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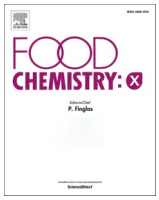

HS-SPME-GC-MS untargeted metabolomics reveals key volatile compound changes during Liupao tea fermentation

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ABSTRACT

This study used headspace solid-phase microextraction-gas chromatography–mass spectrometry and multivariate statistical analysis to comprehensively analyze the volatile components in Liupao tea samples throughout fermentation. In total, 1009 volatile organic compounds were detected and identified, including terpenoids, heterocyclic compounds, esters, ketones, hydrocarbons, alcohols, aromatics, and acids. Principal component and hierarchical cluster analyses, characterize the volatile components of Liupao tea samples were characterized at various fermentation stages. Orthogonal partial least squares discriminant analysis identified 248 differentiating compounds (VIP ≥ 1, $P < 0.05$, and $|\mathrm{Log_2FC}| \geq 1.0$) during fermentation. K-means clustering analysis showed that 11 metabolites increased significantly throughout the fermentation process, whereas 31 metabolites decreased continuously. Annotation of these differential compounds revealed significant changes in sensory flavor characteristics in "green, sweet, fruity, floral, and woody" flavors. The results demonstrated significant variations in the volatile components of Liupao tea fermentation, along with notable changes in flavor characteristics.

1. Introduction

Liupao tea originates from Liubao Town, Cangwu County, Wuzhou City, Guangxi, China (Huang, Chen, et al., 2022). It is a postfermented black tea with a 1500-year history (Mao et al., 2018). This tea, known for its distinctive "red, strong, stale, mellow" quality, is highly favored in Southeast Asian countries (Ding et al., 2019). The production of Liubao tea involves fresh leaves from the local species and Guangxi large-leaf tea trees in Cangwu County as raw materials and included five stages: withering, rolling, fermentation, re-rolling, and drying (Wang, Teng, et al., 2023). Among these, fermentation is crucial for developing its unique flavor (Wang et al., 2021). Tea's bioactive compound enhance immune function and support overall well-being contribute a diverse range of flavor (Bhardwaj et al., 2021; Chaturvedula & Prakash, 2011). Recent studies have identified over 300 organic volatile components in Liupao tea products (Li, Hao, et al., 2022; Mu et al., 2017). Given the complexity and diversity of these volatiles, further research is needed to explore their trends during its crucial fermentation process, to better understand the formation of Liupao tea's distinctive "stale flavor."

Aroma is a key quality characteristic and an important factor influencing consumer choice in tea. It is shaped by various volatile compounds that collectively impact the tea's flavor. Liupao tea is known for two distinct aromas: stale and betelnut (Li, Hong, et al., 2022). Different volatile compounds contribute to specific flavors, such as oxidized linalool, which imparts "floral" and "woody" notes (Pang et al., 2019), benzaldehyde which provides an "almond" flavor (Xu et al., 2016), and α-ionone which gives a violet fruity flavor (Ma et al., 2023). While previous studies have primarily focused on the aromatic components of aged Liupao tea, there has been a lack of research on the key aroma formation process, particularly the changes in volatile components

Abbreviations: EI, electron impact ion source; GC-IMS, gas chromatography-ion mobility spectrometry; GC–MS, and gas chromatography–mass spectrometry; GC × GC-TOFMS, two-dimensional gas chromatography time-of-flight mass spectrometry; HCA, hierarchical cluster analysis; HS-SPME, headspace solid-phase microextraction; HS-SPME-GC–MS, headspace solid-phase microextraction-gas chromatography–mass spectrometry; MSEA, metabolite set enrichment analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; PCA, principal component analysis; PCC, Pearson correlation coefficients; QC, quality control; rOAV, relative odor activity values; RT, retention time; SAFE, solvent-assisted flavor evaporation; SBSE, stir bar sorptive extraction; SIM, selected ion monitoring; TIC, total ion current.

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during fermentation.

From an analytical chemistry perspective, the flavor and quality of tea are closely related to its chemical composition (Zhang et al., 2020). The extraction method is crucial for studying volatile components in tea. Currently, common extraction methods for volatile components include headspace solid-phase microextraction (HS-SPME) (Feng LingRan et al., 2015), stir bar sorptive extraction (SBSE) (Ma, Zhu, et al., 2021), and solvent-assisted flavor evaporation (SAFE) (Pang et al., 2019). HS-SPME integrates the extraction, concentration, and sampling of volatile components. It is simple, time-efficient, environmentally friendly, making it widely used for analyzing volatile components in various types of tea (Mu et al., 2018). Wang et al. found that in the extraction of volatile components from Pu'er tea, the HS-SPME method extracted the highest amount of volatile components compared to other methods (Wang, Li, et al., 2022). In terms of volatile component detection, advanced detection instruments, such as gas chromatography-ion mobility spectrometry (GC-IMS), comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC × GC-TOFMS), and gas chromatography–mass spectrometry (GC–MS), they have always been the main instruments for volatile component detection in tea (Marek et al., 2020; Song & Liu, 2018; Wang et al., 2020). Ma et al. (2023) used stir bar sorptive extraction combined with gas chromatography–mass spectrometry to identify 132 volatile compounds in Liupao tea, of which 24 underwent significant changes after processing.

This study utilized HS-SPME-GC–MS to analyze volatile metabolites in Liupao tea through fermentation. Multivariate statistical analysis, was employed to explore differences in volatile organic and flavor compounds of Liupao tea during fermentation, offering valuable insights into the main volatile components and their trends throughout the fermentation process.

2. Materials and methods

2.1. Samples and chemicals

In this study, three Liupao tea samples were produced using the same processing method, with fresh tea leaves collected from the same garden. The samples underwent traditional pile fermentation for 70 days, with each tea pile measuring 2.3 m long, 1.8 m wide, and 0.5 m high, containing approximately 2 tons of raw tea leaves. Sampling was performed at different fermentation stages: the initial (F0, 0 d), middle (F60, 35 d), and final stages (F240, 70 d). For each sample obtained from one central and four peripheral points. Samples (1 kg) were collected from the top, middle, and bottom of each pile. Subsequently, the tea samples were mixed evenly and stored at −80 °C for metabolomics analysis.

Sodium chloride (NaCl) was purchased from Fisher Scientifific (New York NJ, USA). Methyl tert-butyl ether and pentane (chromatographic purity) were purchased from Aladdin (Shanghai, China). 2-Octanol and ethyl decanoate, with purities of 99.5 % and 99 %, respectively, were also from Aladdin (Shanghai, China). Standard purchased from Sigma-Aldrich as shown in Table 1S (Sigma-Aldrich Louis, MO, USA) were used to identify volatiles by calculating retention indices. Ultrapure water (18.2 MΩ cm) was sourced from a Milli-Q Academic ultrapure water system (Millipore, Billerica, MA, USA).

2.2. Sample preparation and treatment

Sample pretreatment: The sample was ground with liquid nitrogen and vortex-mixed, then, 500 mg of the powder was transferred into a 20 mL headspace vial (Agilent, Palo Alto, CA, USA). To this, 2 mL of saturated NaCl solution and 20 μL of internal standard solution (10 μg/mL) were added before sealing the lid.

SPE Microextraction: The extraction head was preheated at 250 °C for 5 min in a Fiber Conditioning Station before sampling. The headspace vial was then agitated at 60 °C for 5 min. Next, the 120 μm DVB/CWR/PDMS (SPME Arrow Agilent, Palo Alto, CA, USA) was inserted into the headspace vial for a 15-min headspace extraction. Finally, the extraction head is thermally desorbed at 250 °C for 5 min, and the volatile compounds extracted are introduced into the GC–MS instrument for separation and identification.

2.3. GC–MS conditions

Gas chromatography conditions The analysis was performed using an Agilent 880 gas chromatograph and a 7000E mass spectrometer, (Agilent Technologies). These instruments were equipped with A 30 m × 0.25 mm × 0.25 μm DB-5MS capillary column,coated with 5 % phenyl methyl silicone, was used for volatile component identification and quantification of volatile components. High-purity helium gas (purity ≥99.999 %) served as the carrier gas, which flowed at a constant rate of 1.2 mL/min. The injection port was set at 250 °C with no split injection, and a solvent delay of 3.5 min was used. The temperature program was as follows: 40 °C for 3.5 min, ramped to 100 °C at 10 °C/min, then to 180 °C at 7 °C/min, and finally to 280 °C at 25 °C/min, for 5 min (Wang, Shi, et al., 2022).

Mass spectrometry conditions: The analysis used a quadrupole mass spectrometer with an electron impact ion source (EI). The ion source temperature was set at 230 °C, quadrupole temperature at 150 °C, and the mass spectrometry interface temperature at 280 °C, and electron energy at 70 eV. Selected ion monitoring (SIM), mode was employed for both the identification and quantification of analytes.

Qualitative metabolism: An independent database was established using data from various species, literature references, partial standards, and retention indices. The database includes the determination of the retention time (RT) and the selection of qualitative and quantitative ions for precise scanning in the selected ion monitoring mode. For each compound, one quantitative ion and 2–3 qualitative ions were chosen. All the ions requiring detection within each group were scanned at different time intervals according to the elution sequence. A substance is identified if the detected retention time aligns with the standard reference and the selected ions were present in the sample mass spectrum after background subtraction (Yuan et al., 2022).

The internal standard (3-hexanone) semi-quantitative method was used to calculate the relative metabolite contents. The formula used is as follows:

$$X_i = \frac{V_s \times C_s}{M} \times \frac{I_i}{I_s} \times 10^{-3}$$

X_i represents the content (μg/g) of compound i in the test sample; V_s represents the volume (μL) of the internal standard added; C_s represents the concentration (μg/mL) of the internal standard; M represents the amount (g) of the test sample; I_s represents the peak area of the internal standard; I_i represents the peak area of compound i in the test sample.

2.4. Calculation of relative odor activity values (rOAV)

To evaluate the impact of different volatile compounds on the aroma of Liupao tea, we identified the key aromatic components by calculating the rOAV based on the sensory threshold of the compounds (Zhu, Wang, et al., 2018). This method helps to determine the contribution of various compounds to the overall flavor of numerous volatile compounds, thus identifying key compounds. In the literature, the relative concentration of individual volatile compounds is calculated by multiplying the concentration of the internal standard by the ratio of the peak area of the volatile compound to that of the internal standard (Zhang et al., 2021). The rOAV is then defined as the ratio between the relative concentration of a single volatile compound and its sensory detection threshold (Qi et al., 2024).

2.5. Multivaraite statistical analysis

Principal component analysis (PCA) is a statistical method used to reduce the dimensionality of a dataset while retaining most of the variation present in the data. PCA was performed using the statistical function "prcomp" function in R (www.r-project.org). The data were unit variance scaled before PCA. Hierarchical cluster analysis (HCA) is used to classify objects or variables into groups based on their similarity. It creates a tree-like diagram, called a dendrogram, showing the relationships between objects or variables in a hierarchical manner. The HCA results for samples and metabolites were presented as heatmaps with dendrograms, whereas the Pearson correlation coefficients (PCC) between samples were calculated using the "cor" function in R and presented only as heatmaps. Both HCA and PCC were performed using the R package, Complex Heatmap. orthogonal partial least squares discriminant analysis (OPLS-DA) is a supervised multivariate analysis technique used for classification and discrimination. It is particularly useful for separating classes or groups in a dataset while removing variations irrelevant to class separation. Differential metabolites for two-group analysis, were determined by VIP (VIP $\geq$1) and absolute Log_2FC ($|Log_2FC| \geq 1.0$). In log_2FC, FC represents the fold change, which is the ratio of the expression levels between the two groups. Taking the logarithm with base 2 of the ratio gives log_2FC. VIP values were extracted from the OPLS-DA results, which also included score plots and permutation plots, and were generated using the R package MetaboAnalystR. The data were log-transformed (log_2) and mean-centered before OPLS-DA. A permutation test (200 permutations) was performed to avoid overfitting. In the context of changes in volatile profiles, PCA can facilitate the visualization of the overall variability of volatile compounds among samples and the detection of trends or patterns in the data. HCA can also aid in grouping samples based on their volatile profiles, emphasizing the similarities and differences between sample clusters. OPLS-DA can be employed to identify the particular volatile compounds that differentiate various sample categories, offering insights into the factors influencing changes in volatile profiles. KEGG annotation and enrichment analysis: The identified metabolites were annotated using the KEGG Compound database (http://www.kegg.jp/kegg/compound/), and then the annotated metabolites were mapped to the KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html). The pathways associated with significantly regulated metabolites were subsequently input into the metabolite set enrichment analysis (MSEA), with significance determined by the *p*-value of the hypergeometric test.

3. Results and discussion

3.1. Quality control (QC) analysis of samples

Fig. S1A shows the total ion current (TIC) mass spectrum obtained in SIM ion detection mode using HS-SPME-GC/MS technology to detect the quality control samples. The x-axis represents the retention time of the metabolite detection, while the y-axis represents the ion detection ion flow intensity. A database established from multiple species, literature, partial standard substances, and retention indices, was used for the qualitative and relative quantitative study of volatile metabolites in Liupao tea samples at different fermentation stages. Quality control samples, prepared by mixing all samples were used to evaluate repeatability using the same processing method. During instrument analysis, a QC sample was inserted every 10 test samples to monitor the reliability of the analysis. By overlapping and analyzing the TIC of different QC samples in mass spectrometry detection, we evaluated the reproducibility of metabolite extraction and detection. Significant overlap in the total ion chromatograms of different quality control samples indicates good reproducibility of metabolite extraction and detection. The experimental results showed high overlap in the TIC spectra of the metabolites, with consistent RT and peak intensity between the QC samples. The high degree of overlap in the spectra shown in Fig. S1B) indicate that this method has good signal stability and provides reliable data.

3.2. Classification of volatile metabolites

In total, 1009 volatile metabolites were identified in Liupao tea across different fermentation stages. These volatile metabolites were categorized into different classes: 232 terpenoids, 176 heterocyclic compounds, 160 esters, 83 ketones, 73 hydrocarbons, 69 alcohols, 67 aromatics, 25 acids, 19 amines, and 23 others (Fig. S2 and Table S2. Terpenoids are the largest category of natural compounds, significantly impacting tea aroma due to their low odor threshold and pleasant fragrance; terpenoids usually show floral and fruit scents in tea (Chen et al., 2016; Jin et al., 2020). The identified volatile compounds were consistent with previous studies; terpenoids, heterocyclic compounds, esters, and ketones were the main aroma components of Liupao tea (Ma et al., 2020; Ma et al., 2023; Ma, Zhu, et al., 2021). However, the promotion of various volatile substances differed slightly from those reported in the literature, likely due to different extraction methods and aging fermentation times.

3.3. Different volatile metabolites analysis based on PCA and OLPS-DA

The peak areas of the volatile compounds in the samples were analyzed using PCA and OPLS-DA multivariate analysis methods to identify key differential volatile components in Liupao tea samples at different fermentation stages. PCA is commonly used to reduce dataset dimensionality while retaining features that contribute most to its variance (Bro & Smilde, 2014). This method allows for visualization of sample differences in two-dimensional or three-dimensional spaces. In the 2D PCA score plot (Fig. 1A), the proximity of QC samples indicates the robustness of the entire analysis process (Pan et al., 2022). In this study, the first two principal components accounted for a cumulative contribution rate of 64.11 %, with PC1 and PC2 accounted for 47.60 % and 16.51 %, respectively. The PCA score plot clearly shows separation between samples F240, F0, and F60; however, the distinction between samples F0 and F60 was less pronounced. HCA was then conducted based on the identified volatile components, resulting in a heat map (Fig. 1B) where rows represents volatile components and columns represent the tea samples. The HCA results clearly distinguished the three tea samples of F0, F60, and F240. Notably, as fermentation time increased, the relative content of most volatile components also significantly increased, particularly in the F240 group.

Although the PCA method effectively extracts main information, it is insensitive to variables with small correlations. Orthogonal partial least squares discriminant analysis combines orthogonal signal correction and partial least squares discriminant methods to separate the X matrix information into two categories: related and unrelated to Y. By eliminating irrelevant differences, OPLS-DA better reveals significant differences in volatile components during Liupao tea fermentation. An OPLS-DA model was established between samples from each of the two stages (F60 vs. F0, F240 vs. F60, and F240 vs. F0). As shown in Fig. 2A-C and Fig. S3, the score plots clearly divided samples into two groups for each comparison. All R^2Y and Q^2 values of the OPLS-DA model exceeded 0.96 and 0.84, respectively, indicating high accuracy and stability, and confirming that the model reliably explains changes in volatile metabolite spectra during the fermentation. Permutation tests ($n = 200$) were used to assess the goodness of fit (R^2X, R^2Y), predictability (Q^2), and cross-validation of the OPLS-DA model. Where R^2X and R^2Y represent the interpretation rates of the model for the X and Y matrices, respectively, and Q^2 represents the predictive ability of the model. The closer these three indicators are to 1, the more stable and reliable the model. A model is effective with $Q^2 > 0.5$, and outstanding with $Q^2 > 0.9$ (Roy & Roy, 2008)., All Q^2 values exceeding 0.8 As shown in Fig. S3, indicate that all analyses of the model were statistically acceptable and effective.

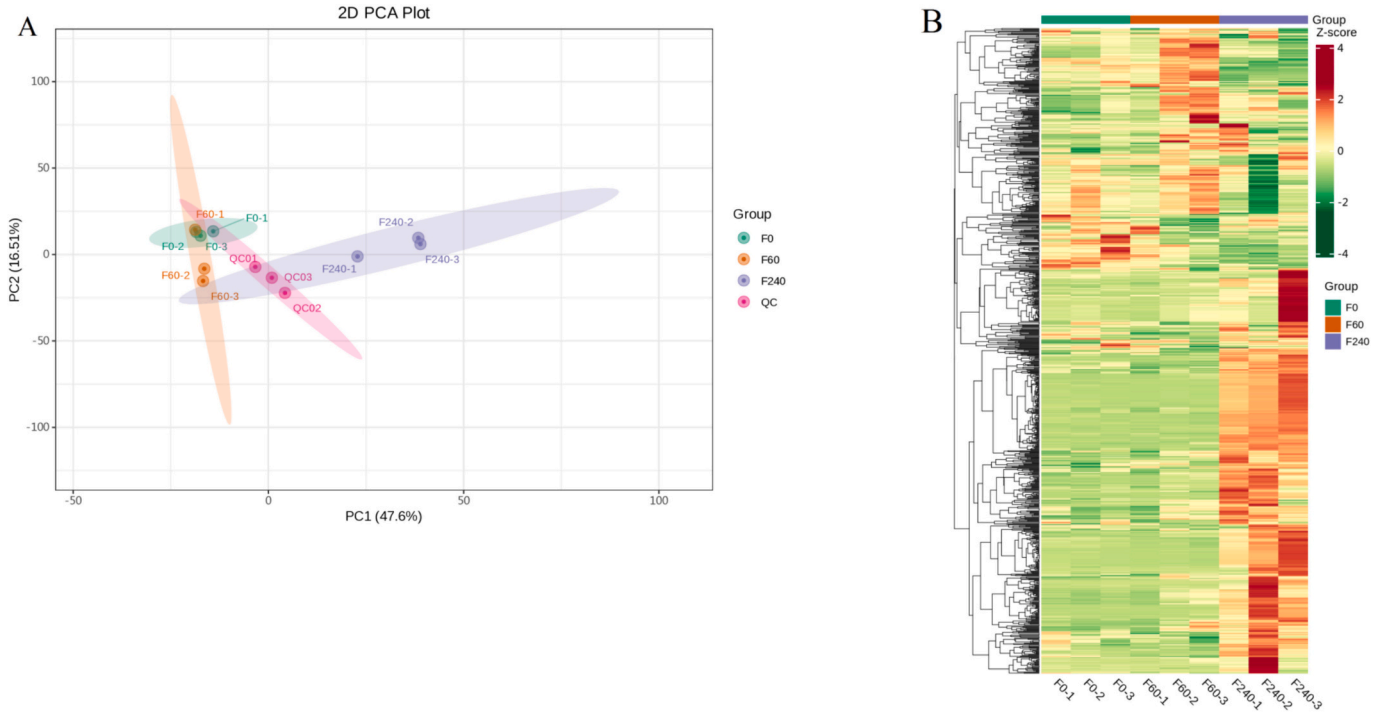

Fig. 1. (A) PCA analysis of volatile metabolites identified from different fermentation stages; (B) HCA heatmap of volatile metabolites from different fermentation stages.

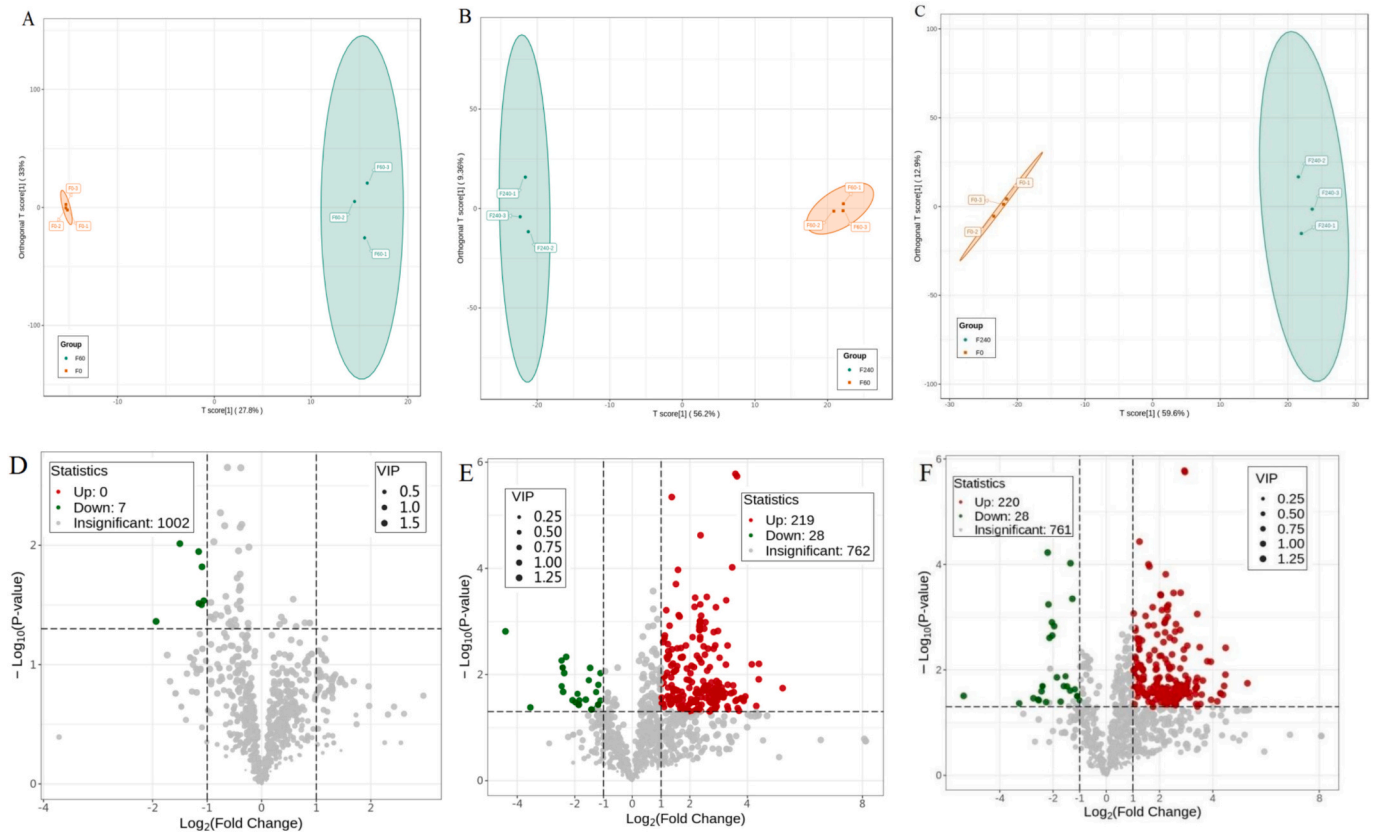

Fig. 2. OPLS-DA analysis of different fermentation stages of Liupao tea: (A) F60 vs. F0, (B) F240 vs. F60, (C) F240 vs. F0 ; volcanic plots of differential metabolites: (D) F0 vs. F60, (F) F60 vs. F240, (E) F0 vs. F240. Each point in the volcano plot represents a metabolite, and the size of the point represents the variable.

Therefore, these models facilitated the identification of differentially significant volatile components in Liupao tea fermentation stage.

The differential volatile components of Liupao tea fermentation stages were screened using VIP ≥ 1, $|\log_2 FC| \geq 1$ and $P < 0.05$. Differences in the expression levels of volatile metabolites between the two groups of tea samples were observed using volcano plots (Fig. 2D-F). In these plots, a larger absolute value on the horizontal axis of indicates more significant differential expression, making the selected metabolites were more reliable. Green dots represent downregulated differential metabolites, red dots represent upregulated differential metabolites, and gray dots represent metabolites that were detected but not significantly different. Compared to the F0 group, seven volatile components were downregulated in the F60 group (Fig. 2D and Table S3).Camphene content significantly decreased with increasing fermentation time during the early stages of fermentation. Camphene is known to be a significant contributor to the "green" flavor in green tea (Lin, Wei, et al., 2021), indicating a transition from green tea to black tea flavor during the fermentation process; 220 volatile components were up-regulated and 28 were down-regulated in the F240 group (Fig. 2E and Table S5). Among them, (2*E*)-2-(Acetylhydrazono)propanoic acid, (2E,4Z)-2,4-decadienal, (5R,8aR)-5-propyloctahydroindolizine, (E)-cinnamaldehyde, and (*S*)-(−)-(4-isopropenyl-1-cyclohexenyl)methanol were the top five volatile compounds with an upregulated $|\log_2 FC|$ value, While (1*R*,2*R*,4*S*,6*S*,7*S*,8*S*)-8-isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decan-4-ol, (2S,4aR,8aR)-4a,8-dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,8a-octahydronaphthalene, 1,2-benzenedimethanol,1,2-ethanediol, monobenzoate,1-butanone, 2-hydroxy-1-phenyl- were the top 5 downregulated volatile compounds with $|\log_2 FC|$ values. (2E,4Z)-2,4-Decadienal is an unsaturated aldehyde known as a key aromatic compound in the fermentation of wheat flour bread that can impart a fatty scent to tea during fermentation (Vermeulen et al., 2007). (E)-Cinnamaldehyde is a volatile compound with a pleasant taste and aroma, and is commonly utilized as a food-flavoring agent (Doyle & Stephens, 2019). Boosting its concentration during tea leaf fermentation can increase the enjoyment of the tea aroma. Compared to the F60 group, 28 volatile components were downregulated, and 219 volatile were upregulated in the F240 group (Fig. 2F and Table S4). During the late stage of tea fermentation, decanal, citronellol, and 3-octanol levels significantly increased. Decanal contributes to the "sweet" and "orange" flavors of tea (Xu et al., 2022); citronellol released by the glycosidase from yeast, is an component of the flavor of tea (Ferremi Leali et al., 2022); 3-Octanol has been proven to be a part of the microbial metabolites during tea fermentation, with a fermented and musty flavor (Kawakami et al., 1987). Isophorone levels showed a downward trend during the late fermentation stages. Isophorone is highly abundant in post-fermented dark tea and is a common product of stuck microbial fermentation (Wen et al., 2023). According to Ma et al., isophorone in Liupao tea is related to core functional microorganisms such as *Aspergillus*, *Wallemia*, *Xeromyces* and *Blastobotrys* (Ma et al., 2023*)*.

Eight common differential compounds were identified among the volatile components (Fig. S4). Information on these common differential metabolites is listed in Table S6. The comparison of the groups indicates that as fermentation time increases, both the differences between samples and the quantity of volatile components also increase. These significant changes in volatile components characteristic aroma components are are likely responsible for the unique flavor of Liupao tea.

3.4. K-means clustering analysis of the differential volatile components

To visually present the trend of metabolite changes during the fermentation process of Liupao tea, K-means clustering analysis method was employed to analyze the relative content of differential metabolites after standardization. K-means clustering is an unsupervised clustering

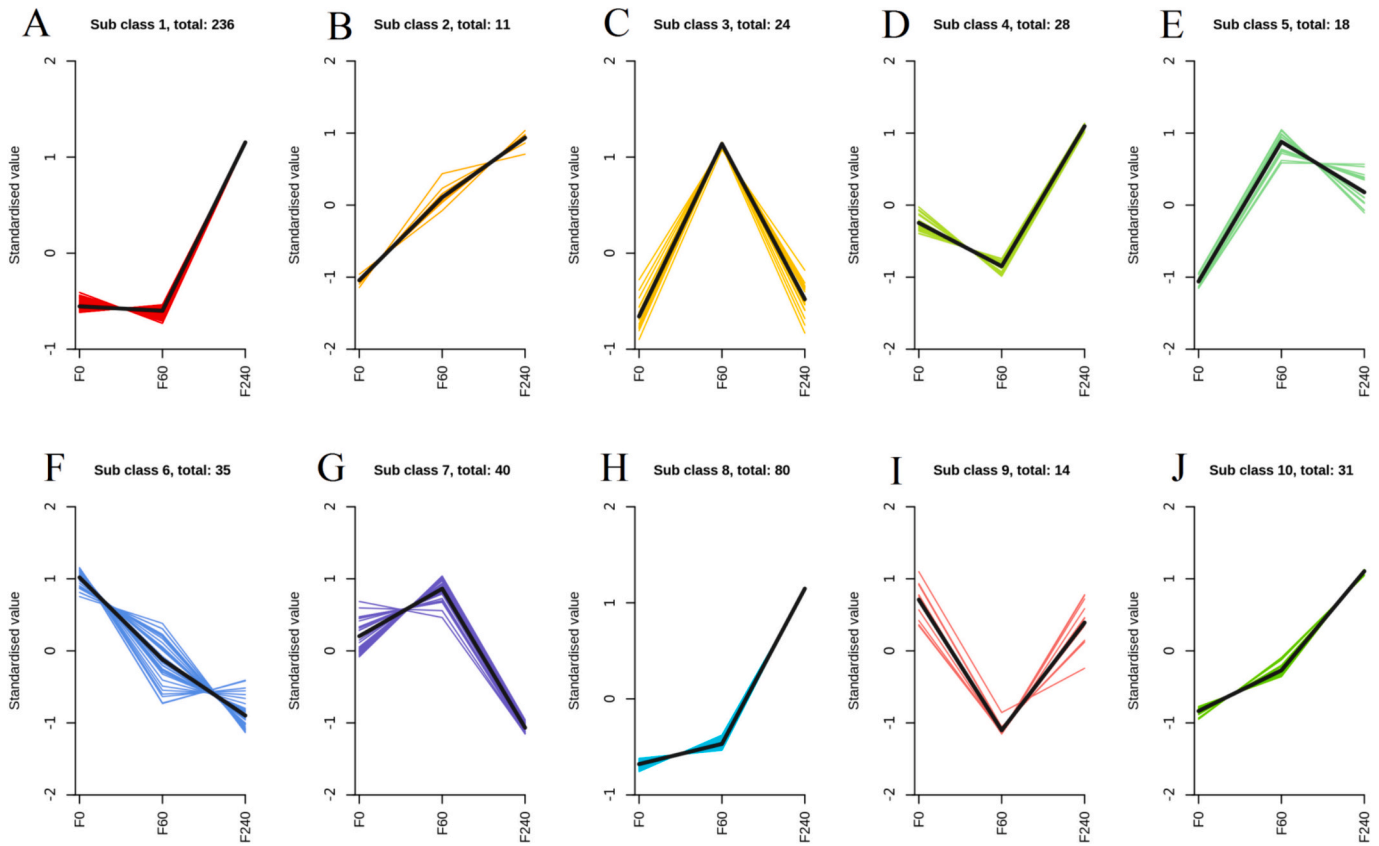

Fig. 3. K-means clustering analysis chart of volatile component differences in Liupao tea with different fermentation times.

algorithm that is designed to partition n data points into k clusters, where each cluster has a centroid that minimizes the distance between its internal data points and the centroid (Coates & Ng, 2012). This is a simple, intuitive, and scalable method. As shown in Fig. 3, volatile metabolites with similar trends during the fermentation period of Liupao tea could be grouped into 10 subclasses. Subclasses 1, 2, 8, and 10 showed notable increases with fermentation time. Specifically, subclass 1 included 236 different metabolites (Fig. 3A), subclass 2 had 11 different metabolites (Fig. 3B), subclass 8 contained 80 different metabolites (Fig. 3H), and subclass 10 had 31 different metabolites (Fig. 3J). The main components in these subclasses were esters, heterocyclic compounds, terpenes, and hydrocarbons. Among the increased volatile components, hexanal, methyl salicylate, and naphthalene had lower odor activity values, were important for forming tea aromas, with grassy, caramel, and peppermint characteristics (Yan et al., 2022; Zhu, Lv, et al., 2018). Subclass 6 exhibited a decreasing trend with fermentation time, and contained 35 different metabolites (Fig. 3F). The other subclasses did not initially increase and then decrease, or vice versa.

K-means clustering analysis indicated a significant and continuous increase in the relative content of the 11 metabolites in subclass 2 throughout the fermentation process (Table 1). 1-Nonanol which imparts floral, fresh, and rose-like flavors. One study detected 1-nonanol in the aroma of black tea, and its content was negatively correlated with the presence of the genus *Bacillus* during the fermentation process (Wen et al., 2023). Levomenthol, known for its minty flavors that exhibit an intensified flavor profile and a calming impact (Anastasia-Sandu et al., 2013). Lilac alcohol C, a primary component of *Syringa oblate* Lindl essential oil, is only found in "Celebrate" and "Virginia," and resembles lilac in fragrance (Yin et al., 2015). The content of these compounds increased as fermentation progressed, which was conducive to the formation of the aroma of Liupao tea.

K-means clustering analysis indicated a significant and continuous increase in the relative content of 35 subclass 6 metabolites during fermentation (Table 2). These compounds were mainly heterocyclic compounds, terpenoids, and esters. Camphene contains woody and herbal flavors, which was considered as main contributors to the "green" note in the white tea (Lin, Ni, et al., 2021). Therefore, a decrease in the relative content of camphene can reduce the taste of "green" in Liupao tea. Benzyl alcohol is considered one of the main sources of important fragrances in tea (Jiang et al., 2021), so it is speculated that the reduction in its relative content is mainly due to the formation of important fragrance components in Liupao tea.

3.5. rOAV analysis of the key aroma component of the finished Liupao tea

To identify the key volatile compounds that influence the overall flavor of finished Liupao tea, rOAV of all volatile compounds were calculated. Typically, an rOAV above 1 indicates the direct impact on the overall flavor of the sample by volatile compounds (Huang, Fang, et al., 2022). As shown in Table S7, the finished Liupao tea (F240) was identified with 165 volatile compounds (rOAV≥1), which were mainly terpenoids, ester, and aldehyde. According to the rOAV analysis method, the five volatile compounds that had the greatest impact on the flavor of Liupao tea after fermentation (F240) were 3-cyclohexene-1-methanethiol. Alpha.,.alpha.,4-trimethyl-, 2(5H)-furanone, 5-ethyl-3-hydroxy-4-methyl-, benzenemethanethiol, β-ionone and 2,6-nonadienal, (E,Z)-. These results are similar to those reported for important aromatic components in black teas, such as Guangdong Yinghong tea (Zhang et al., 2021) and Sichuan Fuzhuan brick tea (Nie et al., 2019). 2(5H)-Furanone, 5-ethyl-3-hydroxy-4-methyl-, is considered one of the main contributors to the aroma of roasted coffee, occurring in fruits such as blackberries, raspberries, and blueberries, as well as in beverages such as beer and wine, imparting "sweet," "fruity," and "caramel" notes (Effenberger et al., 2019). 3-cyclohexene-1-methanethiol,. alpha.,. alpha.,4-trimethyl-, and benzenemethanethiol compounds have odor descriptions, including sulfur and onion, which are rarely found in other tea beverages, except for vine tea (Qi et al., 2024). These compounds had very low concentrations, but had significant prominence in the volatile compounds of the finished Liupao tea. However, there have been no reports on the detection of aromatic components in Liupao tea. It is possible that these compounds degrade during the aging process from finished Liupao tea to Liupao tea. β-ionone, a volatile compound commonly found in tea, contributes floral, sweet, fruity, and berry-like aromas, particularly enriching the floral notes (Xu et al., 2022). According to Ma et al. (2023) believed that β-ionone contributes floral orris aromas in the molecular aroma wheel of Liupao tea. Similarly, 2,6-nonadienal, (E,Z), is known to enhance the "cucumber" and "green" aroma in tea infusion, which is considered as a key odorant in Chinese black tea and Fu brick tea (Chen et al., 2019; Xuexue et al., 2022).

Combined with the results of OPLS-DA (Table S5), 77 volatile compounds (Table 3) were identified as discriminatory aroma compounds in the finished Liupao tea after fermentation (VIP $\geq$ 1, $P < 0.05$, |Log2FC| $\geq$ 1.0, rOAV≥1 and up-regulated). These compounds were mainly aldehyde, aromatics, ester and terpenoids volatile compounds. Similar to the discovery of Ma et al. (2023) in the molecular aroma wheel of

Table 1
Eleven metabolites were significantly increase during the fermentation process.

Index	Compounds	Class	Odor Descriptor	CAS	NIST_RI
KMW0344	1-Nonanol	Alcohol	fresh, clean, fatty, floral, rose, orange, dusty, wet, oily	143-08-8	1170.6
KMW0431	5H-5-Methyl-6,7-dihydrocyclopentapyrazine	Heterocyclic compound	earthy, baked, potato, peanut, roasted	23,747-48-0	1144.0
XMW0255	BenzAldehyde, 2,4-dimethyl-	Aldehyde	naphthyl, cherry, almond, spice, vanilla	15,764-16-6	1182.0
XMW0170*138	BenzAldehyde, 3-ethyl-	Aldehyde	–	34,246-54-3	1168.0
KMW0361*138	BenzAldehyde, 4-ethyl-	Aldehyde	bitter, almond, sweet, anisice	4748-78-1	1180.0
KMW0425	Benzothiazole	Heterocyclic compound	meaty, vegetable, brown, cooked, beefy, coffee	95-16-9	1228.0
D307	Bicyclo[3.1.1]heptan-3-ol, 2,6,6-trimethyl-, (1.alpha.,2. beta.,3.alpha.,5.alpha.)-	Terpenoids	–	27,779-29-9	1179.0
KMW0392*095	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1.alpha.,2. alpha.,5.alpha.)-	Terpenoids	menthol	491-02-1	1187.0
XMW1226	Hexanoic acid, 2-methylpropyl ester	Ester	fruity, pineapple, green, apple skin, sour, tropical, peach, earthy	105-79-3	1149.0
KMW0360*095	Levomenthol	Terpenoids	minty	2216-51-5	1176.9
XMW1136	Lilac Alcohol C	Alcohol	–	33,081-36-6	1219.0

Odor descriptor were obtained from reputable databases: The Good Scents Company (http://www.thegoodscentscompany.com), Perflavory Information System (http://perflavory.com), The LRI & Odor Database - Odor Data (http://www.odour.org.uk/odour/index.html), and Food Flavor Lab (http://foodflavorlab.cn/#/home).

Table 2
Thirty-five metabolites were significantly reduced during the fermentation process.

Index	Compounds	Class I	Odor Descriptor	CAS	NIST_RI
XMW0746*118	(1*R*,2*R*,4*S*,6*S*,7*S*,8*S*)-8-Isopropyl-1-methyl-3-methylenetricyclo [4.4.0.02,7]decan-4-ol	Terpenoids	–	124,753-76-0	1586.0
NMW0324	(*E*)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	Phenol	–	32,811-40-8	1743.0
XMW0750	(*E*)-Tetradec-2-enal	Aldehyde	citrus, waxy, fatty, green, floral	51,534-36-2	1673.0
D400*124	11-Tetradecen-1-ol, (E)-	Alcohol	–	35,153-18-5	1673.0
KMW0644*118	1H-Cycloprop[*e*]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]-	Terpenoids	earthy, herbal, fruity	6750-60-3	1576.0
KMW0181	2,3-Dehydro-1,8-cineole	Terpenoids	minty, lemon	92,760-25-3	992.0
D253	2-Acetyl-3-methylpyrazine	Heterocyclic compound	nutty, flesh, roasted hazelnut, toasted grain, corn, chip, vegetable, nut skin, caramel	23,787-80-6	1082.0
D427	2-n-Heptylfuran	Heterocyclic compound	green, fatty, lactonic, oily, roasted, nutty	3777-71-7	1195.0
D303	2H-Pyran-2-one, tetrahydro-6-nonyl-	Heterocyclic compound	waxy, creamy, buttery, oily, fatty, cheese, milky, dairy	2721-22-4	1929.0
NMW0359	3,7,11-Trimethyl-dodeca-2,6,10-trienoic Acid	Acid	–	7548-13-2	1822.0
XMW1003	4,4-Dimethyl-1,2,3,4-tetrahydro-.gamma.-carboline	Heterocyclic compound	–	22,315-87-3	1893.0
NMW0406	4-(N-Nitroso-*N*-methylamino)-1-(3-pyridyl)-1-butanone	Heterocyclic compound	–	64,091-91-4	1943.0
D424	5-Cyclohexadecen-1-one	Ketone	musky, amber, civet	37,609-25-9	1939.0
XMW0840	6-Methyl-2-(piperidin-1-ylmethyl)-4-pyrimidinylAmine	Heterocyclic compound	–	112,860-60-3	1918.0
XMW0055	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	Heterocyclic compound	–	82,304-66-3	1923.0
D281	Avocadynofuran	Heterocyclic compound	–	24,708-33-6	1827.0
XMW0982	Benzene, [1-[[1-(1-methylethyl)-3-butenyl]oxy]ethyl]-, [S-(R*,R*)]-	Aromatics	–	98,088-51-8	1463.0
KMW0571	Butyl caprate	Ester	whiskey	30,673-36-0	1590.0
KMW0172	Camphene	Terpenoids	woody, herbal, fir, needle, camphor, terpenic	79-92-5	953.0
XMW0396	Cyclobutanecarboxylic acid chloride	Halogenated hydrocarbons	–	5006-22-4	853.0
D285	Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester	Ester	floral, oily, jasmin, green, lactonic	24,851-98-7	1665.1
XMW0627	Diethyl Phthalate	Ester	bitter	84-66-2	1594.0
XMW0620	Dimethyl phthalate	Ester	–	131-11-3	1454.0
WMW0059	E-2-Hexenyl benzoate	Ester	–	76,841-70-8	1588.0
XMW0090	Heptadecane, 3-methyl-	Hydrocarbons	–	6418-44-6	1769.0
XMW0260	Hexadecane, 2-methyl-	Hydrocarbons	–	1560-92-5	1664.0
XMW0218	Hexadecane, 7,9-dimethyl-	Hydrocarbons	–	21,164-95-4	1682.0
XMW0017	Hexadecanoic acid, methyl ester	Ester	oily, waxy, fatty, orris	112-39-0	1928.4
D382*124	Myristoleyl Alcohol	Alcohol	–	34,010-15-6	1678.0
NMW0413	Noruron	Terpenoids	–	18,530-56-8	1960.0
XMW1417	Octanoic acid, 3-hexenyl ester, (*Z*)-	Ester	green, leafy, orchid, kiwi, metallic, fruity, asparagus, lettuce	61,444-41-5	1588.0
NMW0297	Oxirane, tetradecyl-	Hydrocarbons	–	7320-37-8	1676.0
XMW1368	Pentadecanoic acid, ethyl ester	Ester	honey, sweet	41,114-00-5	1894.8
D399*124	cis-9-Tetradecen-1-ol	Alcohol	–	35,153-15-2	1666.0
KMW0323	Benzyl Alcohol	Alcohol	floral, rose, phenol, balsamic	100-51-6	1034.8

Odor descriptor were obtained from reputable databases: The Good Scents Company (http://www.thegoodscentscompany.com), Perflavory Information System (http://perflavory.com), The LRI & Odor Database - Odor Data (http://www.odour.org.uk/odour/index.html), and Food Flavor Lab (http://foodflavorlab.cn/#/home).

Liupao tea, decanal (waxy, orange peel), benzaldehyde (sweet, bitter), and hexanal (grassy, green) were significantly higher in finished Liupao tea than in raw Liupao tea. 2-Octen-1-ol, (*E*)- (green, citrus, vegetable) was noted for its key aromatic compound contributing to the "green" scent characteristic of asam sunti (Xu et al., 2017). Furan, 2-pentyl- (fruity, green, earthy) is a characteristic fragrance component of the betel nut-aroma type of Liupao tea (Li, Hong, et al., 2022). (2E,4Z)-Decadienal and 2,4-decadienal (E,E)- were found to produce new aromatic components after baking Liubao tea, which may be produced by the oxidation of linoleic acid and linolenic acid lipids in tea leaves (Wei et al., 2023). Naphthalene provides pungent, dry, and tarry unpleasant odors with a relatively high odor threshold; however, its concentration

Table 3
The relative odor activity values (rOAVs) aroma-active compounds in F240 Liupao tea samples(VIP ≥ 1, $P < 0.05$, |Log2FC| ≥ 1.0, rOAV≥1 and up-regulated).

Index	Compounds	Class	CAS	Odor Descriptor	NIST_RI	Threshold (mg/L)	Ralative content (mg/L)	Relative Odo Activity Value (rOAV)
KMW0345*032	2,6-Nonadienal, (E,*Z*)-	Aldehyde	557-48-2	cucumber, green	1154.7	0.00001	0.17 ± 0.043	17,017.05
KMW0213	3-Mercapto-3-methylbutyl formate (ester)	Ester	50,746-10-6	sulfury, catty, caramel, onion, roasted coffee, roasted meat, tropical	1023.0	0.000002	0.024 ± 0.005	11,899.96
KMW0399	Decanal	Aldehyde	112-31-2	sweet, aldehydic, waxy, orange peel, citrus, floral	1206.4	0.0001	0.187 ± 0.02	1870.97
KMW0482*049	2,4-Decadienal, (E,*E*)-	Aldehyde	25,152-84-5	dusty, waxy, oily, soapy	1320.1	0.00007	0.092 ± 0.023	1314.22
KMW0475	(2E,4Z)-2,4-Decadienal	Aldehyde	25,152-83-4	fried, fatty, geranium, green, waxy	1295.0	0.00007	0.089 ± 0.024	1277.13
WMW0048	6-Nonenal, (E)-	Aldehyde	2277-20-5	–	1124.0	0.000022	0.025 ± 0.002	1133.15
KMW0608	3-Mercaptohexanol	Alcohol	51,755-83-0	sulfury, fruity, tropical	1127.0	0.00006	0.025 ± 0.008	417.01
WMW0094*032	2,6-Nonadienal, (E,E)-	Aldehyde	17,587-33-6	fresh, citrus, green, cucumber, melon	1153.0	0.0005	0.17 ± 0.043	340.34
NMW0115*091	Anethole	Aromatics	104-46-1	sweet, exotic, flowery, stewed	1287.0	0.015	3.886 ± 3.33	259.09
D245	4-Phenyl-2-butanol	Alcohol	2344-70-9	floral, peony, foliage, sweet, mimosa, heliotrope	1262.4	0.0043	0.962 ± 0.09	223.83
WMW0050	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (*R*)-	Heterocyclic compound	17,092-92-1	musky, coumarin	1532.0	0.0021	0.442 ± 0.109	210.60
XMW0299	Butanoic acid, butyl ester	Ester	109-21-7	fruity, banana, pineapple, green, cherry, tropical fruit, ripe fruit, juicy fruity	996.0	0.028	4.979 ± 1.517	177.84
KMW0299	trans-Rose oxide	Terpenoids	876-18-6	floral	1121.0	0.0005	0.055 ± 0.017	110.15
KMW0294	2-Octen-1-ol, (E)-	Alcohol	18,409-17-1	green, citrus, vegetable, fatty	1067.6	0.02	1.927 ± 0.61	96.33
KMW0457	Ethanone, 1-(2-aminophenyl)-	Ketone	551-93-9	grape, sweet	1308.0	0.00027	0.025 ± 0.006	91.24
QWM0004	Naphthalene, 1,2-dihydro-1,1,6-trimethyl-	Aromatics	30,364-38-6	licorice	1354.0	0.0025	0.194 ± 0.029	77.74
WMW0172*091	TRANS-ANETHOLE	Aromatics	4180-23-8	sweet, anisic, licorice, mimosa	1283.0	0.057	3.886 ± 3.33	68.18
KMW0329	Camphor	Terpenoids	76-22-2	camphor	1151.0	0.016	0.915 ± 0.412	57.16
KMW0182	Furan, 2-pentyl-	Heterocyclic compound	3777-69-3	fruity, green, earthy, beany, vegetable, metallic	992.7	0.006	0.32 ± 0.064	53.40
NMW0156	Benzene, (isothiocyanatomethyl)-	Sulfur compounds	622-78-6	mild, watercress, dusty, medicinal, horseradish, oily	1367.0	0.0007	0.033 ± 0.009	46.55
XMW1310*106	Isobutyl isovalerate	Ester	589-59-3	sweet, fruity, apple, raspberry, green, banana	1005.0	0.034	1.466 ± 0.618	43.11
KMW0540	Eugenol	Phenol	97-53-0	floral, clove	1362.4	0.0025	0.107 ± 0.025	42.88
KMW0344	1-Nonanol	Alcohol	143-08-8	fresh, clean, fatty, floral, rose, orange, dusty, wet, oily	1170.6	0.0053	0.227 ± 0.099	42.85
WMW0076	Benzene, 1,3-bis(1,1-dimethylethyl)-	Aromatics	1014-60-4	–	1258.5	1.1	45.639 ± 4.506	41.49
XMW0888	Butanoic acid, 3-methyl-, propyl ester	Ester	557-00-6	bitter, sweet, apple, fruity	951.3	0.0087	0.299 ± 0.076	34.36
D269*106	Butanoic acid, 2-methyl-, 2-methylpropyl ester	Ester	2445-67-2	sweet, fruity	1004.0	0.043	1.466 ± 0.618	34.09
XMW0662*082	Naphthalene, 2-methyl-	Aromatics	91-57-6	sweet, floral, woody	1297.0	0.004	0.115 ± 0.027	28.82
KMW0488	Tridecane	Hydrocarbons	629-50-5	alkane	1300.0	0.042	1.074 ± 0.265	25.57
WMW0069*077	2-Propenal, 3-phenyl-	Aldehyde	104-55-2	sweet, spicy, aldehydic, aromatic, balsamic, cinnamyl, resinous, honey, powdery	1274.0	0.024	0.548 ± 0.051	22.82
KMW0472	Indole	Heterocyclic compound	120-72-9	animalic, floral, moth, mothball, fecal, naphthelene	1298.6	0.04	0.857 ± 0.688	21.41
KMW0326	2-Nonenal, (Z)-	Aldehyde	60,784-31-8	orris, fatty, waxy, cucumber	1148.0	0.0045	0.093 ± 0.052	20.56
KMW0260	2-Cyclopenten-1-one, 2-hydroxy-3,4-dimethyl-	Ketone	21,835-00-7	strong, caramel	1075.0	0.02	0.406 ± 0.141	20.32

(*continued on next page*)

Table 3 (*continued*)

Index	Compounds	Class	CAS	Odor Descriptor	NIST_RI	Threshold (mg/L)	Ralative content (mg/L)	Relative Odo Activity Value (rOAV)
WMW0181	4-Decenoic acid, methyl ester, *Z*-	Ester	7367-83-1	fruity, pear, mango, fishy, peach skin, green	1323.0	0.003	0.055 ± 0.022	18.46
KMW0435	2-Decenal, (Z)-	Aldehyde	2497-25-8	tallow	1252.0	0.05	0.919 ± 0.089	18.38
WMW0075	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-	Terpenoids	7212-44-4	floral, green, waxy, citrus, woody	1537.0	0.01	0.179 ± 0.06	17.95
KMW0233	Acetophenone	Ketone	98-86-2	sweet, pungent, hawthorn, mimosa, almond, acacia	1067.8	0.065	1.05 ± 0.132	16.16
KMW0308	5-Methyl-2-thiophenecarboxaldehyde	Heterocyclic compound	13,679-70-4	sweet, almond, cherry, furfural, woody, acetophenone	1118.0	0.001	0.016 ± 0.005	15.64
KMW0164	Pentanoic acid, 4-methyl-, ethyl ester	Ester	25,415-67-2	fruity	968.0	0.006	0.092 ± 0.031	15.39
w08*082	Naphthalene, 1-methyl-	Aromatics	90-12-0	naphthyl, chemical, medicinal, camphor	1307.0	0.008	0.115 ± 0.027	14.41
KMW0161*128	3-Octanone	Ketone	106-68-3	fresh, herbal, lavender, sweet, mushroom	986.0	0.0013	0.018 ± 0.007	13.77
KMW0202	3-Octanol	Alcohol	589-98-0	earthy, mushroom, herbal, melon, citrus, woody, spicy, minty	994.0	0.078	1.064 ± 0.349	13.64
NMW0068	Acetic acid, 4-methylphenyl ester	Ester	140-39-6	narcissus, phenol, animalic	1171.0	0.025	0.302 ± 0.032	12.08
KMW0211	2-Acetylthiazole	Heterocyclic compound	24,295-03-2	nutty, popcorn, roasted, peanut, hazelnut	1020.0	0.004	0.048 ± 0.01	11.94
NMW0122	Geranyl formate	Ester	105-86-2	fresh, rose, neroli, tea, rose, green	1301.0	0.2	2.378 ± 0.606	11.89
KMW0612	1,3-Benzodioxole, 4-methoxy-6-(2-propenyl)-	Aromatics	607-91-0	spicy, warm, balsamic, woody	1520.0	0.088	1.007 ± 0.011	11.44
KMW0066	Hexanal	Aldehyde	66-25-1	aldehyde, grassy, green, leafy, vinegar	799.8	0.005	0.049 ± 0.022	9.79
KMW0554*055	.alpha.-Ionone	Terpenoids	127-41-3	sweet, woody, floral, violet, orris, tropical, fruity	1435.1	0.00378	0.035 ± 0.01	9.28
XMW1325	4-Undecanone	Ketone	14,476-37-0	fruity	1208.0	0.041	0.366 ± 0.057	8.94
KMW0276	1-Octanol	Alcohol	111-87-5	intense citrus, rose	1069.8	0.022	0.185 ± 0.061	8.42
KMW0204	Acetic acid, hexyl ester	Ester	142-92-7	fruity, green, apple, banana, sweet	1013.1	0.115	0.815 ± 0.085	7.09
KMW0672	Naphthalene, 2,6-dimethyl-	Aromatics	581-42-0	grassy	1411.9	0.01	0.068 ± 0.022	6.82
KMW0570	2-Dodecenal, (*E*)-	Aldehyde	20,407-84-5	citrus, metallic, mandarin, orange, waxy, aldehydic	1468.0	0.0073	0.049 ± 0.016	6.67
XMW1478	Geranyl isobutyrate	Ester	2345-26-8	sweet, floral, fruity, green, peach, apricot, rose	1514.0	0.013	0.084 ± 0.035	6.47
NMW0166	Biphenyl	Aromatics	92-52-4	pungent, rose, green, geranium	1381.0	0.0033	0.016 ± 0.005	4.84
NMW0789	Benzene, 1-ethyl-3-methyl-	Aromatics	620-14-4	–	957.0	0.088	0.422 ± 0.148	4.79
KMW0404	Naphthalene	Aromatics	91-20-3	pungent, dry, tarry	1189.6	0.05	0.223 ± 0.044	4.47
KMW0395	Estragole	Aromatics	140-67-0	sweet, sassafrass, anisic, spice, green, herbal, fennel	1202.5	0.035	0.13 ± 0.05	3.73
KMW0456	1-Decanol	Alcohol	112-30-1	fatty, waxy, floral, orange, sweet, watery	1271.5	0.023	0.08 ± 0.027	3.46
KMW0218*089	Eucalyptol	Terpenoids	470-82-6	eucalyptus, herbal, camphor, medicinal	1034.3	0.015	0.05 ± 0.016	3.33
XMW0770	Indane	Aromatics	496-11-7	–	1029.0	0.01	0.032 ± 0.003	3.19
NMW0105	Phenol, 4-propyl-	Phenol	645-56-7	medicinal, phenol	1257.5	0.107	0.337 ± 0.228	3.15
KMW0492	2(3H)-Furanone, 5-ethyldihydro-	Ester	695-06-7	sweet, caramel	1054.9	0.26	0.645 ± 0.105	2.48
XMW0385	Carotol	Terpenoids	465-28-1	pleasent, mild	1594.0	0.008	0.02 ± 0.011	2.46
KMW0362	Isoborneol	Terpenoids	124-76-5	balsamic, camphor, herbal, woody	1157.0	0.0085	0.017 ± 0.012	2.01
XMW0213	Isopentyl hexanoate	Ester	2198-61-0	fruity, banana, apple, pineapple, green	1250.0	0.32	0.581 ± 0.267	1.82
NMW0089*116	6-Octen-1-ol, 3,7-dimethyl-, (*R*)-	Terpenoids	1117-61-9	citronella oil, rose, leafy, oily, petal	1220.0	0.04	0.068 ± 0.021	1.71
KMW0430*116	Citronellol	Terpenoids	106-22-9	floral, rose, lime	1228.0	0.04	0.068 ± 0.021	1.71
XMW1345	Octanoic acid, 3-methylbutyl ester	Ester	2035-99-6	sweet, oily, fruity, green, soapy, pineapple, coconut	1446.0	0.07	0.105 ± 0.024	1.50

(*continued on next page*)

Table 3 (*continued*)

Index	Compounds	Class	CAS	Odor Descriptor	NIST_RI	Threshold (mg/L)	Ralative content (mg/L)	Relative Odo Activity Value (rOAV)
WMW0078	2,4-Di-tert-butylphenol	Phenol	96-76-4	phenol	1512.6	0.5	0.692 ± 0.013	1.38
KMW0519	Vanillin	Aldehyde	121-33-5	sweet, vanilla, creamy, chocolate	1404.1	0.053	0.072 ± 0.046	1.35
KMW0158	BenzAldehyde	Aldehyde	100-52-7	sweet, bitter, almond, cherry	962.5	0.35	0.365 ± 0.152	1.04
KMW0444	Carvone	Terpenoids	99-49-0	minty, licorice	1242.0	0.067	0.067 ± 0.019	1.00

Tips:classification of non-volatile compounds mainly comes from US National Institutes of Health (https://pubchem.ncbi.nlm.nih.gov/compound/91712414), 960 Chemical Industry network (https://www.chem960.com/cas/3796701/),ClassyFire (http://classyfire.wishartlab.com/#structure-query); Odor Descriptor and threshold were obtained from reputable databases: The Good Scents Company (http://www.thegoodscentscompany.com), Perflavory Information System (http://perflavory.com), The LRI & Odor Database - Odor Data (http://www.odour.org.uk/odour/index.html), and Food Flavor Lab(http://foodflavorlab.cn/#/home).

in dark teas such as Pu'er tea is lower, resulting in an insignificant overall impact on the aroma of dark tea (Wang, Li, et al., 2022). Carvone has a minor licorice aroma. Studies had found that it was mainly converted from carvelo to carvone by carvelo dehydrogenase during the fermentation process of Pu'er tea (Li et al., 2018). Carvone is a key aroma component of finished liupao tea, indicating that similar reactions also occur during the fermentation process. Furan, 2-pentyl- provided fruity, green, and earthy aroma, which is considered a stale aroma source for Pu'er tea, Liupao tea, and other dark teas, and is also a characteristic aroma component that distinguishes the stale-aroma type from other aroma types of Liupao tea (Li, Hong, et al., 2022; Xu et al., 2016). Benzyl alcohol known for its floral and rose aroma, is the most abundant volatile component in Fu brick tea, contributing significantly to its floral attributes (Li et al., 2020; Xuexue et al., 2022). 4-di-tert-butylphenol, which provides the characteristic phenolic smell, which was previously believed to be an active compound in Pu'er tea and other dark tea (Ma, Gao, et al., 2021; Wang, Li, et al., 2022).

3.6. KEGG functional annotation and enrichment of differential metabolites

To better identify the differential volatile metabolites in the fermentation process of Liupao tea, we conducted a KEGG pathway enrichment analysis. In the present study, 492 volatile differential metabolites were mapped using the KEGG database. First, we focused on metabolic pathways and the discovery that all metabolites were mapped as "metabolites." Enrichment was conducted on the annotation pathway results of differential metabolites according to KEGG pathway types, and the enrichment results are shown in Fig. 4. The analysis revealed that the F60 vs. F0, F240 vs. F60, and F240 vs. F0 groups had 18, 63, and 73 pathways involved in the differential metabolite analysis, respectively. As shown in Fig. 4, the metabolites of all metabolic pathways in the F0 vs F60 group were up-regulated, and in the F240 vs F60 group, except for the "Sesquiterpenoid and triterpenoid biosynthesis" metabolic pathway, all the metabolites of the different metabolic pathways were also upregulated, but in the F240 vs F0 group, all the metabolites of the different metabolic pathways were up-regulated.

During the fermentation process of Liupao tea pile fermentation, the metabolic pathways of volatile aroma components primarily involve arginine biosynthesis, tyrosine metabolism, monoterpenoid biosynthesis, phenylalanine metabolism, and sesquiterpenoid and triterpenoid biosynthesis. The impact of these metabolic pathways on volatile aroma components may be related to changes in microorganisms and intracellular enzymes during fermentation process (Zhang et al., 2021).

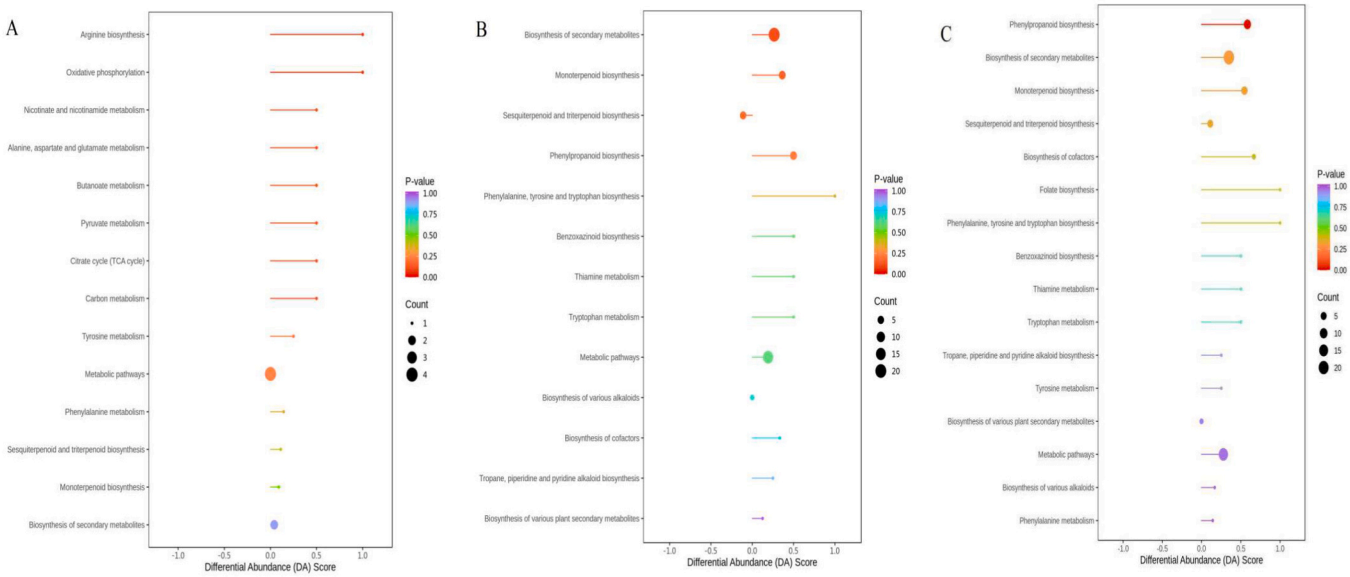

Fig. 4. KEGG enrichment pathway differential abundance score analysis. (A) F60 vs. F0, (B) F240 vs. F60, (C) F240 vs. F0. (Differential abundance score (DA Score) = (the pathway regulates the number of differential metabolites-the pathway downregulates the number of differential metabolites)÷The number of all differential metabolites annotated to this pathway. DA Score reflects the overall changes of all metabolites in the metabolic pathway, with a score of 1 indicating an upregulation trend in the expression of all identified metabolites in the pathway, and a score of −1 indicating a downregulation trend in the expression of all identified metabolites in the pathway.).

During the early stages of fermentation (Day0-Day35), the major enriched and significantly upregulated metabolic pathways were arginine biosynthesis and tyrosine metabolism. Amino acid metabolism generates free amino acids that contribute to the fresh taste of tea and provide nutrients for microorganisms during the subsequent fermentation process. In the late stage of fermentation (Day35-Day70), the main enrichment and significantly increased metabolic pathways were the biosynthesis of secondary metabolites and monoterpene biosynthesis. Terpenoids have a significant impact on tea aroma. L-. Alpha-terpineol is generated through the monoterpene biosynthesis pathway, with lilac, floral, and terpenic odors, and is considered an important aromatic component of Liupao tea (Li, Hong, et al., 2022). Alpha-farnesene is produced through the biosynthesis of secondary metabolite pathways, imparts citrus, herbal, and lavender odors, and is considered a key aroma-active compound in Pu'er tea and Fu brick tea (Lv et al., 2014). During fermentation, Liupao tea produces various volatile compounds via different metabolic pathways. These compounds contribute to a variety of fragrances and provide a sense of pleasure.

3.7. Flavor wheel of volatile differential metabolites in the fermentation process of Liupao tea

Previous research has shown that not all volatile compounds significantly contribute to the formation of tea flavor, however, those with specific aromatic properties can create a unique tea flavor when combined at different concentrations (Zhu, Lv, et al., 2018). Each volatile differential metabolite compound was entered into the website (https://www.thegoodscentscompany.com/index.html) to determine whether it had aroma attributes. The odor description of the selected differential volatile compounds was confirmed by corresponding records from previous literature (Guo et al., 2021; Wang, Dai, et al., 2023; Yao et al., 2023). The differential metabolite flavor characteristics of each group are summarized in Table S8-S10. For each differential comparison group, the metabolites identified based on the selection criteria and their

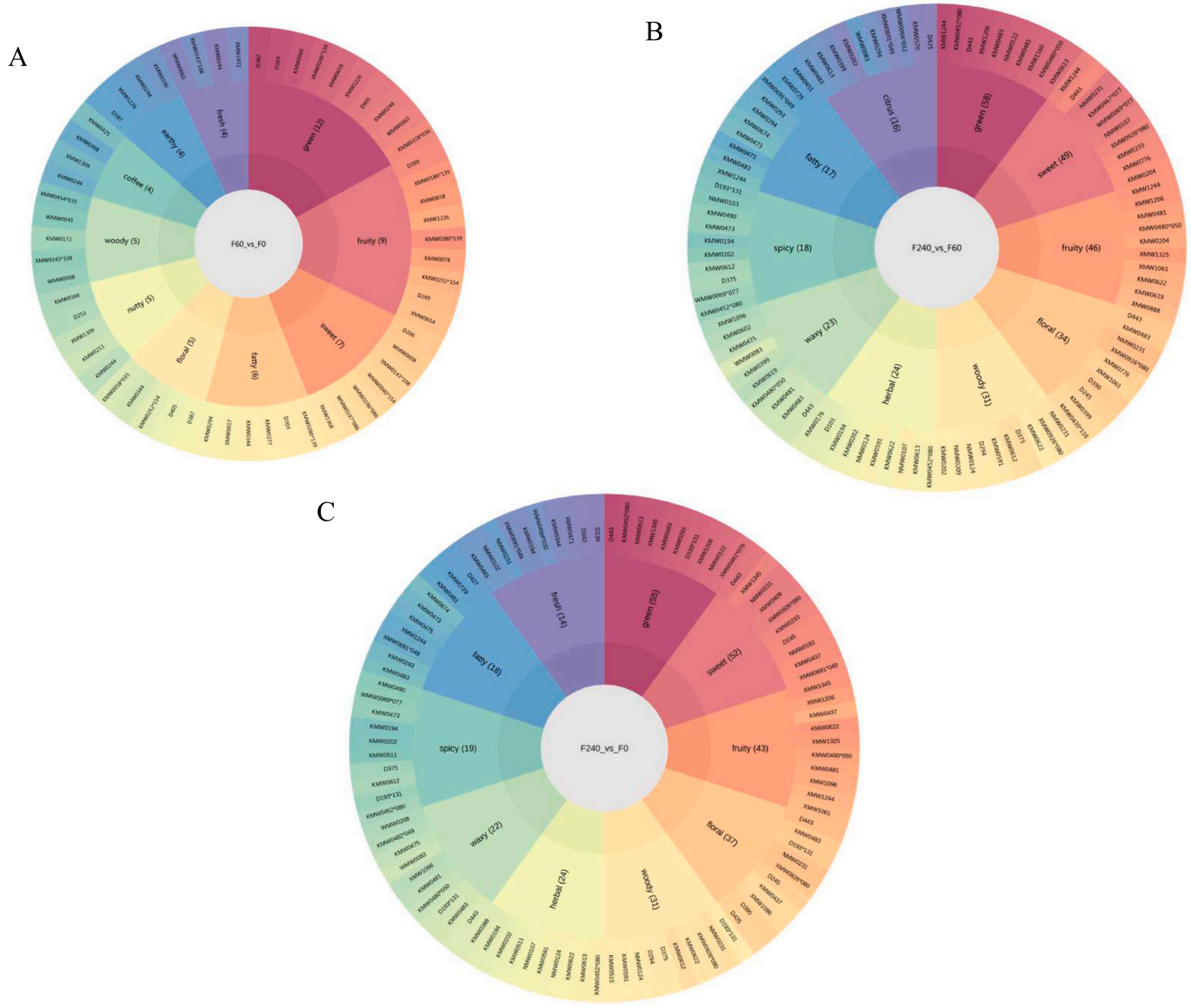

Fig. 5. The volatile components differ in metabolism and flavor wheel during the fermentation process: (A) F60 vs. F0, (B) F240 vs. F60, (C) F240 vs. F0. The innermost circle of the figure shows the differential comparison group, the second circle shows the top 10 sensory flavor characteristics annotated by the differential metabolites in that comparison group, and the number in parentheses indicates the number of differential metabolites annotated to that sensory flavor characteristic. The outermost circle represents the differential metabolites. If the number of differential metabolites annotated to a sensory flavor characteristic exceeds 10, the top 10 differential metabolites with the highest VIP values will be displayed.

related sensory flavor characteristics were annotated, and the top 10 sensory flavors with the highest number of annotations were selected (Fig. 5). As shown in Fig. 5A, in the F60 vs F0 group, exhibited a total of 117 flavor characteristics among differential metabolites, among which "green, fruity, sweet, fatty, and floral" were the differential metabolites annotated to up to 5 flavor characteristics. The F240 vs. F60 (Fig. 5B) and F240 vs. F0 (Fig. 5C) groups showed differences in metabolites with 247 and 236 flavor characteristics, respectively. "Green, sweet, fruity, floral, and woody" were the top five flavor characteristics commonly annotated in both groups of different metabolites.

The differential metabolites identified in the comparison of each group were screened and annotated with their sensory flavor characteristics. The top 10 sensory flavors with the highest numbers of annotations were selected for grid chart drawing. A network diagram of the sensory flavor characteristics associated with differential metabolites is shown in Fig. S5. From the figure, it can be seen that in the early stage of fermentation of Liupao tea (Fig. S5A), there were relatively few annotated differential metabolites for each sensory flavor. However, in the later stage of fermentation (Fig. S5B), a large number of differential metabolites annotated with "green, fruity, sweet, floral, herbal, and woody" sensory flavors increased, indicating that the formation of Liupao tea flavor mainly occurs in the late stage of fermentation; throughout the fermentation process, the annotated differential metabolites were rich in sensory flavor characteristics (Fig. S5C), among which "green, sweet, fruity, floral, and woody" flavors had the most annotated differences, indicating that Liupao tea developed a special flavor towards the above five sensory flavor changes after fermentation.

4. Conclusions

PCA effectively manages high intra-class variability and is widely used metabolomics and transcriptomics, due to its interpretability in multi-class situations (Bylesjö et al., 2006). This study combined HS-SPME-GC–MS with multivariate statistical (PCA and OPLS-DA) analysis to comprehensively analyze the volatile components in Liupao tea samples during fermentation. We employed the high-throughput detection mode of SPME Arrow extraction head and SIM detection mode for precise volatile component analysis. This approach enabled accurate identification of 1009 volatile components predominantly comprising terpenoids, heterocyclic compounds, esters, ketones, hydrocarbons, alcohols, aromatics, acids, and amines that contributed to the overall aroma profile of the sample. Principal component and hierarchical cluster analyses, characterized these volatile components at various fermentation stages. Orthogonal partial least squares discriminant analysis identified 248 differentiating compounds (VIP $\geq$ 1, $P <$ 0.05, and $|\mathrm{Log_2FC}| \geq 1.0$) during the fermentation. Key compounds with an upregulated Log2FC value included, (2*E*)-2-(acetylhydrazono)propanoic acid, (2E,4Z)-2,4-decadienal, (5R,8aR)-5-propyloctahydroindolizine, (E)-cinnamaldehyde, and (*S*)-(−)-(4-isopropenyl-1-cyclohexenyl)methanol. K-means clustering analysis classified these metabolites into 10 sub-clusters, which were further grouped into three groups based on the changing trends of these substances: increasing, decreasing, and irregular. Among these, 11 differential metabolites in subclass 2 exhibited increasing trend throughout the fermentation process, whereas 31 significantly differential metabolites in subclass 6 exhibited decreasing trend during the fermentation process. These compounds are believed to be produced and enhanced during the fermentation process by microbial and intracellular enzymes, giving Liupao tea a unique flavor with a rich and pleasant aroma.

Analysis of rOAV value, identified 77 key volatile compounds (VIP $\geq$ 1, P $<$ 0.05, $|\mathrm{Log_2FC}| \geq 1.0$, rOAV$\geq$1 and up-regulated) in finished Liupao tea. Among these, 3-cyclohexene-1-methanethiol. Alpha.,. alpha.,4-trimethyl- 2(5H)-furanone, 5-ethyl-3-hydroxy-4-methyl-, benzenemethanethiol, β-ionone, and 2,6-nonadienal, (E,Z)- were identified as the primary contributors to the aroma profile of Liupao tea. 3-Cyclohexene-1-methanethiol,. alpha.,.alpha.,4-trimethyl-, 2(5H)-furanone, 5-ethyl-3-hydroxy-4-methyl-, benzenemethanethiol, β-ionone, and 2,6-nonadienal, (E,Z)- may be the major aroma contributors to Liupao tea fermentation. Flavoromic analysis linked these compounds to sensory characteristics, revealing notable shifts in "green, sweet, fruity, floral, and woody" flavors among the samples. These findings underscore the substantial variations in of Liupao tea's volatile components fermentation, accompanied by noticeable changes in the flavor characteristics. HS-SPME-GC–MS combined with multivariate statistical analysis offered valuable insights into primary volatile components and their variation throughout the fermentation of Liupao tea.

CRediT authorship contribution statement

Jianfeng Liang: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Hailin Wu:** Writing – original draft, Methodology, Investigation. **Mingfei Lu:** Writing – original draft, Methodology, Investigation. **Ya Li:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition.

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

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101764.

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