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Effects of fixation temperature on volatile components and key differential aroma compounds of green tea

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Abstract

In the present study, stir bar sorptive extraction (SBSE) coupled with gas chromatography-mass spectrometry (GC-MS) alongside odour activity value (OAV) calculation were employed to investigate the effects of different fixation temperatures (190, 220, 250, 280, 310, 340 °C) on the composition and content of volatile compounds in green tea samples made from fresh tea leaves of Longjing 43 (*Camellia sinensis* var. *sinensis*). A total of 102 volatile components were identified. The total content of these volatiles generally decreased significantly by about 80%–89% after fixation, compared to that in fresh tea leaves, and exhibited a more stable decline with increasing fixation temperature. Among them, 12 key differential aroma compounds were identified, including (*E*)-β-ionone, hexanal, δ-decalactone, linalool, (*Z*)-linalool oxide (furanoid), (*Z*)-3-hexen-1-ol, based on VIP > 1, $p < 0.05$, and OAV ≥ 1, which may greatly influence the formation of green tea aroma quality.

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Introduction

Green tea, known for its pleasant flavour, has been a popular beverage for many centuries, particularly in China and some other Asian countries. Tea aroma is one of the critical evaluation factors for determining green tea quality, which essentially comprises various volatile components at different concentrations and ratios[1,2]. Tea processing technology has a significant impact on the formation of aroma quality[3−5], during which volatile components undergo significant changes. For example, aroma precursors and some non-volatile metabolites in the fresh tea leaves are converted into aroma components via enzymatic reactions and thermochemical reactions, which constitutes the main mechanism of green tea aroma formation[6].

Green tea processing is generally divided into four major steps, including spreading, fixation, rolling, and drying. Among them, fixation has been proven to be the essential stage in the transformation of aroma compounds and therefore shapes the initial aroma profile of green tea[7,8]. In particular, fixation temperature is an important parameter to regulate the fixation process of green tea and has a significant impact on the development of its flavour quality[9]. In this case, the high temperature during the fixation process enables the release of volatile components such as linalool, which has a floral scent, and the volatilization of small molecules, including some aldehydes with a lower boiling point and pungent odor. Therefore, it is essential to comprehend how fixation temperature controls the volatile components of green tea and affects its aroma quality. By regulating different fixation temperatures, the composition and content of volatile components in green tea can be changed greatly, thus achieving the requirements of different aroma types in tea production. A recent study has demonstrated the impact of fixation temperature on the non-volatile metabolites that influence the taste quality of Longjing tea[10]. However, the investigation of the impact of fixation temperature on volatile components in green tea is very scarce, and the explicit effect of fixation temperature on green tea aroma components has yet to be elucidated. Therefore, it is considered that the different fixation temperatures might significantly impact the composition and content of volatile compounds of green tea, thus further affecting its aroma quality.

In this study, various green tea samples with different fixation temperatures were produced (190, 220, 250, 280, 310, 340 °C), using the same batch of fresh tea leaves from Longjing 43 (*Camellia sinensis* var. *sinensis*) as raw materials, and the fixed tea leaves during tea processing were also collected to investigate the effect of fixation temperature on the characteristics of volatile metabolites in green tea. The volatile components of these green tea samples were analyzed using stir bar sorptive extraction combined with gas chromatography-mass spectrometry (SBSE-GC-MS). Furthermore, partial least squares discriminant analysis (PLS-DA), and the odour activity value (OAV) method were employed to screen for the differential volatile compounds in green tea with different fixation temperatures. These results may enhance our understanding of how fixation temperature affects green tea aroma quality, and offer valuable insights for the targeted regulation of aroma profiles during tea processing.

Materials and methods

Chemicals

NaCl was supplied by Merck (Darmstadt, Germany). The *n*-alkane mixtures of C8–C40 were purchased from J&K Scientific Co., Ltd (Beijing, China). The detailed information on aroma standards is provided in Supplementary Table S1.

Sample preparation

Fresh tea leaves (FL) of the Longjing 43 cultivar with one bud and two leaves were picked in Shengzhou City, Zhejiang Province, China (Longitude 120°82', Latitude 29°74'). Part of FL (moisture content 76.85%) was exposed to liquid nitrogen and freeze-dried using a vacuum freeze-dryer (GOLD-SIM) (SIM International Group, California, USA). The remaining fresh tea leaves were spread for 6 h (moisture content 67.75%) and then processed into green tea samples with traditional manufacturing processes as follows.

After spreading, the fresh tea leaves were divided into six portions. Each portion was fixed using a 6CST-50 rolling stir-frying machine (20 rpm; Zhejinag Zhufeng Machinery Co., Ltd, China). Six different fixation temperatures were applied, ranging from 190 to 340 °C, with a gradient of 30 °C (AF: AF190, AF220, AF250, AF280, AF310, AF340). The moisture contents of these fixed leaves were 59.55%, 56.20%, 56.50%, 51.25%, 50.30%, and 44.40%, respectively. The fixation time was kept consistent at 3 min across different fixation temperatures. The fixed leaves were instantly rolled for 1 h (Model 0725, small tea rolling machine, the average moisture content of the rolled leaves after different fixation temperatures is 50.30%). The rolling process comprised 20 min under light pressure, followed by 20 min under heavy pressure, then 10 min under light pressure, and concluding with 10 min without pressure. The samples were then first dried at 110 °C for about 1 h using a 6CHM-901 electric heating dryer (Zhejinag Chunjiang Tea Machinery Co., Ltd, China), and finally second dried at 90 °C for 30 min before being made into green tea samples (TS: TS190, TS220, TS250, TS280, TS310, TS340, with moisture contents of 7.21%, 7.23%, 6.85%, 6.20%, 6.34%, and 6.44%, respectively). In this study, fresh tea leaves (FL), after fixed tea leaves (AFs), and tea samples (TSs) were collected, respectively, with the FL and AF samples directly freeze-dried for preservation. All tea samples were ground into powder for testing.

Sensory evaluation

The sensory evaluation of green tea samples was conducted. All sensory experiments were performed by five well-trained experts from the professional sensory institute 'Tea Quality Supervision and Inspection Center of the Ministry of Agriculture', according to the national standards of tea (GB/T 23776-2018 named 'Tea Sensory Evaluation Method', GB/T 14487–2017 named 'Tea Sensory Evaluation Terminology'). The specific steps are as follows:

First, 100 g of tea samples were placed on a designated white tea evaluation tray. The tea's shape was evaluated by visual inspection and tactile methods. The tea leaves were flipped and repositioned to compare their appearance, and scores and comments were given for each attribute (each attribute had a maximum score of 100 points). Then, 3.0 g of the tea sample was placed in a tea evaluation cup. Boiling water was added at a 1:50 tea-to-water ratio (by mass/volume), the cup was covered, and the tea was steeped for 4 min. Afterward, the tea infusion was filtered at a consistent speed, and the infused leaves were placed into the cup.

The evaluation was performed in the following order: liquor color, aroma, taste, and infused leaves. Corresponding scores and evaluations were provided for each attribute. The five tea experts conducted their assessments independently during the entire evaluation process. The total score was calculated as follows:

Total Score = Score (shape) × 25% + Score (liquor color) × 10% + Score (aroma) × 25% + Score (taste) × 30% + Score (infused leaves) × 10%. The evaluation results are listed in Supplementary Table S2.

GC-MS analysis

Extraction of volatiles

The volatile compounds were extracted using the SBSE method, referring to our previous research[11]. In brief, tea powder (0.6 g) and NaCl (0.5 g) were placed in a 20 mL headspace bottle, and 10 mL of boiling water was added for brewing. The headspace bottle was then put on a multi-position magnetic stirrer (SP200-2T; Miu Instruments Co. Ltd, Hangzhou, China) equipped with a polydimethylsiloxane twister (10 mm × 0.5 mm, 24 μL; Gerstel, Germany) for volatile enrichment. The volatile compounds were extracted for 30 min under the conditions of 80 °C and 1,200 rpm. Subsequently, the twister was removed, and any residual tea powder on the surface was washed off with pure water. The twister was then dried and placed in an injection bottle for analysis.

GC-MS analysis

The sample was injected in the form of gas at an initial temperature of 30 °C and held for 1 min, then increased to 240 °C at a rate of 100 °C/min, and held for desorption for 5 min. Subsequently, the sample was cooled to −100 °C with liquid nitrogen (99.99%) and held for 1 min, then heated up to 280 °C, and held for 3 min at a rate of 12 °C/s.

The volatile compounds in green tea samples were analyzed with an Agilent 7980B GC system coupled with a 5977B MSD mass system (Agilent, Santa Clara, CA, USA), separated using a DB-5 MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent, Santa Clara, CA, USA). The GC oven temperature program was set as follows: the initial temperature was 50 °C for 2 min, increased to 170 °C by 4 °C/min (holding for 5 min), and finally increased to 265 °C at a rate of 10 °C/min and held for 5 min. The injector temperature was 280 °C. Helium (> 99.99%) was used as the carrier gas at a 1.6 mL/min flow rate. The mass spectrometer was recorded in electric impact (EI) mode, with a mass scan range from 50 to 550 Amu at 70 eV. The temperature of the ion source was 220 °C. Each sample was analyzed in triplicate.

Qualitative and quantitative analysis

The volatile compounds were identified by comparing with the NIST 2014 standard spectral library, requiring that the difference between the retention indices (RI) of the compounds measured on the basis of *n*-alkanes and the theoretical retention indices from the spectral library be within 30 RI units. The quantitative method of the compounds was based on their abundance in GC-MS analysis, and the relative contents of the volatile compounds were presented by fold changes of peak area between each tea sample and fresh tea leaves (FL). In addition, the significantly different aroma compounds screened were quantified by establishing standard curves from the corresponding aroma compound standards using the previously established standard addition approach[12] under consistent conditions, as those in tea samples. For compounds where specific standards were lacking, appropriate surrogates with similar chemical structures and properties were used. Mixed standard solutions of different concentrations were prepared, and 10 μL of each solution was added to the blank matrix. The samples were then analyzed under the same extraction and GC-MS conditions as the tea samples. A standard curve was constructed based on the peak areas of the standards, and the concentrations of volatile compounds in the samples were calculated accordingly[12].

The preparation of the blank tea matrix was based on the method previously developed by our group, with slight modifications: 50 g of green tea was brewed in 5,000 mL of boiling water for at least three rounds of infusion. After cooling, the tea liquor was discarded once the tea leaves no longer exhibited any noticeable aroma. The tea leaves were then subjected to rotary evaporation at 60 °C to remove residual moisture, followed by drying in an oven at 60 °C[13].

Odour activity value analysis

The odour thresholds of each volatile compound were referenced from previous literature reports. The odour activity value (OAV) of each compound was calculated according to the equation: $OAV_i = C_i/OT_i$, where C_i represents the concentration of the single compound in the tea sample (ng/mL), and OT_i denotes the odour threshold of the corresponding compound. A compound is generally considered to play a vital role in contributing to tea aroma quality when its OAV is ≥ 1.

Statistical analysis

PLS-DA and permutation tests were performed using SIMCA software version 14.1. Statistical significance was determined using one-way ANOVA with LSD *post hoc* test in *SPSS* software version 27.0. Hierarchical clustering analysis (HCA) was performed using TBtools software.

Results and discussion

Analysis of volatile compounds in green tea samples by GC-MS

Overall profiles of volatile compounds in FL, AFs, and TSs

In this study, a total of 102 volatile compounds were identified from FL, AFs, and TSs using SBSE-GC-MS. These compounds were classified into nine different chemical classes according to their chemical structures, including 30 alcohols, 19 esters, 12 aldehydes, 12 alkenes, nine ketones, seven aromatics, five oxygen heterocyclics, three lactones, and five other volatile compounds, and alcohols, esters, and aldehydes were the major group in these samples (Supplementary Tables S3 & S4). The total ion chromatogram of the samples is shown in Supplementary Fig. S1. As shown in Fig. 1a, FL had the highest total content of volatile components, which was much higher than AFs and TSs. Meanwhile, their total content decreased sharply during the fixation process, and this is primarily due to the high-temperature treatment causing low-boiling-point volatile compounds (e.g., alcohols and aldehydes) to evaporate, which leads to a significant reduction in their levels and, consequently, the observed decrease in volatile compounds. Additionally, some volatile compounds undergo decomposition or polymerization reactions under high temperatures, generating new compounds and further reducing their overall content. For instance, it has been demonstrated that terpenoids can be converted into their glycosidically bound forms at elevated temperatures due to the activity of glycosyltransferases that have not yet been completely inactivated[14,15]. The total content of volatile components decreased by 80.9%, 85.0%, 87.3%, 87.1%, 89.3%, and 89.5% in tea leaves fixed at 190, 220, 250, 280, 310, 340 °C, respectively, as compared to FL. This was in contrast to a prior study that recognized high-temperature fixation as outperforming low-temperature fixation in terms of aroma component accumulation, which might be caused by the use of different fixation temperature ranges and methods[9,16]. In addition, the total content of volatile components continued to decrease in TSs after the drying process. Comparing different fixation temperatures, the total content of volatile components demonstrated a steady downward trend as the fixation temperatures increased. Tea samples fixed at 190 °C had the highest content, while 340 °C was the lowest.

As demonstrated in Fig. 1b, a hierarchical cluster analysis heatmap was used to visualize the variation in the distribution differences of volatile components in samples subjected to varying fixation temperatures. It was observed that FL, AFs, and TSs could be successfully clustered into distinct groups based on the peak areas of volatile components, indicating that significant changes had occurred in volatile components among these groups. Among these, the levels of most volatile components were highest in fresh tea leaves and decreased notably after fixation, which was consistent with the results of a previous study[9]. In addition, the contents of some alkenes, including neophytadiene, β-cymene, γ-terpinene, limonene, isophorone, as well as 3-undecanone, estragole, methyl jasmonate, and 2-ethylhexyl salicylate, were generally higher in the TSs than in the FL and AFs; while 2,3-octanedione, 2,2,6-trimethylcyclohexanone, (*E*)-β-ionone, β-cyclocitral, as well as two lactones (including δ-decalactone and dihydroactinidiolide), generally exhibited higher levels in the AFs. Changes in the contents of all volatile compounds between fresh tea leaves and other samples were expressed as fold changes, as shown in Supplementary Table S4.

After fixation, the contents of almost all alcohols decreased, with benzyl alcohol (FC = 0.06 in AF190), 1-nonanol (FC = 0.09 in AF190), nerol (FC = 0.07 in AF190), and geraniol (FC = 0.05 in AF190) showing a much greater reduction. Among the aldehydes, α-citral (FC = 0.14 in AF190), (*Z*)-3-nonen-2-one (FC = 0.10 in AF190), 7-methyl-3-methylene-6-octen-1-ol (FC = 0.11 in AF190), and β-cyclocitral (FC = 0.11 in AF190) showed more variablility. Esters, including hexanoic acid, ethenyl ester (FC = 3.96 in AF190), (*Z*)-3-hexen-1-yl-3-methylbutanoate (FC = 3.46 in AF190), and methyl salicylate (FC = 0.09 in AF190), also showed significant differences during green tea fixation. Additionally, the contents of some volatile compounds, such as indole (FC = 0.05 in AF190), (*Z*)-linalool oxide (furanoid) (FC = 0.18 in AF190), eugenol (FC = 0.20 in AF190), and (*E*)-linalool oxide (furanoid) (FC = 0.25 in AF190), also varied significantly during fixation.

Notably, heptanol and (*E*)-2-decen-1-ol were only detected in fresh tea leaves in this study; these are common volatile components responsible for green and grassy odours in tea. The disappearance of these two aromas could be attributed to the instability of their chemical properties, which causes them to volatilize or transform their chemical structure during prolonged heat treatment at the fixation stage[17]. Besides, isobutyl benzoate was not detected in the TS group of samples. Furthermore, previous studies have shown that pyrazines and pyrroles, responsible for caramel and baking odors, were easily generated during drying[1], due to the proneness of the Maillard reaction between free amino acids and reducing sugars[18]. In this study, only 3-ethyl-2,5-dimethylpyrazine was identified as a pyrazine compound. This finding may be attributed to our rigorous criteria employed for the identification of each volatile compound, as well as suboptimal conditions for the Maillard reaction, which resulted from the brief fixing time and relatively low drying temperatures. It has been noted that the Maillard reaction typically occurs under conditions of elevated temperature and extended time, especially during processes like drying and roasting[19,20]. During the fixing process in this study, although the temperature is relatively high (190–340 °C), the fixing time is short (approximately 3 min), which leads to an incomplete Maillard reaction. Notably, the temperature mentioned here is the set temperature, not the actual temperature of the tea leaves. Moreover, studies have reported that no pyrazine compounds were detected after the fixing process in green tea or oolong tea[21,22]. In addition, green tea is rich in antioxidant compounds such as epigallocatechin gallate (EGCG), which may inhibit the Maillard reaction to some extent, resulting in lower levels of pyrazine and pyridine compounds[23].

Currently, several studies have investigated the differences in aroma composition of green tea processed using various fixation methods. For example, it was found that there were significant differences in the main aroma components and key aroma-active components between the green tea fixed by steaming and green tea fixed by pan-frying, and steamed green tea had a higher concentration of alkenes and esters than pan-fried green tea[11]. The roller-hot air fixation method favours the formation of the chestnut scent

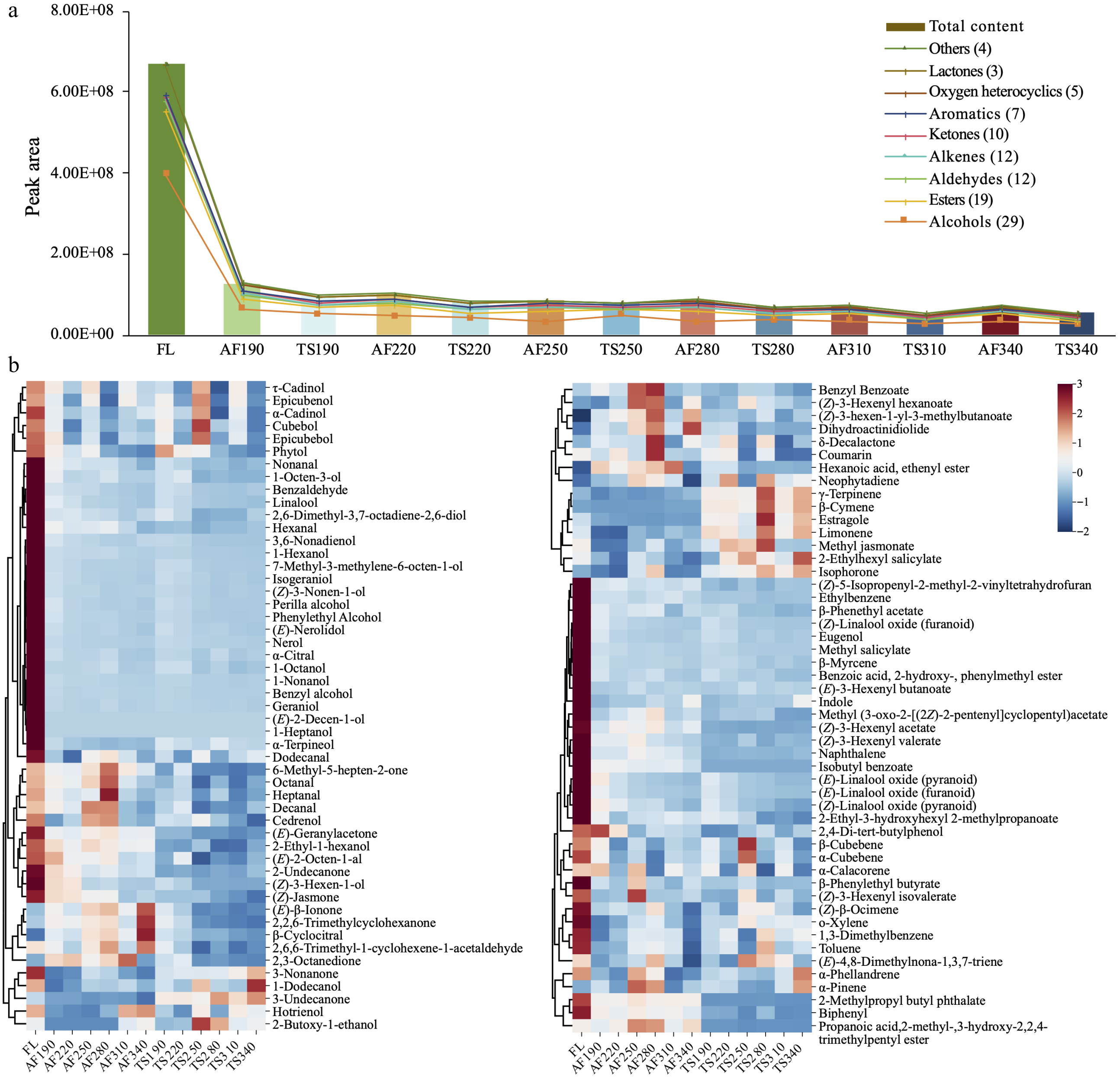

Fig. 1 Diagrams of volatile compounds in green tea samples with different fixation temperatures. (a) Diagrams of the total contents of volatile compounds in all green tea samples. (b) Hierarchical cluster analysis heatmap of volatile compounds in all green tea samples. FL: fresh tea leaves; AFs: after fixed tea leaves; TSs: Tea samples.

in green tea[24]. In comparison, the influence of fixation temperatures on green tea aroma compounds has yet to be thoroughly studied. Most previous research has focused on comparing aroma composition variations in processed green tea samples under different temperature conditions, often overlooking the essential effects of rolling and drying tea leaves for green tea production. Thus, to ascertain the impact of the fixation temperature on the formation of aroma quality of green tea, the following analyses in this study concentrated on comparing the differences in aroma composition between FL and AF samples that were immediately freeze-dried after fixation, aiming to keep variables to a minimum.

Changes in volatile compounds in tea samples with different fixation temperatures

As shown in Fig. 2a, the total content of alcohols and aldehydes showed a continuous decreasing trend with the increase in fixation temperatures. The alcohol content decreased significantly at lower fixation temperatures (190, 220 °C), but their content changes were not significant when the fixation temperature exceeded 250 °C ($p < 0.05$) (Fig. 2a). Fresh tea leaves fixed at 190 °C showed the highest content of aldehydes (Fig. 2b), while at fixation temperatures of 220, 310, and 340 °C, the content of aldehydes significantly decreased ($p < 0.05$). Esters were more abundant at low-temperature fixation and decreased at high-temperature fixation (Fig. 2c). The total content of esters reached the highest at the fixation temperature of 280 °C, while it significantly decreased when the fixation temperature reached 310 °C ($p < 0.05$).

Alkenes and aromatic compounds, in general, changed in response to rising fixation temperatures, namely initially increasing and then decreasing. Low-temperature fixation resulted in higher content levels of alkenes (Fig. 2d), and aromatic compounds (Fig. 2e), reaching their peak at 280 °C, before decreasing with further temperature increases. Furthermore, the fixation temperature

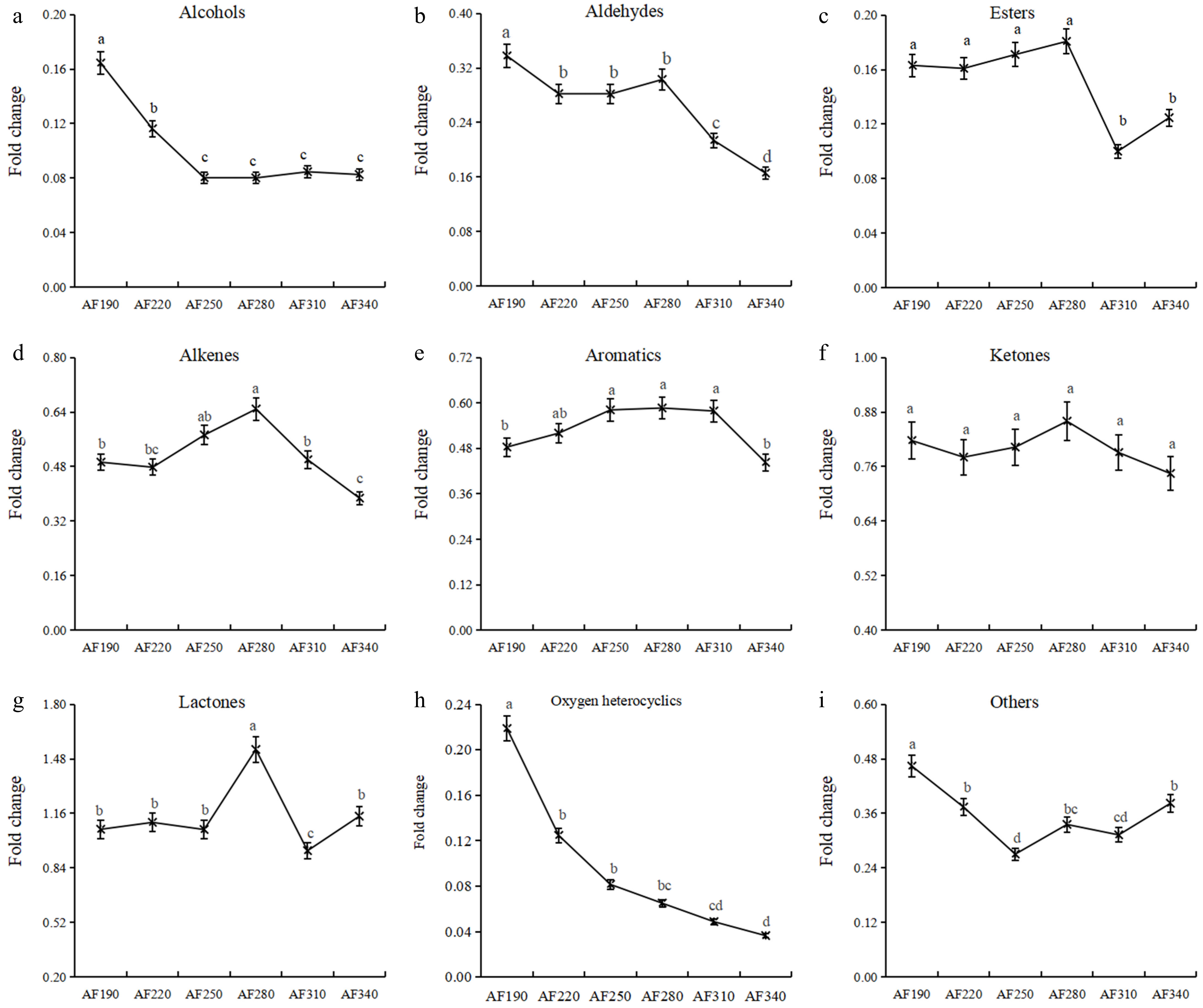

Fig. 2 Changes of volatile compounds in green tea fixed with different temperatures. AF190: tea leaves fixed at 190 °C; AF220: tea leaves fixed at 220 °C; AF250: tea leaves fixed at 250 °C; AF280: tea leaves fixed at 280 °C; AF310: tea leaves fixed at 310 °C; AF340: tea leaves fixed at 340 °C. Different lowercase letters indicate significant differences in the contents of compounds at $p < 0.05$. The temperature range is depicted by a single unit length along the coordinate axis.

significantly impacted fluctuations in the content of oxygen heterocyclic compounds ($p < 0.05$); when the fresh tea leaves were fixed at 220 °C, their content dropped sharply, and was reduced by 43% as compared to fixation at 190 °C. In contrast, the fixation temperature did not significantly affect the total content level of ketones (Fig. 2f). Notably, the lactones showed higher content levels only at 280 °C (Fig. 2b), while at other fixation temperatures, their levels were significantly lower and more similar to each other ($p < 0.05$).

Screening for differential volatile compounds in green teas with different fixation temperatures

To further elucidate the effect of fixation temperatures on the changes in volatile components during green tea processing, a PLS-DA analysis of the samples was conducted based on the relative contents of 102 volatile components using SIMCA 14.1 software. Supplementary Fig. S2a displays the PLS-DA score scatter plot, which showed satisfactory variance explanation ability and high predictive power ($R^2Y = 0.994$ and $Q^2 = 0.990$). The reliability of this model was assessed through 200 permutation tests, yielding fitting parameters of $R^2Y = 0.048$ and $Q^2 = -0.034$, with no overfitting (Supplementary Fig. S2b). FL was successfully discriminated from AFs in the PLS-DA plot, with a distribution in different quadrants. Additionally, as shown in Supplementary Fig. S2c, samples in the AF group could still be clearly discriminated (fitting parameters of $R^2Y = 0.982$ and $Q^2 = 0.944$) when analyzed separately.

In terms of spatial distribution, the flavour compositions of AF220 and AF190 exhibited proximity, while those of AF250 and AF280 showed similar patterns. Conversely, the aroma profile of AF310 was significantly different from the other treated samples. As seen in the PLS-DA validation model in Supplementary Fig. S2d, the y-axis intercept of the Q^2 regression straight line was less than 0. The analysis concluded that the PLS-DA model did not exhibit overfitting, confirming its increased reliability (fitting parameters with $R^2Y = 0.417$ and $Q^2 = -0.767$).

The VIP value of PLS-DA modeling combined with one-way ANOVA further screened 21 volatile compounds that were dramatically affected by fixation temperatures (VIP > 1, $p < 0.05$), including seven esters, five alcohols, two oxygen heterocyclics, two lactones,

Table 1. Analysis of key aroma components of green tea significantly impacted by different fixation temperatures.

No.	Compound	Boiling Point (℃)	Odor threshold (ng/mL)	Content (ng/mL)						Odor active value					
				AF190	AF220	AF250	AF280	AF310	AF340	AF190	AF220	AF250	AF280	AF310	AF340
1	Hexanal	129.6	5	25,890.85	19,811.58	20,684.53	23,509.97	15,314.54	7,401.65	5,178.17	3,962.32	4,136.91	4,701.99	3,062.91	1,480.33
2	(*Z*)-3-Hexen-1-ol	156.0–157.0	13	4,269.35	3,919.25	1,553.05	1,751.49	1,258.30	1,201.35	328.41	301.48	119.47	134.73	96.79	92.41
3	2-Butoxy-1-ethanol	168.4	880	0.00	0.00	0.00	0.00	21,575.90	27,864.85	0.00	0.00	0.00	0.00	24.52	31.66
4	2,3-Octanedione	230.0	–	76.33	80.69	65.47	76.73	98.57	34.60	–	–	–	–	–	–
5	(*Z*)-3-Hexenyl acetate	75.0–76.0	31	86.57	169.63	137.80	192.79	76.12	127.22	2.79	5.47	4.45	6.22	2.46	4.10
6	(*E*)-Linalool oxide (furanoid)	201.0–202.0	320	6,524.00	3,662.21	2,017.66	1,465.78	1,130.51	654.76	20.39	11.44	6.31	4.58	3.53	2.05
7	(*Z*)-Linalool oxide (furanoid)	188.0–192.0	320	12,185.93	5,925.80	3,710.26	2,560.51	1,455.47	909.18	38.08	18.52	11.59	8.00	4.55	2.84
8	Linalool	198.0	6	21,743.76	14,360.20	7,148.75	6,189.82	4,248.04	1,876.03	3,623.96	2,393.37	1,191.46	1,031.64	708.01	312.67
9	Nonanal	195.0	253	423.76	322.79	269.65	270.55	151.59	28.00	1.67	1.28	1.07	1.07	0.60	0.11
10	(*E*)-4,8-Dimethylnona-1,3,7-triene	81 (Press: 18 Torr)	–	56.69	65.15	72.32	100.38	68.70	41.25	–	–	–	–	–	–
11	Phenylethyl Alcohol	218.2	390	7,053.80	2,852.71	1,828.99	1,872.61	216.44	8.52	18.09	7.31	4.69	4.80	0.55	0.02
12	Methyl salicylate	220.0–224.0	40	612.52	452.55	273.70	297.19	164.79	127.66	15.31	11.31	6.84	7.43	4.12	3.19
13	(*Z*)-3-Hexenyl isovalerate	98.0	110	10.64	12.95	71.27	13.59	13.96	18.91	0.10	0.12	0.65	0.12	0.13	0.17
14	Hexanoic acid, ethenyl ester	168.6	–	123.48	104.38	117.74	128.01	150.15	49.92	–	–	–	–	–	–
15	Geraniol	230.0	7.5	777.55	374.60	294.32	834.80	50.34	334.08	103.67	49.95	39.24	111.31	6.71	44.54
16	3-Hydroxy-2,2,4-trimethylpentyl isobutyrate	255.0–260.0	–	103.81	127.55	205.91	198.24	108.56	153.71	–	–	–	–	–	–
17	(*Z*)-3-Hexenyl hexanoate	115.0	16	56.61	72.30	113.90	112.33	61.05	89.18	3.54	4.52	7.12	7.02	3.82	5.57
18	(*Z*)-3-hexen-1-yl-3-methylbutanoate	60.0	–	27.36	33.16	38.42	51.45	24.12	44.00	–	–	–	–	–	–
19	Coumarin	301.7	34	91.96	107.95	91.43	138.29	88.31	86.72	2.70	3.17	2.69	4.07	2.60	2.55
20	(*E*)-β-Ionone	271.0	0.007	46.35	43.19	49.04	50.49	45.17	55.23	6,621.52	6,169.83	7,006.11	7,212.63	6,452.43	7,889.93
21	δ-Decalactone	117.0–120.0	1	2,888.92	2,753.56	2,718.35	4,074.61	2,257.06	3,077.52	2,888.92	2,753.56	2,718.35	4,074.61	2,257.06	3,077.52

The odor thresholds in water were obtained from previous studies[6,12,13]. The boiling points of volatile compounds were cited from www.ncbi.nlm.nih.gov.

and one aldehyde, as shown in Table 1. The more diverse aroma compounds included 2-butoxy-1-ethanol, linalool, (*Z*)-3-hexenyl acetate, geraniol, (*Z*)-3-hexenyl isovalerate, (*Z*)-3-hexenyl hexanoate, vinyl hexanoate, phenylethyl alcohol, 2,3-octanedione, (*Z*)-linalool oxide (furanoid), and methyl salicylate. Subsequently, the 21 significantly distinct aroma compounds were quantified by using the improved standard addition method to establish standard curves, in which aroma standards corresponding to these components were employed (Table 1 & Supplementary Table S5). The quantitative approach adopted in this study is in accordance with the methodology previously established by the present research group. The methodology validation conducted in our previous study revealed that the limits of detection (LOD) and limits of quantitation (LOQ) for most volatile compounds were below nanogram levels[13,25]. Furthermore, the low relative standard deviation (RSD) values observed for most volatiles (< 10%) and the satisfactory recovery rates ranging from 80% to 120% indicate excellent reproducibility and accuracy[16]. In this study, the RSD values for each target volatile compound were also found to be below 10%, indicating the high reliability of the established quantification method.

Among them, 2-butoxy-1-ethanol was only detected in green tea samples fixed at high temperatures (310 and 340 °C). (*Z*)-3-Hexenyl acetate and geraniol had the highest content at 280 °C (192.79 and 834.80 ng/mL, respectively), followed by the second highest content at 190 °C (169.63 and 777.55 ng/mL, respectively). In contrast, (*Z*)-3-hexenyl isovalerate (71.27 ng/mL) was at a much higher content at 250 °C than that at other temperature treatments. The content of (*Z*)-3-hexenyl hexanoate was only 3.54 ng/mL at 340 °C, and its content varied up to 32-fold at different fixation temperatures. Moreover, the content of vinyl hexanoate decreased sharply at 340 °C, dwindling from 150 ng/mL (AF310) to 49.92 ng/mL (AF340). Besides that, the content of phenyl ethanol was more dramatically affected by low and high-temperature fixation. For example, when 220 °C was used, its content decreased by 59.6% compared to 190 °C fixation; and when 340 °C was used, its content decreased by 96.1% compared to 310 °C fixation, indicating that the temperature conditions have a significant influence on the content of phenyl ethanol ($p < 0.05$).

Among these significantly different metabolites, a few compounds have lower boiling points, ranging from 110 to 250 °C. These compounds include (*Z*)-3-hexenyl acetate (75.0–76.0 °C), (*Z*)-3-hexenyl isovalerate (98.0 °C), and (*Z*)-3-hexen-1-yl-3-methylbutanoate (60.0 °C). The low retention of these low boiling point esters in the fixed tea leaves may be due to their high volatilization following the high-temperature treatment. Furthermore, aroma compounds with boiling points frequently around 200 °C, such as (*Z*)-linalool oxide (furanoid) (188.0–192.0 °C), linalool (198.0 °C), nonanal (195.0 °C), and phenylethyl alcohol (218.2 °C), may have content variations that are closely related to boiling point. We found that the content of these compounds radically decreased at 220 °C, as opposed to 190 °C, by 51.4%, 34.0%, 23.8%, and 59.6%, respectively, which may be related to the strong release of the compounds upon reaching their boiling point[26]. It still needs to be clarified how the physical characteristics of other differential metabolites change regarding their content changes. During the fixation process of green tea, the formation of aroma mainly stems from thermal reactions, lipid degradation, and glycoside hydrolysis, among other pathways[27]. In addition, most volatile components are transformed through a series of chemical reactions involving aroma precursors (nonvolatile components) during tea processing, and a single volatile compound may originate from multiple precursors[1]. It was found that roasting at different temperatures has a significant influence on the aroma of oolong tea, with higher temperatures resulting in increased levels of pyrazine compounds[20]. Furthermore, comprehensive studies on the transformations of carotenoid and glycoside precursors during tea processing have indicated that varying degrees of transformation, as well as distinct products, may be generated under different temperature conditions[5,15]. Based on the aforesaid literature and the findings of this study, it was conjectured that their biological reactions and the variations in their metabolic pathways under various fixation temperatures might offer a more explicit explanation for this phenomenon.

Analysis of key differential aroma compounds impacted by fixation temperatures

To evaluate the characteristic aroma components of tea, OAV analysis is commonly utilized, wherein compounds with OAV ≥ 1 are typically identified as key aroma compounds[28]. Based on the absolute contents of the 21 significantly distinct aroma compounds, their respective OAVs were calculated, as outlined in Table 1. Among them, the OAVs of 12 volatile components in all the samples processed at different fixation temperatures were greater than 1. In descending order of their OAVs, these included (*E*)-β-ionone (6,169.83–7,889.93), hexanal (1,480.33–5,178.17), δ-decalactone (2,257.06–4,074.61), linalool (312.67–3,623.96), (*Z*)-3-hexen-1-ol (92.41–328.41), and geraniol (6.71–111.31). Moreover, 2-butoxy-1-ethanol exhibited higher OAV values during high-temperature fixation, with OAV = 24.52 and OAV = 31.66 in AF310 and AF340, respectively. Notably, linalool and phenylethyl alcohol were identified as key aroma compounds only at medium and low fixation temperatures. However, they ceased to be considered key aroma compounds when the fixation temperature reached 310 °C. Linalool and phenylethyl alcohol represent floral aroma and may contribute to the floral attribute of green tea aroma.

Each of these compounds has a unique odour profile, and the pattern of changes in their contents differs greatly, as shown in Fig. 3. The fixation temperature had a consistent effect on (*Z*)-3-hexenol, (*E*)-linalool oxide (furanoid), (*Z*)-linalool oxide (furanoid), methyl salicylate, and linalool, with the highest content at 190 °C, and decreasing with increasing temperature. There was a significant intergroup difference among almost all tea samples with various fixation temperatures ($p < 0.05$). Linalool can be formed from geranyl pyrophosphate precursors (Fig. 4a) under the action of linalool synthase[29], while linalool oxides (furanoid) can be generated either by the oxidation of linalool itself or by hydrolysis of the corresponding glycosidic precursors[24,30]. These aroma compounds all present floral and fruity odour characteristics, have relatively high contents in green tea, and are essential aroma contributors in many teas, such as Keemun black tea[31] and white tea[32]. The exogenous addition of β-glucosidase improved the aroma quality of instant white tea, presumably by increasing the concentration of furan linalool oxides[33]. Therefore, regulating the changes in their content by controlling the fixation temperature may contribute to the improvement of the aroma quality of green tea.

Hexanal and geraniol were detected in higher concentrations in AF280 and AF190. Hexanal tended to be formed during low-temperature fixation and decreased significantly with rising temperatures. It increased greatly at 280 °C and continued to decline with subsequent temperature increases ($p < 0.05$). The oxidative degradation of linoleic acid releases hexanal (Fig. 4b), which can be converted into the corresponding alcohols through isomerization and reduction during the heating process[1]. Hexanal has a low boiling point, and its volatilization due to heat and the isomerization reaction occurring during the fixation process may influence changes in content with variations in greening temperature[34]. The fixation temperature significantly impacted the geraniol content.

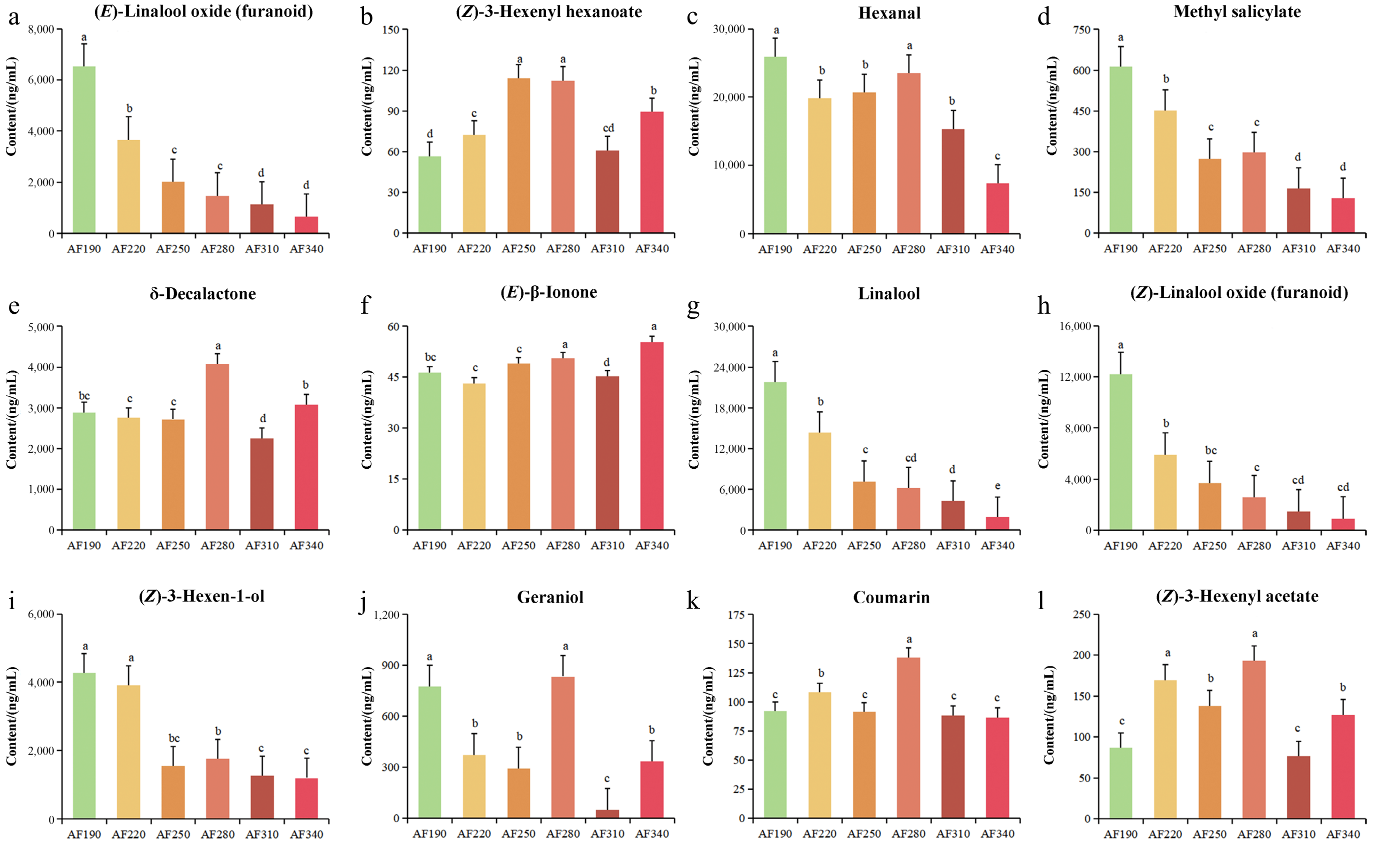

Fig. 3 Changes in the content of key aroma components with various fixation temperatures. AF190: tea leaves fixed at 190 °C; AF220: tea leaves fixed at 220 °C; AF250: tea leaves fixed at 250 °C; AF280: tea leaves fixed at 280 °C; AF310: tea leaves fixed at 310 °C; AF340: tea leaves fixed at 340 °C. Different lowercase letters indicate significant differences in the contents of volatile compounds in green tea samples at $p < 0.05$.

Compared with AF280, the geraniol content in AF310 decreased significantly (50.34 ng/mL), representing a reduction of approximately 94%. It is noteworthy that linalool and geraniol share the same aroma precursor substances; both are floral compounds with similar chemical structures and properties. However, their content change patterns differ significantly. Previous studies have shown that linalool can undergo thermal cleavage to form geraniol[35]. Therefore, it is hypothesized that the varying trends in the content of these two compounds, influenced by temperature, are associated with alterations in the principal non-enzymatic transformation pathways under corresponding manufacturing conditions (Fig. 4b).

As the fixation temperature reached 340 °C (AF340), the content of (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenyl hexanoate increased dramatically, distinct from most key aroma compounds. It is presumed that thermal oxidation and esterification processes of fatty acids occur during the fixation process[36], and changes in these heat-driven processes may influence the accumulation of (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenyl hexanoate. Notably, (*E*)-β-ionone is a vital active component in tea. Although its quantity in AF tea samples is low (43.19–55.23 ng/mL), it considerably impacts the aroma quality of green tea due to its shallow odour threshold (OT = 0.007 ng/mL). (*E*)-β-ionone presents the aroma characteristics with floral and rosy notes, mainly originating from the oxidative degradation of carotenoids during processing (Fig. 4c)[5]. Overall, the content level of (*E*)-β-ionone increased with high-temperature fixation and decreased with low-temperature fixation. In addition, δ-decalactone is a major aroma-active component in peach fruits[37], adding to the odour profile of peach-like and fruity notes. However, the method of synthesis of lactone components in tea remains unclear. Methyl salicylate is generally formed via the shikimic acid pathway. Its concentration varies with the fixation temperature, which may be influenced by salicylic acid carboxymethyltransferases[30] and heat-induced esterification reactions (Fig. 4d).

Analysis of fixation temperatures impacting aroma types of green tea

The primary objectives of fixation are to effectively eliminate enzyme activity in fresh leaves and to halt the enzymatic oxidation of polyphenolic compounds, thereby achieving the characteristic color, aroma, and flavor associated with green tea. This process predominantly relies on elevated temperatures, during which it is essential for the leaf temperature to rapidly exceed 80 °C in order to effectively inhibit enzyme activity. Achieving this requires a specific level of moisture content in the fresh leaves. Long-term practice and research have demonstrated that optimal quality is attained when the leaf moisture content ranges between 68% and 70%. Following fixation, there is a significant reduction in the moisture content of tea leaves (below 60%), accompanied by varying degrees of initial biochemical (enzymatic) and subsequent thermochemical (non-enzymatic) transformations of volatiles and their precursors, contingent upon the applied temperature. Consequently, the fixation temperature has a profound impact on the overall aroma quality of green tea[35]. However, it is important to note that the findings of this study reveal no significant difference in leaf moisture content when measured at fixation temperatures of 220 °C (56.20%) and 250 °C (56.50%), which appears to be inconsistent with theoretical expectations. This phenomenon can be attributed to the relatively

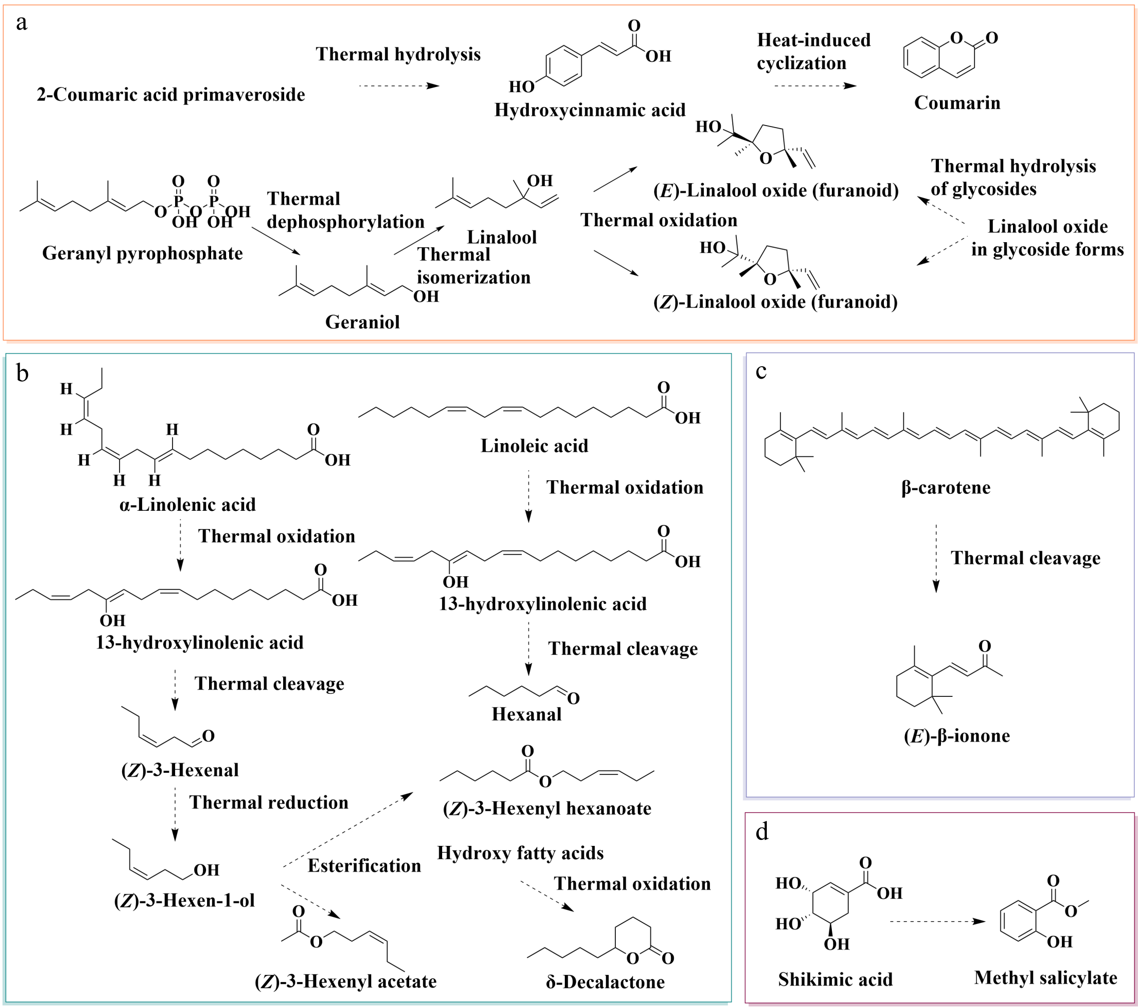

Fig. 4 Main synthetic pathways of key aroma components impacted by various fixation temperatures. (a) Aromatic/terpenoid-related volatiles; (b) Lipid-related volatiles; (c) Carotenoid-related volatiles; (d) Shikimate-related volatiles.

mild conditions present during the fixation process under two specific scenarios, in which the temperature of the tea leaves typically reaches just the critical threshold of 80 °C. Furthermore, the temperature differential within the fixation machine is only 30°C; consequently, there is no significant variation in the actual temperatures experienced by the tea leaves. Additionally, the fixation duration was just 3 min, during which uneven mixing could result in localized areas of higher moisture content within the leaves. Conversely, when the temperature exceeds 250 °C, the leaf temperature reaches approximately 90 °C. At this juncture, the leaves become sufficiently desiccated, leading to a reduced moisture content in comparison to temperatures below this threshold. Moreover, the moisture analyzer requires 20 min to complete a measurement, and if the moisture content of the leaves is relatively high post-fixation, there could be moisture reabsorption during this period.

As shown in Supplementary Table S2, tea samples prepared from tea leaves of one bud and two leaves of Longjing 43 at different fixation temperatures show good aroma quality. The sensory aroma wheel of these green tea samples were also plotted (Fig. 5). According to the sensory evaluation results shown in Supplementary Table S2, the overall sensory quality was better when the fixation was carried out at 190, 250, and 280 °C, with the characteristics of 'tight, slightly curly, deep green' in appearance, 'bright and clear, green' in tea infusion, 'clean and high' in aroma, as well as 'mellow, umami'

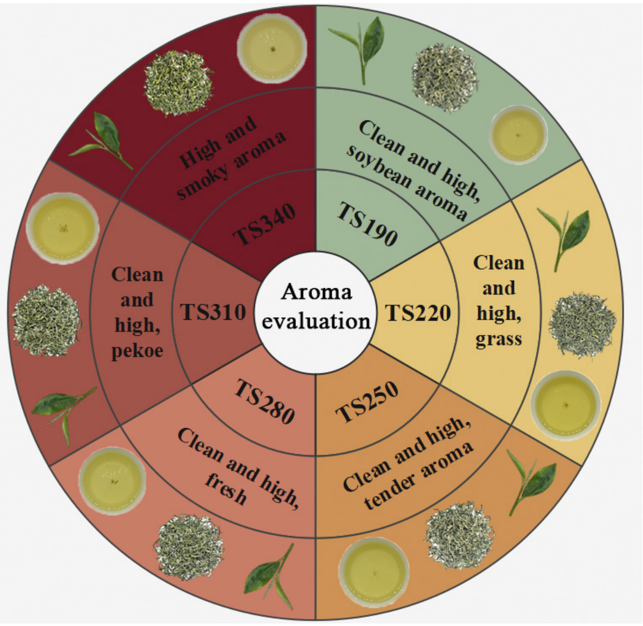

Fig. 5 Aroma characteristics of green tea samples with different fixation temperatures.

in taste, which is a typical characteristic of good green tea flavor quality.

It can be found that the rest of the green tea samples (AFs) subjected to fixation temperatures ranging from 190 to 310 °C, except for AF340, presented a 'clean and high' aroma upon sensory evaluation, which could be abbreviated as 'fresh' aroma. Additionally, some samples also displayed unique aroma characteristics, such as the green tea sample fixed at 190 °C with a bean aroma, the green tea fixed at 250 °C with a tender aroma, and the green tea fixed at 310 °C with a pekoe aroma. These results indicate that tea aroma quality is not a single type but rather a combination of different aromatic profiles.

Meanwhile, 190 °C was a relatively low temperature and is rarely used in actual tea production. At this temperature, due to the limited time for rapid leaf heating, it is possible that not all relevant enzymes (e.g., polyphenol oxidase, peroxidase) in fresh leaves are completely inactivated throughout the entire fixation process. Particularly in the initial stages of fixation or when the internal leaf temperature has not yet fully risen, these enzymatic activities may persist and influence the formation and transformation of certain aroma compounds. Therefore, the aroma and overall sensory quality of the samples were enhanced when fixation temperatures of 250 and 280 °C were employed. It was observed that controlling the fixation temperature within an acceptable range allowed for the development of the fresh aroma in green tea. However, an excessive fixation temperature may lead to the production of a smoky aroma, which adversely affects the tea aroma quality.

Conclusions

Fixation is a vital stage that influences the development of the quality of the green tea aroma, and fixation temperature is the critical process parameter that has considerable effects on the composition and content of green tea volatiles and therefore determines its aroma quality. In this study, it was found that the total content of volatile components showed a more stable decreasing trend with increasing fixation temperature, and also identified 12 key differential aroma components that were significantly affected by the fixation temperature. By adjusting the quantity of these aroma components, the aroma quality of green tea might be improved. Moreover, the fixation temperature of 280 °C might be a suitable reference for parameter setting that can contribute to the formation of better aroma quality in green tea. In particular, it was found that fixation at this temperature was favourable to the 'fresh' aroma quality formation in green tea. The results obtained from this study have enriched our understanding of the effects of various fixation temperatures on the volatiles of green teas and the formation mechanism of their key flavour components. Further studies are needed to explore the modern processing technologies for targeted regulation of green tea aroma types.

Ethical statement

Ethical permission to conduct a human sensory study on tea flavour quality is not a requirement of our institution and country.

The authors ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

The panelists involved in the tea sensory evaluation provided informed consent by affirming the following statement before participating in the survey: 'I have carefully read the information and fully understand my rights as a potential subject in a research experiment. I am aware that my responses are confidential, and I agree to participate in this study'. They had the option to withdraw from the survey at any time without providing a reason. The applied tea samples were safe for consumption.

Author contributions

The authors confirm contributions to the paper as follows: methodology: You Q, Lin Z, Zhu Y, Lv H; investigation: You Q, Yang Y, Yang G, Qiu Z, Lv H; software: You Q, Yang Y, Yang G; formal analysis, visualization: Zhou M, Guo L; sample preparation: Yang Y, Yang G; writing - original draft: You Q; Writing - review & editing: Zhu Y, Lv H; project administration: Lin Z, Lv H. All authors reviewed the results and approved the final version of the manuscript.

Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Conflict of interest

The authors declare that they have no conflict of interest.

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