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Identification of volatile compounds and metabolic pathway during ultrasound-assisted kombucha fermentation by HS-SPME-GC/MS combined with metabolomic analysis

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ABSTRACT

The current work combines headspace solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC/MS) with multivariate analysis fusion metabonomics for examining metabolite profile changes. The correlation with metabolic pathways during the fermentation of kombucha tea were comprehensively explored. For optimizing the fermentation process, ultrasound-assisted factors were explored. A total of 132 metabolites released by fermented kombucha were detected by HS-SPME-GC/MS. We employed the principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) to present the relationship between aroma components and fermentation time, of which the first two principal components respectively accounted for 60.3% and 6.5% of the total variance. Multivariate statistical analysis showed that during the fermentation of kombucha tea, there were significant differences in the phenotypes of metabolites in the samples, and 25 characteristic metabolites were selected as biomarkers. Leaf alcohol was first proposed as the characteristic volatile in the fermentation process of kombucha. Furthermore, we addressed the generation pathways of characteristic volatiles, their formation mechanisms, and the transformational correlation among them. Our findings provide a roadmap for future kombucha fermentation processing to enhance kombucha flavor and aroma.

1. Introduction

Traditional microbially fermented drinks such as tea fungus use sucrose-rich tea water as the substrate for the fermentation process, which is carried out by a symbiotic combination of yeast and bacteria [1]. Black tea was used as the primary raw material for the fermentation of tea bacteria and was referred to as kombucha. Studies indicated that kombucha has the ability to promote gastrointestinal digestion, inhibit harmful microorganisms in the intestinal tract, antioxidant properties, promote vascular diastole, and assist in the prevention of cardiovascular and cerebrovascular disease [2]. The acetic acid bacteria and yeasts symbiotically complete the metabolic activity of the tea fungus, while maintaining the system stability [3]. Invertase, a secreted enzyme by yeast decomposed the sucrose in tea fungus into glucose and fructose. Green tea is fermented using Saccharomyces cerevisiae and it might increase the key aroma compounds(linalool and 2-phenyl ethanol) by 1.3 times and 10 times, respectively, to yield fermented green tea with a fruity aroma [4]. At the same time, it further synthesizes ethanol *via* glycolysis. Acetic acid bacteria use reducing sugars such as glucose to produce gluconic acid and glucuronic acid, and further produce glucaric acid 1, 4-lactone.

Fermentation is a key process in generating kombucha aroma quality. The process administers the combined action of microorganisms, enzymes and humid heat, which generate a large number of aldehydes, acids, ketones and other compounds [5,6]. Several relevant literature reports on the mechanism of aroma quality formation during tea fermentation have been reported. It can be summarized as, hydrolysis of glycosides, oxidative degradation of carotenoids/fatty acids, decarboxylation, decarboxylation/deamination of amino acid compounds and Maillard reactions [7,8]. At present, physical processing technologies such as infrared, microwave and ultrasonic have been successfully applied in food fermentation, which can promote the formation of

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product flavor quality more than traditional processing technology [9,10]. Compared to production by other physical methods, ultrasonically treated tea is highly stable [11]. Physical stress can enhance the expression of genes involved in aroma synthesis, resulting in the formation of distinct and diverse aroma compounds in tea leaves [12].

Vacuum distillation extraction (VDE) and simultaneous distillation extraction (SDE) methods have been used to obtain and capture tea aroma [13]. The VDE method generally requires large sample volumes, prolonged processing cycle with a poor ability to capture esters compounds. The SDE method requires tea samples to be exposed to high temperature and high humidity for an extended time period, and the composition and ratio of the aroma substances can be determined easily. In recent years, the headspace solid-phase microextraction (HS-SPME) sample preparation step has been applied for the extraction of various tea aroma substances [14]. HS-SPME requires exceptionally lower sample volumes without requirements of organic solvents and offers speed, high sensitivity, simplicity in operation, reproducibility and convenience [15,16]. The purpose of ultrasonic technology for extraction was to replace traditional extraction technologies due to its speed, low-cost, simplicity, environmental friendliness and efficiency. Ultrasonic technology is also regarded as a special way to enhance the release of tea aroma [17,18]. Some studies have shown that it can effectively express detailed aroma characteristics at low temperatures, which may be used in future tea aroma evaluation processes [19]. Therefore, HS-SPME combined with ultrasonic technology may be suitable for the analysis of the tea fermentation process.

Metabolomics as research method offers comprehensive qualitative and quantitative measurements of all metabolites collectively and at the same time [20]. The main research components analyzed were metabolites such as substrates and products related to various metabolic pathways [21]. With the advances in metabolomics, many research procedures administer and use these methods for the separation of substances in the food system [22]. The synthesis and metabolism of a large number of volatile and non-volatile substances in many fermented

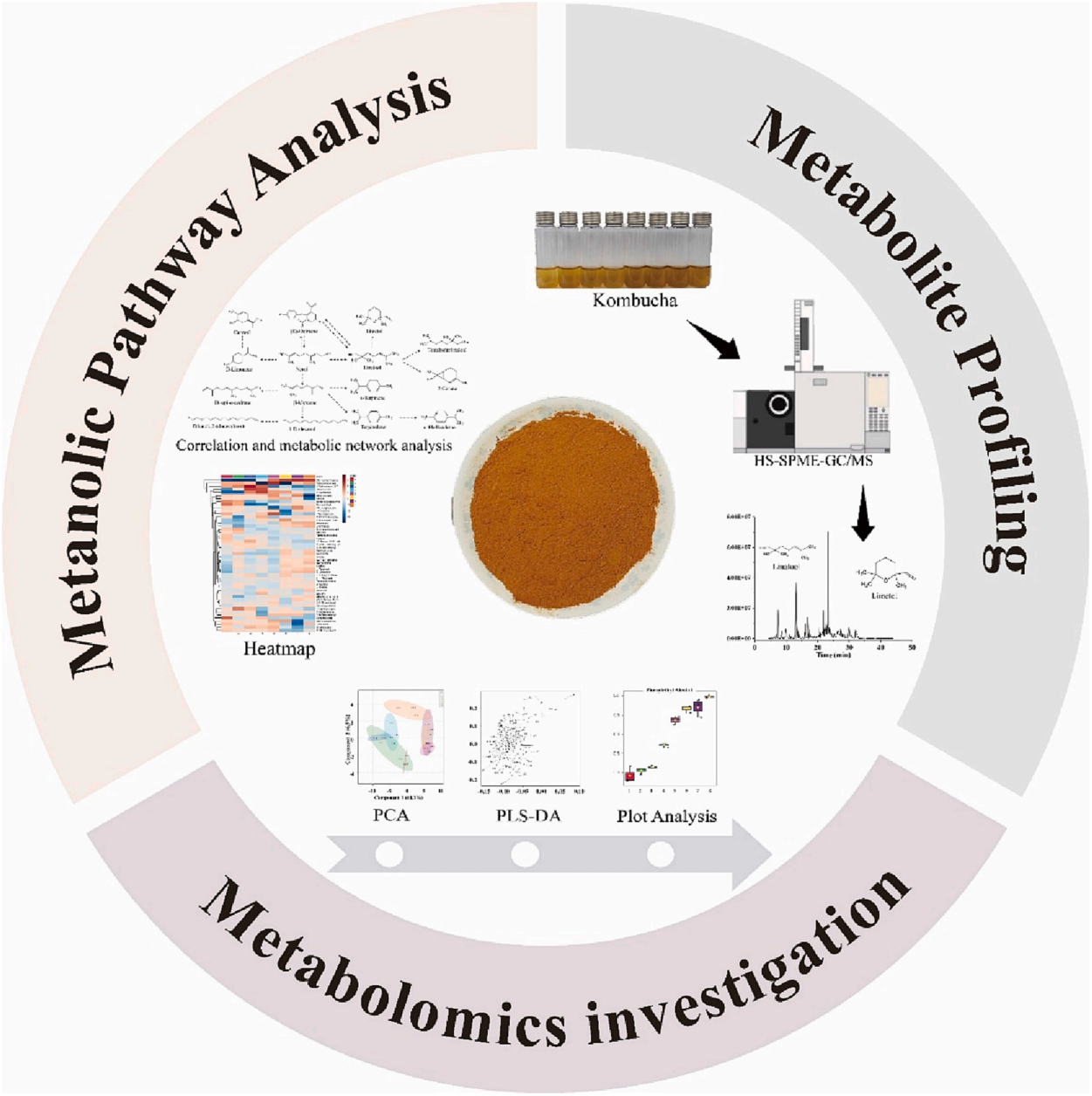

Fig. 1. HS-SPME-GC/MS-based metabolomics investigation combined with multivariate analysis for characteristic volatiles and metabolic pathways during kombucha fermentation.

foods are closely related to the fermentation characteristics of microorganisms [23]. Volatile substances are important and contribute to the odor of fermented substances [24]. At present, HS-SPME combined with gas chromatography-mass spectrometry (GC/MS) technology-based omics methods have been widely used in metabolomics for volatile compound analysis [25,26]. However, there have been fewer reports on the correlation between metabolite changes and metabolic pathways in kombucha using HS-SPME-GC/MS combined with metabolomic methods.

In this comprehensive study, the fermentation process of kombucha was improved and the single-factor and response surface analysis experiment was used to optimize the fermentation process. HS-SPME-GC/MS technology was used to perform metabolomic analysis of kombucha fermentation to comprehend the changes in metabolites during kombucha processing. This can be further used to analyze metabolic pathways and their effects on the nature of volatile compounds (a schematic illustration was displayed in Fig. 1).

2. Materials and methods

2.1. Chemicals and materials

Sugar (sucrose) was purchased from a local market. Instant green tea (IGT) was purchased from Nanjing Rongdian Food Technology Co., Ltd. (Jiangsu, China). Tea fungus was purchased from Anqing Mingxun Tosen Trading Co., Ltd. (Anhui, China). Phosphate Buffered Saline (PBS) was supplied by Macklin (Shanghai, China). Anthrone, glucose, seignette salt, ferrous sulfate, and sulfuric acid were obtained from Sinopharm Group Chemical Reagents Co., Ltd. (Shanghai, China). All the chemicals were analytical grade and were used without further processing. Ultrapure water from Millipore Milli-Q was used in the whole experiment (Millipore, Billerica, MA, USA).

2.2. Instrumentation

An SPME fiber (50/30 μm DVB/CARBOxen-PDMS Automatic StableFlex™ 1 cm; Supelco, PA, USA) was tested and used to identify the volatile compounds in the fermented kombucha samples. Triple quadrupole (TQ-8040) GC–MS equipped with AOC-6000 autosampler was utilized for HS-SPME sampling (SHIMADZU, Japan). The ultraviolet–visible absorption spectra were measured using an 8453 ultraviolet–visible spectrometer (Agilent Technologies Inc., USA). The US device was purchased from Zhenjiang Gerui Biological Engineering Co., Ltd. (Jiangsu, China). A set of self-made ultrasonic stress bioreactors composed of a fermentation tank, ultrasonic generator and steam generator was used to improve the fermentation process.

2.3. Mother liquor preparation

A sucrose medium (sucrose and distilled water in a ratio of 1:10) was formulated for kombucha fermentation. The tea fungus (diameter 10 cm, thickness 1 cm), an appropriate amount of bacteria liquid, and culture medium were placed in a 2 L glass bottle covered with gauze and allowed to ferment at room temperature for 7 days. The time required for fermentation was judged by the consumption rate of total sugar and tea polyphenols.

2.4. Optimization of ultrasonic treatment conditions

Single factor test was carried out under fixed conditions of ultrasonic time (30 min), ultrasonic stirring speed (150 rpm) and mother liquor inoculation amount (10%). The effects of ultrasonic frequency (23, 28, 33, 37 and 40 kHz), pH (3.2, 3.6, 4.0, 4.4 and 4.8) and temperature (28, 30, 32, 34 and 36 °C) on kombucha fermentation were investigated. Then, response surface analysis was performed to obtain the fermentation conditions of kombucha using the above conditions as control factors. The coded and actual values of the process parameters and the response values obtained are shown in Tables S3 and Table S4, and the complete response surface methodology is presented in the supporting material.

The optimized conditions of 30 °C of fermentation temperature, 150 rpm, natural pH, and inoculation amount of 10% were applied in the experiment. Two sets of experiments with and without ultrasound assistance and three parallel experiments for each group were performed.

2.5. HS-SPME-GCMS method

The SPME extraction procedure was performed according to the previously reported literature method [27]. All kombucha samples were filtered with sterile gauze. Kombucha samples (6 mL) were weighed and placed in a 20 mL screw-capped headspace vial. Subsequently, 6 μL of internal standard (ethyl decanoate, 10 μg/mL) was introduced through a micro-injector [28]. The HS-SPME program was carried out using the AOC-6000 autosampler. An SPME fiber was inserted into the sample headspace and then continuously stirred (250 rpm) for 30 min at 70 °C. After extraction, the fiber was removed from the vial and immediately inserted into the GC–MS sampler for desorption (250 °C for 4 min) and further analysis.

The volatiles were studied using Triple quadrupole GC–MS-TQ8040. An Rtx-5MS column (30 m × 0.25 mm × 0.25 μm) was used as the column, with high-purity helium, as the carrier gas at a flow rate of 1.0 mL/min. The column temperature was initially set to 50 °C (hold for 4 min), increased to 290 °C (hold for 5 min) at 6 °C/min, and then further increased to 310 °C at 10 °C/min (kept for 5 min). The MS ion source temperature and the inlet temperature were set to 230 °C and 250 °C, respectively, and the electron energy was 70 eV [29]. The scanning range was 35–650 amu, and the solvent delay time was 4 min. To ensure the independence of the next extraction, the SPME fiber was kept for 30 min after each extraction.

2.6. Determination of total sugar content and polyphenols in kombucha

2.6.1. Determination of residual sugar content

The total sugar content was determined by the anthrone-sulfuric acid method. Briefly, a glucose standard solution (1.0 mg/mL) was prepared by accurately weighing 0.1000 g of glucose and drying it to constant weight at 100 °C before being dissolved in 100 mL of distilled water. Varying volumes (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mL) of the test solution were then transferred to a graduated test tube with a stopper with the addition of 2.0 mL of ultrapure water. Subsequently, 6 mL of 0.1% anthrone sulfate solution was added to the test tube and mixed. The test tube was placed in a 100 °C water bath for 15 min, and then placed in an ice water bath for 15 min after completion. The standard curve was established against the reagent blank reference, and the ultraviolet–visible spectrometer was used to detect the absorbance value at 625 nm [30]. The fitting equation was as follows: $y = 0.5027x - 0.0565$, $R^2 = 0.9900$, where x is the absorbance at 625 nm and y is the glucose concentration (0.5 mg/mL).

2.6.2. Determination of polyphenols

The tea polyphenols were detected using a reference Chinese national standard (GB/T 21733-2008) spectrophotometric method. An accurately weighed amount (1–3 g) of fermentation broth was constituted in a 25 mL volumetric flask. Thereafter, 4 mL of water, 5 mL of ferrous tartrate solution, and PBS buffer were added to the volumetric flask to complete the mark up to 25 mL. A 10 mm cuvette was used to determine the absorbance (A_1) with reagent blank values deducted at 540 nm. At the same time, the same amount of fermentation broth was weighed into a 25 mL volumetric flask, 4 mL of water was added, and the volume was adjusted to 25 mL with PBS buffer. After subtracting the reagent blank value, the absorbance (A_2) was recorded. The formula for

calculating the content of tea polyphenols was as follows:

$$tea\ polyphenol\ content(\%) : X = \frac{(A_1 - A_2) \times 1.957 \times 2 \times K}{m} \times 1000$$

X is the content of tea polyphenols in the sample (mg/kg); A_1 is the absorbance of the test solution after color development; A_2 is the absorbance of the background color of the test solution; 1.957 is that when the absorbance is 0.50, the content of polyphenols in 1 mL of tea infusion is equivalent to 1.957 mg; K is dilution times; m is weighed mass of the test solution during measurement (g).

2.7. Statistical analysis

Firstly, the raw data obtained by GC–MS-TQ8040 was integrated by GCMS solutions software (V 4.52, SHIMADZU, Japan). The integration conditions were set as follows: the initial peak number was kept at 5, and the slope and variable parameter time were set to 600/min and 1000/min, respectively. Next, the data obtained was registered to the NIST 17 Standard Mass Spectrometry Database (NIST, Gaithersburg, Maryland, USA) for the identification of metabolites from selected variables. The exported metabolites list was analyzed by Microsoft Office and the features with similarity (SI) less than 50% in the sample were removed. MetaboAnalyst (https://www.metaboanalyst.ca) is a comprehensive network analysis platform for quantitative metabolomic data [31]. The generated data were preprocessed by K-Nearest Neighbor (KNN) algorithm method for the missing values, and normalization was implemented using logarithmic transformation and pareto scaling (mean is the center divided by the square root of the standard deviation of each variable). Finally, principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were established to model the relationship between aroma components and fermentation time. PCA is an unsupervised recognition pattern, which transforms the original variable into a new set of independent variables by reducing the dimension of the data matrix. This independent variable is called principal components (PCs) [32]. All the analyses were performed in triplicate. One-way ANOVA was performed using SPSS (SPSS Inc., USA), and data clusters and heatmaps were plotted using MetaboAnalyst.

3. Results and discussion

3.1. Determination of fermentation conditions

The consumption rates of total sugar and polyphenols in the fermentation broth under different ultrasonic frequencies have been depicted in Fig. 2A and B. According to these results, a clear difference was noticed in the consumption rate of total sugar and tea polyphenols for the ultrasonic treatment and non-ultrasonicated samples. The consumption rate of polyphenols and total sugar of the fermentation broth without ultrasonic treatment was 35.04%-39.18% and 70.38%-73.33% on the fifth day, respectively. The fermentation broth under ultrasonic treatment consumed the tea polyphenols and total sugar on the third day with respective rates of 32.96%-37.18% and 70.89%-72.88%. Moreover, no noticeable change was observed for the following few days. Therefore, the optimized fermentation time was set to three days. According to the sampling time, the fermentation cycle was recorded as S0-S7. The initial stage of fermentation was recorded as S0.

The influence of ultrasonic frequencies in the range 23, 28, 33, 37, and 40 kHz, was further investigated at the optimized parameters of rotation speed (150 rpm), temperature (28 °C), pH (3.2) and the inoculum size (10%) for 3 days fermentation time. It can be seen from Fig. 2C, as the ultrasonic frequency increased, the rate of consumption of sugar and tea polyphenols by the flora decreased, and reached the lowest point at 40 kHz. Therefore, an ultrasonic frequency of 23 kHz was chosen as optimal for the subsequent experiments.

For enhanced fermentation results other experimental parameters were also optimized. The influence of pH in the range 3.2, 3.6, 4.0, 4.4, and 4.8 were examined under a fixed rotation speed of 150 rpm, inoculation amount of 10% at 30 °C temperature for three days for fermentation time using an optimal ultrasonic frequency of 23 kHz. As shown in Fig. 2D, as the pH increased, the flora's consumption of sugar

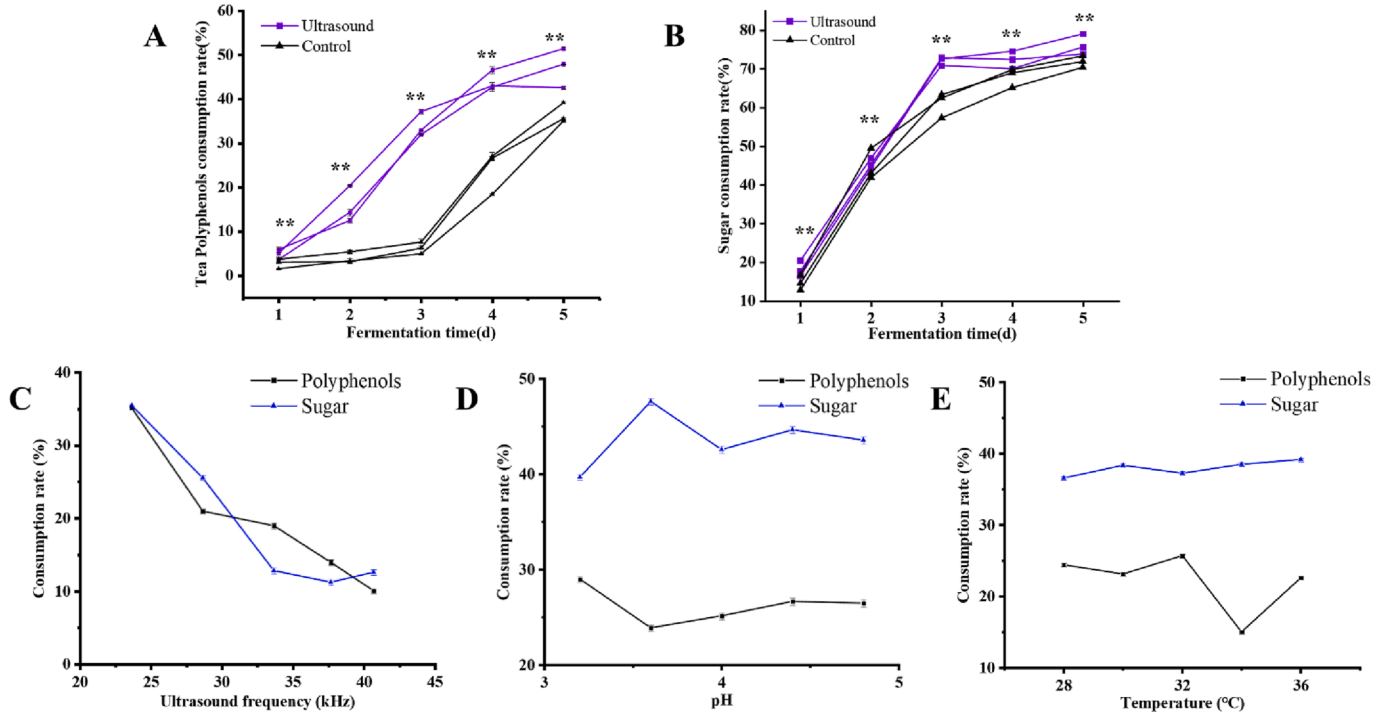

Fig. 2. The influence of ultrasonic treatment against a control group on the consumption rate of tea polyphenols (A) sugar consumption rate (B), effects of five ultrasonic frequencies on the consumption rate of tea polyphenols and sugar (C), effects of five pH on the consumption rate of tea polyphenols and sugars (D), effects of five temperatures on the consumption rate of tea polyphenols and sugar (E).

increased and then decreased, while their consumption of tea polyphenols decreased and then increased. Taking into account these results comprehensively, a pH value of 3.2 was selected as the optimum for subsequent fermentation condition inspection.

In further optimization experiments for fermentation conditions, the rotation speed was kept at 150 rpm, the inoculum amount was 10%, the pH was 3.2, the fermentation time was 3 days, the ultrasonic frequency was 23 kHz and the influence of temperature was evaluated. Different temperatures such as 28, 30, 32, 34, and 36 °C and the impact on fermentation were examined. It can be seen from the Fig. 2E that the temperature rise has a negligible influence on the consumption rate of sugar. The consumption rate of tea polyphenols increased from 24.44 to 25.72 and then decreased to 22.62, showing a trend of first increasing and then decreasing. Therefore, an optimum temperature of 30 °C was selected for subsequent analysis.

After the variables were determined, the consumption rate of total sugar and phenols was used as the response value after fermentation, and were subjected to Box-Behnken design. Fermentation temperature (A), initial pH (B) and ultrasonic frequency (C) were selected, as the independent variables of the model. The total sugar consumption rate (R1) and total polyphenols consumption rate (R2) at the end of the process were selected as the dependent variables in the model to obtain a ternary regression model of tea extract fermentation conditions. The preliminary regression equation is as follows:

$$R1 = 71.12–16.66A-0.0813B-2.03C + 0.7778AB-1.5AC + 6.4BC-15.05A2-5.48B2-5.38C2$$

$$R2 = 30.89–4.18A-1.09B-1.57C + 1.88AB + 1.91AC + 0.2570BC-4.03A2-2.84B2-2.38C2$$

The regression equation R1 was tested with a determination coefficient R2 of 0.9907, $p < 0.01$. The model has a certain significance, quadratic term A^2, B^2 and C^2 which are extremely significant as shown in Table S1. No obvious correlation exists interaction items, indicating that the interaction between the factors was insignificant. The test of mismatched items was insignificant, indicating that the regression equation has a high degree of fit. The regression equation R2 was tested, and the determination coefficient $R2 = 0.9595$, $p < 0.01$. As shown in Table S2, it can be seen from the table that the model of total polyphenols consumption rate has certain significance. Quadratic term A^2, B^2 and C^2 is extremely significant. There is clear correlation between interaction items, indicating that the interaction between factors is not significant. The test of mismatched terms is not significant, indicating that the regression equation has a high degree of fit.

Morphological images of tea fungus in 23 kHz treated and control groups were obtained by scanning electron microscopy (SEM). The results showed that the surface of the control group was smooth and round without any noticeable and obvious change. In contrast, the cell surface after sonication at 23 kHz showed indentations and wrinkles (Fig. 3). These changes may be related to acute cavitation at frequencies between 20 and 40 kHz. Appropriate ultrasound frequencies can increase the temperature, leading to increased cavitation, which can cause microdamage on the cell surface and accelerate the release of cell contents, thus increasing the rate of cellular material transport [33]. Ultrasound treatment can also improve the activity of related enzymes in microorganisms, thereby improving the fermentation efficiency [34].

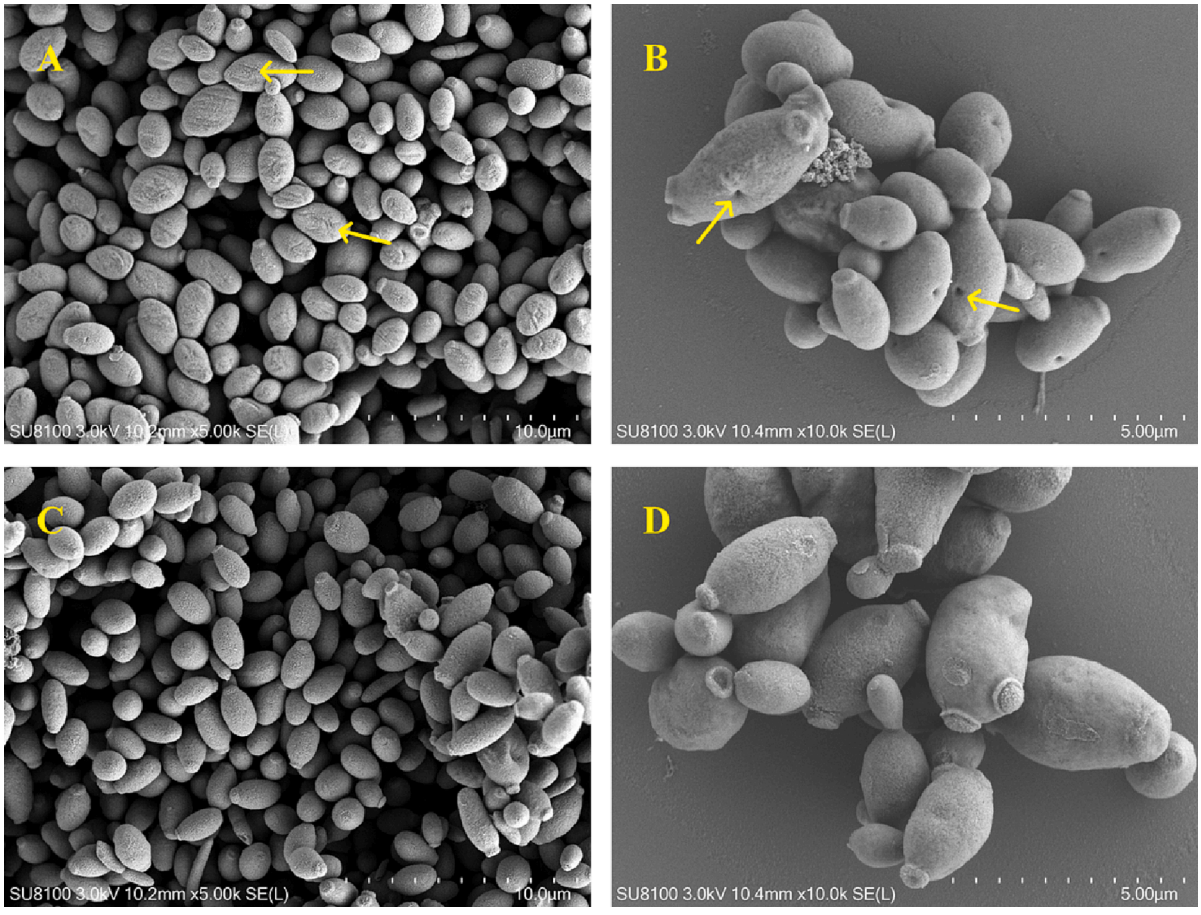

Fig. 3. Scanning electron microscopic observation of the effect of ultrasound on the morphology of tea fungus, Ultrasound treatment (A and B), Control group (C and D).

3.2. Metabolome composition analysis

The GC–MS-TQ8040 used in this study has the built-in characteristics of high-throughput and intelligent operation, with a high-brightness ion source and an efficient collision cell, which can be used for ultra-sensitive analysis. The Table 1 shows retention time, the list of identified compounds, abbreviations, CAS number, and molecular formula. The total ion chromatogram of volatile compounds during kombucha fermentation examined using HS-SPME-GC/MS is shown in Supplementary Fig. S1. The volatile components and area percentages of kombucha fermentation obtained by GC/MS analysis are shown in Table S5 [35].

Apparently, a total of 132 odor-active compounds were organized into 10 groups (32 alcohols, 13 ketones, 16 alkenes, 18 esters, 14 alkanes, 11 aromatics, 9 acids, 7 ethers, 4 nitrogen volatile compounds and one sulfide). The initial stage of fermentation was recorded as S0. According to the sampling time, the fermentation cycle was recorded as S0-S7. The total relative peak area of volatile compounds at S0 was 162.63 ± 31.49, and then increased to 448.86 ± 137.07, with an increase of 176%. Subsequently, the total amount of volatile compounds began to decrease gradually, and the content at S7 was observed to be 349.56 ± 145.63. Some uncontrollable factors were responsible for the unusually higher value of SD, such as the difference of microbial activity at the beginning of fermentation and the different efficiency of nutrient utilization during the fermentation process. However, despite the higher SD value, the experimental trend of the results remained unaffected. At the end of fermentation, the relative peak area content of volatile compounds was alcohols (34.1%) > aromatics (14.1%) > alkanes (9.9%) > esters (9.8%) > alkenes (9.5%) > nitrogen compounds and others (6.8%) > ketone (5.5%) > acid (4.0%) > aldehyde (3.4%) > ether (3.0%). In contrast, metabolic profiles of volatiles during kombucha fermentation indicate that the identified compounds were well separated. The significance of the above 132 volatile compounds in the fermentation process was calculated using one-way ANOVA and Tukey's post-test to validate the selected characteristic volatiles. The ANOVA statistics for 132 high-contributing volatiles are shown in Table 2.

3.3. Analysis of iconic volatile substances

PCA was used to distinguish fermentation samples into different groups (a-h: S0-S7). From the dispersion and aggregation distribution of sample points on the PCA score plot, the S0 sample aggregation distribution can be seen at the bottom and on the left half of the vertical axis. It is far from fermentation, indicating that fermented and unfermented tea have different volatile matter compositions (Fig. 4A). S1-S3 were spatially clustered at the junction of the second and fourth quadrants, whilst S4, S5, and S6 were spatially clustered at the intersection of the first and fourth quadrants. The sample points differed significantly from S0 and were spread widely apart. As the fermentation process continued, it became clear from the spatial distribution of sampling points that the volatiles in tea also underwent periodic changes as a result of the fermentation process of kombucha. According to the PCA score plot, the first two principal components account for 66.8% of the total variance, with PC1 and PC2 constituting for 60.3% and 6.5%, respectively. The variance accounted for the second component appeared to be significantly lower. However, the separation trend between S0-S7 was obvious that was examined carefully. Therefore, these findings did not affect further screening of characteristic metabolites during kombucha fermentation.

Furthermore, the contribution of the original variables to the principal components can be used to identify some potential associations between metabolites, and each PC also reflected their importance in the principal components [36,37]. The loading plot's values for the variables on the x- and y-axes represent their contribution to the model fit, the farther away from the origin, the more that component contributes to the sample variance [38]. The loading plot of PCA proficiently explains the specific components that caused the difference in the changes of S0-S7 metabolites, as shown in Fig. 4B (see Table 1 for the codes of A1, E14, L14, etc.). According to the loading plot (Fig. 4B), the positive portion of the PC1 axis was related primarily to Ethylhexyl (A1) and to a lesser extent to 2-Ethylhexyl salicylate (E14) and Eicosane (L14). The positive part of the PC2 axis was related to 2-methyl Butanoic acid (C2), Physical Alcohol (A5), D-Limonene (O5) and Heneicosane (L10). Some of these aroma compounds contribute to the overall floral, sour sweet and lemon flavor of the kombucha tea (i.e., 2-methyl Butanoic acid, D-Limonene and phenyl ethanol) [39]. These compounds were far from the zero point, contributed significantly to PC1 and PC2, and thus to the odor profile of the fermentation broth.

PCA is an unsupervised learning model that transforms multiple variables into a few comprehensive variables through orthogonal transformation to represent the information of the original variables [40,41]. However, the performance of discriminating components with insignificant differences between groups still needs to be improved. To address this, it is necessary to use PLS-DA supervised algorithm. Compared to PCA, the PLS-DA score plot showed better model fit (with more significant between group differences) with PC1 and PC2 accounting for 59.1% and 7.6%, respectively (Fig. 4C). As shown in Fig. 4D, twenty-five volatile compounds were selected, and were ranked (VIP value > 1.0) as leaf alcohol, 2-ethylhexyl salicylate, 3,7-dimethyl-1,5,7-octatrien-3-ol, phenylethyl alcohol, 3-*tert*-butyl-2-pyrazolin-5-one, eicosane, carveol, (−)-4-terpineol, 2-methyl-butanoic acid, nonanoic acid, isovaleric acid, ethyl caproate, p-xylene, 1-ethyl-1 h-pyrrole, methyl triacontyl ether, dehydro-b-ionone, heneicosane, isopropyl palmitate, cedrol, 1-decanol, 10-amino-10,11-dihydro-5-acetyldibenz [b,f] azepine, octanoic acid, dichloroacetic acid, decyl ester, isopropyl myristate, 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (A1, E14, A4 and other codes are explained in Table 1). The 3,7-dimethyl-1,5,7-octatrien-3-ol is musky, and the ethyl acetate is synthesized from ethanol and acetic acid produced by yeast metabolism. Phenylethyl alcohol enhances the "floral" flavor, improves the overall sensory aroma quality, and enhances the "sweet" aroma characteristic of kombucha [42]. The unpleasant odor in kombucha is probably caused by 2-methylbutyric acid, which has been described as a sweaty odor. P-xylene is the main aromatic component of another type of oolong tea called Phoenix Mono, which has a greenish or irritating odor [43]. The results of volatile components differentiation indicate the significant influence of the fermentation process on the volatile components in kombucha. Furthermore, these volatile compounds were considered to be the main characteristic aroma components during the fermentation of kombucha.

3.4. Identification of characteristic metabolites

Characterized metabolites were further screened by identifying characteristic volatiles that significantly contributed to PCA and PLS-DA. PCA model reflects the original state of the data, which is conducive to distinguish the differences between groups to comprehend the distribution of metabolic data from the overall overview. PLS-DA model can identify the key volatiles and screen the important volatile substances in different fermentation stages [44]. By combining the analysis results of loading plots and VIP scores, the corresponding boxplots (Fig. 5) and cluster heatmaps (Fig. S2) of the metabolites were first drawn. Some of these differential metabolites were linearly related to the kombucha fermentation process. The contents of leaf alcohol, eicosane, 2-ethylhexyl salicylate, 2-methyl-butanoic acid, o-cymene, methyl triacontyl ether, phenylethyl alcohol and isopropyl palmitate were positively correlated with the fermentation time of kombucha. The remaining compounds (oxime-, methoxy-phenyl, linalool, cedaryl alcohol, dichloroacetic acid, decyl ester) shows an opposite correlation with the storage time.

Table 1
List of metabolites during fermentation of kombucha identified by HS-SPME-GC/MS.

Class	R.T(min)	Metabolite	abbreviation	CAS	Formula	SI
Alcohols	5.868	Leaf alcohol	A1	928-96-1	$C_6H_{12}O$	78
	11.268	Benzyl alcohol	A2	100-51-6	$C_{10}H_{18}O$	78
	13.225	Linalool	A3	78-70-6	C_7H_8O	89
	13.316	3,7-dimethyl-1,5,7-octatrien-3-ol	A4	29957-43-5	$C_{10}H_{18}O$	97
	13.57	Phenylethyl Alcohol	A5	60-12-8	$C_{10}H_{16}O$	90
	13.839	Carveol	A6	99-48-9	$C_8H_{10}O$	96
	15.214	1-Decanol	A7	112-30-1	$C_{10}H_{16}O$	77
	15.31	(3R,6S)-2,2,6-Trimethyl-6-vinyltetrahydro-2H-pyran-3-ol	A8	39028-58-5	$C_{10}H_{22}O$	93
	15.499	Terpinen-4-ol	A9	562-74-3	—	83
	15.503	(-)-4-Terpineol	A10	20126-76-5	$C_{10}H_{18}O$	89
	15.879	L-α-Terpineol	A11	10482-56-1	$C_{10}H_{18}O$	90
	15.88	α-Terpineol	A12	98-55-5	$C_{10}H_{18}O$	95
	21.095	2,4,7,9-Tetramethyl-5-decyn-4,7-diol	A13	126-86-3	$C_{10}H_{18}O$	95
	21.37	2-Isopropyl-5-methyl-1-heptanol	A14	91337-7-4	$C_{14}H_{26}O_2$	88
	22.332	Ethanol, 2-[2-(2-butoxyethoxy)ethoxy]-	A15	143-22-6	$C_{11}H_{24}O$	74
	22.596	Tetrahydrolinalool	A16	78-69-3	$C_{10}H_{22}O_4$	84
	22.616	1-Dodecanol	A17	112-53-8	$C_{10}H_{22}O$	69
	22.686	2,4-diethyl-1-Heptanol	A18	80192-55-8	$C_{12}H_{26}O$	89
	22.878	2-methyl-1-Decanol	A19	18675-24-6	$C_{11}H_{24}O$	77
	23.423	1-Hexadecanol	A20	36653-82-4	$C_{11}H_{24}O$	87
	23.792	4-Methyl-dodecan-1-ol	A21	86234-17-5	$C_{16}H_{34}O$	82
	23.804	1-Octanol, 2-butyl-	A22	3913-02-8	$C_{12}H_{26}O$	84
	23.97	1,2-Dihydrolinalool	A23	18479-51-1	$C_{12}H_{26}O$	70
	24.472	Nerol	A24	106-25-2	$C_{10}H_{20}O$	63
	25.662	Cedrol	A25	77-53-2	$C_{15}H_{26}O$	94
	26.359	2-hexyl-1-Decanol	A26	2425-77-6	$C_{15}H_{26}O$	53
	26.619	3,7,11-Trimethyl-1-dodecanol	A27	6750-34-1	$C_{16}H_{34}O$	77
	27.693	2-(eicosyloxy)-Ethanol	A28	2136-73-4	$C_{15}H_{32}O$	76
	27.705	2-(octadecyloxy)-Ethanol	A29	2136-72-3	$C_{22}H_{46}O_2$	86
	27.751	2D,4D,6D,8D-tetramethyloctacosanol	A30	27829-63-6	$C_{20}H_{42}O_2$	64
	27.875	2-(tetradecyloxy)-Ethanol	A31	2136-70-1	$C_{32}H_{66}O$	80
	30.619	n-Pentadecanol	A32	629-76-5	$C_{16}H_{34}O_2$	75
Esters	10.205	Ethyl caproate	E1	123-66-0	$C_8H_{16}O_2$	69
	12.093	Octadecanoic acid,phenyl ester	E2	637-55-8	$C_{24}H_{40}O_2$	71
	12.721	Terephthalic acid, bis(hydroxyethyl) ester	E3	959-26-2	$C_{12}H_{14}O_6$	71
	15.211	Dichloroacetic acid, decyl ester	E4	83005-0-9	$C_{12}H_{22}Cl_2O_2$	90
	15.376	Succinic acid, ethyl 2-ethylhexyl ester	E5	—	—	72
	15.79	Methyl salicylate	E6	119-36-8	$C_8H_8O_3$	95
	17.37	Phenethyl acetate	E7	103-45-7	$C_{10}H_{12}O_2$	90
	19.96	gamma-Nonanolactone	E8	104-61-0	$C_9H_{16}O_2$	94
	20.281	(3-hydroxy-2,2,4-trimethylpentyl) 2-methylpropanoate	E9	77-68-9	$C_{12}H_{24}O_3$	67
	22.085	Acetic acid, trifluoro-, undecyl ester	E10	53800-1-4	$C_{13}H_{23}F_3O_2$	87
	23.06	methyl 4-heptylbenzoate	E11	6892-80-4	$C_{15}H_{22}O_2$	59
	23.07	Methoprene	E12	40596-69-8	$C_{19}H_{34}O_3$	65
	25.028	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	E13	6846-50-0	$C_{16}H_{30}O_4$	86
	29.291	2-Ethylhexyl salicylate	E14	118-60-5	$C_{15}H_{22}O_3$	59
	29.568	Isopropyl myristate	E15	110-27-0	$C_{17}H_{34}O_2$	82
	30.234	Diisobutyl phthalate	E16	84-69-5	$C_{16}H_{22}O_4$	84
	31.912	Dibutyl phthalate	E17	84-74-2	$C_{16}H_{22}O_4$	94
	33.044	Isopropyl palmitate	E18	142-91-6	$C_{19}H_{38}O_2$	91
Olefin	9.945	β-Myrcene	O1	123-35-3	$C_{10}H_{16}$	91
	10.095	α-Terpinen	O2	99-86-5	$C_{10}H_{16}$	80
	10.446	α-Phellandrene	O3	99-83-2	$C_{10}H_{16}$	81
	10.774	Terpinolene	O4	586-62-9	$C_{10}H_{16}$	93
	11.15	D-Limonene	O5	5989-27-5	$C_{10}H_{16}$	94
	11.344	(E)-Ocimene	O6	3779-61-1	$C_{10}H_{16}$	96
	11.661	(Z)-β-ocimene	O7	3338-55-4	$C_{10}H_{16}$	95
	12.019	g-Terpinene	O8	99-85-4	$C_{10}H_{16}$	93
	12.826	2-Carene	O9	554-61-0	$C_{10}H_{16}$	93
	15.216	1-Decene	O10	872-5-9	$C_{10}H_{20}$	89
	21.474	(Z)-1-Methyl-4-(6-methylhept-5-en-2-ylidene)cyclohex-1-ene	O11	13062-0-5	—	84
	21.476	Di-epi-.alpha.-cedrene	O12	50894-66-1	$C_{15}H_{24}$	81
	22.126	(E)-β-Farnesene	O13	18794-84-8	$C_{15}H_{24}$	74
	22.805	Curcumene	O14	644-30-4	$C_{15}H_{22}$	71
	22.994	(E)-5-Icosene	O15	74685-30-6	$C_{20}H_{40}$	90
	34.081	1-Nonadecene	O16	18435-45-5	$C_{19}H_{38}$	75
alkane	10.282	Undecane	L1	1120-21-4	$C_{11}H_{24}$	91
	10.944	4-methyl-Decane	L2	2847-72-5	$C_{11}H_{24}$	82
	10.998	o-Cymene	L3	527-84-4	$C_{10}H_{14}$	93
	13.83	Cosmene	L4	460-1-5	$C_{10}H_{14}$	81
	16.001	Dodecane	L5	112-40-3	$C_{12}H_{26}$	92
	20.937	Tetradecane	L6	629-59-4	$C_{14}H_{30}$	95
	23.174	Heptadecane	L7	629-78-7	$C_{17}H_{36}$	96
	24.677	phytane	L8	638-36-8	$C_{20}H_{42}$	80

(*continued on next page*)

Table 1 (*continued*)

Class	R.T(min)	Metabolite	abbreviation	CAS	Formula	SI
	24.681	8-hexyl-Pentadecane	L9	13475-75-7	$C_{21}H_{44}$	83
	25.284	Heneicosane	L10	629-94-7	$C_{21}H_{44}$	92
	26.615	1-methoxy-13-methylpentadecane	L11	56196-9-9	$C_{17}H_{36}O$	86
	27.756	1-Methoxyhexacosane	L12	237742-59-5	$C_{17}H_{36}$	69
	28.623	3-methyl-Heptadecane	L13	6418-44-6	$C_{18}H_{38}$	70
	29.164	Eicosane	L14	112-95-8	$C_{20}H_{42}$	90
aromatic compound	5.993	Ethylbenzene	R1	100-41-4	C_8H_{10}	98
	8.172	3,5-Dimethylphenol	R2	108-68-9	$C_8H_{10}O$	89
	17.263	1,3-bis(1,1-dimethylethyl)-Benzene	R3	1014-60-4	$C_{14}H_{22}$	80
	23.31	2,4-Di-*tert*-butylphenol	R4	96-76-4	$C_{14}H_{22}O$	95
	26.062	3,5-Di-*tert*-butylcatechol	R5	1020-31-1	$C_{14}H_{22}O_2$	65
	26.865	Cadalin	R6	483-78-3	$C_{15}H_{18}$	65
	26.876	2,4-Ditert-butyl-6-nitrophenol	R7	20039-94-5	$C_{14}H_{21}NO_3$	61
	6.257	p-Xylene	R8	106-42-3	C_8H_{10}	86
	6.922	1,2-xylene	R9	95-47-6	C_8H_{10}	94
	8.178	Oxepine, 2,7-dimethyl-	R10	1487-99-6	$C_8H_{10}O$	89
	25.087	Phenol, 2,4,6-tri-*tert*-butyl-	R11	732-26-3	$C_{18}H_{30}O$	65
Ketones	6.84	2-Heptanone	K1	110-43-0	$C_7H_{14}O$	95
	8.308	4-Methyl-2-heptanone	K2	6137-6-0	$C_8H_{16}O$	95
	10.869	Acetophenone	K3	98-86-2	C_8H_8O	80
	12.943	2-Nonanone	K4	821-55-6	$C_9H_{18}O$	78
	14.603	6,10-dimethyl-2-Undecanone	K5	1604-34-8	$C_{13}H_{26}O$	89
	18.119	Ionone	K6	8013-90-9	$C_{13}H_{20}O$	81
	18.342	2-Undecanone	K7	112-12-9	$C_{11}H_{22}O$	90
	18.841	β-Ionone	K8	14901-7-6	$C_{13}H_{20}O$	87
	18.97	3-*tert*-Butyl-2-pyrazolin-5-one	K9	29211-68-5	$C_7H_{12}N_2O$	76
	22.69	Dehydro-b-ionone	K10	1203-8-3	$C_{13}H_{18}O$	72
	22.264	2,6-Di-*tert*-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one	K11	10396-80-2	$C_{15}H_{24}O_2$	70
	30.974	2-Nonadecanone	K12	629-66-3	$C_{19}H_{38}O$	70
	31.062	Oxaspiro	K13	82304-66-3	$C_{17}H_{24}O_3$	86
Acids	5.425	Isovaleric acid	C1	503-74-2	$C_5H_{10}O_2$	88
	5.59	2-methyl-Butanoic acid	C2	116-53-0	$C_5H_{10}O_2$	77
	9.654	Hexanoic acid	C3	142-62-1	$C_6H_{12}O_2$	92
	10.568	2-Hexenoic acid	C4	1191-4-4	$C_6H_{10}O_2$	68
	15.171	Octanoic acid	C5	124-7-2	$C_8H_{16}O_2$	92
	15.213	Nonanoic acid	C6	112-5-0	$C_9H_{18}O_2$	87
	18.033	3-Nonenoic acid	C7	4124-88-3	$C_9H_{16}O_2$	86
	19.661	Neric acid	C8	4613-38-1	$C_{10}H_{16}O_2$	92
	28.331	Tetradecanoic acid	C9	544-63-8	$C_{14}H_{28}O_2$	89
Aldehydes	9.059	Benzaldehyde	D1	100-52-7	C_7H_6O	99
	10.35	Octanal	D2	124-13-0	$C_8H_{16}O$	83
	11.537	Benzeneacetaldehyde	D3	122-78-1	C_8H_8O	97
	12.728	2-methyl-Benzaldehyde	D4	529-20-4	C_8H_8O	92
	13.339	Nonanal	D5	124-19-6	$C_{14}H_{28}O$	82
	17.928	2-oxo-Benzenepropanal	D6	56485-4-2	$C_9H_8O_2$	74
	19.133	1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl-	D7	116-26-7	$C_{10}H_{14}O$	66
Ethers	9.37	Limetol	T1	7392-19-0	$C_{10}H_{18}O$	93
	14.685	Neroloxide	T2	1786-8-9	$C_{10}H_{16}O_2$	69
	17.517	Edulan I	T3	41678-29-9	$C_{22}H_{24}O_3$	68
	26.132	Methyl triacontyl ether	T4	—	—	87
	27.697	Ethanol, 2-(dodecyloxy)-	T5	4536-30-5	$C_{14}H_{30}O_2$	74
	28.238	Ethanol, 2-(hexadecyloxy)-	T6	2136-71-2	$C_{18}H_{38}O_2$	92
	32.049	1-octoxyoctane	T7	629-82-3	$C_{16}H_{34}O$	
Nitrogen compound	4.632	1-Ethyl-1H-pyrrole	N1	617-92-5	C_6H_9N	92
	7.074	methoxy-phenyl-Oxime	N2	1000222-86-6	—	81
	8.708	10-Amino-10,11-dihydro-5-acetyldibenz[b,f]azepine	N3	28291-57-8	$C_{16}H_{16}N_2O$	72
	29.97	Caffeine	N4	58-08-2	$C_8H_{10}N_4O_2$	76
Sulfur compound	16.233	1-ethylsulfanyl-2-(2-ethyl sulfanylethoxy) ethane	S1	5648-30-6	$C_8H_{18}OS_2$	67

3.5. Metabolic pathway analysis

In this study, generation pathways of characteristic volatiles, their formation mechanisms, and the transformational correlation among them have been presented. The metabolic pathways of the characteristic metabolites found during kombucha fermentation were shown in Fig. 6.

Linalool contributed greatly to the floral aroma and was found to be abundant during the early fermentation stage [45], which declined further at later stages of the process. As linalool and nerol are isomers, their structures are modified by the action of biological enzymes [46,47]. On the other hand, linalool was converted into tetrahydrolinalool through a reduction reaction. It was also discovered that terpenoids such as limonene and linalool were converted into 4-terpineol type terpenoids through double bond hydration, dehydration and cyclization reactions under acidic conditions [48]. This series of reactions changed the ratio of terpenoids in tea leaves, which in turn affected the aroma of kombucha. The floral and fruity aroma of kombucha was attributed to the presence of aromatic alcohols with benzene rings such as benzyl and phenylethyl alcohol [49].

The aldehydes were mainly aromatic aldehydes and saturated aldehydes. Saturated aldehyde includes octanal and nonanal which are described as having green and grass, clean and fruit aromas, and are mostly related to the degradation of unsaturated fatty acids [50]. The aromatic aldehydes mainly contain 2-methylbenzaldehyde and benzaldehyde. Phenylalanine is pyrolyzed to produce benzaldehyde under higher temperature and humidity conditions [51]. Among aromatic aldehydes, benzaldehyde and phenylacetaldehyde have strong almond and fruit flavors [52]. The carbonyl compounds produced during the

Table 2
The relative peak area change (mean ± SD) of volatile components in kombucha during fermentation and its correlation with fermentation time.

RT (min)	Compound	S0	S1	S2	S3	S4	S5	S6	S7	Coefficient of correlation
13.225	Linalool	9.55 ± 3.55^{c}	30.4 ± 13.82^{a}	37.36 ± 1.27^{a}	26.5 ± 2.05^{ab}	13.19 ± 0.59^{c}	17.21 ± 1.22^{bc}	15.9 ± 2.29^{bc}	33.56 ± 9.33^{a}	
10.095	α-Terpinen	0.39 ± 0.05^{bc}	0.79 ± 0.32^{ab}	0.99 ± 0.34^{a}	0.71 ± 0.15^{ab}	0.39 ± 0.14^{bc}	0.13 ± 0.01^{c}	0.4 ± 0.19^{bc}	0.94 ± 0.3^{a}	
12.019	g-Terpinene	0.09 ± 0.02^{b}	0.2 ± 0.09^{ab}	0.23 ± 0.03^{ab}	0.19 ± 0.02^{ab}	0.09 ± 0^{ab}	0.22 ± 0.24^{ab}	0.13 ± 0.03^{ab}	0.28 ± 0.08^{ab}	
10.446	α-Phellandrene	1.38 ± 0.02^{a}	1.47 ± 0.87^{a}	2.35 ± 1.98^{a}	1.26 ± 1.16^{a}	1.43 ± 0.09^{a}	1.56 ± 0.14^{a}	1.11 ± 0.85^{a}	3.02 ± 1.13^{a}	
13.839	**Carveol**	**1.38 ± 0.02^{bc}**	**2.51 ± 0.96^{ab}**	**3.65 ± 0.85^{a}**	**1.76 ± 1.24^{bc}**	**0.57 ± 0.81^{c}**	**1.56 ± 0.14^{bc}**	**0.5 ± 0.82^{c}**	**0.9 ± 1.43^{bc}**	**-0.834***
10.869	Acetophenone	0.09 ± 0.02^{b}	0.05 ± 0.01^{b}	0.07 ± 0.03^{b}	0.07 ± 0.03^{b}	0.06 ± 0.01^{b}	0.06 ± 0.01^{b}	0.07 ± 0.01^{b}	0.13 ± 0.03^{a}	
15.88	α-Terpineol	0.98 ± 0.06^{e}	2.9 ± 1.21^{bc}	3.63 ± 0.15^{e}	2.75 ± 0.23^{bcd}	1.27 ± 0.28^{e}	1.68 ± 0.41^{de}	1.8 ± 0.24	4.09 ± 1.21^{cde}	
23.31	2,4-Di-*tert*-butylphenol	20.5 ± 1.29^{c}	37.79 ± 16.19^{ab}	45.8 ± 8.53^{a}	22.03 ± 1.31^{c}	27.13 ± 1.28^{bc}	27.41 ± 1.61^{bc}	27.67 ± 5.2^{bc}	30.08 ± 11.73^{bc}	
12.721	**Terephthalic acid, bis (hydroxyethyl) ester**	**0.59 ± 0.05^{b}**	**1.59 ± 0.67^{a}**	**1.77 ± 0.26^{a}**	**0.75 ± 0.38^{b}**	**0.51 ± 0.04^{b}**	**0.56 ± 0.07^{b}**	**0.46 ± 0.09^{b}**	**0.76 ± 0.26^{b}**	**-0.911****
6.922	**1,2-xylene**	**0.47 ± 0.23^{b}**	**0.87 ± 0.33^{ab}**	**1.27 ± 0.43^{a}**	**0.57 ± 0.16^{b}**	**0.32 ± 0.02^{b}**	**0.54 ± 0.35^{b}**	**0.6 ± 0.4^{b}**	**0.43 ± 0.13^{b}**	**-0.717***
5.868	**Leaf alcohol**	**0.05 ± 0.01^{c}**	**0.09 ± 0.02^{c}**	**0.1 ± 0.02^{c}**	**0.88 ± 1.46^{bc}**	**1.43 ± 0.09^{b}**	**1.56 ± 0.14^{b}**	**1.56 ± 0.15^{b}**	**3.02 ± 1.13^{a}**	**0.844****
21.37	2-Isopropyl-5-methyl-1-heptanol	0.5 ± 0.25^{a}	0.7 ± 0.27^{a}	6.25 ± 9.07^{a}	0.6 ± 0.38^{a}	0.76 ± 0.56^{a}	0.51 ± 0.08^{a}	2.04 ± 2.91^{a}	2.46 ± 1.46^{a}	
15.216	1-Decene	1.38 ± 0.02^{e}	2.51 ± 0.96^{a}	1.8 ± 1.25^{a}	1.26 ± 1.22^{a}	1.43 ± 0.09^{a}	0.9 ± 0.69^{a}	1.56 ± 0.15^{a}	1.39 ± 1.04^{a}	
23.792	4-Methyl-dodecan-1-ol	1.1 ± 0.47^{b}	2.14 ± 1.44^{b}	4.32 ± 2.01^{a}	2.5 ± 0.21^{b}	1.25 ± 0.41^{b}	1.56 ± 0.14^{b}	1.7 ± 0.24^{b}	3.02 ± 1.13^{ab}	
31.912	Dibutyl phthalate	0.24 ± 0.2^{ab}	0.27 ± 0.12^{ab}	0.33 ± 0.17^{a}	0.19 ± 0.03^{ab}	0.16 ± 0.06^{ab}	0.08 ± 0.01^{b}	0.1 ± 0.01^{b}	0.35 ± 0.12^{a}	
30.234	Diisobutyl phthalate	0.59 ± 0.09^{d}	1.44 ± 0.65^{ab}	2.08 ± 0.25^{a}	1.39 ± 0.18^{abc}	0.79 ± 0.06^{bcd}	0.64 ± 0.13 ^{cd}	0.75 ± 0.14^{bcd}	1.86 ± 0.89^{a}	
15.211	**Dichloroacetic acid, decyl ester**	**1.38 ± 0.02^{bc}**	**2.51 ± 0.96^{ab}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{ab}**	**1.43 ± 0.09^{bc}**	**1.56 ± 0.14^{bc}**	**0.41 ± 0.07^{c}**	**2.38 ± 1.84^{ab}**	**-0.740***
31.062	**Oxaspiro**	**0.3 ± 0.17^{ab}**	**0.38 ± 0.16^{ab}**	**0.46 ± 0.2^{a}**	**0.39 ± 0.07^{ab}**	**0.27 ± 0.1^{ab}**	**0.15 ± 0.06^{b}**	**0.18 ± 0.05^{ab}**	**0.33 ± 0.21^{ab}**	**-0.756***
12.943	2-Nonanone	0.42 ± 0.08^{d}	3.36 ± 1.56^{b}	9.15 ± 0.25^{a}	8.36 ± 0.65^{a}	1.27 ± 0.11 ^{cd}	1.01 ± 0.15 ^{cd}	0.76 ± 0.17 ^{cd}	1.64 ± 0.51^{c}	
22.686	2,4-diethyl-1-Heptanol	1.22 ± 0.26^{b}	2.51 ± 0.96^{ab}	5.03 ± 3.23^{a}	1.21 ± 1.17^{b}	1.31 ± 0.33^{b}	1.56 ± 0.14^{b}	2.37 ± 2.25^{ab}	2.76 ± 0.69^{ab}	
18.119	Ionone	0.54 ± 0.06^{c}	1.25 ± 0.54^{ab}	1.64 ± 0.21^{a}	1.36 ± 0.11^{a}	0.61 ± 0.03^{c}	0.79 ± 0.09^{bc}	0.72 ± 0.04^{bc}	1.69 ± 0.59^{a}	
22.596	**Tetrahydrolinalool**	**1.33 ± 0.09^{a}**	**2.51 ± 0.96^{ab}**	**3.64 ± 0.88^{a}**	**2.57 ± 1.23^{ab}**	**1.43 ± 0.09^{b}**	**1.56 ± 0.14^{b}**	**1.56 ± 0.15^{b}**	**3.02 ± 1.13^{a}**	**-0.760***
20.281	(3-hydroxy-2,2,4-trimethylpentyl) 2-methylpropanoate	0.69 ± 0.02^{d}	1.29 ± 0.5^{bcd}	2.2 ± 0.47^{a}	1.94 ± 0.27^{ab}	1.22 ± 0.21^{bcd}	1.54 ± 0.15^{abc}	1.07 ± 0.12 ^{cd}	2.27 ± 0.91^{a}	
25.662	**Cedrol**	**1.38 ± 0.02^{c}**	**2.51 ± 0.96^{b}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{b}**	**1.43 ± 0.09^{c}**	**1.56 ± 0.14^{bc}**	**1.23 ± 0.64^{c}**	**1.01 ± 0.6^{c}**	**-0.878****
22.994	(E)-5-Icosene	1.44 ± 0.13^{b}	2.54 ± 0.93^{ab}	5.66 ± 4.33^{a}	2.5 ± 0.21^{ab}	2.05 ± 0.98^{b}	1.56 ± 0.14^{b}	2.03 ± 1.59^{b}	3.41 ± 1.38^{ab}	
9.37	Limetol	0.09 ± 0.01	0.27 ± 0.22^{a}	0.1 ± 0.05^{ab}	0.08 ± 0.02^{ab}	0.04 ± 0.01^{b}	0.07 ± 0.02^{b}	0.09 ± 0.07^{ab}	0.17 ± 0.18^{ab}	
25.087	**Phenol, 2,4,6-tri-*tert*-butyl-**	**1.38 ± 0.02^{bc}**	**2.51 ± 0.96^{ab}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{ab}**	**1.33 ± 0.26^{bc}**	**0.94 ± 0.54^{b}**	**0.79 ± 0.64^{c}**	**2.58 ± 1.57^{ab}**	**-0.824***
23.06	**methyl 4-heptylbenzoate**	**1.38 ± 0.02^{bc}**	**2.51 ± 0.96^{ab}**	**3.61 ± 0.89^{a}**	**2.5 ± 0.21^{ab}**	**0.89 ± 0.61^{c}**	**1.02 ± 0.35^{c}**	**1.36 ± 0.45^{bc}**	**2.75 ± 1.38^{ab}**	**-0.719***
25.028	**2,2,4-Trimethyl-1,3-pentanediol diisobutyrate**	**1.38 ± 0.02^{bc}**	**2.51 ± 0.96^{ab}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{ab}**	**1.16 ± 0.44^{bc}**	**0.75 ± 0.7^{c}**	**0.57 ± 0.06^{c}**	**2.56 ± 1.61^{ab}**	**-0.794***
26.619	3,7,11-Trimethyl-1-dodecanol	1.57 ± 1.46^{ab}	2.51 ± 0.96^{ab}	2.14 ± 2.18^{ab}	2.5 ± 0.21^{ab}	0.74 ± 0.63^{b}	1.56 ± 0.14^{ab}	1.34 ± 0.46^{ab}	3.02 ± 1.13^{a}	
22.805	Curcumene	1.38 ± 0.02^{a}	2.51 ± 0.96^{a}	2.18 ± 2.12^{a}	0.66 ± 0.15^{a}	1.43 ± 0.09^{a}	0.67 ± 0.64^{a}	1.56 ± 0.15^{a}	1.94 ± 2.15^{a}	
28.623	3-methyl-Heptadecane	1.38 ± 0.02^{a}	2.1 ± 1.39^{a}	2.79 ± 2.1^{a}	1.15 ± 1.31^{a}	1.43 ± 0.09^{a}	1.56 ± 0.14^{a}	1.13 ± 0.85^{a}	1.61 ± 2.35^{a}	
24.677	phytane	0.67 ± 0.61^{c}	1.56 ± 0.72^{abc}	2.44 ± 1.25^{ab}	1.85 ± 1.08^{abc}	1.12 ± 0.62^{bc}	0.7 ± 0.62^{c}	1.18 ± 0.74^{bc}	3.02 ± 1.13^{a}	
12.093	Octadecanoic acid,phenyl ester	0.94 ± 0.76^{b}	1.26 ± 2.03^{b}	3.65 ± 0.85^{a}	0.94 ± 1.49^{b}	1.01 ± 0.82^{b}	1.01 ± 0.82^{b}	0.55 ± 0.82^{b}	0.85 ± 1.17^{b}	
25.284	Heneicosane	0.86 ± 0.11^{b}	1.06 ± 0.96^{b}	3.43 ± 1.03^{b}	3.28 ± 0.99^{b}	1.17 ± 0.2^{b}	2.24 ± 0.24^{b}	1.35 ± 0.42^{b}	6.01 ± 3.31^{a}	
32.049	1-octoxyoctane	0.46 ± 0.12^{c}	1.3 ± 0.56^{abc}	2.13 ± 0.31^{ab}	2.04 ± 0.24^{ab}	2.66 ± 2^{a}	0.5 ± 0.16^{c}	0.86 ± 0.29^{bc}	1.51 ± 0.15^{abc}	
23.174	Heptadecane									

(*continued on next page*)

Table 2 (*continued*)

RT (min)	Compound	S0	S1	S2	S3	S4	S5	S6	S7	Coefficient of correlation
		1.07 ± 0.02^{c}	2.43 ± 1.91^{abc}	3.32 ± 0.21^{ab}	2.31 ± 0.53^{abc}	1.74 ± 0.78^{abc}	1.68 ± 0.36^{bc}	1.95 ± 0.5^{abc}	3.37 ± 0.99^{a}	
30.619	n-Pentadecanol	0.53 ± 0.24^{b}	0.84 ± 0.4^{b}	1.52 ± 0.65^{b}	1.68 ± 0.58^{b}	0.65 ± 0.08^{b}	0.61 ± 0.18^{b}	0.54 ± 0.42^{b}	3.14 ± 2.04^{a}	
30.974	2-Nonadecanone	1.38 ± 0.02^{bcd}	2.51 ± 0.96^{ab}	3.65 ± 0.85^{a}	0.29 ± 0.06 ^{cd}	1.43 ± 0.09^{bc}	0.12 ± 0.08^{d}	1.13 ± 0.85 ^{cd}	3.02 ± 1.13^{a}	
20.937	Tetradecane	0.59 ± 0.07^{ab}	1.17 ± 0.6^{a}	2.19 ± 0.87^{ab}	1.18 ± 0.2^{ab}	0.77 ± 0.15^{b}	0.91 ± 0.14^{b}	1.04 ± 0.32^{ab}	2.09 ± 0.8^{ab}	
4.632	1-Ethyl-1H-pyrrole	0.06 ± 0^{b}	0.12 ± 0.04^{b}	0.08 ± 0.01^{a}	0.04 ± 0^{b}	0.02 ± 0.01^{b}	0.01 ± 0^{b}	0.03 ± 0.03^{b}	0.09 ± 0.12^{a}	
8.308	4-Methyl-2-heptanone	0.09 ± 0.01^{c}	0.19 ± 0.12^{ab}	0.21 ± 0.02^{a}	0.09 ± 0.01^{c}	0.11 ± 0.01^{bc}	0.14 ± 0.01^{abc}	0.11 ± 0.02^{bc}	0.09 ± 0.02^{c}	
13.57	**Phenylethyl Alcohol**	**0.58 ± 0.34^{d}**	**1.96 ± 1.06 ^{cd}**	**3.12 ± 0.15^{bc}**	**3.92 ± 0.4^{bc}**	**3.68 ± 0.4^{bc}**	**4.61 ± 0.64^{b}**	**4.98 ± 0.92^{b}**	**11.48 ± 3.54^{a}**	**0.960****
11.15	**D-Limonene**	**0.63 ± 0.11^{d}**	**2.11 ± 1^{bc}**	**2.73 ± 0.06^{ab}**	**2.06 ± 0.22^{bc}**	**0.97 ± 0.05^{d}**	**1.36 ± 0.2 ^{cd}**	**1.47 ± 0.32 ^{cd}**	**3.31 ± 0.91^{a}**	**0.759***
10.774	Terpinolene	0.1 ± 0.03^{b}	0.24 ± 0.12^{ab}	0.78 ± 0.7^{a}	0.5 ± 0.48^{ab}	0.09 ± 0.02^{b}	0.15 ± 0.07^{b}	0.17 ± 0.07^{b}	0.41 ± 0.13^{ab}	
29.97	Caffeine	1.38 ± 0.02^{c}	2.51 ± 0.96^{abc}	3.65 ± 0.85^{a}	2.5 ± 0.21^{abc}	3.22 ± 0.63^{ab}	2.44 ± 0.79^{abc}	1.96 ± 0.38^{bc}	2.93 ± 1.38^{ab}	
17.928	2-oxo-Benzenepropanal	1.38 ± 0.02^{bc}	2.51 ± 0.96^{ab}	3.65 ± 0.85^{a}	2.5 ± 0.21^{ab}	0.75 ± 0.64^{c}	0.8 ± 0.53^{c}	0.8 ± 0.61^{c}	3.02 ± 1.13^{a}	
16.233	1-ethylsulfanyl-2-(2-ethylsulfanylethoxy)ethane	2.87 ± 0.23^{b}	5.23 ± 3.18^{a}	5.79 ± 0.35^{a}	2.85 ± 0.25^{b}	3.95 ± 0.24^{ab}	5.09 ± 0.33^{ab}	4.15 ± 0.96^{ab}	2.85 ± 0.63^{b}	
15.499	Terpinen-4-ol	0.08 ± 0.02^{c}	0.06 ± 0.07^{abc}	0.19 ± 0.01^{ab}	0.12 ± 0.02^{bc}	0.07 ± 0^{c}	0.1 ± 0.03^{c}	0.09 ± 0.02^{c}	0.22 ± 0.08^{a}	
26.615	1-methoxy-13-methylpentadecane	0.7 ± 0.4^{b}	0.84 ± 0.31^{b}	2.28 ± 1.78^{a}	0.99 ± 0.43^{ab}	1.03 ± 0.65^{ab}	0.53 ± 0.17^{b}	0.72 ± 0.21^{b}	1.61 ± 0.55^{ab}	
12.826	2-Carene	0.52 ± 0.04^{c}	1.23 ± 0.55^{ab}	1.53 ± 0.02^{a}	0.96 ± 0.04^{bc}	0.55 ± 0.04^{c}	0.68 ± 0.09^{c}	0.66 ± 0.15^{c}	1.41 ± 0.39^{a}	
28.331	**Tetradecanoic acid**	**0.26 ± 0.07^{bc}**	**0.39 ± 0.2^{ab}**	**0.58 ± 0.18^{a}**	**0.36 ± 0.02^{bc}**	**0.17 ± 0.06^{c}**	**0.15 ± 0.01^{c}**	**0.16 ± 0.03^{c}**	**0.4 ± 0.12^{ab}**	**-0.769***
22.085	Acetic acid, trifluoro-, undecyl ester	1.38 ± 0.02 ^{cd}	2.51 ± 0.96^{abc}	2.75 ± 0.71^{ab}	2.5 ± 0.21^{abc}	1.16 ± 1.07^{d}	1.56 ± 0.14^{bcd}	1.56 ± 0.15^{bcd}	3.13 ± 1.02^{a}	
12.728	2-methyl-Benzaldehyde	1.38 ± 0.02^{a}	1.96 ± 1.59^{a}	2.8 ± 1.99^{a}	1.79 ± 1.17^{a}	1.07 ± 0.71^{a}	1.56 ± 0.14^{a}	1.56 ± 0.15^{a}	1.17 ± 1.21^{a}	
10.998	**o-Cymene**	**0.17 ± 0.05^{d}**	**0.33 ± 0.08 ^{cd}**	**0.58 ± 0.05^{ab}**	**0.44 ± 0.07^{bc}**	**0.21 ± 0.01^{d}**	**0.3 ± 0.06 ^{cd}**	**0.35 ± 0.09 ^{cd}**	**0.74 ± 0.21^{a}**	**0.796***
21.476	Di-epi-.alpha.-cedrene	1.38 ± 0.02^{c}	2.3 ± 1.22^{abc}	2.88 ± 1.11^{ab}	1.41 ± 0.76^{c}	1.43 ± 0.09^{c}	1.56 ± 0.14^{bc}	1.56 ± 0.15^{bc}	3.02 ± 1.13^{ab}	
5.425	**Isovaleric acid**	**0.21 ± 0.12^{c}**	**0.42 ± 0.17^{bc}**	**0.55 ± 0.08^{bc}**	**0.23 ± 0.12^{c}**	**0.19 ± 0.04^{c}**	**0.39 ± 0.12^{bc}**	**0.64 ± 0.38^{b}**	**2.26 ± 0.34^{a}**	**0.781***
26.865	Cadalin	1.03 ± 0.4^{bc}	1.18 ± 0.32^{bc}	1.81 ± 0.39^{ab}	1.33 ± 0.19^{bc}	1.08 ± 0.08^{bc}	0.6 ± 0.05^{c}	0.89 ± 0.28^{bc}	2.34 ± 1.14^{a}	
19.661	**Neric acid**	**0.94 ± 0.73^{c}**	**1.44 ± 0.92^{bc}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{ab}**	**1 ± 0.71^{c}**	**1.11 ± 0.77^{c}**	**0.72 ± 0.87^{c}**	**0.48 ± 0.22^{c}**	**-0.811***
13.83	**Cosmene**	**0.07 ± 0.03^{c}**	**0.19 ± 0.08^{ab}**	**0.23 ± 0.04^{a}**	**0.11 ± 0.03^{bc}**	**0.05 ± 0.03^{c}**	**0.07 ± 0.07^{c}**	**0.06 ± 0.02^{c}**	**0.12 ± 0.08^{bc}**	**-0.873****
27.697	Ethanol, 2-(dodecyloxy)-	1.38 ± 0.02^{b}	2.04 ± 1.61^{b}	5.43 ± 3.93^{a}	2.5 ± 0.21^{ab}	0.65 ± 0.59^{b}	1.56 ± 0.14^{b}	1.56 ± 0.15^{b}	2.47 ± 1.89^{ab}	
17.517	Edulan I	0.23 ± 0.03^{b}	0.51 ± 0.2^{ab}	0.62 ± 0.05^{ab}	0.78 ± 0.58^{a}	0.23 ± 0.05^{b}	0.25 ± 0.01^{b}	0.2 ± 0.03^{b}	0.54 ± 0.3^{ab}	
18.033	3-Nonenoic acid	1.03 ± 0.6^{b}	1.55 ± 0.73^{ab}	2.3 ± 1.49^{ab}	1.13 ± 1.25^{b}	1.43 ± 0.09^{ab}	1.56 ± 0.14^{ab}	1.56 ± 0.15^{ab}	3.02 ± 1.13^{a}	
24.472	Nerol	0.77 ± 0.09^{c}	1.42 ± 0.4^{bc}	2.02 ± 0.3^{ab}	1.79 ± 0.2^{ab}	0.86 ± 0^{c}	0.81 ± 0.05^{c}	0.72 ± 0.14^{c}	2.35 ± 0.96^{a}	
23.07	Methoprene	1.25 ± 0.23^{b}	2.37 ± 1.12^{ab}	3.26 ± 0.53^{a}	1.98 ± 0.52^{ab}	1.29 ± 0.33^{b}	1.23 ± 0.69^{b}	1.31 ± 0.28^{b}	3.1 ± 1.3^{a}	
23.804	1-Octanol, 2-butyl-	1.14 ± 0.42^{b}	2.46 ± 1.01^{ab}	4.03 ± 1.5^{b}	2.5 ± 0.21^{ab}	0.88 ± 0.69^{b}	1.03 ± 0.79^{b}	1.17 ± 0.93^{b}	3.02 ± 1.13^{b}	
15.31	**(3R,6S)-2,2,6-Trimethyl-6-vinyltetrahydro-2H-pyran-3-ol**	**0.12 ± 0.03^{bcd}**	**0.16 ± 0.06^{ab}**	**0.22 ± 0.01^{a}**	**0.11 ± 0.07^{bcd}**	**0.07 ± 0.01^{d}**	**0.08 ± 0.02 ^{cd}**	**0.07 ± 0^{d}**	**0.15 ± 0.03^{bc}**	**-0.767***
11.344	(E)-Ocimene	0.34 ± 0.06^{c}	1.01 ± 0.72^{ab}	1.1 ± 0.14^{a}	0.8 ± 0.12^{abc}	0.4 ± 0.09^{c}	0.54 ± 0.07^{bc}	0.54 ± 0.12^{bc}	1.3 ± 0.17^{a}	
23.423	1-Hexadecanol	1.38 ± 0.02^{b}	2.14 ± 1.45^{ab}	2.18 ± 1.24^{ab}	2.37 ± 0.42^{ab}	1 ± 0.48^{b}	1.56 ± 0.14^{b}	1.18 ± 0.54^{b}	3.29 ± 0.91^{a}	
11.661	(Z)-β-ocimene	0.48 ± 0.07^{d}	1.15 ± 0.52^{ab}	1.3 ± 0.03^{ab}	0.97 ± 0.1^{bc}	0.47 ± 0.01^{d}	0.64 ± 0.09 ^{cd}	0.65 ± 0.15 ^{cd}	1.48 ± 0.42^{a}	
13.316	**3,7-dimethyl-1,5,7-octatrien-3-ol**	**0.98 ± 0.15^{c}**	**2.81 ± 1.52^{b}**	**5.46 ± 0.47^{a}**	**3.54 ± 0.43^{b}**	**0.25 ± 0.4^{c}**	**0.3 ± 0.35^{c}**	**0.08 ± 0.01^{c}**	**0.25 ± 0.05^{c}**	**-0.765***
18.97	**3-*tert*-Butyl-2-pyrazolin-5-one**	**1.38 ± 0.02^{bc}**	**2.51 ± 0.96^{ab}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{ab}**	**0.26 ± 0.15^{c}**	**0.33 ± 0.16^{c}**	**0.22 ± 0.03^{c}**	**1.51 ± 2.43^{bc}**	**-0.873****
10.944	4-methyl-Decane	0.93 ± 0.79^{ab}	2.51 ± 0.96^{a}	0.09 ± 0.01^{b}	2.5 ± 0.21^{a}	1.43 ± 0.09^{ab}	1.09 ± 0.93^{ab}	1 ± 0.83^{ab}	2.3 ± 2.16^{a}	
8.708										**-0.943****

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Table 2 (*continued*)

RT (min)	Compound	S0	S1	S2	S3	S4	S5	S6	S7	Coefficient of correlation
	10-Amino-10,11-dihydro-5-acetyldibenz [b,f] azepine	**3.06 ± 0.51^{de}**	**5.42 ± 2.08^{b}**	**8.88 ± 0.64^{a}**	**4.95 ± 0.7^{bc}**	**2 ± 0.13^{de}**	**2.08 ± 0.13^{de}**	**1.67 ± 0.2^{e}**	**3.51 ± 1.2 ^{cd}**	
27.751	**2D,4D,6D,8D-tetramethyloctacosanol**	**1.16 ± 0.36^{b}**	**2.37 ± 1.12^{b}**	**4.4 ± 2.14^{a}**	**2.5 ± 0.21^{b}**	**1.11 ± 0.84^{b}**	**1.11 ± 0.65^{b}**	**1.27 ± 0.68^{b}**	**2.47 ± 0.63^{b}**	**-0.771***
26.359	2-hexyl-1-Decanol	1.38 ± 0.02^{ab}	2.51 ± 0.96^{ab}	5.5 ± 6.33^{a}	1.78 ± 1.11^{ab}	0.75 ± 0.63^{b}	1.56 ± 0.14^{ab}	1.56 ± 0.15^{ab}	1.87 ± 0.9^{ab}	
27.756	1-Methoxyhexacosane	1.38 ± 0.02^{b}	2.02 ± 1.62^{ab}	3.63 ± 0.81^{a}	1.37 ± 1.08^{b}	1.52 ± 0.2^{b}	1.56 ± 0.14^{b}	1.6 ± 0.14^{b}	2.64 ± 1.63^{ab}	
27.693	2-(eicosyloxy)-Ethanol	0.79 ± 0.54^{c}	1.98 ± 1.69^{abc}	3.2 ± 0.1^{a}	2.5 ± 0.21^{ab}	2.04 ± 1.07^{abc}	1.56 ± 0.14^{bc}	1.46 ± 0.28^{bc}	1.92 ± 0.81^{abc}	
27.705	**2-(octadecyloxy)-Ethanol**	**1.38 ± 0.02^{b}**	**2.51 ± 0.96^{ab}**	**3.65 ± 0.85^{a}**	**1.73 ± 1.2^{b}**	**1.18 ± 0.38^{b}**	**1.56 ± 0.14^{b}**	**1.1 ± 0.86^{b}**	**2.52 ± 1.81^{ab}**	**-0.796***
28.238	Ethanol, 2-(hexadecyloxy)-	1.45 ± 0.13^{b}	2.14 ± 1.45^{ab}	3.65 ± 0.85^{a}	1.86 ± 1.3^{ab}	1.43 ± 0.09^{b}	1.56 ± 0.14^{b}	0.56 ± 0.77^{b}	2.44 ± 1.93^{ab}	
27.875	2-(tetradecyloxy)-Ethanol	1.48 ± 1.36^{b}	2.15 ± 1.43^{b}	5.65 ± 4.32^{a}	1.31 ± 1.17^{b}	1.43 ± 0.09^{b}	1.56 ± 0.14^{b}	2.15 ± 0.97^{b}	2.14 ± 1.37^{b}	
15.503	**(-)-4-Terpineol**	**0.94 ± 0.75^{c}**	**2.51 ± 0.96^{ab}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{ab}**	**1.43 ± 0.09^{bc}**	**0.59 ± 0.85^{c}**	**0.55 ± 0.82^{c}**	**0.15 ± 0.05^{c}**	**-0.842****
26.876	2,4-Ditert-butyl-6-nitrophenol	1.34 ± 0.08^{b}	2.9 ± 1.82^{ab}	3.87 ± 1.69^{a}	1.82 ± 0.19^{b}	1.43 ± 0.09^{b}	1.56 ± 0.14^{b}	1.56 ± 0.15^{b}	3.02 ± 1.13^{ab}	
22.126	(E)-β-Farnesene	0.57 ± 0.14^{b}	1.05 ± 0.46^{b}	1.58 ± 0.63^{b}	1.11 ± 0.08^{b}	0.79 ± 0.26^{b}	0.64 ± 0.1^{b}	0.74 ± 0.17^{b}	2.83 ± 1.5^{a}	
22.878	2-methyl-1-Decanol	1.38 ± 0.02^{b}	2.51 ± 0.96^{b}	4.57 ± 2.44^{a}	2.5 ± 0.21^{b}	1.86 ± 0.79^{b}	1.56 ± 0.14^{b}	1.93 ± 0.6^{b}	2.83 ± 0.8^{ab}	
23.97	1,2-Dihydrolinalool	1.38 ± 0.02^{b}	2.51 ± 0.96^{ab}	2.4 ± 1.33^{ab}	2.5 ± 0.21^{ab}	1.12 ± 0.63^{b}	1.56 ± 0.14^{b}	1.56 ± 0.15^{b}	3.02 ± 1.13^{a}	
34.081	**1-Nonadecene**	**0.22 ± 0.16 ^{cd}**	**0.57 ± 0.28^{b}**	**0.88 ± 0.22^{a}**	**0.48 ± 0.12^{bc}**	**0.29 ± 0.17^{bcd}**	**0.18 ± 0.01 ^{cd}**	**0.16 ± 0.03^{d}**	**0.26 ± 0.12 ^{cd}**	**-0.881****
14.685	Neroloxide	0.09 ± 0.01^{d}	0.22 ± 0.11^{ab}	0.21 ± 0.03^{abc}	0.13 ± 0.06^{bcd}	0.08 ± 0.01^{d}	0.12 ± 0.01 ^{cd}	0.1 ± 0.02^{d}	0.26 ± 0.09^{a}	
14.603	6,10-dimethyl-2-Undecanone	0.05 ± 0.02^{b}	0.07 ± 0.04^{b}	0.1 ± 0.05^{b}	0.08 ± 0.04^{b}	0.04 ± 0.01^{b}	0.06 ± 0.01^{b}	0.05 ± 0.01^{b}	0.2 ± 0.09^{a}	
18.841	β-Ionone	0.57 ± 0.16^{c}	1.23 ± 0.53^{ab}	1.34 ± 0.12^{a}	0.97 ± 0.06^{abc}	0.55 ± 0.15^{c}	0.68 ± 0.16^{bc}	0.67 ± 0.03^{bc}	1.38 ± 0.65^{a}	
8.178	Oxepine, 2,7-dimethyl-	0.97 ± 0.71^{a}	1.88 ± 1.7^{a}	2.19 ± 1.69^{a}	1.79 ± 1.43^{a}	1.43 ± 0.09^{a}	1.56 ± 0.14^{a}	0.56 ± 0.81^{a}	1.67 ± 1.24^{a}	
22.332	Ethanol, 2-[2-(2-butoxyethoxy) ethoxy]-	2.33 ± 0.18^{b}	3.26 ± 0.93^{b}	5.63 ± 1.87^{a}	3.91 ± 0.26^{b}	3.02 ± 0.92^{b}	2.47 ± 0.5^{b}	2.9 ± 0.63^{b}	5.95 ± 1.11^{a}	
33.044	**Isopropyl palmitate**	**0.66 ± 0.62^{a}**	**1.93 ± 1.78^{a}**	**2.73 ± 2.2^{a}**	**1.78 ± 1.44^{a}**	**1.43 ± 0.09^{a}**	**1.56 ± 0.14^{a}**	**1.56 ± 0.15^{a}**	**3.02 ± 1.13^{a}**	**0.804***
9.654	Hexanoic acid	0.66 ± 0.21^{bc}	0.6 ± 0.19^{bc}	0.73 ± 0.33^{b}	0.54 ± 0.11^{bc}	0.32 ± 0.05^{c}	0.5 ± 0.19^{bc}	0.46 ± 0.15^{bc}	1.44 ± 0.22^{a}	
24.681	**8-hexyl-Pentadecane**	**1.04 ± 0.59^{a}**	**2.51 ± 0.96^{a}**	**2.85 ± 1.91^{a}**	**1.93 ± 1.19^{a}**	**1.43 ± 0.09^{a}**	**1.18 ± 0.78^{a}**	**0.6 ± 0.78^{a}**	**2.33 ± 1.92^{a}**	**-0.750***
21.474	(Z)-1-Methyl-4-(6-methylhept-5-en-2-ylidene)cyclohex-1-ene	0.66 ± 0.14^{c}	1.13 ± 0.55^{abc}	1.55 ± 0.15^{ab}	1.23 ± 0.15^{abc}	0.7 ± 0.16^{c}	0.54 ± 0.07^{c}	0.85 ± 0.35^{bc}	1.66 ± 0.82^{a}	
21.095	2,4,7,9-Tetramethyl-5-decyn-4,7-diol	1.94 ± 0.22^{b}	2.55 ± 1.54^{b}	6.37 ± 2.49^{a}	5.24 ± 0.61^{a}	1.24 ± 0.22^{b}	1.56 ± 0.35^{b}	2.08 ± 0.29^{b}	4.97 ± 2.26^{a}	
15.171	**Octanoic acid**	**0.32 ± 0.02^{c}**	**0.47 ± 0.04^{bc}**	**0.99 ± 0.33^{a}**	**0.68 ± 0.16^{b}**	**0.33 ± 0.13^{c}**	**0.21 ± 0.02^{c}**	**0.13 ± 0.01^{c}**	**0.44 ± 0.31^{bc}**	**-0.831***
13.339	**Nonanal**	**1.8 ± 0.66^{bc}**	**3.51 ± 1.43^{a}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{abc}**	**1.32 ± 0.16^{c}**	**1.56 ± 0.14^{c}**	**1.56 ± 0.15^{c}**	**3.02 ± 1.13^{ab}**	**-0.825***
10.35	**Octanal**	**0.33 ± 0.05^{cde}**	**0.44 ± 0.19^{bcd}**	**0.74 ± 0.11^{a}**	**0.48 ± 0.07^{bc}**	**0.19 ± 0.08^{e}**	**0.23 ± 0.04^{de}**	**0.2 ± 0.09^{e}**	**0.6 ± 0.23^{ab}**	**-0.723***
10.205	**Ethyl caproate**	**1.38 ± 0.02 ^{cd}**	**2.51 ± 0.96^{b}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{b}**	**1.43 ± 0.09 ^{cd}**	**1.08 ± 0.71 ^{cd}**	**1.56 ± 0.15^{bc}**	**0.5 ± 0.22^{d}**	**-0.797***
9.945	β-Myrcene	2.17 ± 0.34^{c}	3.16 ± 1.05^{c}	5.15 ± 0.91^{ab}	3.62 ± 0.08^{bc}	2.49 ± 0.18^{c}	3 ± 0.56^{c}	3.15 ± 0.51^{c}	6.59 ± 2.05^{a}	
11.537	Benzeneacetaldehyde	0.25 ± 0.03^{ab}	0.34 ± 0.14^{a}	0.26 ± 0.04^{ab}	0.2 ± 0.04^{bc}	0.08 ± 0.02^{d}	0.09 ± 0.01 ^{cd}	0.14 ± 0.02 ^{cd}	0.26 ± 0.07^{ab}	
22.69	**Dehydro-b-ionone**	**1.38 ± 0.02^{bc}**	**2.51 ± 0.96^{ab}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{ab}**	**1.43 ± 0.09^{bc}**	**0.66 ± 0.8^{c}**	**0.85 ± 0.57^{bc}**	**2.2 ± 2.11^{abc}**	**-0.859****
15.79	Methyl salicylate	0.22 ± 0.06^{a}	0.13 ± 0.06^{abc}	0.16 ± 0.06^{ab}	0.13 ± 0.09^{abc}	0.03 ± 0.01^{c}	0.07 ± 0.06^{bc}	0.09 ± 0.01^{bc}	0.21 ± 0.05^{a}	
10.568	**2-Hexenoic acid**	**1.38 ± 0.02^{a}**	**2.03 ± 1.48^{a}**	**2.03 ± 2.25^{a}**	**1.85 ± 1.08^{a}**	**1.01 ± 0.68^{a}**	**0.67 ± 0.77^{a}**	**0.66 ± 0.72^{a}**	**1.78 ± 1.05^{a}**	**-0.725***
29.291	**2-Ethylhexyl salicylate**	**0.13 ± 0.03^{b}**	**1.3 ± 2^{ab}**	**0.27 ± 0.08^{b}**	**0.87 ± 1.21^{b}**	**1.43 ± 0.09^{ab}**	**1.56 ± 0.14^{ab}**	**1.56 ± 0.15^{ab}**	**3.02 ± 1.13^{a}**	**0.841****
5.59	**2-methyl-Butanoic acid**	**0.05 ± 0.03^{d}**	**0.09 ± 0.04^{d}**	**0.22 ± 0.03^{b}**	**0.2 ± 0.02^{bc}**	**0.11 ± 0.01 ^{cd}**	**0.16 ± 0.02^{bcd}**	**0.21 ± 0.08^{bc}**	**0.6 ± 0.12^{a}**	**0.919****
19.133	1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl-	1.38 ± 0.02^{bc}	2.51 ± 0.96^{ab}	3.65 ± 0.85^{a}	2.5 ± 0.21^{ab}	0.8 ± 0.6^{c}	1.56 ± 0.14^{bc}	0.76 ± 0.64^{c}	3.02 ± 1.13^{a}	
29.164	**Eicosane**	**0.75 ± 0.92^{b}**	**0.94 ± 1.08^{b}**	**1.21 ± 1.17^{b}**	**1.87 ± 0.41^{b}**	**1.81 ± 0.57^{b}**	**1.58 ± 0.48^{b}**	**2.72 ± 0.3^{b}**	**7 ± 4.49^{a}**	**0.867****
22.616	1-Dodecanol									

(*continued on next page*)

Table 2 (*continued*)

RT (min)	Compound	S0	S1	S2	S3	S4	S5	S6	S7	Coefficient of correlation
		1.61 ± 0.42 ^{cd}	2.52 ± 0.98^{bc}	3.65 ± 0.85^{ab}	2.5 ± 0.21^{bc}	1.43 ± 0.09 ^{cd}	0.85 ± 0.75^{d}	1.37 ± 0.43 ^{cd}	3.98 ± 1.29^{a}	
15.213	**Nonanoic acid**	**8.27 ± 1.85^{ab}**	**7.06 ± 4.6^{abc}**	**12.19 ± 6.84^{a}**	**8.6 ± 1.71^{ab}**	**1.81 ± 0.82^{c}**	**1.82 ± 0.84^{c}**	**1.44 ± 0.12^{c}**	**3.47 ± 1.93^{bc}**	**-0.834***
16.001	Dodecane	0.5 ± 0.18^{b}	0.72 ± 0.28^{ab}	0.88 ± 0.19^{a}	0.61 ± 0.09^{ab}	0.38 ± 0.13^{b}	0.54 ± 0.08^{b}	0.48 ± 0.05^{b}	0.92 ± 0.29^{a}	
15.214	**1-Decanol**	**1.38 ± 0.02^{bc}**	**2.6 ± 1.19^{ab}**	**3.65 ± 0.85^{a}**	**2.45 ± 0.16^{ab}**	**1 ± 0.43^{c}**	**0.62 ± 0.43^{c}**	**0.25 ± 0.03^{c}**	**3.02 ± 1.13^{a}**	**-0.739***
18.342	2-Undecanone	1.38 ± 0.02^{c}	2.3 ± 0.82^{bc}	3.38 ± 0.35^{a}	1.43 ± 0.11^{c}	1.43 ± 0.09^{c}	1.56 ± 0.14^{c}	1.56 ± 0.15^{c}	3.02 ± 1.13^{ab}	
10.282	Undecane	0.27 ± 0.02^{b}	0.45 ± 0.23^{b}	0.94 ± 0.19^{a}	0.67 ± 0.05^{ab}	0.32 ± 0.08^{b}	0.28 ± 0.11^{b}	0.32 ± 0.08^{b}	1.01 ± 0.62^{a}	
6.84	2-Heptanone	0.26 ± 0.32^{b}	0.68 ± 0.39^{b}	3.58 ± 1.04^{a}	4.16 ± 1.33^{a}	0.84 ± 0.1^{b}	0.85 ± 0.05^{b}	0.6 ± 0.11^{b}	1.04 ± 0.26^{b}	
29.568	**Isopropyl myristate**	**0.09 ± 0.03^{ab}**	**0.14 ± 0.05^{a}**	**0.15 ± 0.01^{a}**	**0.08 ± 0.01^{ab}**	**0.06 ± 0^{b}**	**0.04 ± 0.02^{b}**	**0.05 ± 0.02^{b}**	**0.12 ± 0.1^{ab}**	**-0.800***
8.172	**3,5-Dimethylphenol**	**0.11 ± 0.03 ^{cd}**	**0.22 ± 0.11^{ab}**	**0.23 ± 0.05^{a}**	**0.13 ± 0.04^{bcd}**	**0.08 ± 0.01^{d}**	**0.09 ± 0.01^{d}**	**0.08 ± 0.01^{d}**	**0.18 ± 0.05^{abc}**	**-0.853****
6.257	p-Xylene	0.74 ± 0.57^{b}	1.64 ± 1.71^{ab}	1.06 ± 0.07^{b}	1.89 ± 1.25^{bc}	1.43 ± 0.09^{bc}	1.56 ± 0.14^{bc}	1.56 ± 0.15^{bc}	3.02 ± 1.13^{a}	
15.879	**L-α-Terpineol**	**1.28 ± 0.18^{c}**	**2.51 ± 0.96^{ab}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{ab}**	**1.43 ± 0.09^{bc}**	**1.64 ± 0.21^{bc}**	**1.56 ± 0.15^{bc}**	**3.02 ± 1.13^{a}**	**-0.733***
19.96	**gamma-Nonanolactone**	**1.38 ± 0.02^{c}**	**2.51 ± 0.96^{b}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{b}**	**0.36 ± 0.27^{c}**	**1.24 ± 0.43^{c}**	**1.29 ± 0.57^{c}**	**1.42 ± 0.58^{c}**	**-0.742***
22.264	**2,6-Di-*tert*-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one**	**1.38 ± 0.02^{bc}**	**2.51 ± 0.96^{ab}**	**3.65 ± 0.85^{a}**	**2.5 ± 0.21^{ab}**	**1.6 ± 0.21^{bc}**	**1.15 ± 0.1^{c}**	**1.44 ± 0.33^{bc}**	**2.91 ± 1.22^{a}**	**-0.760***
17.37	Phenethyl acetate	1.38 ± 0.02^{bc}	2.51 ± 0.96^{ab}	3.65 ± 0.85^{a}	2.5 ± 0.21^{ab}	0.38 ± 0.15^{c}	0.78 ± 0.68^{c}	1.56 ± 0.15^{bc}	3.02 ± 1.13^{a}	
26.062	**3,5-Di-*tert*-butylcatechol**	**0.45 ± 0.04^{c}**	**1 ± 0.44^{b}**	**1.45 ± 0.08^{a}**	**0.93 ± 0.15^{b}**	**0.5 ± 0.17^{c}**	**0.5 ± 0.03^{c}**	**0.42 ± 0.03^{c}**	**0.85 ± 0.15^{b}**	**-0.948****
17.263	1,3-bis(1,1-dimethylethyl)-Benzene	2.16 ± 0.19^{c}	4.57 ± 1.27^{b}	6.5 ± 0.07^{a}	3.43 ± 0.45^{bc}	2.61 ± 0.3^{c}	3.04 ± 0.3^{bc}	2.56 ± 0.26^{c}	4.54 ± 1.94^{b}	
9.059	Benzaldehyde	0.66 ± 0.11^{ab}	0.74 ± 0.08^{a}	0.51 ± 0.07^{abc}	0.3 ± 0.01^{c}	0.31 ± 0.03^{c}	0.37 ± 0.07^{c}	0.41 ± 0.11^{bc}	0.73 ± 0.38^{a}	
11.268	Benzyl alcohol	0.19 ± 0.03^{c}	0.36 ± 0.06^{abc}	0.52 ± 0.06^{a}	0.31 ± 0.12^{bc}	0.19 ± 0.06^{c}	0.22 ± 0.08^{c}	0.26 ± 0.04^{c}	0.46 ± 0.19^{ab}	
5.993	Ethylbenzene	0.27 ± 0.02^{b}	0.56 ± 0.12^{b}	1.51 ± 0.97^{a}	0.36 ± 0.07^{b}	0.66 ± 0.06^{b}	0.62 ± 0.28^{b}	0.74 ± 0.17^{b}	0.47 ± 0.11^{b}	
15.376	Succinic acid, ethyl 2-ethylhexyl ester	1.38 ± 0.02^{bc}	1.39 ± 1^{bc}	3.65 ± 0.85^{a}	2.5 ± 0.21^{ab}	0.58 ± 0.77^{c}	1.03 ± 0.79^{c}	0.1 ± 0.01^{c}	3.02 ± 1.13^{a}	
7.074	**methoxy-phenyl-Oxime**	**8.8 ± 1.77 ^{cd}**	**18.4 ± 9.08^{ab}**	**24.7 ± 3.89^{a}**	**16.99 ± 2.49^{b}**	**6.86 ± 0.76^{d}**	**7.87 ± 1.02 ^{cd}**	**7.24 ± 0.62 ^{cd}**	**14.28 ± 2.4^{bc}**	**-0.933****
26.132	Methyl triacontyl ether	0.61 ± 0.47^{c}	1.38 ± 0.69^{bc}	1.84 ± 1.1^{bc}	2.5 ± 0.21^{ab}	1.43 ± 0.09^{bc}	1.56 ± 0.14^{bc}	1.56 ± 0.15^{bc}	3.02 ± 1.13^{a}	

a–eDifferent letters means the S0-S7 of storage differ at $P < 0.05$.
SD: standard deviation; $n = 3$.
r: Correlation between relative content of volatile components and fermentation time.
Significance of model:*, $P < 0.05$; **, $P < 0.01$.

oxidative degradation of sugars and fatty acids react with amino groups in amino acids to form Strecker aldehydes. Benzaldehyde formed showed an upward trend initially and gradually decline as the fermentation process continues. The yeast in kombucha contained the Gre2 protein that can function as an effective aldehyde reductase [53]. This protein has the ability to catalyze the reduction of some aromatic aldehydes and to convert isovaleraldehyde to isoamyl alcohol [54], which may be the reason for the increase in the aldehyde content at the late stage of kombucha fermentation was not obvious.

During the course of fermentation, the ketones showed a fluctuating and rising trend. The characteristic floral aroma of kombucha originated from ionone compounds. Carotenoids undergo primary and secondary oxidation and produce β-ionone. In the enzymatic oxidation reaction, the generation pathway of β-ionone was as follows: β-carotene generated β-ionone under the action of dioxygen lyase, and β-carotene undergoes photooxidation and pyrolysis to produce β-ionone. The content of catechins dropped significantly during the fermentation process and this decline was also aided by to the oxidation products of catechins [55]. The oxidation of catechins during the middle and late stages of fermentation led to the oxidation and degradation of carotene, etc., and the production of β-ionone and related structural aroma compounds increased the amount of ionone compounds.

Esters aroma components levels such as isopropyl palmitate, methyl salicylate, Phenethyl acetate, 2-ethylhexyl salicylate increased and declined afterward. Among the aroma components of esters methyl salicylate helps in identifying unfermented tea from fermented tea [15]. It has green and burnt herbal, holly-like aroma, and some warm sweet root fruit aroma. Phenethyl acetate has a rose and honey-like aroma. Diisobutyl phthalate is an uncommon and rare volatile aroma component in tea responsible for the sour tea unique aroma. The content of alkanes was low, the overall content was increased due to the different types of volatile components contained in them. The volatile component levels coordinated with each other with an overall effect and contribution to the net aroma of kombucha. The yeast in kombucha can oxidize the methyl groups at both ends of alkanes to carboxyl groups through their own metabolism to produce long-chain dibasic acids with corresponding carbon atoms [56].

The olefinic aromatic compounds are mainly terpenes, and their content was increased during the fermentation process. Fungal microorganisms can produce and convert terpenes and the main two pathways are mevalonate pathway (MVA) and methylerythritol 4-phosphate pathway (MEP). The MVA pathway mainly synthesizes sesquiterpenes,

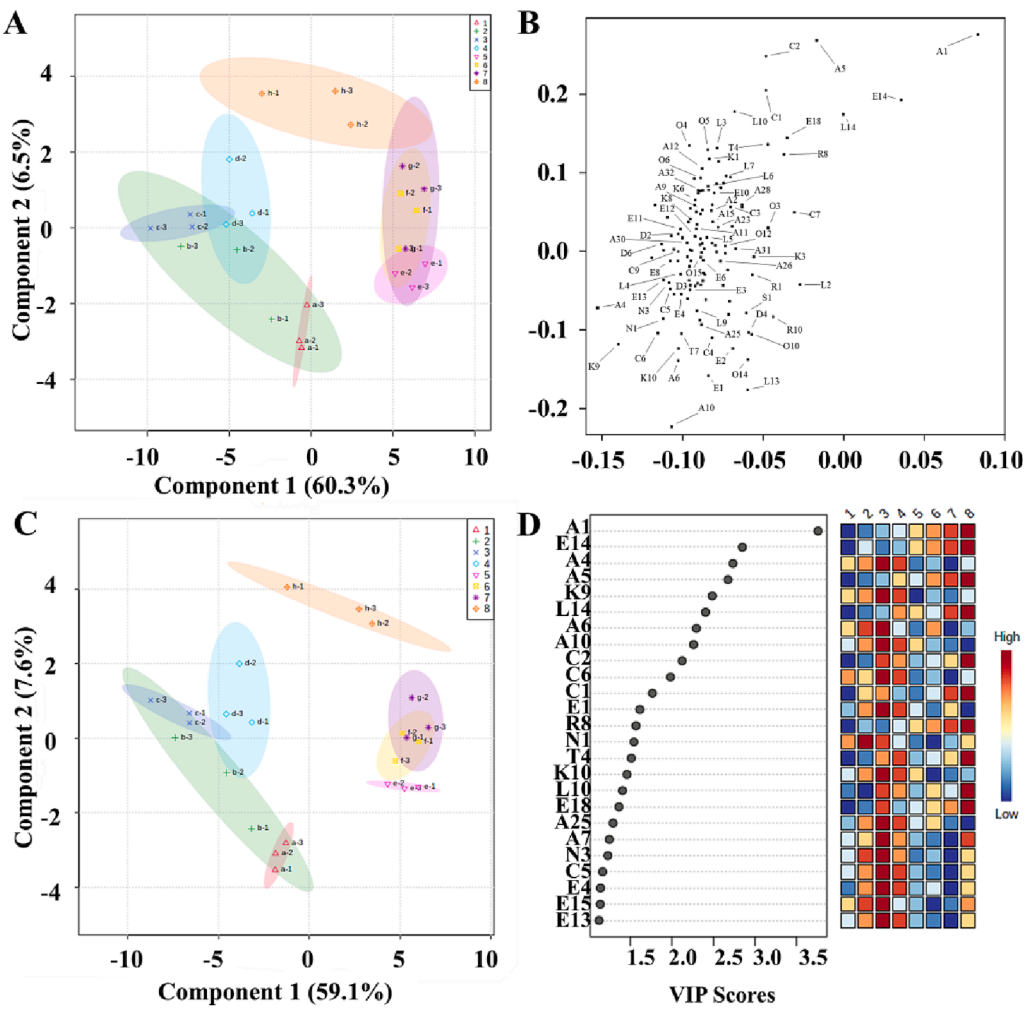

Fig. 4. Multivariate statistical analysis of kombucha tea samples and mass spectrometry data set PCA score plot (A), the contribution rates of PC1 and PC2 are 60.3% and 6.5%, loading plots of variables in kombucha samples based on PCA model (B) (see Table 1 for the codes of A1, E14, L14, etc.), PLS-DA score plot (C), PC1 and PC2 contribute 59.1% and 7.6%, top twenty-five volatile compounds were selected by PLS-DA, which were ranked (VIP value > 1.0) (D). Colors represent relative concentrations of each metabolite from different sample groups.

triterpenes and sterols (such as brassinolides). The MEP pathway generates monoterpenes, diterpenes and precursors of phytol, gibberellin and carotenoids [57]. In the Kombucha fermentation process, terpenes with high content are β-Myrcene (woody, floral), D-Limonene (lemony), α-Phellandrene (citrus), Terpinolene (pine) and E-β-Farnesene (Fragrant, floral and fruity). The β-myrcene content raised initially but afterward declined as the fermentation process continued attributed to the reduction of dihydro linalool and α-Terpineol by microorganisms.

Aromatic compounds present were mainly 2,4-di-*tert*-butylphenol, 2,4,6-tri-*tert*-butylphenol, p-xylene and 1,2-xylene. Phenols are composed of phenolic acids and lignins generated through microbial heating. A compound formed after degradation or enzymatic degradation 2,4-Di-*tert*-butylphenol is a yeast metabolite [58]. The findings in this study revealed that during the fermentation process of kombucha, 1,2-xylene levels will increase due to the methylation of catechins by microbial action.

4. Conclusions

In the present work, the fermentation conditions of kombucha were determined by single factor optimization experiment and response surface methodology as 30 °C, pH 3.2, 23 kHz. The integrated changes of volatile properties during ultrasound-assisted treatment were monitored using metabolomics technique. Generally, a comprehensive metabonomic profile was identified consisting of 132 components, such as alcohols, aldehydes, ketones, aromatic hydrocarbons, sulfur and nitrogen compounds. The multivariate statistical analysis was successfully executed, and 25 characteristic metabolites with VIP > 1 were identified, as biomarkers. In addition, the participation of metabonomic pathways and the transformation of each volatile was studied in detail. The results showed that in the late fermentation period, there was a metabolic pathway of conversion from volatile substances. The results indicate that the process can be optimized and the reaction process can be accelerated and improved during the fermentation processing of kombucha. The study might provide an important basis for future investigations into the changes and impact mechanisms of kombucha-fermented metabolites.

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CRediT authorship contribution statement

Zhen Wang: Methodology, Investigation, Writing – original draft. **Waqas Ahmad:** Software, Formal analysis. **Afang Zhu:** Investigation, Writing – review & editing. **Wenhui Geng:** Conceptualization, Investigation. **Wencui Kang:** Validation, Data curation. **Qin Ouyang:** Data curation. **Quansheng Chen:** Supervision.

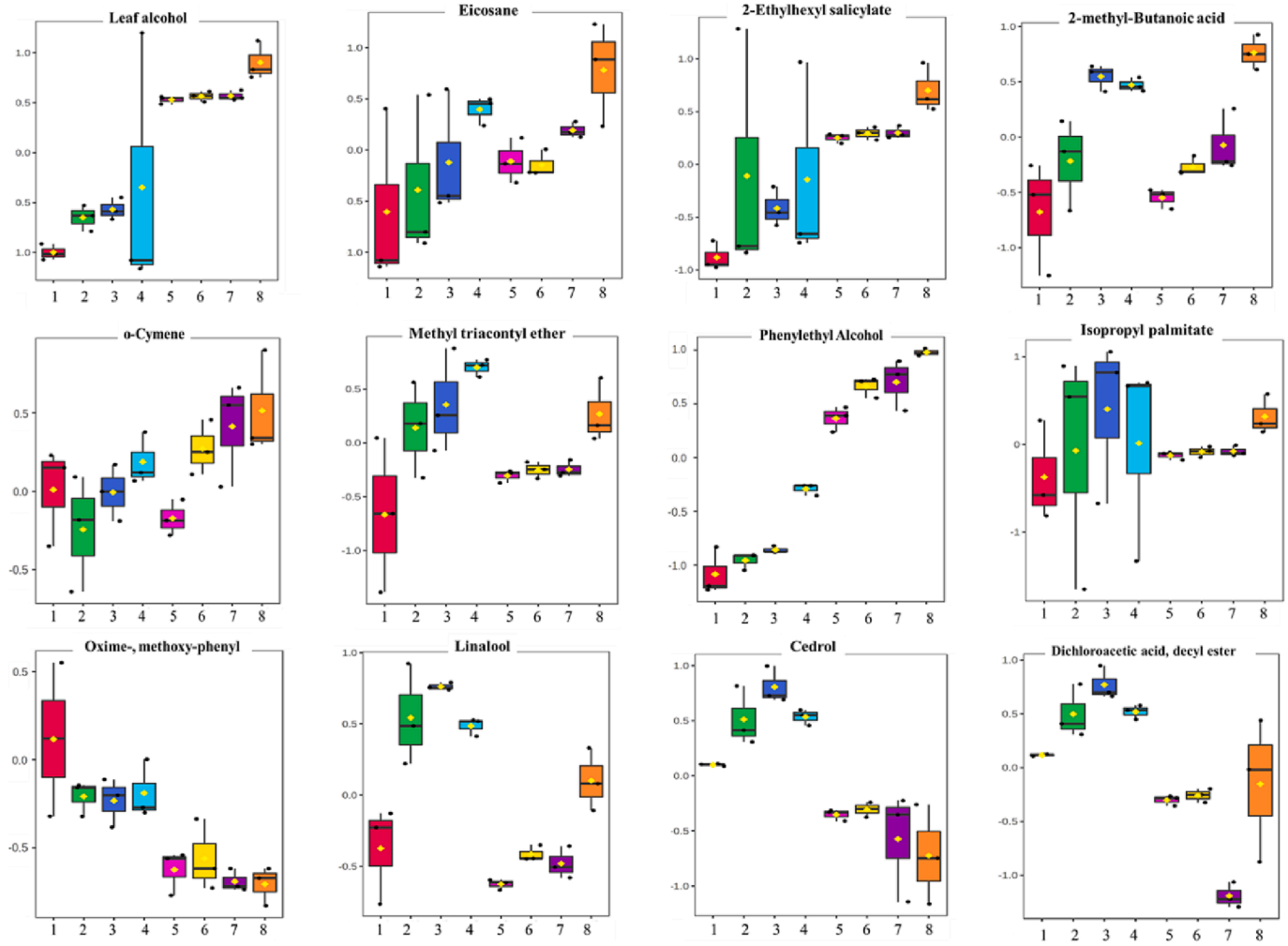

Fig. 5. Boxplots of 12 example metabolites showing significant differences between fermentations.

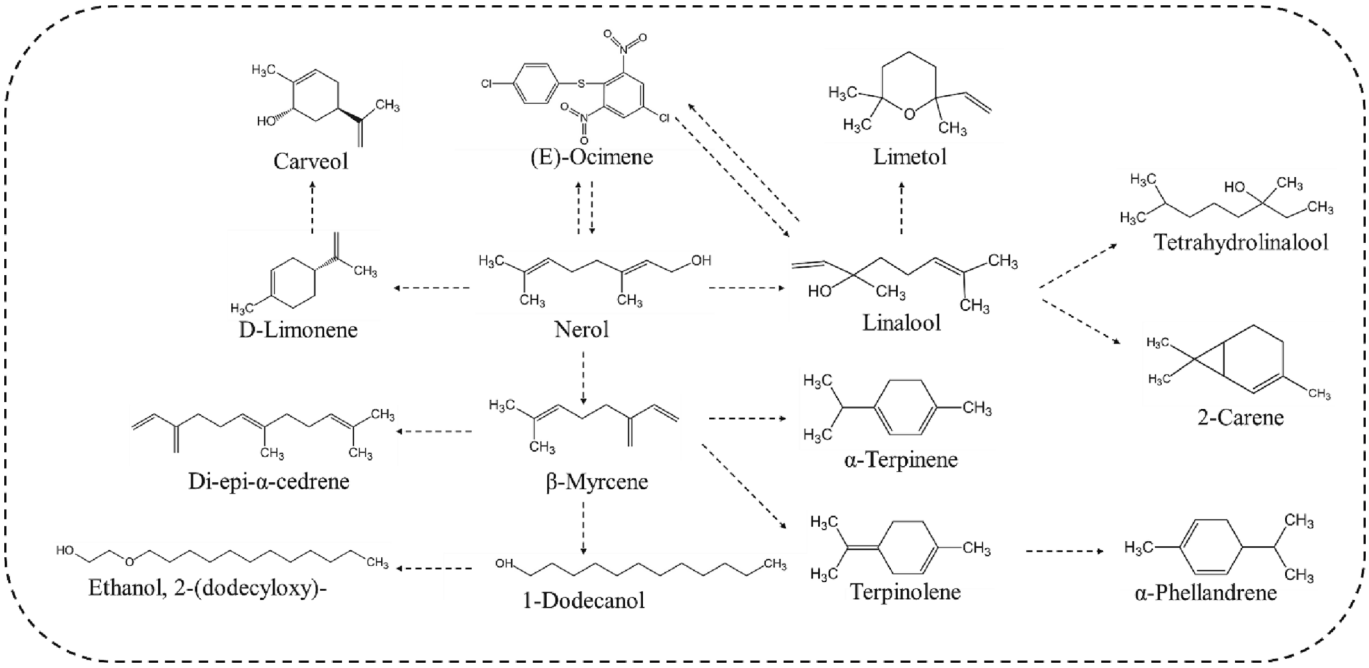

Fig. 6. The metabolic pathways of the characteristic metabolites found during kombucha fermentation.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2023.106339.

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