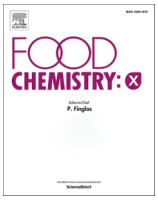
Food Chemistry: X 20 (2023) 100991

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Analysis of aroma quality changes of large-leaf black tea in different storage years based on HS-SPME and GC–MS

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ARTICLE INFO

Keywords:
Aroma quality
Black tea
Volatile metabolites
HS-SPME
GC–MS
rOAV

ABSTRACT

The reasons for the change in volatile metabolites and aroma of black tea during storage remain unclear. Therefore, we used HS-SPME and GC–MS methods to analyze the aroma compounds of new tea (2021) versus aged tea groups (2015, 2017, and 2019). A total of 109 volatile components were identified. During storage, 36 metabolites mainly with floral and fruity aromas decreased significantly, while 18 volatile components with spicy, sour, and woody aromas increased significantly. Linalool and beta-ionone mainly contributed to sweet and floral aromas of freshly-processed and aged black tea, respectively. Isovaleric acid and hexanoic acid mainly caused sour odor of aged black tea. The monoterpene biosynthesis and secondary metabolic biosynthesis pathways might be key metabolic pathways leading to changes in the relative content of metabolites during storage of black tea. Our study provides theoretical support for fully understanding the changes in the aroma quality of black tea during storage.

1. Introduction

Black tea is the most commonly served type of tea, which occupies a very important proportion of world tea consumption. The world's four major high-flavor black teas include China's Keemun black tea, India's Darjeeling black tea and Assam black tea, and Sri Lanka's Ceylon black tea. Taste and aroma are two key quality factors for black tea. The aroma of black tea mainly comes from the complex degradation of carotenoids, fatty acid hydrolysis, glycoside hydrolysis, amino acid degradation during the withering, rolling, and fermentation of fresh leaves (Feng, et al., 2019). During the withering process, the activity of β -glucosidase gradually reached a peak, but it mainly exists in the cell wall of tea leaves, therefore, only a small part of the enzyme in the cell can hydrolyze glycosides, producing some aroma substances (Supriyadi, Nareswari, Fitriani, & Gunadi, 2021; Yang, Baldermann, & Watanabe, 2013). During the rolling process, the cell wall of tea leaves is mostly destroyed, releasing a large amount of β -glucosidase, and many glycosides are hydrolyzed to produce rich aroma substances (Zeng, Watanabe, & Yang, 2019). During the fermentation process, amino acid derivatives and carotenoid derivatives increased significantly, giving black tea its

Abbreviations: GC–MS, gas chromatography-tandem mass spectrometry; HS-SPME, headspace solid-phase microextraction; TIC, total ions current; PCA, principal component analysis; VIP, variable importance projection; rOAV, relative odor activity value; QC, quality control; EI, electron impact; NIST, National Institute of Standards and Technology; HCA, hierarchical cluster analysis; PCC, Pearson correlation coefficients; OPLS-DA, the orthogonal projection to the latent structure discriminant analysis.

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https://doi.org/10.1016/j.fochx.2023.100991
Received 23 June 2023; Received in revised form 11 October 2023; Accepted 8 November 2023
Available online 17 November 2023

unique sweet and fruity aroma. At the same time, the composition of fatty acid derivatives and terpenoids also changed, which jointly promoted the formation of black tea flavor (Chen et al., 2022).

The aroma compounds of black tea mainly include alcohols, aldehydes, ketones, acids, esters, terpenes, hydrocarbons, aromatic hydrocarbons, sulfur-containing compounds, heterocyclic compounds, etc. Rawat et al. identified a total of 101 aromatic compounds in Kangra orthodox black tea, among which the main component compounds were linalool, linalool oxide, geraniol, methyl salicylate, 3,7-dimethyl base-1,5,7-octatrien-3-ol, epoxy alcohol, 2-hexenal, 1-penten-3-ol, (*Z*)-3-hexenol, etc (Rawat, et al., 2007). Joshi & Gulati found that the main aromatic compounds in Kangra orthodox black tea were geraniol, linalool, (*Z/ E*)-linalool oxide, 2-Hexenal, phytol, b-violet ketones, trienols, methyl pyrazine, and methyl salicylate, etc (Joshi & Gulati, 2015).

Aged tea has wonderful organoleptic and health effects. In China, dark tea has the reputation of "the more it ages, the better it smells" (Xu, et al., 2019). Aged white teas are also collected by many due to their outstanding health benefits (Hazra, Dasgupta, Sengupta, Saha, & Das, 2020). However, tea storage is not limited to white tea or dark tea, all six major tea types can be stored. For example, huang et al. showed that black tea can be stored for long periods of time and its bitterness and astringency significantly reduce during storage (Huang, et al., 2021). In areas such as Guangdong in China tea farmers have the habit of collecting black tea because of the special flavor quality and certain health effects of aged black tea. Compared to new black teas, aged black teas lose their freshness, but have a stronger woody and sweet aroma. Little is known about the changes in volatile components and aroma quality of black tea with different storage times. To address these issues, we used headspace-solid phase micro-extraction (HS-SPME) and gas chromatography-mass spectrometry (GC–MS) to extract the aroma compounds of black tea at different storage time (2015, 2017, 2019, and 2021). We systematically outlined the changes in the main volatile compounds during various stages of storage. These results help to understand the changes of aroma compounds, especially the main volatile compounds that affect storage, and provide a reference for selecting the appropriate storage time for black tea products.

2. Materials and methods

2.1. Tea samples and regents

The Yinghong NO.9 black tea in 2015, 2017, 2019, and 2021 were provided by the Tea Research Institute of Guangdong Academy of Agricultural Sciences (Guangdong province, China), and the tea gardens are asexual cuttings with the same cultivation and management practices. All teas were grown in the same tea plantation, picked in the same season and prepared by the same processing. All samples were stored for 0, 2, 4 and 6 years in a light-proof tea warehouse at a temperature of 20–25 °C and a relative humidity of less than 50 %. A total of 24 tea samples were included in this study and grouped according to the above-described 4 groups, so each group contained 6 biological replicates. Analytical grade sodium chloride was purchased from China National Pharmaceutical Group Co., Ltd. (Sinopharm). GC–MS grade pure hexane was purchased from Merck company and chromatographic standards of GC–MS grade were purchased from the Sigma-Aldrich company.

2.2. Sensory evaluation of aroma quality of tea samples

To determine the objective perception and professionally evaluate the black tea of 4 different years, a sensory evaluation of tea samples was conducted by the trained panelist. The aroma factor of each tea was evaluated according to the Chinese national standard (Methodology for sensory evaluation of tea, GB/T 23776–2018). Ten trained panelists (5 males and 5 females) were invited for sensory evaluation of black tea. The sensory evaluation was conducted in a professional panel room with controlled temperature and relative humidity. Tea samples (3 g) were infused with 150 mL boiled water (m:V = 1:50) in a specified column cup for five minutes and tea infusion was collected in a specified white tea tasting bowl. Objective and comprehensive aroma quality assessment for the lid, tea liquor, and infused leaves of each tea were recorded.

2.3. Headspace-solid phase microextraction

The HS-SPME of volatile metabolites from Yonghong NO.9 tea samples was extracted according to the following guidelines. The tea leaves were first taken out from the −80 °C freezer and immediately frozen and ground to a powder in liquid nitrogen. Then, 1 g of the dry powder was immediately placed into a 20 mL head-space vial (Agilent, Palo Alto, CA, USA) containing 1–2 mL saturated NaCl (Analytical Grade, supplied by China National Pharmaceutical Group Co., Ltd. [Sinopharm]) solution to inhibit any enzyme reaction. A 10 μL internal stand solution (Benzaldehyde-d6, 50 μg/mL) was also added to facilitate downstream data quality control. The internal standard was set to six duplications. The vials were sealed using crimp-top caps with TFE-silicone headspace septa (Agilent, Palo Alto, CA, USA). At the stage of HS-SPME (AllowCond, CTC Analytics AG, Zwingen, Switzerland), each vial was incubated at 100 °C for 15 min with a SPME fiber (a 120 μm coating fiber DVB/CAR/PDMS; Agilent, Palo Alto, CA, USA) placed in the CombiPal. The fiber was initially conditioned by heating in the SPME Fiber Cleaning and Conditioning Station (placed in the CombiPal, CTC Analytics AG, Zwingen, Switzerland) at the temperature of 270 °C for 5 min. SPME extracts were desorbed from the coating fiber in the gas chromatography injector at the temperature of 250 °C for 5 min and all the volatile components on the coating fiber were then analyzed using gas chromatography-tandem mass spectrometry. Besides, quality control (QC) samples were prepared by mixing all 24 sample extracts to analyze the repeatability of samples under the same processing method.

2.4. Gas chromatography-tandem mass spectrometry analysis

The identification and quantification of volatile compounds were carried out using GC–MS equipment (8890-5977B, Agilent J&W Scientific, Folsom, CA, USA). A DB-5MS (5 % phenyl-polydimethylsiloxane) capillary column (30 m × 0.25 mm × 1.0 μm, Agilent J&W Scientific, Folsom, CA, USA) was used for chromatographic analysis. High purity (99.999 %) helium was used as the carrier gas at a linear flow rate of 1.0 mL/min. The injector temperature was kept at 250 °C and the detector at 280 °C. The GC oven temperature was programmed from 40 °C (held for 3.5 min), increased at 10 °C/min to 100 °C, 7 °C/min to 180 °C, and 25 °C/min to 180 °C, held for 5 min. Mass spectra were recorded in electron impact (EI) ionization mode at 70 eV. The quadrupole mass detector, ion source, and transfer line temperatures were set at 150, 230, and 280 °C, respectively. Mass spectra were scanned in the range *m/z* 50–500 amu at 1 s intervals. Identification of volatile compounds was achieved by comparing the mass spectra with the public National Institute of Standards and Technology (NIST) database library.

2.5. Qualitative and quantitative analysis of volatile metabolites

During instrument sampling, a QC sample was inserted into every 10 test samples to monitor the repeatability of the analysis process. The raw mass spectrometry data obtained by GC–MS analysis were first deconvoluted by Masshunter Qualitative Analysis Workflows (Agilent, Palo Alto, CA, USA) to obtain information such as *m/z*, retention time, and peak area. Based on the NIST database and the MWGC metabolic database, qualitative analyses of the volatile metabolites were carried out. To facilitate downstream analysis, data pre-treatment procedures, such as nonlinear retention time alignment, peak alignment, grouping, and identification were performed. Next, we filtered peaks that were quantified in less than 50 % of a single group or all groups and replaced the missing value with half of the minimum positive value. Then, internal

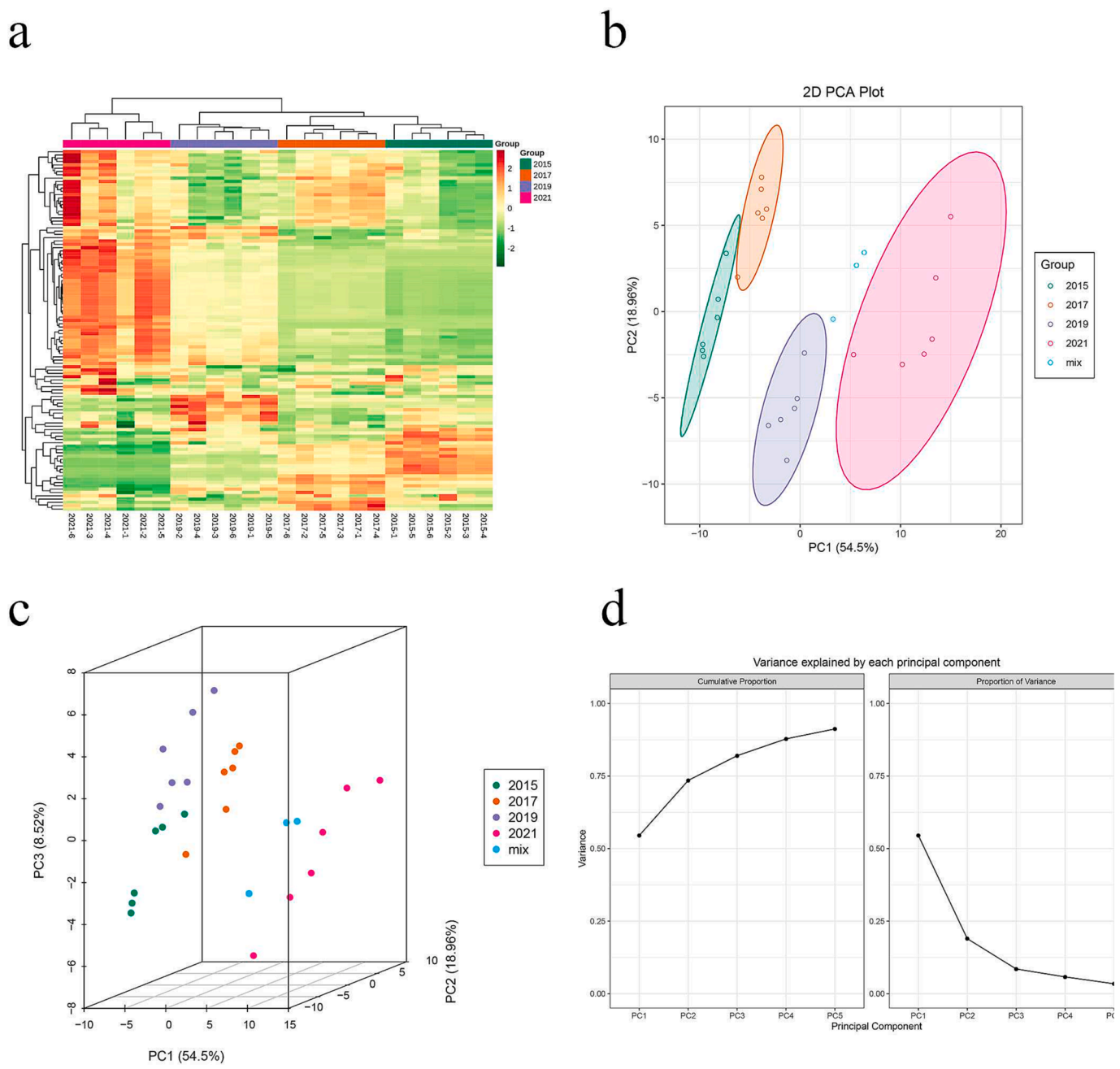

Fig. 1. The overall metabolic profile distribution among different storage years in Yinghong NO.9 Tea samples: (A) Heatmap and dendrogram of volatile components from different storage time groups; (B) 2D PCA plots of sample distribution in all 24 tea samples; (C) 3D PCA plots of sample distribution in all tea samples; (D) The proportion of variance is explained by the first five principal components.

standards were used to normalize data processing and the integrated data of all chromatographic peak areas were exported for subsequent statistical analysis. In addition, we also used internal standard method for quantitative analysis of volatile metabolites.

2.6. Calculation of relative odor activity value

The relative odor activity value (rOAV) was calculated to evaluate the contribution of individual volatile compounds to the overall aroma. The rOAV used to identify key volatile compounds from tea was calculated according to the formula (Yifan Zhu, 2020):

$$rOAV_i = 100 \times OAV_i / OAV_{max}$$

$$OAV_i = C_i / OT_i$$

where OAV_i represents the odor activity value of a certain volatile compound. OAV_{max} represents the highest odorant activity value among the volatile compounds. C_i represents the concentration of a certain volatile compound in the sample. OT_i represents the odor threshold of a certain volatile compound.

2.7. Clustering and correlation analysis

Unsupervised principal component analysis (PCA) was performed by statistics function prcomp in R (https://www.r-project.org/) to identify the within-groups and between-groups variations. The hierarchical cluster analysis (HCA) results of samples and metabolites were presented as heatmaps with dendrograms, while Pearson correlation coefficients (PCC) between samples were calculated by the cor function in

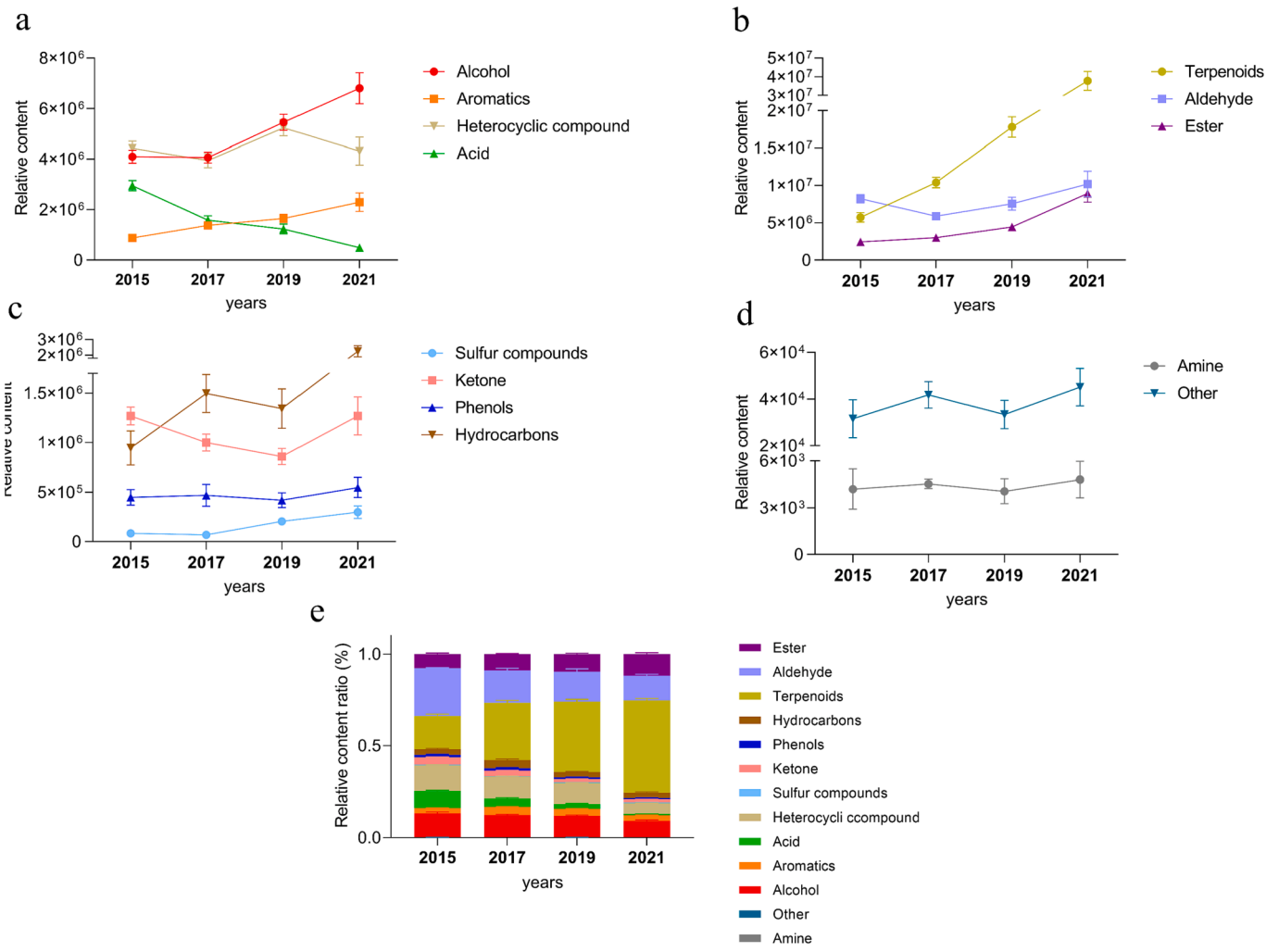

Fig. 2. The relative content and relative content proportion of various volatile metabolites: (A-D) Line graphs of relative contents of various volatile metabolites in samples from different storage years; (E) Histograms of relative content proportions of various volatile metabolites in samples from different storage years.

R and presented as only heatmaps. For PCA and HCA, normalized signal intensities of metabolites by unit variance scaling were used for visualization and calculation, respectively.

The peak data were log2 transformed (log2) and mean-centered before the orthogonal projection to the latent structure discriminant analysis (OPLS-DA) analysis. To avoid overfitting, a 200-bootstrapped permutation test was performed. The variable importance projection (VIP) value was extracted from the OPLS-DA model, and score plots and permutation plots were generated using the MetaboAnalystR package (version 1.0.1) (Chong, Yamamoto, & Xia, 2019). The reference standard for screening differential metabolites were VIP $\geq$ 1 and absolute Log2FC (fold change) $\geq$ 2.0 or Log2FC $\leq$ 0.5 and P value $<$ 0.05. Meanwhile, to study the relative change trends of metabolites among different groups, data from significantly regulated metabolites were firstly normalized and clustered by K-means analysis.

2.8. Statistical analysis

Volatile metabolites were firstly functionally annotated using the KEGG database (https://www.kegg.jp/kegg/compound/). Then, metabolite set enrichment analysis (MSEA) by the hypergeometric test was performed by the MetaboAnalystR package (version 1.0.1) (Chong, et al., 2019) to identify significantly enriched functional pathways. All continuous variables were presented as the mean $\pm$ standard deviation (SD). Statistical significance was defined as P $<$ 0.05. The R (version 4.0.2, https://www.r-project.org/) software was used for statistical analysis.

3. Results and discussion

3.1. Sensory evaluation of aroma quality of black tea in different years

We first conducted a sensory evaluation on the aroma quality of black tea from four different years and integrated the professional comments from 10 panelists. The results were shown in Table S1. The black tea processed in 2021 had a fairly pure and rich sweet floral aroma; in 2019 processed, the black tea was fairly pure with a sweet floral aroma; the aroma of tea processed in 2017 was pure, slightly sweet, and sour; in 2015 processed, the aroma of black tea was pure, sour, and less sweet. The sensory evaluation results indicated that the sweetness and floral aroma of black tea gradually weakened and the sourness gradually increased with the increase of storage years. The changes in aroma quality of black tea during storage are consistent with those reported in the previous literature (Meng, et al., 2021a).

3.2. The volatile metabolites profile of black tea in different years

To gain a panoramic view of volatile component changes, we used GC–MS to compare black tea samples with different storage times. As shown in Table S2, a total of 109 volatile metabolites were identified in

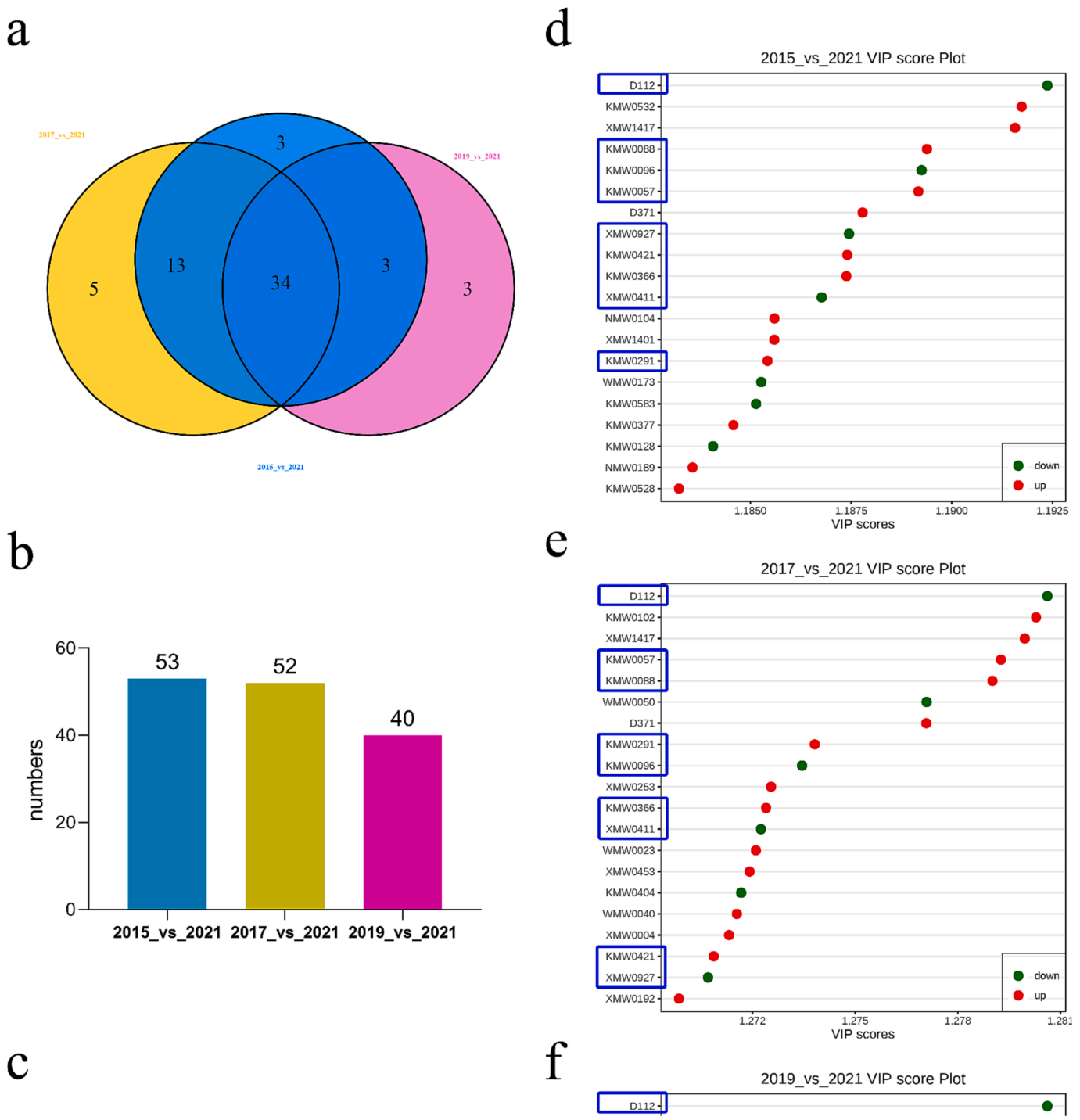

Fig. 3. Differential metabolite analysis between freshly-processed tea group (2021) and aged tea groups: (A) Venn diagram of differential metabolites of fresh tea (2021) versus aged tea; (B) Numbers of differential metabolite between fresh tea (2021) and aged tea; (C) The number of differential metabolites at all levels; (D-F) Dot plot of VIP scores among the comparison of 2015 vs. 2021, 2017 vs. 2021, and 2019 vs. 2021, respectively.

all 24 tea samples. The 109 aromatic compounds can be divided into 13 categories, including 25 hydrocarbons, 19 terpenoids, 18 esters, 11 aldehydes, 9 heterocyclic compounds, 8 alcohols, 8 aromatics, 5 ketones, 2 acids, 1 amine, 1 halogenated hydrocarbon, 1 phenol, and 1 sulfur-containing compound. Major volatile compounds such as alcohols, aldehydes, ketones, esters, and hydrocarbons were also detected in black tea processed from Yinghong NO.9 grafted to other tea trees (Chen, Qi, Wang, Miao, & Ma, 2021). Volatile compounds such as terpenes, pyrazines, furans, and acids were also detected in Turkish black tea (Alasalvar, et al., 2012). Aromatic substances such as aldehydes, alcohols, ketones, esters, acids, terpenes, furans, and pyrroles can be detected in instant black tea (Kraujalyte, Pelvan, & Alasalvar, 2016).

Next, we visualized the total ion chromatogram (TIC) profile of the mixed QC samples in Fig. S1A. The overlapped TIC profiles indicated the good stability and repeatability of the instrumental system. We also performed PCC to assess the biological duplication between samples and groups (Fig. S1B). The correlations between samples within the same group were close to 1, also indicated that the reproducibility of samples within one group was very good.

We then used HCA to extract information on differences among

different storage years. From Fig. 1A, we can see that there are significant differences in metabolites in 4 different storage years. Both the freshly-processed tea group (2021) and the other aged tea groups have good within-group consistency. We then used unsupervised PCA analysis to investigate the overall sample distributions from four different storage years and the variability within each group. From the 2D PCA (Fig. 1B) and 3D PCA plot (Fig. 1C), the extracted first three principal components PC 1, PC 2, and PC 3 were 54.5 %, 18.96 %, and 8.52 %, respectively. The mixed QC samples (cyan) in the PCA plot also showed good stability of the instrumental system. The total accumulative contribution rate of PC 1 to PC 5 reached nearly 90 % (Fig. 1D). These results also showed that black teas in 2015, 2017, 2019, and 2021 were distinguished from one another, and all four groups showed good within-group consistency. Notably, the freshly-processed tea group (2021) differs from other non-fresh processed tea groups (2015, 2017, and 2019), implying that differences in tea storage time will lead to differences in the composition and content of volatile components. The aroma profile of tea leaves changes significantly during storage. For example, the aroma of white tea gradually changes from sweet, fruity and floral to stale, woody and herbal during long-term storage (Wang, et al., 2022). The woody, floral and fruity aroma components were accumulated year by year, result to the woody, floral, and fruity character observed in aged oolong tea (Zhang, et al., 2023).

Furthermore, to summarize the chemical composition changes, the relative content trends of 13 types of volatile metabolites were analyzed over 4 storage years (Fig. 2). The relative contents of terpenoids, aromatics, and ester decreased gradually with the increase of storage years, while the relative content of acids increased with the increase of storage years. The relative contents of alcohols and sulfur-containing compounds gradually decreased with the increase of storage years, but then showed a small upward trend between 2017 and 2015 (Fig. 2A–D). In terms of the relative content ratio (Fig. 2E), terpenoids and ester also showed a downward trend with the increase in storage years. However, aldehydes, ketones, heterocyclic compounds, acids, and alcohols showed the opposite trend. Among them, both the relative content and the relative content ratio of terpenes and esters showed a decreased trend with the increase in storage years, whereas the relative content and relative content ratio of acid showed a reverse trend. Some volatiles in tea such as alcohols, aldehydes and ketones, which were easily affected by long-duration oxidation during storage, so that they may contribute to the decrease of sweet and fresh odors (Meng, et al., 2021b).

3.3. Differential analysis of volatile metabolites between storage time

We use both univariate and multivariate statistical analysis to accurately screen the differential volatile metabolites between the freshly-processed tea group (2021) and other remaining aged tea groups. 62 significantly different volatile metabolites were identified (Table S3). These metabolites can be divided into 10 categories, including 15 terpenoids, 14 esters, 6 aromatics, 6 aldehydes, 5 alcohols, 5 hydrocarbons, 4 ketones, 4 heterocyclic compounds, 2 acids, and 1 sulfur-containing compound. As shown in Fig. S2, with the increase in storage years, the relative contents of terpenes, esters, alcohols, and aromatics gradually decreased, while the relative content of acids increased, which were similar to the above results (Fig. 2). Some studies reported that methoxy benzenes volatiles, lipids derivatives and carotenoids derivatives increasing, while glycoside-derived volatiles decreasing during black tea storage (Meng, et al., 2021b).

We then conducted a K-means clustering analysis to study the relative change trends of metabolites among four different groups. A total of 62 differential metabolites were divided into 4 clusters (Fig. S3). Thirty-six metabolites of subclass 1 from K-means clustering analysis showed a sharp downward trend with the increase of storage years (Fig. S3A). By classifying the 36 volatile compounds, we can group the subclass 1 metabolites into 10 esters, 9 terpenes, 4 alcohols, 4 aromatics, and 3 aldehydes, 2 hydrocarbons, 2 ketones, 1 sulfur-containing compounds, and 1 heterocyclic compound; the odor description of these metabolites was mainly floral-fruity (Table S4). Contrary to the above trend, 18 volatile components from subclass 2 were with the increase of storage years (Fig. S3B). These volatile metabolites include 4 terpenoids, 3 esters, 2 acids, 2 ketones, 2 aldehydes, 2 aromatic hydrocarbons, 1 heterocyclic compound, 1 alcohol, and 1 hydrocarbon. And the dominant odor type of subclass 2 was spicy, sour, and woody (Table S5). The relative content of the 5 metabolites of subclass 3 first increased with the increase of storage years and then decreased after reaching the peak in 2019 (Fig. S3C). Three volatile metabolites from subclass 4 showed a wave distribution as the storage time increased (Fig. S3D). Studies have shown that the degradation of carotenoids during the fermentation of black tea will produce terpenes, alcohols, aldehydes, and other aroma compounds, suggesting that the production of various aroma compounds in black tea may be closely related to the fermentation process (Mei, et al., 2021).

3.4. Differential analysis of volatile metabolic components among different storage years

To identify volatile metabolites related to storage time, we first calculate differential volatile metabolites between freshly-processed tea (2021) and vintage tea (2015, 2017, and 2019). As shown in Fig. 3A–C, a total of 53, 52, and 40 differential metabolites were found in 2015 vs. 2021, 2017 vs. 2021, and 2019 vs. 2021, respectively. There are 11 unique differential metabolites and 34 common differential metabolites among the three comparison groups (Fig. 3A–C).

Then, we used the VIP scores to further screen the differential metabolites and found that, of the top 20 differential metabolites sorted by the VIP score, 9 differential metabolites were identified in the three comparison groups. The nine differential metabolites included 3 esters, 2 alcohols, 1 aldehyde, 1 acid, 1 terpenoid, and 1 ketone. Among them, 4 volatile metabolites were significantly down-regulated in freshly-processed tea (2021) compared with aged tea (2015, 2017, and 2019), including methyl phenylacetate, isovaleric acid, 2,6, 6-trimethyl-2-cyclohexene-1,4-dione, and 2,6-dimethylcyclohexanol (VIP score $>$ 1.18; Fig. 3D–F). Methyl phenylacetate, was detected as the main volatile component in Antrodia cinnamomea (Chiang, Huang, Lin, Chen, & Chiang, 2013). Isovaleric acid is a short-chain fatty acid, mainly presenting a rotten, pungent sour odor, and is a volatile substance with the characteristic flavor of Japanese buckwheat (Sakurai, Tomiyama, Yaguchi, & Asakawa, 2020). 2,6,6-Trimethyl-2-cyclohexene-1,4-dione is one of the important aroma substances in kanuka (Beitlich, Koelling-Speer, Oelschlaegel, & Speer, 2014). 2,6-Dimethylcyclohexanol exhibits a certain anesthetic effect by regulating GABAA receptors, so it can be used in anesthetics research in medicine (Chowdhury, et al., 2016).

Compared with aged tea, 5 compounds were significantly up-regulated in freshly-processed tea, which were *cis*-3-hexenyl butyrate, methyl salicylate, linalool, *cis*-2-penten-1-ol, 2-hexenal (VIP score $>$ 1.18; Fig. 3D–F). Collectively, these results indicated that the 9 different metabolites shared by the three comparisons maybe the key volatile compounds that distinguish freshly-processed black tea from aged tea. Cis-3-hexenyl butyrate is one of the key odor compounds that distinguish the aroma of three white tea (Silver needle with white hair, White peony, and Shoumei), mainly showing floral, fruity, and sweet flavors (Chen, et al., 2020). During the processing of white tea, methyl salicylate is synthesized by the substrate salicylic acid under the action of salicylic acid carboxymethyltransferas, which imparts a special floral aroma to white tea (Deng, Wang, Yang, Jiang, & Zhang, 2017). It was found that linalool, geraniol, and methyl salicylate exhibited high aroma contribution to the three black teas (Jinjunmei, Keemun, and Dianhong), and methyl salicylate could interact with nerol, benzyl alcohol, α-terpineol, and β-ocimene compounds and exhibited synergy interactions in aroma perception (Niu, et al., 2022). Linalool is also an important evaluation for the aroma quality of many green teas (Kato &

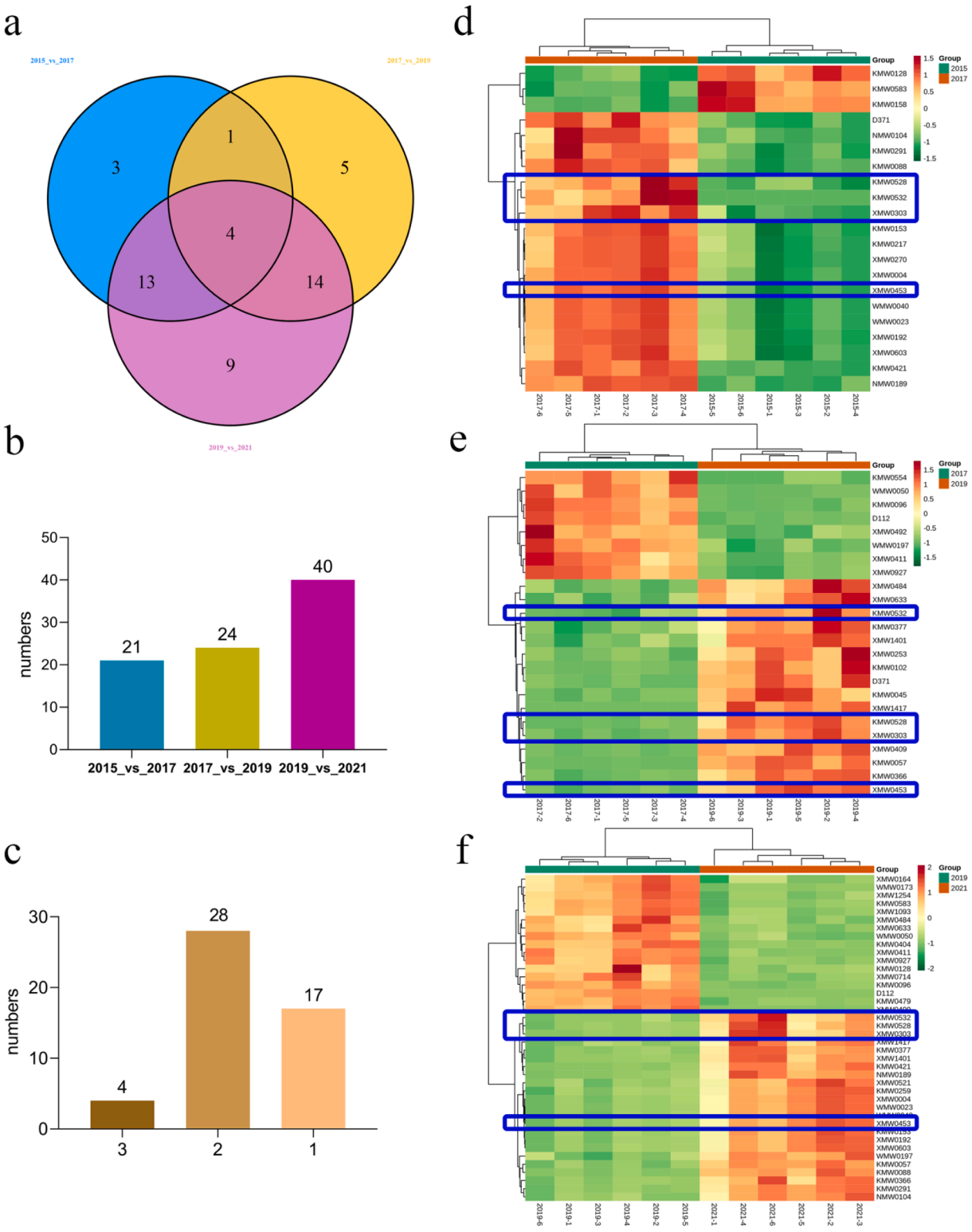

Fig. 4. Differential metabolite analysis between adjacent storage years: (A) Venn diagram of differential metabolites between adjacent storage years; (B) Numbers of differential metabolite between adjacent storage years; (C) The number of differential metabolites at all levels; (D-F) Heatmap of differential metabolites among the comparison of 2015 vs. 2017, 2017 vs. 2019, and 2019 vs. 2021, respectively.

Shibamoto, 2001). The tea leaves eaten by the green leafhopper can significantly up-regulate linalool synthase (CsLIS1 and CsLIS2), promote the formation and release of linalool in tea leaves, and make the tea leaves have a strong floral aroma (Mei, et al., 2017). *cis*-2-Penten-1-ol can be used as one of the important aroma components to distinguish green tea grades, as well as a walnut oxidation marker and one of the important volatile compounds to distinguish walnut oxidation levels (Grilo & Wang, 2021; Kato & Shibamoto, 2001). 2-Hexenal contributes greatly to the green aroma of tea leaves. The latest research found that (*Z*)-3-hexenal can be converted into 2-hexenal by the enzymatic action of (*Z*)-3:(*E*)-2-hexenal isomerase during tea processing, indicating that this isomerase may play a key regulatory role in the corresponding

Table 1
Information of aroma substances in different years of black tea.

Compounds	CAS	Odor descriptor	Threshold (mg/m3)	Contents (mg/L)				rOVA			
				2015	2017	2019	2021	2015	2017	2019	2021
gamma-terpinene	99–85-4	lemon, gasoline, terpene, turpentine, herbal, woody, oily, lime (†)	2.5	4.01 ± 0.507^{c}	7.43 ± 0.58^{c}	12.88 ± 1.73^{b}	27.22 ± 4.58^{a}	<0.01	<0.01	<0.01	<0.01
naphthalene	91–20-3	strong mothball odor, dry, pungent, tarry (#, †, *)	0.45	12.59 ± 1.09^{b}	20.27 ± 1.69^{a}	10.61 ± 1.36^{c}	2.33 ± 0.37^{d}	<0.01	0.03	0.01	<0.01
beta-ionone	79–77-6	violet, orange, jam, seaweed, orris, raspberry, cedar wood odor (†, *)	0.00004	14.83 ± 1.74^{a}	7.17 ± 0.69^{b}	3.90 ± 0.44^{c}	1.29 ± 0.22^{d}	100.00	100.00	51.47	7.09
linalool	78–70-6	lemon, citrus, orange, floral, sweet, woody, blueberry, bois de rose, lavender (#, †)	0.0024	113.69 ± 15.81^{d}	240.28 ± 8.86^{c}	468.06 ± 21.57^{b}	1140.55 ± 13.88^{a}	12.78	55.86	102.96	104.40
(+)-alpha-pinene	7785–70-8	–	0.0053	22.85 ± 4.11^{c}	51.23 ± 3.21^{c}	108.32 ± 7.66^{b}	260.85 ± 16.01^{a}	1.16	5.39	10.79	10.81
2-hexenal	6728–26-3	apple, cheesy, vegetable, banana, rancid, fatty, plum, almond (#, †,*)	0.0031	3.98 ± 0.55^{d}	11.56 ± 1.07^{c}	22.63 ± 1.94^{b}	84.93 ± 9.02^{a}	0.35	2.08	3.85	6.02
hexanal	66–25-1	grass, sweaty, tallow, fresh, fatty, fruity, aldehydic (#, †)	0.23	5.63 ± 0.20^{c}	4.26 ± 0.18^{d}	7.08 ± 1.29^{b}	11.67 ± 1.14^{a}	<0.01	0.01	0.02	0.01
hexyl hexanoate	6378–65-0	peach, vegetable, herbal, apple peel, fresh, cut grass (#, †, *)	41.89	<0.01	<0.01	0.01 ± 0.10^{b}	0.04 ± 0.39^{a}	<0.01	<0.01	<0.01	<0.01
tetradecane	629–59-4	alkane, waxy, mild (†)	5	4.43 ± 0.42^{b}	6.92 ± 1.12^{a}	5.17 ± 0.61^{b}	5.49 ± 0.86^{b}	<0.01	<0.01	<0.01	<0.01
tridecane	629–50-5	alkane (†)	42	2.28 ± 0.42^{b}	2.70 ± 0.31^{b}	3.01 ± 0.54^{b}	3.96 ± 0.68^{a}	<0.01	<0.01	<0.01	<0.01
dimethyl disulfide	624–92-0	citrus, cabbage, sulfurous, putrid, wine_like, onion, fatty, floral, woody, herbaceous, nutty, spicy (#, †,*)	0.0084	5.17 ± 0.56^{c}	4.14 ± 0.42^{c}	12.95 ± 1.90^{b}	19.47 ± 4.09^{a}	0.54	0.46	0.81	0.51
2-phenylethanol	60–12-8	lilac, rose, rose water, honey, rose flower, rose dried (†)	0.0012	65.88 ± 4.46^{b}	62.88 ± 6.54^{b}	75.90 ± 3.91^{a}	78.85 ± 7.30^{ab}	47.90	48.90	33.39	14.43
D-limonene	5989–27-5	mint, lemon, citrus, orange, fresh, sweet (†, *)	0.045	23.69 ± 4.40^{d}	47.45 ± 3.38^{c}	75.79 ± 7.02^{b}	153.53 ± 10.16^{a}	0.46	0.98	0.89	0.75
2-heptanol	543–49-7	lemon, mushroom, sweet, floral, green, herbal, fresh, mild alcohol odor (†, *)	0.1	35.65 ± 5.46^{c}	49.71 ± 4.32^{c}	84.26 ± 5.34^{b}	156.33 ± 2.56^{a}	0.31	0.28	0.44	0.34
M−cymene	535–77-3	–	3.51	20.45 ± 3.94^{d}	33.60 ± 2.16^{c}	50.39 ± 5.06^{b}	79.80 ± 5.48^{a}	<0.01	0.01	0.01	<0.01
isovaleric acid	503–74-2	sour, sweat, stinky, animal, rancid, cheese, penetrating odor (#, †, *)	0.0018	91.14 ± 5.79^{a}	63.09 ± 5.64^{b}	20.86 ± 1.60^{c}	4.56 ± 0.70^{d}	13.66	19.56	6.12	0.56
beta-cyclocitral	432–25-7	mint, saffron, damascone, sweet, fruity, rose oxide (†)	0.015	4.17 ± 0.35^{c}	4.66 ± 0.25^{c}	8.33 ± 0.75^{a}	6.67 ± 1.05^{b}	0.07	0.17	0.29	0.10
linalool oxide (*trans*-pyranoid)	39028–58-5	woody (†)	36.100	44.70 ± 4.26^{a}	41.60 ± 5.04^{a}	44.66 ± 4.6^{a}	47.03 ± 1.74^{a}	<0.01	<0.01	<0.01	<0.01
beta-ocimene	3779–61-1	citrus, herbal, sweet, woody, terpene, herb (†)	0.0187	14.49 ± 2.75^{c}	32.72 ± 2.13^{c}	66.74 ± 6.64^{b}	154.68 ± 10.47^{a}	0.21	0.98	1.88	1.82
trans-linalool oxide (furanoid)	34995–77-2	–	0.330	28.48 ± 2.78^{b}	22.76 ± 1.83^{c}	36.62 ± 3.15^{a}	27.65 ± 3.38^{b}	0.02	0.04	0.06	0.02
2-methylisoborneol	2371–42-8	must, earth (†)	0.00048	49.34 ± 6.60^{b}	46.44 ± 6.57^{b}	55.00 ± 8.19^{b}	76.89 ± 6.86^{a}	27.72	53.98	60.50	30.73
(-)-terpinen-4-ol	20126–76-5	–	3	1.32 ± 0.16^{c}	1.08 ± 0.06^{b}	1.65 ± 0.16^{a}	1.86 ± 0.20^{a}	<0.01	<0.01	<0.01	<0.01
(-)-beta-pinene	18172–67-3	resinous, fresh, green, hay, woody, pine, dry (†)	2	60.57 ± 10.8^{d}	134.82 ± 7.30^{c}	270.91 ± 19.22^{b}	594.64 ± 26.75^{a}	<0.01	0.04	0.07	0.07
cis-3-hexenyl butyrate	16491–36-4	apple, wine, fresh, fruity, metallic, buttery (†)	2.78	0.31 ± 0.03^{c}	0.48 ± 0.06^{c}	1.76 ± 0.24^{b}	5.57 ± 1.01^{a}	<0.01	<0.01	<0.01	<0.01
cis-2-penten-1-ol	1576–95-0	rubber, ethereal, green, fruity, plastic (†)	2.02	4.39 ± 0.68^{c}	3.47 ± 0.36^{c}	30.99 ± 3.24^{b}	74.05 ± 5.31^{a}	<0.01	<0.01	0.01	0.01
hexanoic acid	142–62-1	fatty, sour, sweat, cheese (†)	0.0048	88.74 ± 7.71^{a}	33.16 ± 5.64^{c}	57.30 ± 10.98^{b}	27.45 ± 2.39^{c}	4.99	3.85	6.30	1.26
2-naphthol	135–19-3	–	0.23	3.44 ± 0.36^{b}	2.59 ± 0.18^{c}	5.33 ± 0.66^{a}	3.14 ± 0.68^{bc}	<0.01	<0.01	0.01	<0.01
alpha-ionone	127–41-3	floral, sweet, woody, fruity, orris, raspberry, violet (†)	0.02	6.76 ± 0.80^{b}	10.12 ± 0.81^{a}	4.89 ± 0.44^{c}	5.02 ± 1.24^{c}	0.09	0.28	0.13	0.06
nonanal	124–19-6	citrus, lime, orange peel, rose, aldehydic, orris, grapefruit (†)	0.0031	4.94 ± 0.45^{ab}	4.35 ± 0.63^{b}	3.65 ± 0.69^{b}	5.70 ± 0.90^{a}	0.43	0.78	0.62	0.40
benzeneacetaldehyde	122–78-1	hyacinth, honey, clover, hawthorne, cocoa, grapefruit, peanut, floral (†)	0.0017	194.84 ± 11.82^{b}	182.16 ± 8.35^{b}	231.85 ± 8.21^{b}	391.90 ± 12.95^{a}	30.91	59.06	75.01	49.37
methyl salicylate	119–36-8	mint, wintergreen, peppermint (#, †, *)	0.016	12.31 ± 1.68^{c}	48.91 ± 3.14^{bc}	87.97 ± 8.59^{b}	324.21 ± 56.01^{a}	0.21	1.71	2.90	4.90

(*continued on next page*)

Table 1 (*continued*)

Compounds	CAS	Odor descriptor	Threshold (mg/m3)	Contents (mg/L)				rOVA			
				2015	2017	2019	2021	2015	2017	2019	2021
dodecane	112–40-3	alkane (†)	0.77	3.62 ± 0.68^{b}	$5.88\infty \pm 0.80^{a}$	4.13 ± 0.80^{b}	7.06 ± 1.61^{a}	<0.01	<0.01	<0.01	<0.01
decanal	112–31-2	citrus, soap, orange peel, tallow, waxy, floral, sweet, aldehydic (†)	0.0026	0.23 ± 0.04^{ab}	0.19 ± 0.05^{b}	0.22 ± 0.06^{b}	0.31 ± 0.08^{a}	0.02	0.04	0.04	0.03
1-hexanol	111–27-3	oil, alcoholic, ethereal, resin, sweet, fruity, flower, green (†)	0.034	2.35 ± 0.64^{c}	2.27 ± 0.34^{c}	15.75 ± 3.04^{b}	21.23 ± 2.80^{a}	0.02	0.04	0.24	0.14
phenol	108–95-2	phenol, plastic, rubber, phenolic (†)	0.021	27.35 ± 4.78^{a}	28.43 ± 6.79^{a}	26.63 ± 4.74^{b}	35.55 ± 6.52^{a}	0.35	0.76	0.67	0.37
mesitylene	108–67-8	–	0.83	6.51 ± 0.82^{b}	9.31 ± 1.23^{a}	7.86 ± 1.49^{ab}	10.28 ± 2.17^{a}	<0.01	<0.01	0.01	<0.01
(-)-alpha-terpineol	10482–56-1	floral, lilac (†)	46.23	6.38 ± 0.59^{c}	7.02 ± 0.45^{c}	8.59 ± 0.55^{b}	11.51 ± 1.60^{a}	<0.01	<0.01	<0.01	<0.01
methyl phenylacetate	101–41-7	spice, waxy, floral, sweet, honey, almond, jasmine odor (†, *)	0.61	2.64 ± 0.21^{a}	1.33 ± 0.11^{b}	0.38 ± 0.03^{c}	<0.01	<0.01	<0.01	<0.01	<0.01
benzaldehyde	100–52-7	cherry, almond, sweet, burnt sugar, sharp, strong (#, †, *)	0.1	261.87 ± 6.36^{a}	122.97 ± 8.46^{c}	188.31 ± 6.29	140.99 ± 8.12^{c}	0.71	0.69	1.05	0.34

abiotic stress of fresh tea leaves (Chen et al., 2022).

Moreover, we also calculate differential volatile metabolites between adjacent storage years, i.e., 2015 vs. 2017, 2017 vs. 2019, 2019 vs. 2021, respectively. As shown in Fig. 4A–C, a total of 21, 24, and 40 differential metabolites were found, respectively. There were 17 unique differential metabolites and 4 common among the three comparison groups (Fig. 4A–C). The 4 shared compounds were *cis*-3-hexenyl hexanoate, hexyl hexanoate, hexanoic acid, 5-hexenyl ester, and (*E*, *Z*)-2,6-dimethylocta-2,4,6-triene, including 3 esters and 1 hydrocarbon. Their relative content decreased significantly with the increase in storage years (Fig. 4D–F). Cis-3-hexenyl hexanoate is identified as one of the key aromatic compounds in Xinyang Maojian green tea (Cui, et al., 2022). Hexyl hexanoate is one of the representative aroma compounds detected in a variety of strawberries, and together with geraniol constitute the main volatiles that distinguish strawberry varieties (González-Domínguez, Sayago, Akhatou, & Fernández-Recamales, 2020). (*E*, *Z*)-2,6-Dimethylocta-2,4,6-triene volatiles were detected at a relative content of 6.21 % in *Pistacia Atlantic* leaves (Falahati, et al., 2015). These results suggested that the four different volatile metabolites may distinguish black tea in the comparison of adjacent years.

3.5. Odor contribution of volatile metabolites

To evaluate the odor contribution of the determined volatile metabolites, we have performed a literature search to retrieve the odor threshold of each volatile metabolite. In total, the odor threshold of 39 volatile metabolites were retrieved, and the contents of metabolites were calculated by internal standard method, which can be used for rOAV calculation. Generally, volatile metabolites can only be perceived by the human noes when the rOAV > 1 (Wei, et al., 2020). The number of metabolites with rOAV > 1 in 2021, 2019, 2017, 2015 black tea were 10, 11, 10, 8, respectively (Table 1). With the increase of storage years (2021 to 2015), the rOAV values of methyl salicylate, 2-hexenal, linalool, (+)-alpha-pinene, beta-ocimene, and 2-methylisoborneol showed a decreasing trend; while the rOAV values of beta-ionone, 2-phenylethanol, isovaleric acid, and hexanoic acid were gradually raised (Table 1). Linalool had the highest rOAV activity in the two years of 2021 and 2019, respectively, which mainly showed the floral and sweet flavors of lemon and orange odor type (Table 1). The volatile compound with the highest rOAV in 2017 and 2015 was beta-ionone (rOAV: 100), which was mainly characterized by the fragrance of violet flowers (Table 1). But isovaleric acid and hexanoic acid were present sour odor, and the rOAV of these were increased with the storage years extend (Table 1). Therefore, it can be found that the floral and fruity aroma of black tea were always dominant with the increase of storage years, and the sour taste gradually increased, which was consistent with the sensory evaluation of the aroma quality in different years.

Notably, methyl salicylate, 2-hexenal, and linalool are also important volatile aromatic substances in Kangra orthodox black tea (Joshi & Gulati, 2015). Isovaleric acid has been widely used in Swiss cheese (Thierry, Richoux, & Kerjean, 2004), it was an isomer of 2-methylbutyric acid and they all contributed prominently to the unique flavor of Swiss cheese. During the fermentation process, *Propionibacterium freudenreichii* uses L-isoleucine and α-ketoglutarate as substrates, and under the action of the corresponding cofactors, firstly generates α-ketopentaisocaproic through transamination, and then generates isovaleric acid by enzymatic reaction (Thierry, Maillard, & Yvon, 2002).

3.6. KEGG functional annotation and enrichment analysis of differential metabolites

The differential metabolites obtained above often have similar or complementary biological functions and are usually regulated by the same metabolic pathway, thus showing unique characteristics among different groups. So, we performed KEGG functional annotation and pathway enrichment analysis for these differential metabolites. Four

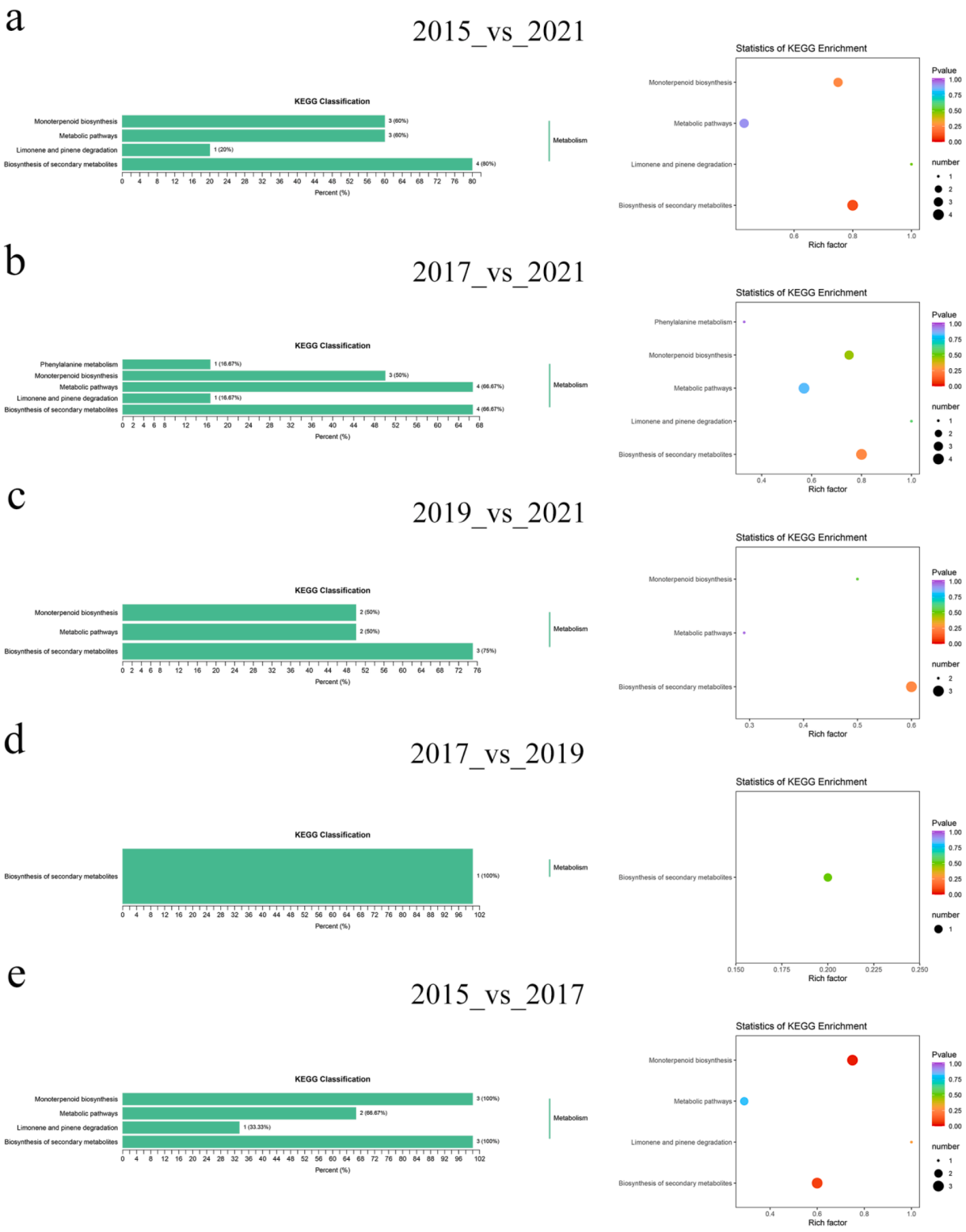

Fig. 5. KEGG enrichment plots of differential metabolites: (A-C) KEGG enrichment classification map and bubble map of differential metabolites between fresh tea group (2021) and aged tea groups; (D-E) KEGG enrichment classification and bubble map of differential metabolite between adjacent storage years.

pathways are enriched in 2015 vs. 2021 comparison (Fig. 5A); 5 pathways are enriched in the 2017 vs. 2021 comparison (Fig. 5B); 3 pathways are enriched in 2019 vs. 2021 comparison (Fig. 5C). The shared enrichment pathway between the freshly-processed tea and aged tea was monoterpene biosynthesis, indicating the difference in the relative content of some volatile metabolites that distinguish freshly-processed black tea from aged tea might be caused by the reaction of monoterpenoid biosynthesis. The changes in the relative contents of some volatile metabolites may be regulated by the biosynthesis of monoterpenes, thereby changing the original aroma quality of black tea. The monoterpene biosynthetic pathway involves the generation of linalool and linalool derivatives (Qiu, et al., 2019). Many studies have shown that linalool has obvious antibacterial activity against bacteria such as Streptococcus spoilus, Aeromonas hydrophila, etc., suggesting

that it can be applied in the preservation of aquatic seafood (Guo, et al., 2021; Zhong, et al., 2021). In addition, it has also been reported that linalool has an antioxidant effect and has a relieving effect on carbon tetrachloride-induced liver injury in rats (Altinok-Yipel, et al., 2020).

Besides, we also applied KEGG enrichment analysis of differential volatile metabolites between adjacent storage years. Overall, 3, 1, and 4 pathways were enriched in 2019 vs. 2021, 2017 vs. 2019, and 2015 vs. 2017, respectively (Fig. 5C–E). Among them, the secondary metabolic biosynthesis pathway was shared by the three comparison groups. Many aroma compounds from tea are generated and degraded through secondary metabolic biosynthetic pathways. Isovaleric acid is a short-chain fatty acid produced by a secondary metabolic biosynthetic pathway, which relaxes colonic smooth muscle via the cAMP/PKA pathway (Blakeney, Crowe, Mahavadi, Murthy, & Grider, 2019). Isovaleric acid inhibits osteoclast differentiation, thereby improving osteoporosis induced by oophorectomy (Cho, Kim, Lee, Lee, & Bae, 2021). Isovaleric acid has a similar structure to γ-aminobutyric acid, and exhibits significant inhibitory activity on γ -aminobutyric acid aminotransferase, which is expected to be an anxiolytic or sedative-related preparation (Park, Lee, Lee, Kwon, & Chun, 2020). In addition, many studies have found that the quantity and richness of various types of short-chain fatty acids, mainly isovaleric acid, in feces are closely related to the occurrence and development of proctitis, colitis, and other diseases (Rotondo-Trivette, et al., 2021; Tian, Ma, Zhang, Mi, & Fan, 2020). Collectively, the secondary metabolic biosynthesis pathway might be the key metabolic pathway leading to changes in the relative content of metabolites during the storage of black tea.

4. Conclusions

The results of the sensory review show a marked difference in the aroma qualities of new and vintage black teas. Black teas have a strong sweet floral aroma, while black teas increase in acidity aroma and decrease in sweetness aroma after a period of storage. We used HS-SPME and GC–MS methods to analyze the aroma compounds of new tea (2021) versus aged tea groups (2015, 2017, and 2019). A total of 109 volatile metabolites were detected in black tea, and 62 significantly different volatile metabolites were identified in different years of processed tea (2015, 2017, 2019, and 2021). And there were 36 metabolites showed a downward trend and 18 volatile components presented an upward pattern with the increase of storage years. Linalool and beta-ionone were mainly volatile metabolites that were contribute to the floral and fruity aroma of black tea at various storage years, which were beneficial for its aroma quality. However, isovaleric acid and hexanoic acid were the main compounds that have bad effects on its aroma quality, because they present sour odor. In addition, the monoterpene biosynthesis and secondary metabolic biosynthesis pathways might be key metabolic pathways leading to changes in the relative content of metabolites during storage of black tea. These findings have certain practical significance for guiding the storage of black tea.

CRediT authorship contribution statement

Suwan Zhang: Writing – original draft, Writing – review & editing, Data curation. **Lingli Sun:** Writing – original draft, Writing – review & editing, Funding acquisition, Data curation. **Shuai Wen:** Investigation. **Ruohong Chen:** Investigation. **Shili Sun:** Investigation. **Xingfei Lai:** Investigation. **Qiuhua Li:** Investigation. **Zhenbiao Zhang:** Investigation. **Zhaoxiang Lai:** Investigation. **Zhigang Li:** Investigation. **Qian Li:** Investigation. **Zhongzheng Chen:** Resources, Methodology, Supervision. **Junxi Cao:** Resources, Methodology, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

This study was funded by Food nutrition and health Collaborative Innovation Center of GDAAS [Grant No. XT202229]; Agricultural competitive industry discipline team building project of Guangdong Academy of Agricultural Sciences [Grant No. 202126TD]; Guangdong Basic and Applied Basic Research Foundation [Grant Nos. 2021A1515010958, 2022A1515110190, 2023A1515010264]; Guangzhou Science and Technology Plan Projects [Grant Nos. 202102020047, 20212100005, 202002030202, 202201011455, 2023E04J0781]; Key-Area Research and Development Program of Guangdong Province [Grant No. 2020B0202080003]; Innovation Fund projects of Guangdong Academy of Agricultural Sciences [Grant Nos. 202115, 202135]; Special fund for scientific innovation strategy-construction of high level Academy of Agriculture Science [Grant Nos. R2019PY-JX004, R2022PY-QY010]; the Innovation Fund projects of Guangdong Key Laboratory of Tea Plant Resources Innovation and Utilization [Grant No. 2021CX02]; Special fund project for introduction of scientific and technological talents of Guangdong Academy of Agricultural Sciences [Grant No. R2021YJ-YB3014]. Funders did not have any role in study design, data collection, and data analysis.

Ethical statements

This study does not contain any human or animal experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100991.

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