0602	0.0199	0.3487
B5	thymol	1.7	1215.72^{a}	590.69^{c}	1011.75^{b}	715.13	347.46	595.14	0.0921	0.0112	0.1542
B6	2,4-di-tert-butylphenol	0.5	–	120.60^{b}	955.01^{a}	–	241.20	1910.02	–	0.0078	0.5259
C1	prenol	0.25	1.78^{b}	2.88^{a}	–	7.12	11.54	–	0.0009	0.0004	–
C2	linalool	0.00022	150.37^{a}	69.68^{b}	58.20^{c}	683488.63	316718.51	264532.69	88.0482	10.2151	72.8359
C3	α-terpineol	1.2	1139.07^{a}	883.02^{b}	694.31^{c}	949.23	735.85	578.59	0.1223	0.0237	0.1593
C4	Z-carveol	0.25	1732.26^{c}	2991.85^{a}	2243.32^{b}	6929.04	11967.40	8973.28	0.8926	0.3860	2.4707
C5	p-cymenol	ND	607.73^{b}	713.37^{a}	445.52^{c}	–	–	–	–	–	–
C6	(-)-carveol	0.25	–	285.04^{a}	274.93^{b}	–	1140.16	1099.72	–	0.0368	0.3028
C7	limonene glycol	ND	610.42^{a}	148.96^{c}	474.19^{b}	–	–	–	–	–	–
C8	perillalcohol	1.1	169.07^{b}	279.38^{a}	147.65^{c}	153.70	253.98	134.23	0.0198	0.0082	0.0370
C9	benzyl alcohol	2.54	–	69.75^{a}	48.40^{b}	–	27.46	19.05	–	0.0009	0.0052
C10	2-ethylhexanol	0.3	333.98^{a}	42.32^{b}	–	1113.25	141.07	–	0.1434	0.0045	–
C11	terpinen-4-ol	1.2	282.27^{a}	220.39^{b}	155.74^{c}	235.22	183.66	129.78	0.0303	0.0059	0.0357
C12	(-)-pinocarveol	ND	–	93.72^{a}	78.28^{b}	–	–	–	–	–	–
C13	phenylethyl alcohol	0.086	177.55^{a}	–	–	2064.56	–	–	0.2660	–	–
C14	(E)-p-mentha-2,8-dien-1-ol	ND	1658.63^{b}	1349.01^{c}	1829.85^{a}	–	–	–	–	–	–
D1	vanillin	0.053	–	402.51^{a}	356.40^{b}	–	7594.58	6724.47	–	0.2449	1.8515
D2	nonanal	0.0011	–	28.40^{a}	–	–	25817.10	–	–	0.8327	–
D3	decanal	0.003	54.60^{a}	–	–	18200.57	–	–	2.3446	–	–
D4	citronellal	0.006	–	58.38^{a}	–	–	9729.28	–	–	0.3138	–
D5	benzaldehyde	0.75	98.19^{a}	23.93^{b}	–	130.92	31.91	–	0.0169	0.0010	–
D6	perillaldehyde	0.03	–	134.70^{a}	75.84^{b}	–	4490.11	2528.15	–	0.1448	0.6961
D7	2-pyrrolaldehyde	65	225.12^{a}	–	147.44^{b}	3.46	–	2.27	0.0004	–	0.0006
E1	dihydrocarvone	3.25	–	82.27^{b}	128.87^{a}	–	25.31	39.65	–	0.0008	0.0109
E2	β-ionone	0.000007	–	18.17^{a}	–	–	2595440.13	–	–	83.7102	–
E3	piperitone	0.68	–	55.93^{a}	28.08^{b}	–	82.25	41.29	–	0.0027	0.0114
E4	(+)-carvone	0.16	1285.06^{b}	1406.29^{a}	1215.37^{c}	8031.61	8789.32	7596.06	1.0346	0.2835	2.0915
E5	benzophenone	ND	149.77^{c}	591.43^{a}	157.09^{b}	–	–	–	–	–	–
E6	4-methoxyacetophenone	ND	76.68^{b}	–	94.61^{a}	–	–	–	–	–	–
F1	methyl methanthranilate	0.349	2611.54^{b}	2902.11^{a}	2352.80^{c}	7482.93	8315.50	6741.56	0.9640	0.2682	1.8562
F2	methyl anthranilate	0.003	116.02^{b}	307.63^{a}	105.30^{c}	38672.96	102541.93	35101.20	4.9819	3.3073	9.6647
G1	octanoic acid	3	27.78^{b}	4.16^{c}	64.19^{a}	9.26	1.39	21.40	0.0012	0.0000	0.0059
G2	lauric acid	10	175.08^{b}	–	431.46^{a}	17.51	–	43.15	0.0023	–	0.0119
G3	palmitic acid	10	–	337.30^{b}	1437.30^{a}	–	33.73	143.73	–	0.0011	0.0396
H1	dimethyl sulfone	ND	243.11^{b}	145.86^{c}	492.16^{a}	–	–	–	–	–	–
H2	2,3-dimethyl pyrazine	0.8	20.18^{a}	–	–	25.23	–	–	0.0033	–	–
H3	1,2-dimethoxybenzene	ND	389.68^{a}	–	92.04^{b}	–	–	–	–	–	–

[A] The odor detection thresholds in water were obtained from previous studies[12,45] and online database (www.vcf-online.nl/VcfHome.cfm). [B] Concentration (mg/kg), The concentration of each volatile compound was calculated based on the calibration equation in Supplemental Table S3. [C] OAV (Odor activity value). [D] ACI (Aroma character impact value). All results were expressed as mean value (n = 3). Values bearing different lowercase letters (a, b, c) were significantly different ($p \leq 0.05$).

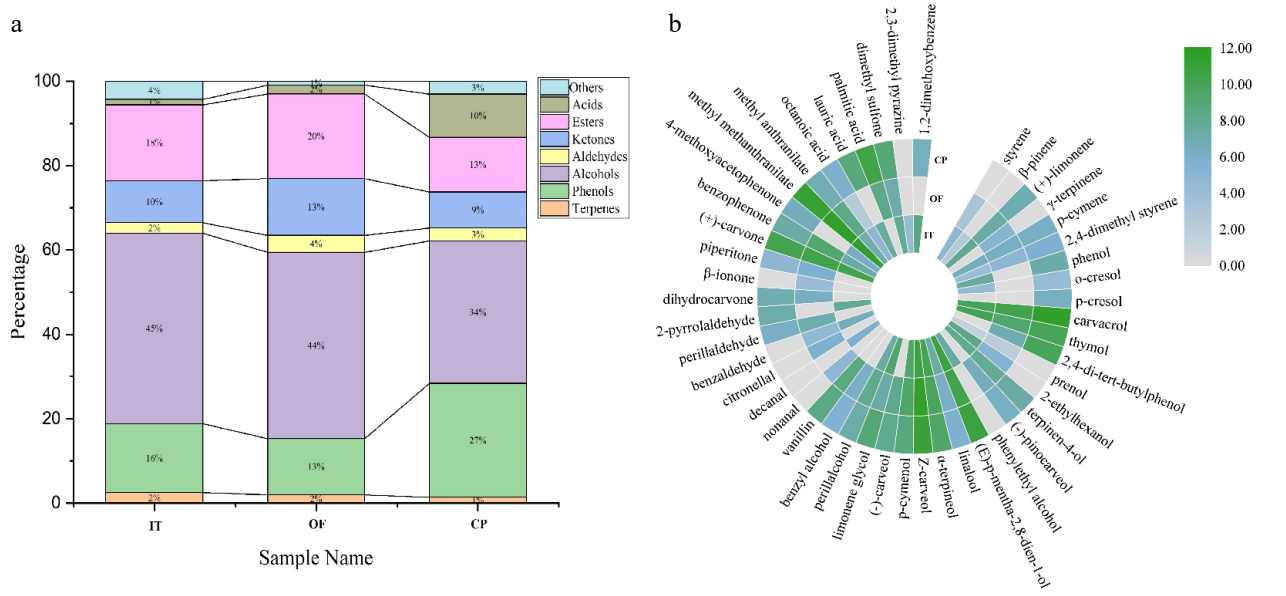

Fig. 2 Distribution map of volatile aroma substances, (a) species distribution profile of volatile compounds, (b) concentration distribution of each volatile compounds in three CPT infusion samples.

in Table 2. Figure 2a presents the chemical profiles of the 47 compounds. There was a same trend in categories of alcohols, esters, ketones and alkenes, with the lowest percentage levels in the CP samples among the three samples. Alcohols, including straight-chain and branched alcohols derived from the reduction of Strecker aldehydes or the hydrolysis of glycoside precursors were regarded as the third largest group of volatiles detected in teas[13]. Under the influence of citrus peel, it occupies the largest proportion among the detected compounds in the three samples, with a percentage of 45% (IT), 44% (OF), and 34% (CP). Alkenes are the most important volatile components in citrus fruits[10]. Phenols (27%) and acids (10%) had the highest percentage levels in the CP samples among all three samples. As shown in Fig. 2b, the color coding changed from green to gray, reflecting the chemical concentration decreasing from high to low levels. The concentration of the same compound varied considerably among different samples, which also reflected the differences of the FD factors of compounds in different samples in the qualitative results mentioned above.

Evaluation and validation of key aroma-active compounds

Odor activity value (OAV) and aroma character impact value (ACI) were calculated for quantitative assessment of the contribution of key aroma-active compounds to the overall aroma for a particular sample (Table 2)[27,31]. OAV ≥ 1 were considered to contribute significantly to the overall aroma of the samples[32]. The number of compounds with OAV values greater than one was 27, 30 and 27 for IT, OF and CP, respectively. The top ten key aroma-active compounds in the CP sample were linalool (OAV = 264,532), methyl anthranilate (OAV = 35,101), p-cresol (OAV = 21,311), Z-carveol (OAV = 8,973), (+)-carvone (OAV = 7,596), methyl methanthranilate (OAV = 6,741), vanillin (OAV = 6,724), (+)-limonene (OAV = 3,531), perillaldehyde (OAV = 2,528), 2,4-di-tert-butylphenol (OAV = 1,910). Notably, compounds with high concentration does not necessarily have a high OAV value, which is determined by its odor threshold. Although the content of linalool was far below than others, its olfactory detection threshold of 0.00022 mg/kg made it as the key aroma-active compound in all three samples[8]. This phenomenon was also observed in the IT and OF samples. For example, the extremely low odor thresholds of ionone (0.007 μg/kg), resulted in a high FD (8) and OAV. Three more substances were also ranked in the top ten aroma-impact volatiles in the OF samples based on their OAV values: β-ionone (OAV = 2,595,440), nonanal (OAV = 25,817), and citronellal (OAV = 9,729). Similarly, decanal (OAV = 18,200), phenylethyl alcohol (OAV = 2,064), 2-ethylhexanol (OAV = 1,113) and α-terpineol (OAV = 949) were considered to have played important roles in the aroma contribution in IT. ACI results revealed more information of volatiles in three samples, linalool (88, 10, 72), β-ionone (–, 84, –), methyl anthranilate (5, 3, 10), (+)-carvone (1, 0.3, 2), methyl methanthranilate (1, 0.3, 1.9), (+)-limonene (0.9, 0.1, 1), p-cresol (–, –, 5.7), Z-carveol (0.9, 0.4, 2.5), phenylethyl alcohol (0.3, –, –), perillaldehyde (–, 0.1, 0.7), citronellal (–, 0.3, –), decanal (2, –, –), nonanal (–, 0.8, –), vanillin (–, 0.2, 1.9), α-terpineol (0.1, 0.02, 0.2), 2-ethylhexanol (0.1, 0.005, –), (–)-carveol (–, 0.04, 0.3), 2,4-di-tert-butylphenol (–, 0.01, 0.5), thymol (0.1, 0.01, 0.1), carvacrol (0.1, 0.02, 0.3) and 2,4-dimethyl styrene (0.02, 0.01, 0.2). The aforementioned 21 volatile compounds had high ACI values in the corresponding samples and their corresponding OAV values were also high. They could be categorized as the key aroma-active compounds corresponding to the three samples.

To further confirm the key aroma-active compounds in the CPT samples, aroma recombination was conducted to initially simulate the aroma of each sample based on quantitative results (Fig. 3)[26]. Statistical analysis revealed a significant difference on one or two odor attributes ($p \leq 0.05$). On this basis, further aroma omission experiments were carried out to verify the contribution of a specific group or individual aroma compounds to the overall aroma (Table 3). Significance results were derived from the frequency of sniffing by the evaluators. For the CP samples, the volatile compounds that had a significant effect on the overall aroma were methyl methanthranilate

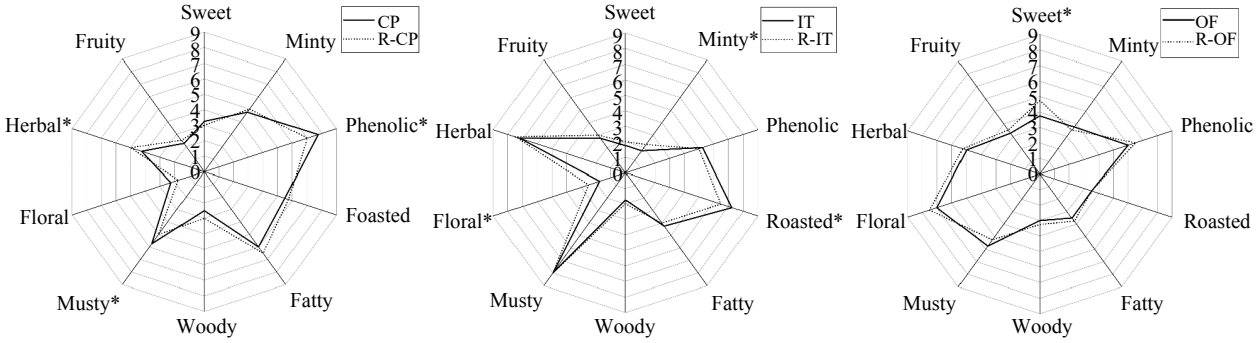

Fig. 3 Descriptive sensory analysis radar diagram of recombination model and corresponding CPT samples. Note: The sensorial parameters indicated with * are significantly different between samples ($p \leq 0.05$).

(N = 15, N means the number of being recognized), methyl anthranilate (N = 15), linalool (N = 14), (+)-carvone (N = 13), vanillin (N = 13), Z-carveol (N = 13), 2,4-dimethyl styrene (N = 11), (+)-limonene (N = 9), (−)-carveol (N = 8), terpinen-4-ol (N = 8), 2,4-di-tert-butylphenol (N = 8), and thymol (N = 8). For the OF sample, β-ionone (N = 13), citronellal (N = 12) and nonanal (N = 8) also had significant effects on the overall aroma. In the IT sample, styrene (N = 11), decanal (N = 9) and benzaldehyde (N = 8) were more deficient. The result showed that the key aroma-active compounds of all three CPT samples were captured in the absence experiment, with a more consistent overall aroma but also more distinct individual characteristics. The results of this aroma omission experiment are consistent with the conclusions of the top ranked compounds calculated from the OAV values and ACI values.

Relationship between key aroma-active compounds and sensory evaluation

After confirming the key aroma-active compounds, the correlation between the sensory evaluation scores and the quantitative results of each substance obtained from the instrumental analysis were established using the PLSR model (Fig. 4). Three parallel experiments showed good reproducibility of results, and the same group of samples clustered in similar positions in the results. The ten sensory evaluation descriptors can also be better distinguished from each other. The experimental results have clearly reflected the correlation between the sensory evaluation results and the compounds, according to the correlation coefficient R^2, $X_1 = 0.488$, $X_2 = 0.436$, and the summation reach 0.924[2].

The CP samples were mainly located in the upper right corner and associated closely with aroma attributes such as fatty, phenolic and minty flavors. Among them, compound G1 (octanoic acid) has a strong correlation with fatty. Pang et al.[20] also identified octanoic acid in Pu-erh tea and described its flavor as 'sweaty' with a higher concentration. The perception is indeed different depending on the concentration. Phenols were considered a major class of volatiles giving smoky, and phenolic characteristics to Pu-erh tea[13]. Compounds B4 (carvacrol), B6 (2,4-di-tert-butylphenol), and B3 (o-cresol) are more inclined to present phenolic aroma attributes. B5 (thymol) is responsible for phenolic, roasted or woody flavor and was also detected in oolong tea. E1 (dihydrocarone) has a strong association with minty flavor. The OF samples were mainly located in the lower right corner, and close to the four aroma attributes of fruity, floral, woody, and sweet notes. This result is consistent with its higher scoring of those aroma attributes in the sensory evaluation (Fig. 1a). In particular, F1 (methyl methanthranilate, alternate name dimethyl anthranilate) is reported as a volatile marker in citrus peel with a fruity and sweet note[10,33]. F2 (methyl anthranilate) was widely recognized as a grape flavor compound, and has been detected widely in teas deriving from anthranolic acid[10,34,35]. In this experiment, both F1 and F2 were considered as the top five key aroma-active compound of CPT samples with high FD values and high OAV values. E5 (benzophenone) and E2 (β-ionone) have a more significant floral aroma. β-ionone is obviously a significant contributor to the aroma of dark teas formed from carotenoid degradation[36]. D1 (vanillin) has a strong correlation with sweet. In the IT sample located in the lower left corner of Fig. 3, the main aroma characteristics were roasted, musty, and herbal notes. Three sensory attributes are close to each other and the flavor compounds are clustered. A3 ((+)-limonene) was determined to contribute the most to the aroma quality of the corresponding citrus[8,33]. C2 (linalool) is a nearly ubiquitous aroma compound in plants which shows a herbal-like note in the specific concentration in this study[35,36]. Combining the aroma descriptions of single compound standards, it can be concluded that compounds like C11 (terinen-4-ol) and C10 (2-ethylhexanol) are more associated with herbal aroma attributes. Terpinen-4-ol and α-terpineol are isomers, both have a pleasant herbal-like odor similar to lilacs[32]. Compounds like A5 (p-cymene) are the main compounds that cause the samples to produce musty aroma attributes. Wang et al. supposed that p-cymene may come from both citrus and tea leaves, and there might be the simple additive effects between the volatile compounds of pure tea and citrus[8]. The p-cymene content was inversely related to the maturity of the citrus fruit, that is, the lower the citrus maturity, the higher the content of this component[10]. Some compounds located in the upper left corner of Fig. 3, which were less relevant in terms of aroma matching, probably because these compounds were detected in only one sample (IT or CP or OF).

Among these compounds, combining the FD factors, OAV and ACI values calculated earlier, the key aroma-active compounds can be categorized into the following aroma notes, such as sweet (vanillin); floral (β-ionone); fruity (methyl anthranilate, methyl methanthranilate, citronellal, (+)-carvone, Z-carveol); roasted (thymol); musty (p-cymene), woody (perillaldehyde); herbal (linalool, (+)-limonene, α-terpineol, 2-ethylhexanol); phenolic (2,4-di-tert-butylphenol, p-cresol, carvacrol, 2,4-dimethyl styrene); minty (dihydrocarvone); fatty (octanoic acid).

Table 3. Omission tests of three citrus Pu-erh tea based on aroma recombination model.

No.	Odorants omitted from the complete recombinant	Number[a]			Significance[b]		
		IT	OF	CP	IT	OF	CP
1	octanoic acid, lauric acid, palmitic acid	6	9	11	*	**	**
1–1	octanoic acid	5	6	5	*	*	*
1–2	lauric acid	5	2	5	*	–	*
1–3	palmitic acid	2	6	7	–	*	*
2	thymol, carvacrol, phenol, o-cresol, p-cresol, 2,4-di-tert-butylphenol	8	7	9	**	*	**
2–1	thymol	9	8	8	**	**	**
2–2	carvacrol	6	7	7	*	*	*
2–3	phenol	4	1	5	*	–	*
2–4	o-cresol	5	3	4	*	–	*
2–5	p-cresol	2	4	8	–	*	*
2–6	2,4-di-tert-butylphenol	8	7	8	**	*	**
3	linalool, perillalcohol, p-cymenol, limonene glycol, terpinen-4-ol, prenol, 2-ethylhexanol	15	15	15	***	***	***
3–1	linalool	14	15	14	***	***	***
3-2	perillalcohol	5	6	5	*	*	*
3–3	p-cymenol	2	3	2	–	–	–
3–4	limonene glycol	1	2	3	–	–	–
3–5	terpinen-4-ol	8	9	8	**	**	**
3–6	prenol	5	4	1	*	*	–
3–7	2-ethylhexanol	6	5	3	*	*	–
4	phenylethyl alcohol, α-terpineol, (E)-p-mentha-2,8-dien-1-ol, (–)-pinocarveol, benzyl alcohol, (–)-carveol, Z-carveol	15	14	15	***	***	***
4–1	phenylethyl alcohol	6	1	2	*	–	–
4–2	α-terpineol	8	7	7	**	*	*
4–3	(E)-p-mentha-2,8-dien-1-ol	1	2	2	–	–	–
4–4	(–)-pinocarveol	2	2	3	–	–	–
4–5	benzyl alcohol	3	7	6	–	*	*
4–6	(–)-carveol	3	7	8	–	*	**
4–7	Z-carveol	14	13	13	***	***	***
5	p-cymene, β-pinene, styrene, 2,4-dimethyl styrene, (+)-limonene, γ-terpinene, 1,2-dimethoxybenzene	13	11	11	***	**	**
5–1	p-cymene	6	6	5	*	*	*
5–2	β-pinene	6	3	3	*	–	–
5–3	styrene	11	3	3	**	–	–
5–4	2,4-dimethyl styrene	10	9	11	**	**	**
5–5	(+)-limonene	15	10	9	***	**	**
5–6	γ-terpinene	3	7	2	–	*	–
5–7	1,2-dimethoxybenzene	3	2	1	–	–	–
6	benzaldehyde, 2-pyrrolaldehyde, perillaldehyde, decanal, nonanal, citronellal, vanillin	9	12	13	**	***	***
6–1	benzaldehyde	8	4	1	**	*	–
6–2	2-pyrrolaldehyde	6	3	7	*	–	*
6–3	perillaldehyde	2	7	6	–	*	*
6–4	decanal	9	2	2	**	–	–
6–5	nonanal	3	8	4	–	**	*
6–6	citronellal	4	12	5	*	***	*
6–7	vanillin	5	12	13	*	***	***
7	2,3-dimethyl pyrazine, methyl methanthranilate, methyl anthranilate, dimethyl sulfone	14	15	15	***	***	***
7–1	2,3-dimethyl pyrazine	6	3	4	*	–	*
7–2	methyl methanthranilate	14	15	15	***	***	***
7–3	methyl anthranilate	13	14	15	***	***	***
7–4	dimethyl sulfone	5	3	3	*	–	–
8	(+)-carvone, dihydrocarvone, piperitone, benzophenone, 4-methoxyacetophenone, β-ionone	13	14	15	***	***	***
8–1	(+)-carvone	12	13	13	***	***	***
8–2	dihydrocarvone	3	6	7	–	*	*
8–3	piperitone	4	7	8	*	*	**
8–4	benzophenone	4	5	4	*	*	*
8–5	4-methoxyacetophenone	2	3	4	–	–	*
8–6	β-ionone	4	13	5	*	***	*

[a] The number of panelists who perceived the aroma difference by means of a triangle test. Fifteen panelists were invited for aroma omission experiment. [b] Levels of significance, defined based on the number of panelists who were able to determine the difference in aroma omission. –, not significant (0–3, $p > 0.05$); *, significant (4–7, $p \leq 0.05$); **, highly significant (8-11, $p \leq 0.01$); ***, very highly significant (12–15, $p \leq 0.001$).

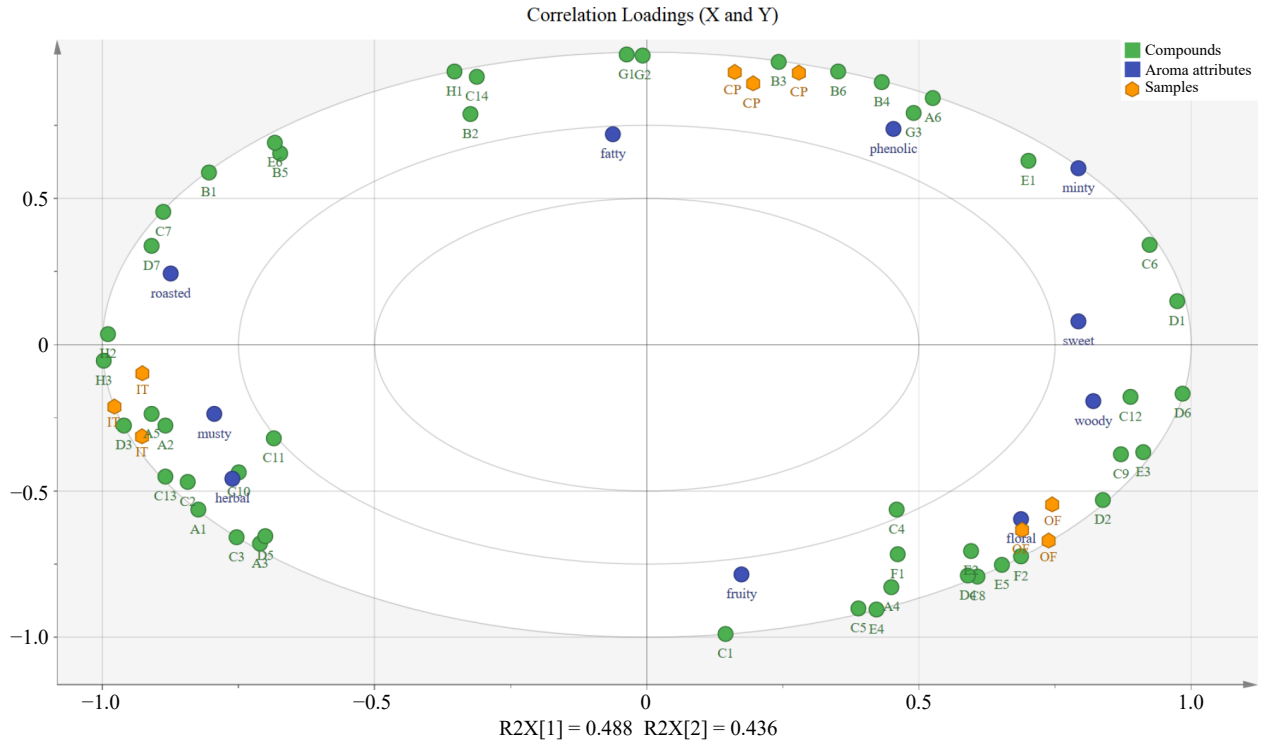

Fig. 4 Correlation loadings plot for aroma-active compounds (X-matrix) and sensory attributes (Y-matrix) of three CPT samples.

Identification of non-volatiles related to the taste of CPT

Non-volatile substances in three CPT samples were identified by non-targeted metabolomics. Each sample was repeated by six times, and analyzed by the multivariate statistical analysis (Supplemental Fig. S1), which showed that the three samples had significant differences, and a total of 2,743 metabolites were detected, of which a total of 1,890 were the same metabolites. Tea flavonoids are widely recognized as critical flavor contributors and crucial health-promoting bioactive compounds, and have long been the focus of research worldwide in food science[37]. Figure 5 is a heat map showing the KEGG metabolic pathway enrichment, in which each dot represents a metabolic pathway, and shows the top 20 metabolic pathways participating in the experimental project. Among them, glucosinolate biosynthesis and flavonoid biosynthesis showed significant differences form others ($p \leq 0.05$). A total of 29 compounds were involved in the two metabolic pathways, of which 14 compounds contributed to taste (Fig. 6). Four compounds in the CP samples were derived from the citrus peel. For example, neohesperidin is a flavanone glycoside, which has been found in different citrus fruits, is widely used as a natural source to produce neohesperidin dihydrochalcone, a semisynthetic sweetener used in the food industry[38]. Leucopelargonidin belongs to anthocyanidin, compared with leucoanthocyanidin and leucodelphinidin, it often as a minor component was established and contribute both bitterness and astringency[39,40]. Some amino acids, such as Tryptophan, Tyrosine, homomethionine were increased after withering, which contributed to the umami and sweet, mellow taste of tea[41,42]. Most flavan-3-ols (mainly EGCG, ECG, GC, and other catechins) were found to be strongly correlated with the bitter and puckering astringent tastes, different from the mouth-drying or velvety-like astringent taste of flavanols glycosides. Aromadendrin levels decreased significantly as yellowing duration, and the umami and sweetness also decreased at the same time[43]. Chlorogenic acid and desmethylxanthohumol contribute to bitterness[44,45]. Sakuranetin and taxifolin have the potential of bitter-masking, which can enhance 'sweet' and suppressed 'sour', 'bitter', 'astringent' and 'aftertaste'[46, 47]. Since flavan-3-ols are thought to be associated with the bitterness and astringent tastes, Xu et al.[2] supposed that citrus peel could speed up the fermentation of Pu-erh tea so as to contribute to the unique flavor of citrus Pu-erh tea.

Conclusions and outlook

The 47 flavor substances with their flavor description (35 aroma-active compounds and 12 taste-related compounds) in the CP samples were plotted on the flavor wheel (Fig. 7). Of which, 15 substances in the upper left corner were considered as the main flavor substances provided by IT. O-cresol, p-cresol, and phenol in box A shared similar structures, with cresol as the basic structure which has a methyl group in the para or adjacent position and have phenolic as the main aroma profile. The 13 compounds in the upper right corner were considered to be the flavor substances provided by citrus peel, three of them in box B were structurally similar and provided mainly minty or woody aroma characteristics. The two aldehydes (perillaldehyde and vanillin) in box C contribute to the aroma characteristics of woody and sweet for CP. The substances in the yellow part at the bottom of Fig. 7 can be divided into three main categories. The substances in box F are mainly the sources of musty, roasted and phenolic aroma characteristics. The compounds in box E are mainly the sources of floral, herbal, and fruity aromas, and some correlations are also found in their chemical structural formula, and there is more cis-trans isomerism. The two esters in box D are particularly similar in structure, both with a

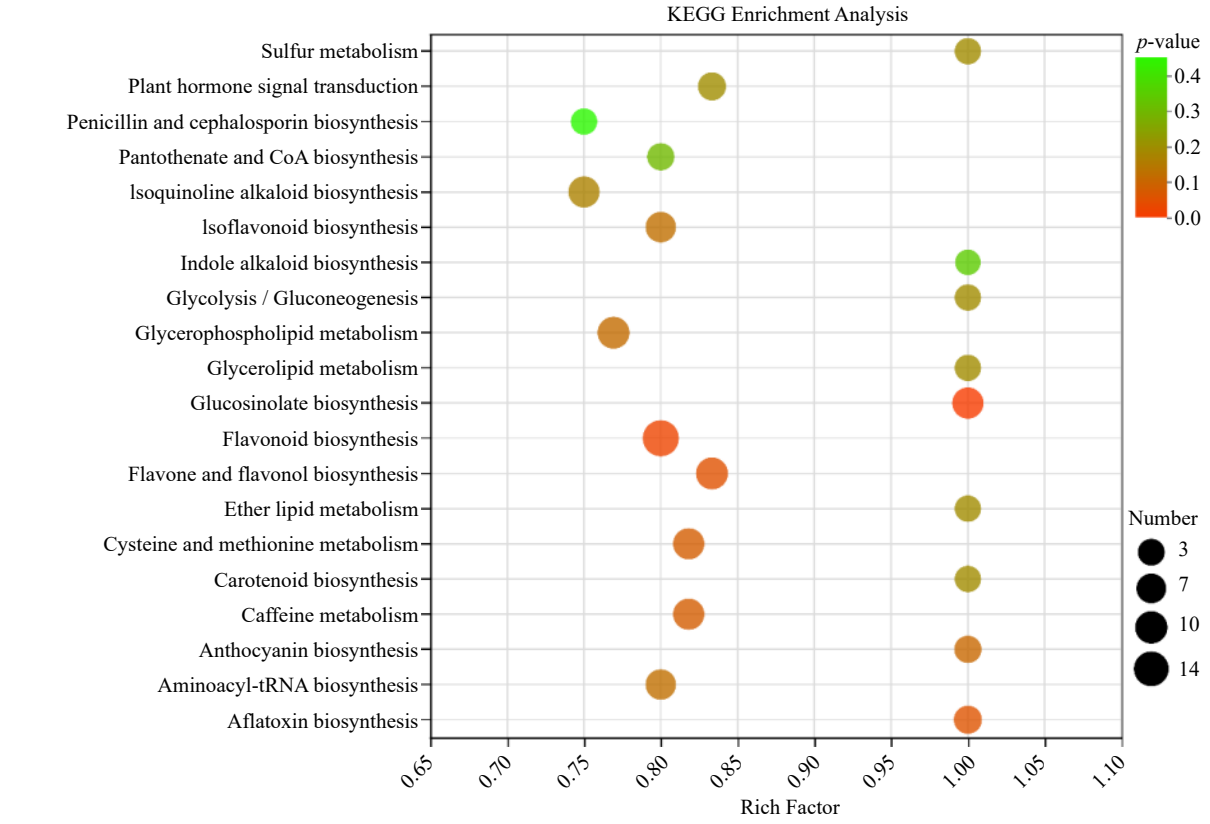

Fig. 5 KEGG enrichment analysis of TOP20 metabolic pathways in untargeted metabolomics.

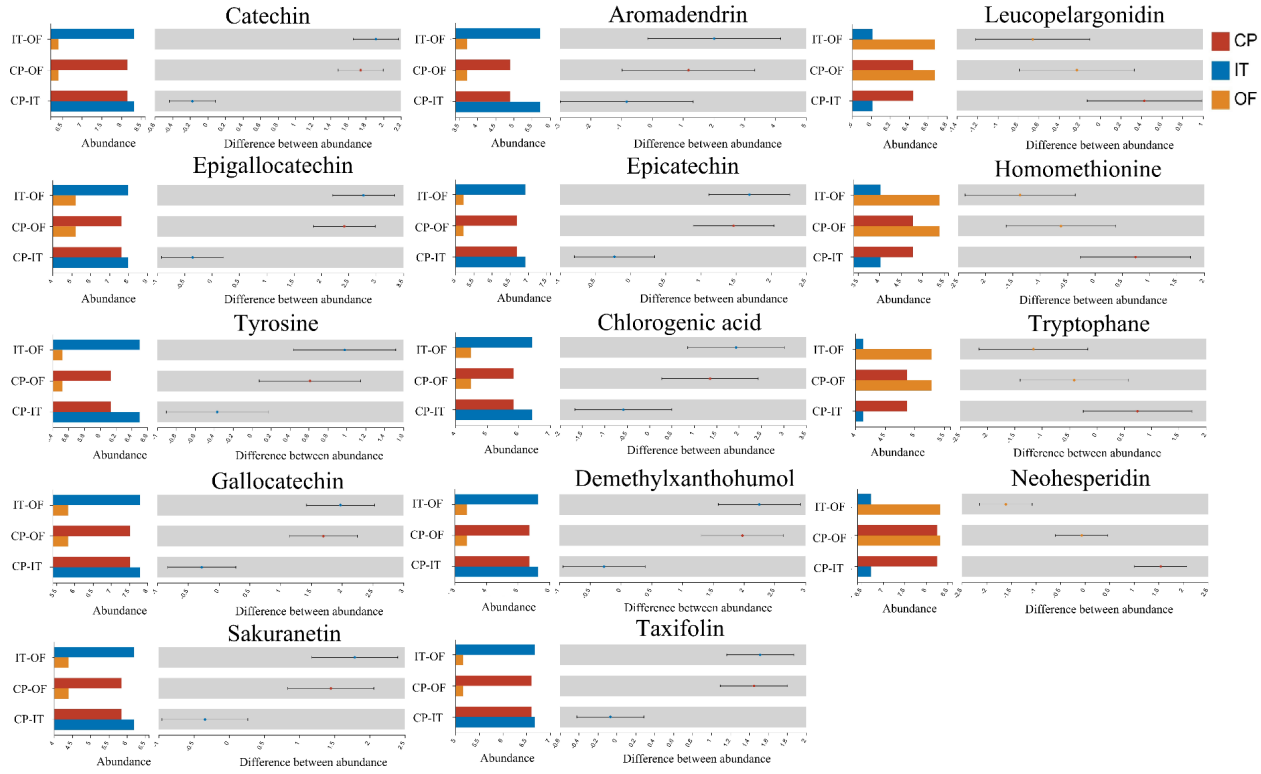

Fig. 6 Non-volatile compounds that significantly contributed to taste of three CPT samples.

carboxyl substituent linked to the benzene ring, differing only in the number of carbon atoms attached to the amino substituent of methyl methanthranilate in the benzene ring neighboring substituent, which is considered to be more relevant for floral and fruity aromas (Fig. 3). Flavanol glycosides with low thresholds are important flavor substances in tea

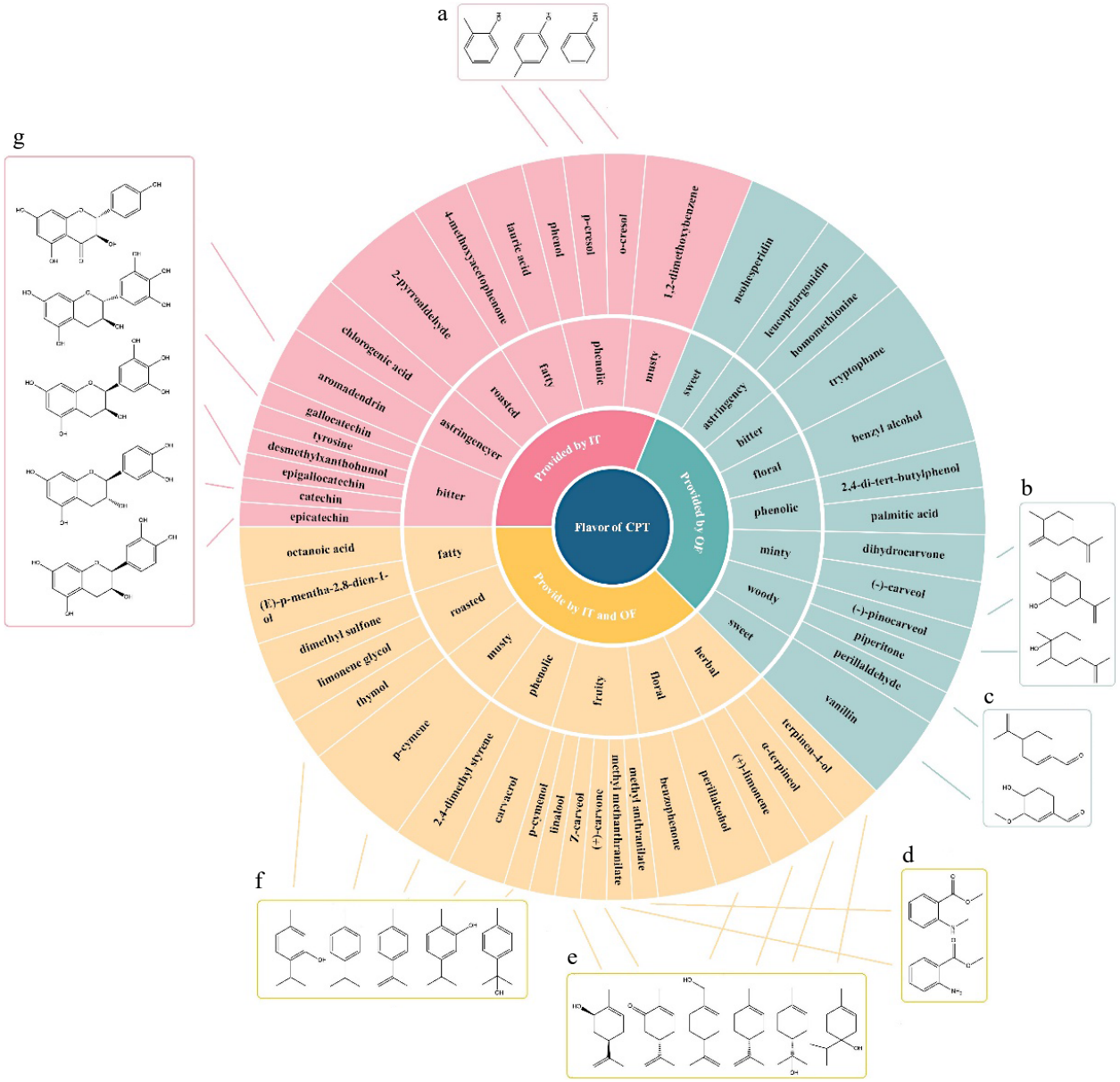

Fig. 7 Flavor wheel of key flavor compounds of CPT infusion samples. Compounds marked with molecular structure in frames (a)–(g) were specific to the IT, OF or both IT and OF.

leaves[48]. The five compounds in the G box are mainly derived from the inner tea and provide bitterness and astringency.

These findings have given some insights on the relationship between structure and aroma characteristics and yielded the main source of flavor compounds of the CP samples. Even after five years of co-fermentation under sunlight, the flavor substances in three samples were still not identical. Most of the aroma substances of the whole fruit sample of citrus tea (CP) were found in both the OF and IT samples, which can be considered to be attributed by both Pu-erh tea and citrus peel. Based on the flavor wheel and the results of the previous screening of key aroma-active compounds, it can be concluded that some of the main characteristics aromas were provided by both IT and OF, and their corresponding key aroma-active compounds were fatty (octanoic acid), roasted (thymol), musty (p-cymene), phenolic (2,4-dimethyl styrene), fruity (methyl methanthrenilate), floral (benzophenone) and herbal (terpinene-4-ol, α-terpinol, (+)-limonene). These detected components can preliminarily explain ingredient-ingredient relation and ingredient-compound relation, which were proposed to support food pairing and further derived flavor pairing[18,49]. For example, compounds in CP that were affected by the OF and IT samples were floral (benzyl alcohol), phenolic (2,4-di-tert-butylphenol), minty (dihydrocarone), woody (perillaldehyde) and sweet (vanillin, neohesperidin) notes. In the OF part, the citrus peel mainly provided fruity (methyl anthranilate) note, floral (β-ionone, benzophene) notes, while in the IT part, Pu-erh tea mainly provided musty(p-cymene) note and herbal (α-terpineol) note and most of the FD values of these aroma components are higher than in the CP part. Generally, the higher FD values, the greater the contribution of the volatile components to the overall aroma. Benzophene provides

rose-like or geranium-like floral notes and β-ionone is the representative component of violet aroma that may cooperate with benzyl alcohol so that enhanced faint floral note in CP, musty note and herbal note can also enhance and match the woody (perillaldehyde) note cause their aroma types are close to each other. It can be seen that OF part improved the flavor of Pu-erh tea and gave citrus Pu-erh tea a unique and coordinated flavor.

In summary, this research studied the flavor profile and revealed taste metabolites of citrus Pu-erh tea, which is expected to provide some useful information for the quality control of the citrus Pu-erh tea.

Author contributions

The authors confirm contribution to the paper as follows, original draft writing: Sun J, Cai W; data analysis: Sun J, Cai W; methodology: Yao L, Song S, Wang H; conceptualization: Feng T, Yu C; manuscript review and editing: Feng T, Chen D, Yao L, Lu J, Wang H, Liu Q; partial funds and consultant: Lu J, Feng T . All authors read and approved the final manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Acknowledgments

The support of the Key Laboratory of cigarette flavoring Technology in Tobacco Industry (TX2018001), Shanghai Gaofeng & Gaoyuan Project for University Academic Program Development (1021GN203004005) and Royal Society of New Zealand Catalyst Seeding Fund (21-AUT-005-CSG).

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Information accompanies this paper at (https://www.maxapress.com/article/doi/10.48130/bpr-0024-0001)

Dates

Received 4 November 2023; Revised 3 December 2023; Accepted 8 December 2023; Published online 1 March 2024

References

1. Zheng Y, Zeng X, Chen T, Peng W, Su W. 2020. Chemical profile, antioxidative, and gut microbiota modulatory properties of Ganpu tea: A derivative of Pu-erh tea. *Nutrients* 12(1):224
2. Xu Y, Liang PL, Chen XL, Gong MJ, Zhang L, et al. 2021. The impact of *Citrus*-tea cofermentation process on chemical composition and contents of Pu-erh tea: An integrated metabolomics study. *Frontiers In Nutrition* 8:737539
3. Spence C. 2020. Food and beverage flavour pairing: A critical review of the literature. *Food Research International* 133:109124
4. Xiao S, Huang J, Huang Y, Lai HQ, Zeng Y, et al. 2021. Flavor characteristics of Ganpu tea formed during the sun-drying processing and its antidepressant-like effects. *Frontiers In Nutrition* 8:647537
5. Yu X, Sun S, Guo Y, Liu Y, Yang D, et al. 2018. *Citri reticulatae pericarpium* (chenpi): Botany, ethnopharmacology, phytochemistry, and pharmacology of a frequently used traditional chinese medicine. *Journal of Ethnopharmacology* 220:265–82
6. Wang C, Gao ZP, Qian YJ, Li X, Wang JY, et al. 2021. Effects of different concentrations of Ganpu tea on fecal microbiota and short chain fatty acids in mice. *Nutrients* 13(11):3715
7. Zheng Y, Zeng X, Guan M, Xie S, Peng W, et al. 2020. UHPLC-Q-TOF-MS/MS-based metabolite profiling of Ganpu tea in rat urine and feces. *Natural Product Communications* 17(3):1–16
8. Wang J, Zhu Y, Shi J, et al. 2020. Discrimination and identification of aroma profiles and characterized odorants in citrus blend black tea with different citrus species. *Molecules* 25(18):4208
9. Qi H, Ding S, Pan Z, Li X. Fu FH. 2020. Characteristic volatile finger prints and odor activity values in different citrus-tea by HS-GC-IMS and HS-SPME-GC-MS. *Molecules* 25(24):6027
10. Wang JT, Shi J, Zhu Z, Ma WJ, Yan H, et al. 2022. Insights into crucial odourants dominating the characteristic flavour of citrus-white teas prepared from *citrus reticulata* Blanco 'Chachiensis' and *Camellia sinensis* 'Fudingdabai'. *Food Chemistry* 377:132048
11. Zhang Y, Jin S, Wang G, Yu L, Zhou S, et al. 2015. Flavor analysis and flavor wheel establishment of ten top famous tea in Zhejiang province. *Journal of Tea Science* 35(3):225–32
12. Zhang Y, Liu X, Lu C. 2019. Study on primitive morpheme in sensory terminology and flavor wheel construction of Chinese tea. *Journal of Tea Science* 39(4):474–83
13. Zhai X, Zhang L, Granvogl M, Ho CT Wan X. 2022. Flavor of tea (*Camellia sinensis*): A review on odorants and analytical techniques. *Comprehensive Reviews in Food Science and Food Safety* 21(5):3867–909
14. Suffet IH, Brady BM, Bartels JHM, Burlingame G, Mallevialle J, et al. 1988. Development of the flavor profile analysis method into a standard method for sensory analysis of water. *Water Science & Technology* 20(8–9):1–9
15. Spencer M, Sage E, Velez M, Guinard JX. 2016. Using single free sorting and multivariate exploratory methods to design a new coffee taster's flavor wheel. *Journal of Food Science* 81(12):S2997–S3005
16. Mirarefi S, Menke SD, Lee SY. 2004. Sensory profiling of chardonel wine by descriptive analysis. *Journal of Food Science* 69(6):S211–S217
17. Lee KYM, Paterson A, Piggott JR, Richardson GD. 2001. Origins of flavour in whiskies and a revised flavour wheel: A review. *Journal of the Institute of Brewing* 107(5):287–313
18. Park D, Kim K, Kim S, Spranger M, Kang J. 2021. FlavorGraph: a large-scale food-chemical graph for generating food representations and recommending food pairings. *Scientific Reports* 11:931–43
19. Ahn Y, Ahnert S, Bagrow J, Barabási A. 2011. Flavor network and the principles of food pairing. *Scientific Reports* 1:196
20. Pang X, Yu W, Cao C, Yuan X, Qiu J, et al. 2019. Comparison of potent odorants in raw and ripened pu-erh tea infusions based on odor activity value calculation and multivariate analysis: understanding the role of pile fermentation. *Journal of Agricultural and Food Chemistry* 67(47):13139–49
21. Zhai X, Granvogl M. 2020. Key odor-active compounds in raw green and red *Toona sinensis* (A. Juss.) Roem. and their changes during blanching. *Journal of Agricultural and Food Chemistry* 68(27):7169–83
22. Dias LG, Hacke A, Bergara SF, Villela OV, Mariutti LRB, et al. 2021. Identification of volatiles and odor-active compounds of aromatic rice by OSME analysis and SPME/GC-MS. *Food Research International* 142:110206
23. Li C, Al-Dalali S, Zhou H, Xu B. 2022. Influence of curing on the metabolite profile of water-boiled salted duck. *Food Chemistry* 397:133752
24. Feng T, Shui MZ, Song SQ, Zhuang HN, Sun M, et al. 2019. Characterization of the key aroma compounds in three truffle varieties from China by flavoromics approach. *Molecules* 24(18):3305–21

25. Yao L, Mo Y, Chen D, Feng T, Song S, et al. 2021. Characterization of key aroma compounds in Xinjiang dried figs (*Ficus carica* L.) by GC-MS, GC-olfactometry, odor activity values, and sensory analyses. *LWT* 150:111982
26. Feng T, Sun J, Wang K, Song S, Chen D, et al. 2022. Variation in Volatile Compounds of Raw Pu-Erh Tea upon Steeping Process by Gas Chromatography-Ion Mobility Spectrometry and Characterization of the Aroma-Active Compounds in Tea Infusion Using Gas Chromatography-Olfactometry-Mass Spectrometry. *Journal of Agricultural and Food Chemistry* 70(42):13741−53
27. Yang X. 2008. Aroma constituents and alkylamides of red and green huajiao (*Zanthoxylum bungeanum* and *Zanthoxylum schinifolium*). *Journal of Agricultural and Food Chemistry* 56(5):1689−96
28. Wang Z, Xiao Q, Zhuang J, Feng T, Ho CT, et al. 2019. Characterization of aroma-active compounds in four yeast extracts using instrumental and sensory techniques. *Journal of Agricultural and Food Chemistry* 68(1):267−78
29. Chang J, Li X, Liang Y, Feng T, Sun M, et al. 2023. Novel umami peptide from *Hypsizygus marmoreus* hydrolysate and molecular docking to the taste receptor T1R1/T1R3. *Food Chemistry* 401:134163
30. Zhou B, Ma B, Xu C, Wang J, Wang Z, et al. 2022. Impact of enzymatic fermentation on taste, chemical compositions and in *vitro* antioxidant activities in Chinese teas using E-tongue, HPLC and amino acid analyzer. *LWT* 163:113549−58
31. Feng Z, Li M, Li Y, Yin J, Wan X, et al. 2022. Characterization of the key aroma compounds in infusions of four white teas by the sensomics approach. *European Food Research and Technology* 248(5):1299−309
32. Zhang W, Cao J, Li Z, Lai X, Sun L, et al. 2021. HS-SPME and GC/MS volatile component analysis of Yinghong No. 9 dark tea during the pile fermentation process. *Food Chemistry* 357:129654
33. Lv W, Lin T, Ren Z, Jiang Y, Zhang J, et al. 2020. Rapid discrimination of *Citrus reticulata* 'Chachi' by headspace-gas chromatography-ion mobility spectrometry fingerprints combined with principal component analysis. *Food Research International* 131:108985
34. Luo ZW, Cho JS, Lee SY. 2019. Microbial production of methyl anthranilate, a grape flavor compound. *Proceedings of the National Academy of Sciences of the United States of America* 116(22):10749−56
35. Rigling M, Liu ZB, Hofele M, Prozmann J, Zhang C, et al. 2021. Aroma and catechin profile and in vitro antioxidant activity of green tea infusion as affected by submerged fermentation with *Wolfiporia cocos* (Fu Ling). *Food Chemistry* 361:130065
36. Pradhan S, Dubey RC. 2021. GC-MS analysis and molecular docking of bioactive compounds of *Camellia sinensis* and *Camellia assamica*. *Archives of Microbiology* 203(5):2501−10
37. Shi J, Yang G, You Q, Sun S, Chen R, et al. 2021. Updates on the chemistry, processing characteristics, and utilization of tea flavonoids in last two decades (2001−2021). *Critical Reviews in Food Science and Nutrition* 63(20):4757−84
38. Gong N, Zhang B, Yang D, Gao Z, Du G, et al. 2015. Development of new reference material neohesperidin for quality control of dietary supplements. *Journal of the Science of Food and Agriculture* 95(9):1885−91
39. Dadic M. 1971. Analytical methods for polyphenols in brewing. Part I. Polyphenols in Brewing and Their Estimation, Proceedings. Annual meeting- American Society of Brewing Chemists. *Taylor & Francis* 29(1):149−58
40. Ramírez-Martínez JR, Luh BS. 1973. Phenolic compounds in frozen avocados. *Journal of the Science of Food and Agriculture* 24(2):219−25
41. Kohl S, Behrens M, Dunkel A, Hofmann T, Meyerhof W. 2013. Amino acids and peptides activate at least five members of the human bitter taste receptor family. *Journal of Agricultural and Food Chemistry* 61(1):53−60
42. Zou L, Shen S, Wei Y, Jia H, Li T, et al. 2022. Evaluation of the effects of solar withering on nonvolatile compounds in white tea through metabolomics and transcriptomics. *Food Research International* 162:112088
43. Wei Y, Li T, Xu S, Ni T, Deng W, et al. 2021. The profile of dynamic changes in yellow tea quality and chemical composition during yellowing process. *LWT* 139:110792
44. Nagel CW, Herrick IW, Graber WR. 1987. Is chlorogenic acid bitter? *Journal of Food Science* 52(1):213
45. De Keukeleire J, Ooms G, Heyerick A, Roldan-Ruiz I, Van Bockstaele E, et al. 2003. Formation and accumulation of α-acids, β-acids, desmethylxanthohumol, and xanthohumol during flowering of hops (*Humulus lupulus* L.). *Journal of Agricultural and Food Chemistry* 51(15):4436−41
46. Stompor M. 2020. A review on sources and pharmacological aspects of sakuranetin. *Nutrients* 12(2):513
47. Raithore S, Kiefl J, Manthey JA, Plotto A, Bai J, et al. 2019. Mitigation of off-flavor in Huanglongbing-affected orange juice using natural citrus non-volatile compounds. *Journal of Agricultural and Food Chemistry* 68(4):1038−50
48. Fang ZT, Song CJ, Xu HR, Ye JH. 2019. Dynamic changes in flavonol glycosides during production of green, yellow, white, oolong and black teas from *Camellia sinensis* L. (cv. Fudingdabaicha). *International Journal of Food Science & Technology* 54(2):490−98
49. Galmarini MV. 2020. The role of sensory science in the evaluation of food pairing. *Current Opinion in Food Science* 33:149−55