



## A comprehensive metabolomics analysis of volatile and non-volatile compounds in matcha processed from different tea varieties

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### ABSTRACT

Tea varieties play a crucial role on the quality formation of matcha. This research aimed to examine the impact of four specific tea plant varieties (Okumidori, Longjing 43, Zhongcha108, and E'Cha 1) on various aspects of matcha, including sensory evaluation, major components, color quality, volatile and non-volatile metabolomic profiles. The findings revealed that the levels of tea polyphenols, ester catechins, nonester catechins, and amino acids varied among these four varieties. Notably, 177 significant different metabolites, such as phenolic acids, flavonoids, tannins, alkaloids were identified among 1383 non-volatile compounds. In addition, 97 key aromatic compounds were identified based on their odor activity value exceeding 1. Aldehydes, heterocyclic compounds, and ketones were closely associated with the formation of volatile metabolites. Overall, this study enhances our understanding of how different tea plant varieties impact the quality of matcha, and can provide valuable guidance for improving matcha varieties in a favorable direction.

### 1. Introduction

Matcha refers to a finely powdered tea product derived from the fresh leaves of the tea tree, harvested and ground after the covering and shading treatment (Rezaeian & Zimmermann, 2022). These leaves are steamed or heated with hot air and then dried, serving as the raw materials for further processing through grinding technology (Kaneko et al., 2006; Dietz, Dekker, & Piqueras-Fiszman, 2017). While matcha's history dates back to the Tang Dynasty in China, its refinement and widespread popularity began in Japan during the 12th century (Cheadle & Kilby, 2016). It has since become an integral part of Japanese tea ceremony traditions, and is now extensively used in various domains, including food, beverages, health products, and daily goods (Kurauchi et al., 2019; Kochman, Jakubczyk, Antoniewicz, Mruk, & Janda, 2020). In recent times, the consumption of matcha has witnessed a gradual surge due to its delectable taste, pleasurable flavour and potential health benefits. (Townsend, Maitin, Chesnut, & Vatter, 2011; Devkota et al., 2021; Sokary, Al-Asmakh, Zakaria, & Bawadi, 2023).

Matcha is a type of green tea characterized by its vibrant green color,

fragrant aroma and roast, vegetable-like taste sensations (Phuah et al., 2023). Unlike traditional green tea, matcha minimizes excessive kneading or shaping processes to enhance the bright green color (Qin et al., 2020). The distinctive shading process utilized during cultivation and straightforward processing methods make matcha quality heavily reliant on the inherent biochemical characteristics of the tea variety (Mao et al., 2020). Premium matcha raw materials are characterized by high levels of amino acids and chlorophyll, along with low tea polyphenol and caffeine contents (Devkota et al., 2021; Jiang et al., 2023). Chlorophyll contributes to the appealing green color of green tea, which can influence consumer preferences (Suzuki & Shioi, 2003). A study reported high levels of chlorophyll-a (2733.33 µg/g) and chlorophyll-b (1467.50 µg/g) contents in matcha (Koláčková et al., 2020). Tea polyphenols (TPs) are related to the astringency of tea, with catechins, myricetin-3-O-galactoside, quercetin-3-O-rutinoide (rutin), and tannins being major astringent compounds (Zhang et al., 2020). Caffeine, on the other hand, contributes to the bitterness of green tea infusion (Zhang et al., 2015). The presence of free amino acids (FAAs) in tea, including theanine, glutamate, threonine, and aspartate, synergistically

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contributes to the umami flavor of green tea (Jiang et al., 2019). In all, these components contribute significantly to the flavor and interior quality of matcha.

In Japan, matcha is primarily sourced from varieties within the 'Uji', 'Chaoru', and 'Yabunhok' series (Yin et al., 2020). In China, a previous study highlighted that among eight cultivars, 'Longjing 43' exhibited the best overall performance in terms of appearance, seaweed-like fragrance, and umami taste in tencha (Mao, Wang, Yin, & Xu, 2020). Another comprehensive evaluation and cluster analysis of 11 quality indexes from 36 tea cultivars indicated that matcha produced from 'Zhongcha 102', 'Zhongcha 108', 'Fuding Dabaicha' had superior color and taste quality (Yuan et al., 2023). However, our understanding of metabolite and aroma differences in matcha from different tea varieties, especially between Chinese and Japanese varieties, remains limited. In the present study, we focused on four tea varieties (including three Chinese and one Japanese variety), which underwent three weeks of shade treatment before being processed into matcha using identical techniques. The objective of this research was to assess how different varieties influence matcha quality in terms of color, taste, and aroma. This evaluation was conducted using methods such as colorimetry, spectrophotometry, UPLC MS/MS, and GC-MS. Our study aims to provide valuable insights into the improvement of matcha quality and the breeding of tea varieties suitable for producing high-quality matcha products.

## 2. Materials and methods

### 2.1. Chemical reagents

Folinphenol, ninhydrin and methanol (AR grade) were purchased from China national pharmaceutical group, Shanghai Chemical Reagent Co., Ltd. (-)-Epicatechin (EC), (-)-epigallocatechin (EGC), catechin (C), (-)-gallocatechin (GC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), catechin gallate (CG) gallocatechin gallate (GCG), standards (purity  $\geq 95\%$ ) were supplied by Sigma-Aldrich (MO, USA). HPLC-grade acetic acid, methanol and acetonitrile were obtained from Aladdin Biological (Shanghai, China).

### 2.2. Preparation of matcha samples

The experiment was conducted between April and May 2022. Four different varieties, namely, Okumidori (Oku), Longjing 43 (LJ43), Zhongcha 108 (ZC108), and E'cha 1 (EC1), were cultivated in Hekou Town, Xiaogan city, Hubei Province, where they had been growing for a period of six years. The designated sun-shaded experimental plots covered an approximate area of 40 m<sup>2</sup> each, and these plots were randomly replicated three times. To construct the shading conditions, a shading net with around 90 % shade intensity was positioned approximately 15 cm<sup>2</sup> above the uppermost leaves of the tea plants. This shading was initiated when the new shoots had developed to a stage of one bud and two leaves. The shading process extended over a period of 21 days. Following the shading period, the experiment involved the collection of new shoots consisting of one bud and five leaves. These collected shoots were then subjected to a series of processing steps in accordance with matcha production technology. This processing sequence included steam detoxification, wind-based separation, drying, color separation, and ultimately grinding. The resulting matcha powder was stored at  $-20\text{ }^{\circ}\text{C}$  until further use.

### 2.3. Characterization of matcha samples

The sensory evaluation of each matcha sample was assessed by three trained panelists: appearance of tea powder (30 %), brew color (20 %), taste (30 %), and aroma (20 %), according to the "Technological specification of matcha sensory evaluation (DB33/T 2279-2020)" established by market supervision and administration of Zhejiang province,

China. All participants gave informed consent via the statement "I am aware that my responses are confidential, and I agree to participate in this survey" before the sensory evaluation. The rights and privacy of all participants were utilized during the execution of the research. They were able to withdraw from the survey at any time without giving a reason. The products tested were safe for consumption.

The content of free amino acids (FAAs) was quantified using the hydrated ninhydrin colorimetric method as per the China national institute of standardization CNIS GB/T 8314-2013 standard for determining FFA content in tea. The amino acid is co-heated with ninhydrin at pH 8.0 to form a purple complex with a maximum absorption wavelength at 570 nm. The measurement of tea polyphenols (TPs) content was conducted by the colorimetric method with the Folin-Ciocalteu reagent described by CNIS GB/T 8313-2018 for determining TPs and catechins content in tea. The tea polyphenols were extracted by 70 % methanol aqueous solution in 70 °C water bath, and then the -OH group in tea polyphenols was oxidized by Folin-Ciocalteu reagent, the maximum absorption wavelength was 765 nm, and the tea polyphenols were quantified by gallic acid as calibration standard. The analysis of caffeine and catechin components was performed using the HPLC method as described previously (Huang et al., 2022). The experiments of each group were repeated three times.

The levels of chlorophyll and carotenoid were extracted using 95 % ethanol and then analyzed using a spectrophotometer at specific wavelengths (665 nm, 649 nm and 470 nm). For the infusion, 3 g of matcha powder was brewed with 150 mL of boiling pure water for 5 min. The resulting supernate was filtered, and its color quality was measured using a spectrophotometer (CM-5 Konica Minolta, Inc., Tokyo, Japan) equipped with a special colorimetric dish (light source D65, angle 4°). The L, a, and b values were used to represent the luminance, red-green degree, and yellow-blue degree, respectively. Each set of tests and analyses was conducted in triplicate for accurate and reliable results.

### 2.4. UPLC MS/MS analysis of non-volatile metabolites in different matcha varieties

#### 2.4.1. Extraction of non-volatiles

Matcha powder (0.1 g) was added with 1.2 mL methanol solution (70 %) and subjected to vortexing for 30 s at 30-minute intervals, totaling 6 cycles. Subsequently, all samples were incubated overnight at 4 °C. After centrifugation (12,000 rpm, 10 min), the extracts underwent filtration prior to UPLC-MS/MS analysis.

#### 2.4.2. UPLC conditions and ESI-MS/MS

The extracted samples were subjected to analysis using an UPLC-ESI-MS/MS system comprising a Shimadzu Nexera X2 UPLC (Kyoto, Japan) and an Applied Biosystems 4500 Q TRAP Mass Spectrometer (Thermo-Fisher-Scientific, Waltham, USA), following the experimental parameters established in a prior study (Shi et al., 2022). The chromatographic conditions utilized were as follows: column, Agilent SB-C18 (1.8  $\mu\text{m}$ , 2.1 mm $\times$ 100 mm); column temperature, 40 °C; mobile phase, solvent A (0.1 % formic acid) and solvent B (acetonitrile with 0.1 % formic acid); flow rate, 0.35 mL $\cdot\text{min}^{-1}$ ; injection volume, 4  $\mu\text{L}$ . Gradient program for sample measurements was as follows: linear gradient to 5 % A, 95 % B for 9 min; 5 % A, 95 % B for 1 min; 95 % A, 5.0 % B within 11.10 min and hold for 2.9 min. The effluent was alternatively directed to an ESI-Q-TRAP-MS system.

Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired using an AB4500 Q TRAP UPLC/MS/MS system, equipped with an ESI Turbo Ion-Spray interface, operated in both positive and negative ion modes and managed through Analyst 1.6.3 software (AB Sciex). The operating parameters for the ESI source were adopted from the previously described method (Shi et al., 2022): turbo spray; ion source, ion spray voltage (IS) 5500 V and  $-4500\text{ V}$  (positive and negative ion modes, respectively); source temperature 550 °C; ion source gas I and II

(GSI and GSII, respectively); curtain gas (CUR) of 25, 50 and 60 psi; and high collision-activated dissociation (CAD). For instrument calibration and tuning, 10 and 100  $\mu\text{mol/L}$  polypropylene glycol solutions were employed in QQQ and LIT modes, respectively. QQQ scans were carried out as Multiple Reaction Monitoring (MRM) experiments, with the collision gas (nitrogen) maintained at a medium level. Optimization of Declustering Potential (DP) and Collision Energy (CE) was performed for each individual MRM transition. A specific array of MRM transitions was continuously monitored for each designated timeframe, aligned with the elution of metabolites within that timeframe.

#### 2.4.3. Quantitative and qualitative analyses of non-volatiles

For qualitative analysis, the retention time, fragmentation pattern, and precise  $m/z$  value of the metabolites were cross-referenced against the standards within the self-compiled metabolite database (MetWare, Wuhan, China) for accurate metabolite identification. The quantitative analysis was carried out according to the signal intensity of metabolites derived from distinctive ions. Repeated MS signals, such as isotope,  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{NH}_4^+$  adduct ions, were excluded. Chromatographic peaks were meticulously integrated and adjusted using MultiQuant software. The area under each chromatographic peak was indicative of the relative content of the corresponding substance.

### 2.5. Analysis of volatile metabolites in different matcha varieties by GC-MS

#### 2.5.1. Sample pretreatment

The matcha powder (0.5 g) was promptly transferred to a 20 mL head-space vial (Agilent, Palo Alto, CA, USA), followed by the addition of 2 mL of saturated sodium chloride solution and 10  $\mu\text{L}$  of 3-hexanone (50  $\mu\text{g/mL}$ ) as an internal standard. Automated solid-phase micro-extraction (SPME) was employed for sample extraction, while detection was carried out using gas chromatography-mass spectrometry (GC-MS) utilizing a Model 7890B GC system and a 7000 d MS system, both from Agilent. During the SPME analysis, each vial was positioned at a temperature of 60  $^\circ\text{C}$  for 5 min, following which a 120  $\mu\text{m}$  DVB/CWR/PDMS fiber (Agilent, CA, USA) was exposed to the headspace of the sample at 60  $^\circ\text{C}$  for 15 min. Replicating the process, each sample underwent this procedure four times.

#### 2.5.1. GC-MS analysis

Agilent 8890-7000D (Agilent, CA, USA) was used to detect volatile organic compounds (VOCs). The capillary column included DB-5MS (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ , 5 % phenyl-polymethylsiloxane) with high-purity helium (99.999 %) and linear velocity (1.2 mL/min). The temperatures of injector and detector were set at 250  $^\circ\text{C}$  and 280  $^\circ\text{C}$ , respectively. The oven was initially set to 40  $^\circ\text{C}$  for 3.5 min, then gradually raised at a rate of 10  $^\circ\text{C}$  per minute to reach 100  $^\circ\text{C}$ . Subsequently, the temperature was increased at a rate of 7  $^\circ\text{C}$  per minute until it reached 180  $^\circ\text{C}$ . To achieve a final temperature of 280  $^\circ\text{C}$ , the oven temperature was ramped up at a rapid rate of 25  $^\circ\text{C}$  per minute. This peak temperature was maintained for 5 min. The mass spectra were acquired using electron impact (EI) ionization mode with an energy of 70 eV. The temperatures of the quadrupole mass detector, ion source, and transfer line were set to 150  $^\circ\text{C}$ , 230  $^\circ\text{C}$ , and 280  $^\circ\text{C}$ , respectively. The identification and quantification analysis were performed using the selected ion monitoring (SIM) mode.

The VOCs were characterized by matching their mass spectra against the data system library (MWGC) and linear retention index. For each compound, one quantitative ion and two or three qualitative ions were carefully chosen. Each group of ions was sequentially detected during their respective time segments based on their elution order. To confirm the identification, the observed retention time was compared with the standard reference, and the presence of all selected ions in the sample spectrum after background subtraction was verified (Yuan et al., 2023; Zhang et al., 2022). For improved quantitative accuracy, specific

quantitative ions were chosen for integration and calibration. The internal standard, 3-hexanone (10  $\mu\text{L}$ , 50  $\mu\text{g/mL}$ ), was employed, and the relative content of each VOC was computed as follows:

$$C_j = \frac{A_j \times m_i}{A_i \times m_s}$$

where  $C_j$  denotes the mass concentration of each compound ( $\mu\text{g/kg}$ );  $A_j$  and  $A_i$  represent the chromatographic peak areas of each compound and internal standard, respectively;  $m_i$  and  $m_s$  indicate the mass of the internal standard ( $\mu\text{g}$ ) and sample powder (kg), respectively.

The OAV of the odor-active compounds was determined by comparing the concentrations of these compounds in water to their respective odor thresholds. The OAV value was computed using the following equation:

$$OAV_j = \frac{C_j}{OT_j}$$

where  $C_j$  ( $\mu\text{g/kg}$ ) represents the concentrations of VOCs, and  $OT_j$  ( $\mu\text{g/kg}$ ) denotes the aroma threshold of VOCs in water (Yue et al., 2023).

### 2.6. Statistical analysis

For the identification of differentially accumulated metabolites (DAMs) between the two cultivars, selection criteria were established with variables in project importance (VIP) scores  $\geq 1$  and fold change  $\geq 2$  or  $\leq 0.5$ . VIP values were extracted from the partial least squares discriminant analysis (PLS-DA) results, which also contained score plots and permutation plots, and were generated using the R package MetaboAnalystR. Heatmap analysis and principal component analysis (PCA) were also conducted using the R package MetaboAnalystR. Venn diagrams were generated through the online platform (<https://cloud.metware.cn/>) to visualize common and unique elements. Annotation and classification of DAM functions were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. GraphPad Prism 9 was employed for graphical representation and one-way ANOVA analysis to indicate data significance ( $p < 0.05$ ). All values are presented as mean  $\pm$  standard deviation.

## 3. Results and discussion

### 3.1. Sensory evaluation in different matcha varieties

To manufacturers, dealers, and consumers, information regarding the color, taste and aroma quality of matcha plays a crucial role in influencing the production, selection or purchase of the products to cater to their specific preferences and demands (Guo et al., 2021). Fig. 1 and Table S1 present the visual appearance of matcha powder and liquid, along with the results of color sensory evaluations for four different matcha varieties. Among these, the Oku variety demonstrated the highest values for appearance and infusion color, characterized by vibrant and glossy shades of green. With regards to taste, LJ43 obtained the highest score (95.30), followed closely by Oku (93.3), ZC108 (91.17), and EC1 (85.8). All four varieties exhibited the typical seaweed fragrance that is characteristic of matcha. Notably, Oku displayed a superior characteristic aroma, described as clean, fresh, and possessing a strong and persistent seaweed fragrance. LJ43 also exhibited a favorable aroma profile, aligning with the previous evaluation of tencha prepared by Oku and LJ43 (Mao, Wang, Yin, & Xu, 2020). In contrast, the aroma of EC1 was relatively less clean and fresh, had seaweed fragrance, but less high and lasting with a lowest score of 85.93. In summary, the total score ranking of the four matcha varieties, from highest to lowest, was Oku, LJ43, ZC108 and EC1. No obvious difference was found in overall performance between Oku and LJ43.

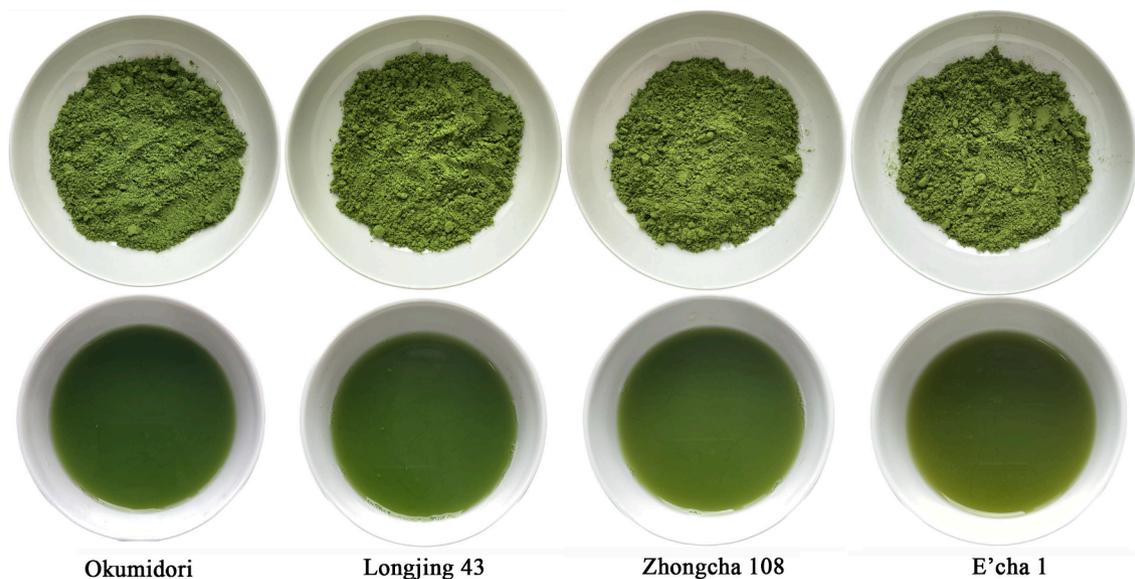


Fig. 1. The appearance and brew color of the four different matcha varieties.

### 3.2. Analysis of major components and color quality in different matcha varieties

The taste quality of matcha is determined by the balance of major components such as tea polyphenols (TPs), catechin, caffeine (CAF), and amino acids (Jiang et al., 2019). As shown in Table 1, the content of TPs was remarkably highest ( $p < 0.05$ ) in EC1, with no significant variation among the other three varieties. The total amount of catechins (TAC) and ester catechins (TEC) were both significantly lower in Oku than the other varieties. The bitterness and astringency of the ester-type

**Table 1**  
Analysis of the main components in four different matcha varieties.

Compounds (%)	Oku	LJ43	ZC108	EC1
TPs	8.82 ± 0.03 <sup>b</sup>	9.31 ± 0.90 <sup>b</sup>	8.93 ± 0.10 <sup>b</sup>	11.42 ± 0.32 <sup>a</sup>
EC	0.32 ± 0.03 <sup>bc</sup>	0.35 ± 0.01 <sup>ab</sup>	0.28 ± 0.01 <sup>c</sup>	0.39 ± 0.04 <sup>a</sup>
EGC	0.90 ± 0.05 <sup>b</sup>	0.69 ± 0.02 <sup>c</sup>	0.67 ± 0.03 <sup>c</sup>	1.23 ± 0.13 <sup>a</sup>
GC	0.07 ± 0.002 <sup>a</sup>	0.08 ± 0.003 <sup>a</sup>	0.07 ± 0.01 <sup>a</sup>	0.08 ± 0.01 <sup>a</sup>
C	0.20 ± 0.01 <sup>a</sup>	0.06 ± 0.003 <sup>c</sup>	0.09 ± 0.01 <sup>b</sup>	0.02 ± 0.002 <sup>d</sup>
EGCG	4.03 ± 0.14 <sup>a</sup>	4.31 ± 0.17 <sup>a</sup>	4.76 ± 0.27 <sup>a</sup>	4.80 ± 0.57 <sup>a</sup>
ECG	0.69 ± 0.05 <sup>c</sup>	1.21 ± 0.04 <sup>a</sup>	0.93 ± 0.05 <sup>b</sup>	0.95 ± 0.11 <sup>b</sup>
GCG	0.11 ± 0.02 <sup>ab</sup>	0.19 ± 0.06 <sup>a</sup>	0.14 ± 0.03 <sup>ab</sup>	0.08 ± 0.01 <sup>b</sup>
CG	0.17 ± 0.02 <sup>a</sup>	0.10 ± 0.04 <sup>b</sup>	0.04 ± 0.003 <sup>c</sup>	0.09 ± 0.03 <sup>b</sup>
TAC	6.49 ± 0.24 <sup>b</sup>	7.13 ± 0.02 <sup>a</sup>	7.18 ± 0.03 <sup>a</sup>	7.64 ± 0.13 <sup>a</sup>
TNEC	1.48 ± 0.05 <sup>a</sup>	1.18 ± 0.03 <sup>b</sup>	1.11 ± 0.05 <sup>b</sup>	1.72 ± 0.18 <sup>a</sup>
TEC	4.72 ± 0.19 <sup>b</sup>	5.51 ± 0.21 <sup>a</sup>	5.69 ± 0.32 <sup>a</sup>	5.75 ± 0.69 <sup>a</sup>
TNEC/TEC	0.31 ± 0.003 <sup>a</sup>	0.21 ± 0.003 <sup>b</sup>	0.20 ± 0.003 <sup>b</sup>	0.30 ± 0.005 <sup>a</sup>
CAF	3.04 ± 0.18 <sup>a</sup>	2.91 ± 0.09 <sup>a</sup>	2.83 ± 0.10 <sup>a</sup>	2.80 ± 0.19 <sup>a</sup>
FAA	4.56 ± 0.06 <sup>d</sup>	5.99 ± 0.14 <sup>b</sup>	6.53 ± 0.12 <sup>a</sup>	5.53 ± 0.11 <sup>c</sup>

Note: All values are shown as mean ± SD. Distinct letters (a-d) within the identical row signify significant variations among the four matcha varieties, as established by the Duncan test ( $p < 0.05$ ). Oku, Okumidori; LJ43, Longjing 43; ZC108, Zhongcha 108; EC1, E'cha 1. TPs, tea polyphenol; EC, (-)-epicatechin; EGC, (-)-epigallocatechin; C, catechin; GC, (-)-gallocatechin; ECG, (-)-epicatechin gallate; EGCG, (-)-epigallocatechin gallate; CG, catechin gallate; GCG, gallocatechin gallate; TAC, total catechins; TNEC, total nonester catechins; TEC, total ester catechins; TNEC/TEC, The ratio of TNEC/TEC; CAF, caffeine; FAA, free amino acid.

catechins were higher than those of the nonester type, leading to a significant correlation between the ratio of nonester catechins to ester catechins (TNEC/TEC) and sensory taste (Huang & Xu, 2021). Oku and EC1 had higher TNEC/TEC values, followed by LJ43 and ZC108. CAF plays a key role in the bitter taste of green tea, also positively impacting catechin astringency (Wang et al., 2022). On the contrary, umami amino acids inhibit the bitter and astringent taste of catechins, which is crucial for green tea taste modulation. In this study, the contents of CAF did not differ significantly among the four varieties, while those of FFAs demonstrated significant variability ( $p < 0.05$ ), following a trend of ZC108 > LJ43 > EC1 > Oku.

The contents of chlorophyll and carotenoid, as well as the color quality are provided in Table S2. Oku exhibited significantly higher chlorophyll *a*, chlorophyll *b*, and total chlorophyll content (*a* + *b*) than the other varieties. However, the difference between LJ43 and ZC108 was not significant. The content of carotenoid followed the order EC1 > LJ43 > ZC108 > Oku. The color quality was further evaluated in terms of *L* (brightness), *a* (green/red chromaticity), and *b* (yellow chromaticity). The *L* values of matcha powder and soup were not significantly different between Oku, LJ43 and ZC108, but were significantly higher than EC1. The *a* value of matcha powder also showed the same pattern, while matcha infusion showed no significant difference between Oku and LJ43, as well as ZC108 and EC1. As for the *b* value, there was no significant difference in tea powder between Oku and EC1. However, the infusion of EC1 displayed significantly higher yellowness and lower brightness than the other varieties, which was consistent with the color appearance of tea infusion. Thus, despite EC1 having higher chlorophyll content, its tea powder color was yellowish due to elevated carotenoid content, with the matcha soup exhibiting reduced brightness and increased yellowness.

### 3.3. Characterization of non-volatile metabolites in four matcha varieties

#### 3.3.1. Comprehensive analysis of non-volatile metabolites across four matcha varieties

A comprehensive assessment of non-volatile metabolites encompassing 12 categories, totaling 1383 compounds, was provisionally identified in the four matcha samples derived from varying tea cultivars. This identification was achieved through a comparative analysis utilizing MS/MS spectrum data from established databases and standards contained within the self-constructed metWare metabolite database. These categories included 12 quinones, 31 terpenoids, 34 tannins, 53

lignans and coumarins, 74 nucleotides and derivatives, 97 organic acids, 129 alkaloids, 139 lipids, 197 amino acids and derivatives, 232 phenolic acids, 263 flavonoids, and 122 other metabolites (Fig. 2A). The outcomes underscored the prevalence of amino acids and derivatives, phenolic acids, flavonoids, and lipids as the dominant non-volatile metabolites in the diverse matcha samples derived from distinct tea varieties. Furthermore, PCA was executed to discern overarching dissimilarities in non-volatile metabolite profiles among the four varieties. The score plot distinctly illustrates substantial disparities among all four matcha varieties (Fig. 2B). This divergence is partially elucidated by the heatmap (Fig. 2C), which highlights metabolites with elevated content in one variety while exhibiting reduced levels in the other three varieties.

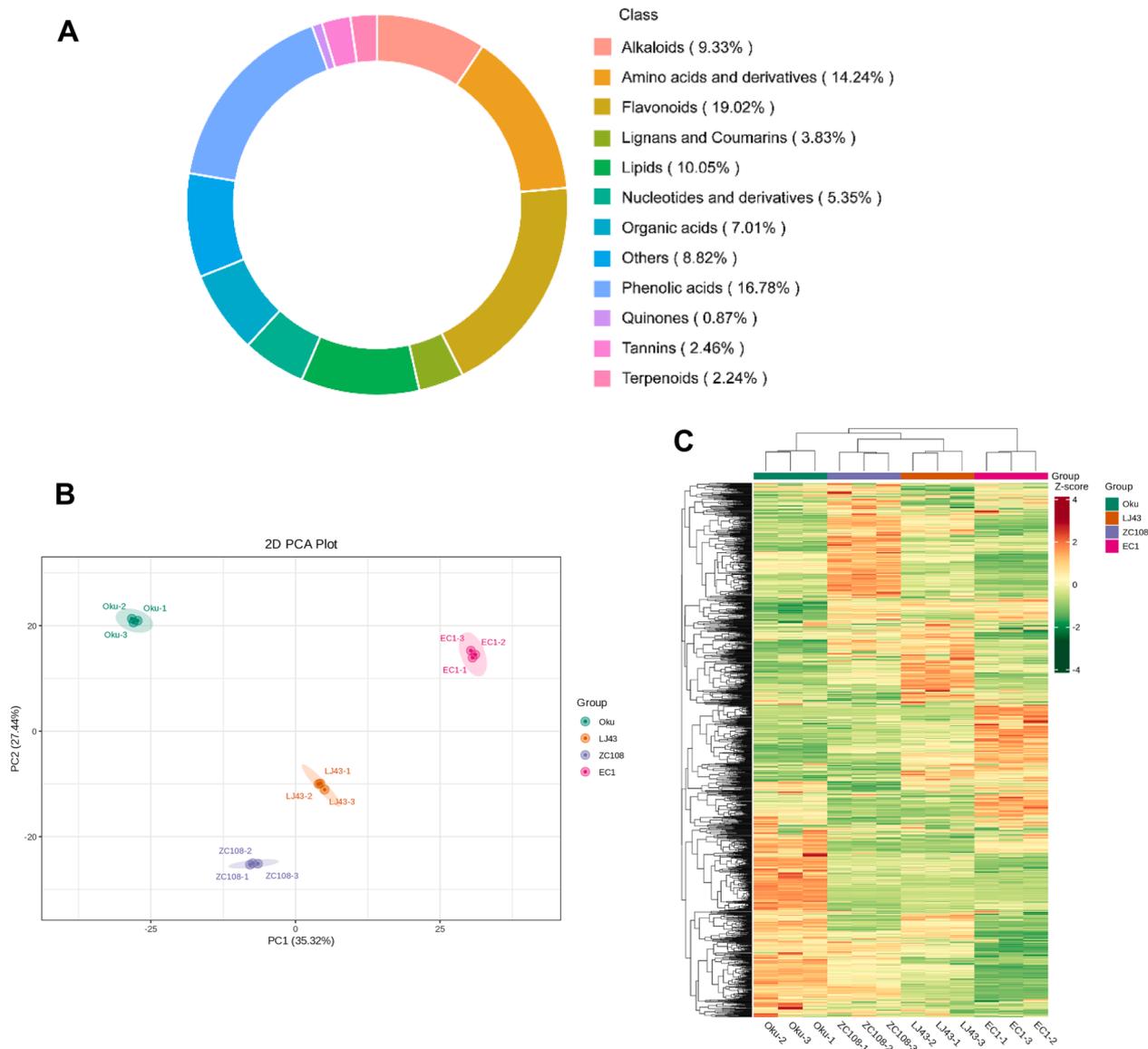
### 3.3.2. Characterization of differential non-volatile metabolites in the four matcha varieties

To gain a deeper insight into the variations in non-volatile compounds among the four matcha varieties, we employed criteria of VIP  $\geq 1$  and fold change  $\geq 2$  or  $\leq 0.5$  to screen for differential metabolites in Oku when compared to LJ43, ZC108, and EC1 (Fig. 3A, Table S3). The

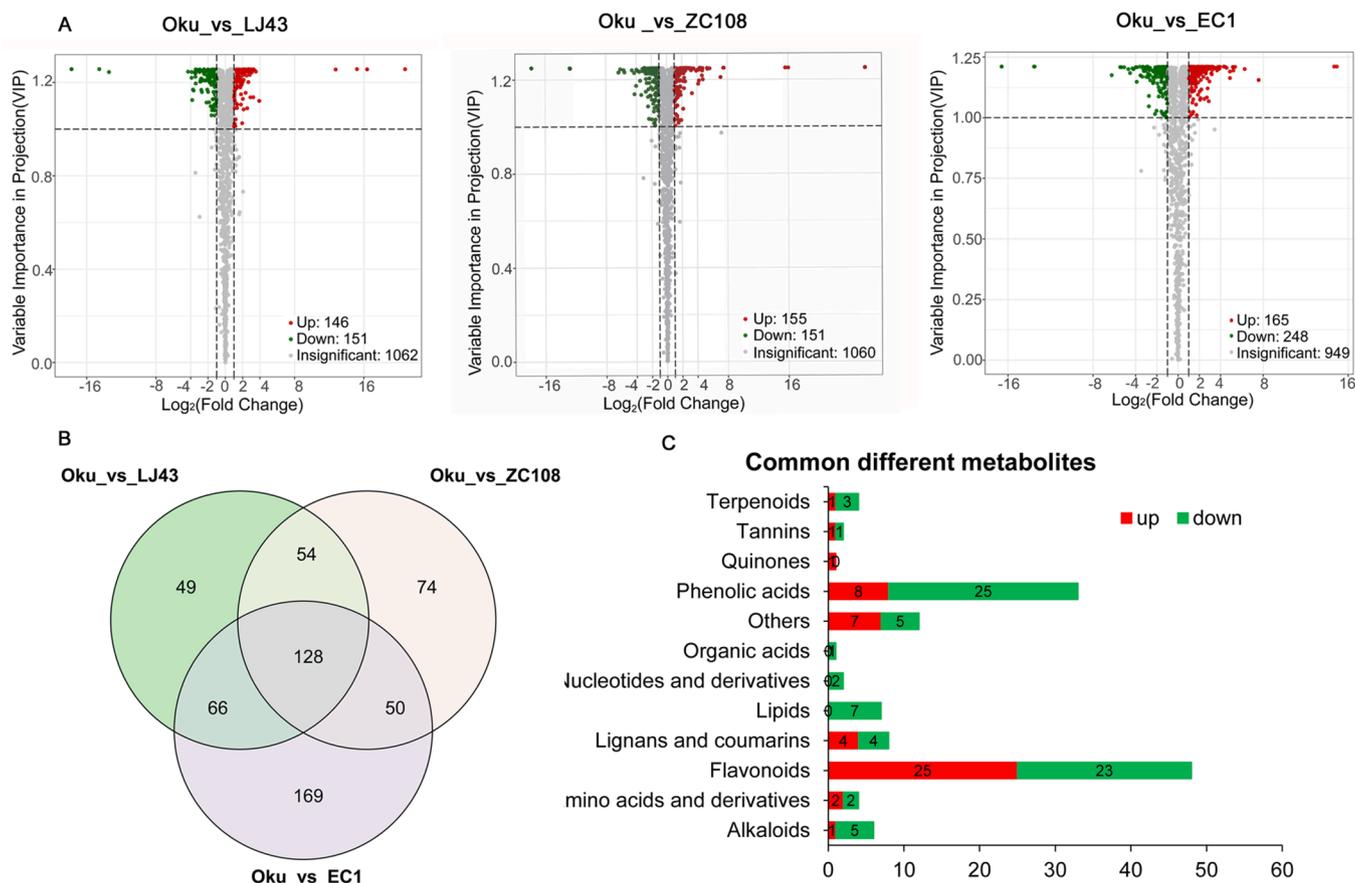
results revealed distinct numbers of differential metabolites for each comparison: LJ43 (297 in total, with 146 upregulated and 151 downregulated), ZC108 (306 in total, with 155 upregulated and 151 downregulated), and EC1 (413 in total, with 165 upregulated and 248 downregulated) (Fig. 3A). Notably, the most pronounced metabolite differences were observed in EC1, indicating that the matcha quality produced by EC1 differed the most from that of Oku among the three Chinese cultivars. This observation was further underscored by the Venn diagram (Fig. 3B), where unique metabolites within each comparison exhibited the following order: EC1 (169), ZC108 (74), and LJ43 (49). Additionally, there were 128 identical metabolites present across the three comparison groups, comprising 49 commonly upregulated metabolites, 76 commonly downregulated metabolites, and 3 metabolites displaying differing trends (Fig. 3C). Notably, the categories of flavonoids and phenolic acids constituted a significant portion, accounting for 60.9 % of the total shared metabolites.

### 3.3.3. Key differential metabolites in different matcha varieties

Based on a fold change threshold of  $\geq 5$  or  $\leq 0.2$ , a total of 177 non-volatiles with significant alterations were identified. These compounds



**Fig. 2.** Classification, hierarchical cluster analysis (HCA), and principal component analysis (PCA) of metabolic profiles in four matcha varieties. (A) Classification of the 1383 metabolites of matcha samples; (B) PCA score plot; (C) HCA map of metabolites. Note: Oku, Okumidori; LJ43, Longjing 43; ZC108, Zhongcha 108; EC1, E'cha 1.



**Fig. 3.** Analysis of differential non-volatile metabolites among the four matcha varieties. (A) In the volcano plot, upregulated metabolites are depicted as red dots, while downregulated metabolites are indicated by green dots; (B) The Venn plot illustrates the numbers of identified differential metabolites in the three comparison groups; (C) The numbers of commonly upregulated and downregulated differential metabolites in variety Oku\_vs\_LJ43, Oku\_vs\_ZC108 and Oku\_vs\_EC1. Note: Oku, Okumidori; LJ43, Longjing 43; ZC108, Zhongcha 108; EC1, E'cha 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are crucial for the characteristics of the tea and primarily belong to categories such as alkaloids, phenolic acids, flavonoids, lignans and coumarins, and tannins metabolites (Table S4, Fig. 4). Notably, three metabolites, namely, quercetin-5-O-glucuronide, secoisolariciresinol diglucoside and theaflavin-3-gallate displayed a higher content in three Chinese varieties.

Flavonoids are essential components not only for the taste quality of tea but also for its coloration (Wang et al., 2006; Zhang et al., 2019). In this study, 63 differential flavonoids were categorized into five subclasses, i.e. flavonols (35), flavones (20), flavanonols (4) flavanols (3) and anthocyanidins (1) (Table S3, Fig. 4A). The greatest number of differential flavonols was observed in Oku and LJ43, with higher contents compared to ZC108 and EC1. Flavonols primarily exist in the form of glycoside in tea, and contribute to the greenish-yellow color of green tea infusion (Wang et al., 2006). They also confers a soft, astringent taste, and can enhance the bitterness of caffeine in tea infusion (Scharbert, Holzmann, & Hofmann, 2004). Among the 13 flavonoids with high contents in ZC108, 7 belong to the flavone sub-class, highlighting the important role of flavones in the quality of matcha from ZC108. The contents of three flavanols, epigallocatechin-3-O-(3,5-O-dimethyl) gallate and epicatechin-3-(3'-O-methyl) gallate were higher in Oku, while galocatechin-(4 $\alpha$  → 8)-galocatechin was more abundant in LJ43. Cyanidin-3-O-glucoside is associated with the red coloration of plant extracts (Zhang et al., 2008). It is worth noting that cyanidin-3-O-glucoside, the only differential anthocyanidins, displays a higher content in EC1, thereby contributing to its liquid color.

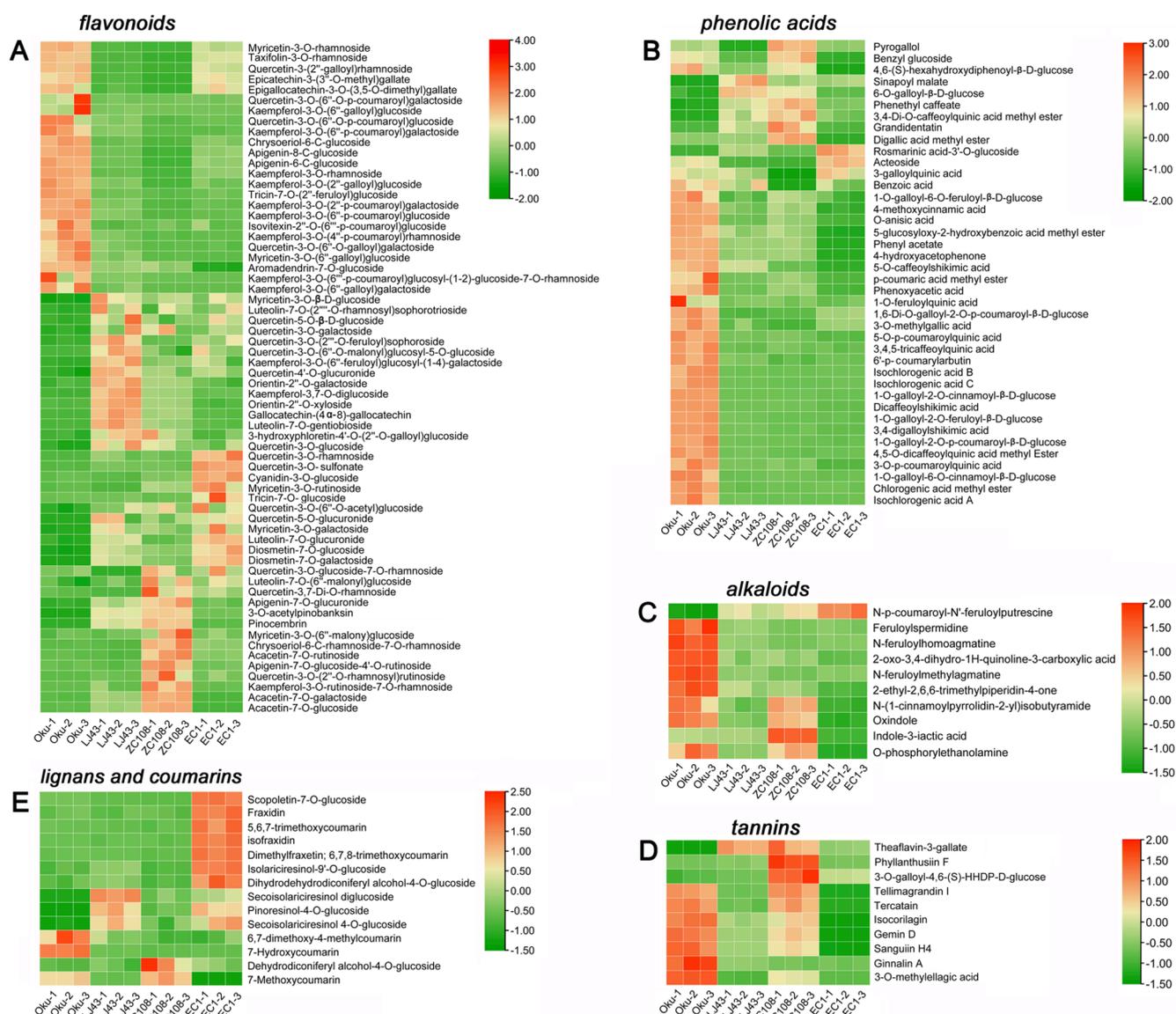
Phenolic acids can enhance the umami taste of tea infusion, and play a significant role in forming the typical taste of matcha (Kaneko et al.,

2006). In this study, 29 differential phenolic acid metabolites were significantly higher in Oku (Fig. 4B), accounting for 72.5 % of the total differential phenolic acids, including shikimic acid, quinic acid, and gallic acid, as well as several phenolic acid glucosides and phenolic acid methyl esters. Pyrogallol was lower only in LJ43, while 3-galloylquinic acid and benzoic acid were relatively lower in ZC108. Digallic acid methyl ester and 4,6-(S)-hexahydroxydiphenoyl- $\beta$ -D-glucose were particularly lower in EC1.

Alkaloids are widely distributed in beverage plants such as tea, cocoa and coffee (Zhang et al., 2022). A total of 10 differential alkaloid metabolites were identified, mainly belonging to the phenolamine category (Fig. 4C). The levels of alkaloid metabolites were generally higher in Oku, except for *N*-p-coumaroyl-*N'*-feruloylputrescine, which was exceptionally lower in Oku compared to the other three varieties. Indole is a key aroma component in Japanese matcha (Tan et al., 2019), and could be a potential marker for evaluating the overall quality of Sencha (Jumtee, Komura, Bamba, & Fukusaki, 2011). Among indole and its derivatives, oxindole and indole-3-lactic acid were found to be significantly lower in EC1.

A previous study indicated that the high tannin content was not conducive to the storage of green tea (Lu, Chu, Yan, & Chen, 2009). Herein, we identified 10 differential tannin metabolites. Most tannins exhibited high levels in Oku and ZC108 (Fig. 4D), suggesting that more rigorous preservation methods should be employed for matcha produced from these two varieties.

The term "sweetness" refers to a delightful richness of flavor, and coumarin contributes to the sweet quality of green tea due to its aromatic sweetness characteristic (Baba & Kumazawa, 2014). Dissimilar to



**Fig. 4.** Heat map of differential (A) flavonoids, (B) phenolic acids, (C) alkaloids, (D) tannins, and (E) lignans and coumarins from different varieties. Green and red segments denote the low and high abundance of metabolites, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the above-mentioned types of metabolites, lignans and coumarins were mainly found at high levels in EC1, including 5 coumarins and 2 lignans (Fig. 4E). These compounds might contribute to the formation of aroma in EC1.

Additionally, notable differences were observed in the concentrations of four amino acids and derivatives, four terpenoids, four organic acids and five lipids among the four matcha varieties (Table S3). Amino acids and derivatives, including *N*-methylglycine, displayed high levels in Oku, while *N*-acetyl-*L*-tryptophan in ZC108 and *L*-lysine-butanoic acid in EC1 increased more than 5-fold compared to Oku. Lipids can participate in Strecker degradation reactions to generate new flavour compounds (Fereidoon & Won, 2020; Rizzi, 2008), and some of them may serve as crucial precursors for tea aroma, such as  $\alpha$ -linolenic acid (Chen et al., 2022). Across the four matcha varieties, lipids including 1-stearidonoyl-glycerol, 2- $\alpha$ -linolenoyl-glycerol, 1- $\alpha$ -linolenoyl-glycerol were all decreased in LJ43, ZC108, and EC1 compared to Oku, along with three terpenoids. In contrast, the fold change values for the four organic acids were lower for both LJ43 and EC1. Nevertheless, the contents of phenylpyruvic acid, 3-hydroxybutyric acid, and malonic acid in ZC108

showed no significant difference compared to Oku.

In particular, we screened 10 most upregulated and 10 most down-regulated non-volatile compounds with the highest  $\text{Log}_2\text{FC}$  values in the three Chinese varieties relative to Oku (Fig. S1). Among the upregulated metabolites in the Chinese varieties, four compounds were consistent: theaflavin-3-gallate, secoisolaricresinol diglucoside, quercetin-5-O-glucuronide, and 3-methyl-1-pentanol. Moreover, three metabolites in LJ43 matched those in ZC108, including 3-O-acetylpinobanksin, *L*-ascorbic acid, and myricetin-3-O-rhamnoside. Only one downregulated metabolite (kaempferol-3-O-(6'-galloyl) glucoside) was identical between ZC108 and EC1.

### 3.3.4. Correlation analysis of sensory score, color quality and significant metabolites

It is well known that the vivid green color and high brightness of matcha and its infusion, along with its appealing taste and aroma, is well-recognized in enhancing consumer acceptance. To establish connections between metabolites and these quality attributes, we conducted a correlation analysis involving sensory evaluation scores, color quality,

and significant metabolites (Fig. S2). Based on the heatmap of Pearson correlation coefficients ( $r$ ), the indicators were categorized into three clusters. The first cluster consisted of appearance, infusion color and aroma, which showed a highly positive correlation ( $r > 0.90$ ) with four phenolic acids (including 5-O-caffeoylshikimic acid, phenyl acetate, 4-hydroxyacetophenone and 5-glucosyloxy-2-hydroxybenzoic acid methyl ester) and one flavonoid (aromadendrin-7-O-glucoside). On the contrary, carotenoid, TEC, TAC and EGCG demonstrated a significant negative correlation with these three indicators. The second cluster included taste, L (powder), b (powder) and L (infusion). This group of substances exhibited a significant negative correlation with some saccharides, lignans and coumarins, as well as TPs, EGC, quercetin-3-O-sulfonate, quercetin-3-O-rhamnoside, and cyanidin-3-O-glucoside. Due to the bitterness and astringency properties, TPs were negatively correlated with taste scores (Massounga Bora, Ma, Li, & Liu, 2018). In rice, cyanidin-3-O-glucoside was inclined to accumulate in the dark purple seeds instead of brown or white seeds (Rahman et al., 2013). Our results indicated that cyanidin-3-O-glucoside had a negative influence on the brightness of matcha's appearance and infusion color. The third cluster encompassed a (powder), a (infusion) and b (infusion). This set of components exhibited an inverse relationship with the first cluster. Significant co-positive correlations included TPs, carotenoid, EGC, TAC, coumarins, *L*-lysine-butanoic acid, 8 flavonoids, 5 coumarins and 7 saccharides. In contrast, the substances with significant negative correlation were mainly phenolic acids such as phenyl acetate, 5-O-caffeoylshikimic acid, 4-methoxycinnamic acid and so on. Interestingly, the correlation between a (powder) and a (infusion) within this cluster was positive, indicating that certain compounds influencing the greenness of both appearance and infusion could be consistent.

### 3.3.5. KEGG analysis of differential metabolites

The KEGG database can provide biological pathway information of metabolite accumulation (Kanehisa & Goto, 2000). KEGG analysis revealed that in the Oku\_vs\_LJ43, Oku\_vs\_ZC108, and Oku\_vs\_EC1 comparisons, the differential metabolites were involved in 67, 53, and 79 KEGG pathways, respectively (Table S3). The differential abundance (DA) scores of twenty KEGG pathways with low P-value are shown in Fig. S3. The findings showed that metabolic processes associated with flavone and flavonol biosynthesis exhibited notable enrichment ( $p < 0.05$ ) and up regulated in the comparisons between Oku\_vs\_LJ43 and Oku\_vs\_ZC108. Flavone and flavonols primarily contribute to the yellow color of green tea infusion (Zhang et al., 2020), this may explain the high score of Oku about the infusion color with vivid brilliant green characteristics. In these three comparisons, the pathways of photosynthesis were all demonstrated downregulated, which was consistent with the high content of chlorophyll in Oku than other three varieties. In the case of the Oku\_vs\_EC1 comparison, distinct enrichment was observed in metabolic pathways such as vitamin B6 metabolism, photosynthesis, anthocyanin biosynthesis, and glycolysis/gluconeogenesis (all  $p < 0.05$ ). Particularly, the expression of anthocyanin biosynthesis was dramatically upregulated in the EC1 comparison. Anthocyanins are a group of natural water-soluble pigments in plants that contribute to the pink-purple color of a range of tissues (He et al., 2021). Abundant accumulation of anthocyanins not only causes the purple color of tea leaves, but also greatly enhances the bitterness of tea (Jiang et al., 2013). Thus, the upregulated of anthocyanin biosynthesis in EC1 may play a vital role of the lowest brightness (Table S2) and "little bitter" taste characteristic (Table S1) of tea infusion.

## 3.4. Alterations of volatile organic compounds (VOCs) in four matcha varieties

### 3.4.1. VOCs identified from four matcha varieties

A total of 488 VOCs were detected and the majority of these compounds were found to be present in all four samples (Table S1), including 97 heterocyclic compound, 82 terpenoids, 76 ester, 49

hydrocarbons, 39 ketone, 39 alcohol, 38 aldehyde, 29 aromatics, 8 phenol, 8 amine, 7 acid, 6 nitrogen compounds, 4 sulfur compounds, 2 halogenated hydrocarbons, 1 ether and 3 others (Fig. 4A). Among them, the number of heterocyclic compound, terpenoids, and ester accounted for 52.2 % of the total VOCs. The total content of volatiles was significantly lower in LJ43 (138.16  $\mu\text{g/g}$ ), ZC108 (121.83  $\mu\text{g/g}$ ) and EC1 (122.43  $\mu\text{g/g}$ ) when compared to Oku (161.88  $\mu\text{g/g}$ ) (Fig. 4A). To delve further into the composition, the distribution of different classes of volatiles within the contents was analyzed (Fig. 4B). The proportions of aldehyde and ketone in LJ43, ZC108 and EC1 were significantly lower than those in Oku. Additionally, the proportions of most VOCs classes did not differ significantly between LJ43 and ZC108, aligning with the results obtained from PCA (Fig. 4B) and PLS-DA (Fig. 4C), while LJ43 and ZC108 were clustered closely with each other. However, there remained significant differences in the upregulated and downregulated VOCs between Oku\_vs\_LJ43 and Oku\_vs\_ZC108 (Fig. S5D). In order to identify the key variations in VOCs, a criterion of VIP  $> 1$  was applied from the PLS-DA model, resulting in the selection of 194 crucial VOCs. Most of these VOCs were clustered into different groups such as heterocyclic compound, ester, terpenoids, hydrocarbons, ketone, alcohol, and aldehyde (Table S6) (Fig. 5 and Fig. 6).

### 3.4.2. Key aroma-active volatiles in four matcha varieties

The influence of volatiles on the overall aroma profile of tea is not only determined by the contents and quantitative abundance of VOCs, but also relies on the odor threshold (Ni et al., 2021). As a result, the odor activity values (OAVs) were employed to delve deeper into the key VOCs of four matcha varieties. Referring to the odor thresholds established in previous studies (Gemert., 2011; Xue et al., 2022; Yang et al., 2022), a total of 195 VOCs were identified as potential aroma-active compounds due to their significant OAVs, with 97 VOCs (OAV  $> 1.0$ ) potentially playing a crucial role in the aroma formation of different matcha varieties (Table S7). The level of aldehydes accounted for the highest percentage at 19.6 %. Aldehydes are closely associated with the formation of specific aroma profiles; for instance, fatty aldehydes primarily impart a robust fruity fragrance, often accompanied by hints of bread-like aromas (Zhu et al., 2015). In this study, six fatty aldehydes, including (*E*)-2-nonenal, (*Z*)-4-heptenal, (*E*)-6-nonenal, 2-nonenal, heptanal and decanal, displayed high OAVs in matcha.

Through comparisons, a total of 20 VOCs emerged as the predominant compounds in matcha with an OAV exceeding 100 across all four varieties, including aldehyde (6), heterocyclic compound (5), ketone (3), terpenoids (2), nitrogen compounds (1), phenol (1), ester (1) and alcohol (1). Among them, (*E*)-2-nonenal, characterized by a fatty and sweet fragrance had the highest OAV (ranging from 13,055–14,598) due to its relatively low threshold (0.08  $\mu\text{g/kg}$ ). Not far behind were (*Z*)-4-heptenal (OAV: 4655–7943) with a hay-like aroma, and fureneol (OAV: 3789–4195) with a caramel aroma. These three compounds appeared to play a pivotal role in contributing to the distinct "seaweed aroma" quality of matcha, aligning with previous research (Ryoko, Yohei, Yoshiyuki, & Kenji, 2017). In addition, several compounds such as 2,2,6-trimethyl-cyclohexanone, 3-octen-2-one, dihydroactinidiolide, hexanal, (*E*, *E*)-2,4-undecadienal, 1-octen-3-ol, 1-nonanol, and acetate-4-hexen-1-ol exhibited significantly higher OAVs in the Oku variety. For example, the OAV value of (*E*,*E*)-3,5-octadien-2-one with fruity aroma was 6.0, 8.9 and 13.1 times greater in Oku compared to LJ43, ZC108 and EC1, respectively. Similarly, the OAV value of dihydroactinidiolide, with a musky or coumarin odor was 5.5, 8.7 and 8.5 times greater in Oku compared to LJ43, ZC108 and EC1, respectively. This suggests that these compounds significantly contribute to the distinct clean, fresh, and seaweed fragrance characteristics found in matcha aroma.

## 4. Conclusions

In the present study, we conducted a comprehensive analysis on volatile and non-volatile metabolites in matcha derived from four

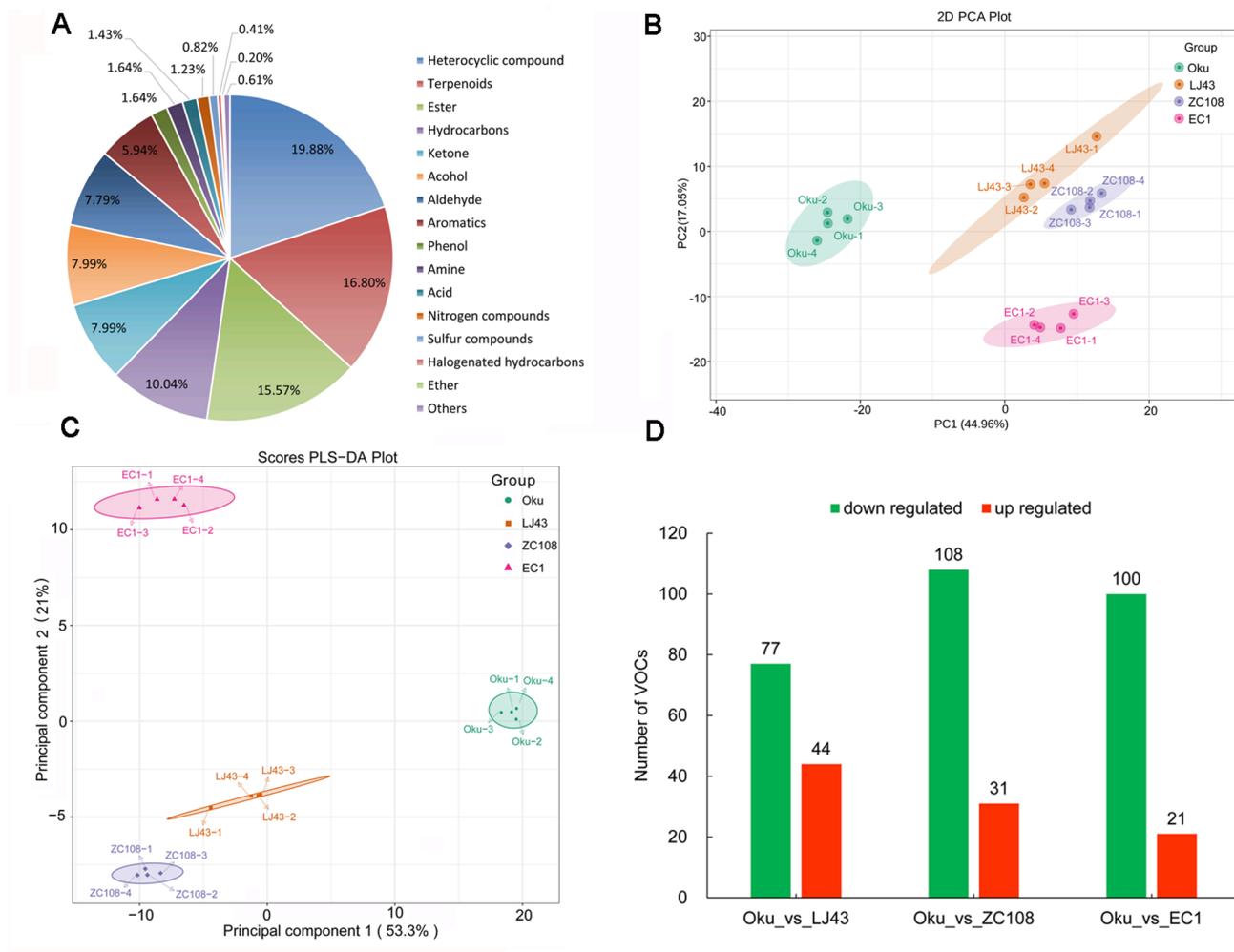


Fig. 5. Multivariate statistical analysis of VOCs in in four matcha varieties. (A) Classification of the identified VOCs; (B) Principal component analysis (PCA); (C) Score plot of PLS-DA; (D) Upregulated and downregulated VOCs in Oku\_vs\_LJ43, Oku\_vs\_ZC108 and Oku\_vs\_EC1, respectively.

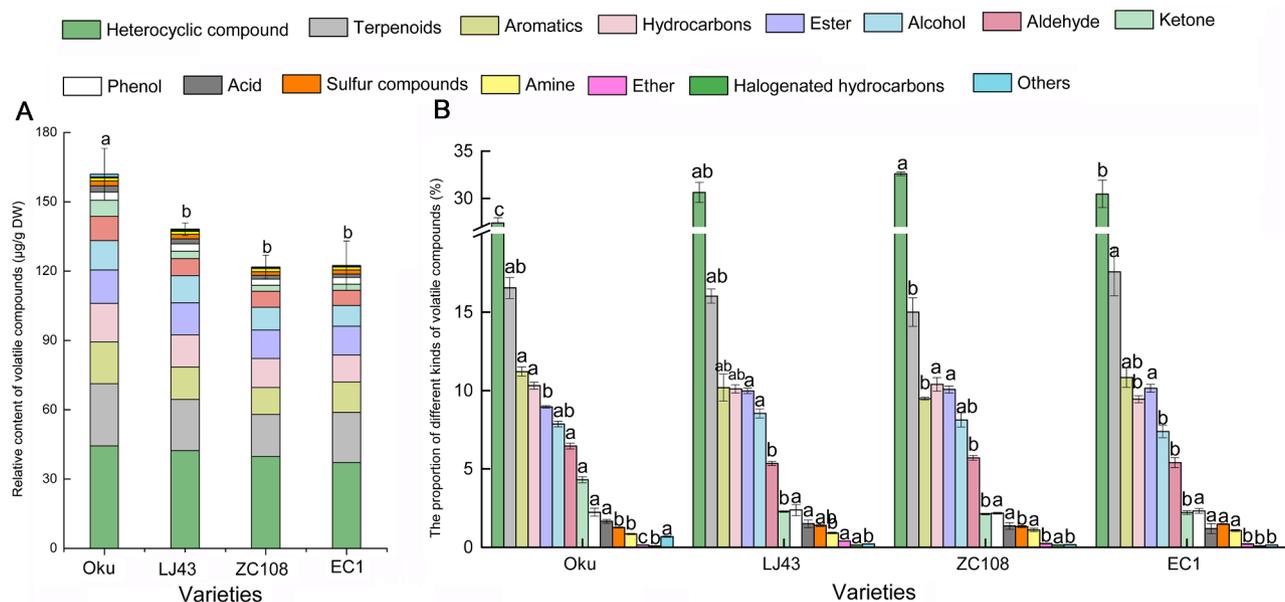


Fig. 6. The relative content (A) and proportion (B) of different classes of volatiles in four matcha varieties. Values sharing identical letters within the same color column exhibit no significant differences ( $p < 0.05$ ). All values represent the mean  $\pm$  SD of four tea samples.

distinct tea varieties using UPLC MS/MS and GC–MS techniques. A total of 177 non-volatile compounds with significant alterations were detected, primarily encompassing flavonoids, phenolic acids, alkaloids, tannins, and lignans and coumarins metabolites. Moreover, our screening identified 97 key aroma-active compounds (OAV > 1.0), with aldehydes constituting the largest proportion at 19.6 %, indicating their significant role in shaping the aroma quality of matcha. This study underscores the substantial impact of tea varieties on the composition of both volatile and non-volatile metabolites in matcha. Nevertheless, further research is warranted to delve into the dynamic changes of essential taste, flavor, and aroma components throughout the matcha processing journey.

#### CRedit authorship contribution statement

**Danjuan Huang:** Writing – original draft, Software, Methodology, Investigation. **Xun Chen:** Writing – review & editing, Data curation. **Rongrong Tan:** Validation, Software. **Hongjuan Wang:** Software, Methodology, Investigation. **Long Jiao:** Software, Methodology, Investigation. **Haiyan Tang:** Software, Methodology, Investigation. **Qingbo Zong:** Resources, Methodology, Investigation. **Yingxin Mao:** Writing – review & editing, Project administration.

#### Declaration of competing interest

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

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2024.101234>.

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