

Integrated UPLC–Q-TOF/MS and GC–IMS Metabolomics Reveals Dynamic Metabolic Remodeling of Tobacco Leaves during Industrial Threshing and Redrying

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Abstract

Threshing and redrying significantly influence the chemical composition, aroma profile, and physical properties of flue-cured tobacco. However, the stage-specific mechanisms underlying tobacco quality formation remain unclear. In this study, ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF/MS) and gas chromatography-ion mobility spectrometry (GC–IMS) were integrated to characterize dynamic alterations in non-volatile and volatile metabolites in tobacco leaves collected at four processing stages: before hot-air moistening, after hot-air moistening, after threshing and pneumatic separation, and after redrying. A total of 568 non-volatile metabolites and 77 volatile compounds were identified. Among the processing stages, hot-air moistening induced the most pronounced metabolic changes, followed by redrying, whereas threshing and pneumatic separation caused relatively minor alterations. Hot-air moistening promoted amino acid metabolism and secondary metabolic pathways, facilitating the accumulation of aroma precursors. Threshing and pneumatic separation mainly triggered antioxidant responses associated with mechanical stress, whereas redrying promoted the transformation of secondary metabolites and enrichment of aroma-active compounds. The volatile profile underwent substantial remodeling during hot-air moistening, shifting from green and grassy odors toward caramel-like, sweet, and roasted aromas, which were further enhanced and balanced during redrying. Collectively, these results demonstrate that hot-air moistening, threshing and pneumatic separation, and redrying represent sequential and complementary processing stages that jointly shape the final quality attributes of tobacco strips. This study provides mechanistic insights into metabolite regulation during industrial threshing and redrying and offers a theoretical basis for optimizing processing parameters to improve tobacco quality.

1. Introduction

Threshing and redrying are essential industrial processes that transform flue-cured tobacco leaves from agricultural products into standardized raw materials for cigarette manufacturing (Breen, 2001; Yang, et al., 2010). In commercial redrying facilities, cured tobacco leaves undergo multiple processing steps, including hot-air moistening, threshing and pneumatic separation, and redrying (Fig. 1). These operations achieve efficient separation of lamina from midrib and regulate the moisture content of the processed tobacco strips (Wang et al., 2025). During hot-air moistening, tobacco leaves are simultaneously heated and humidified. This process increases their flexibility and reduces brittleness, thereby facilitating subsequent lamina-stem separation. Redrying subsequently removes excess moisture and promotes uniform moisture distribution within tobacco strips. Variations in temperature and humidity during these processes may influence the degradation, transformation, and accumulation of endogenous chemical constituents and aroma-related compounds, ultimately influencing the quality of tobacco raw materials. Following threshing and redrying, tobacco leaves generally exhibit improved aroma, taste, and sensory harmony. Moreover, redrying can modify aroma-active compound profiles, enhance storage stability and aging suitability, reduce batch-to-batch variation, and improve the consistency of cigarette formulations (Liu et al., 2022).

Threshing and redrying involve complex heat and mass transfer processes that substantially influence the physical properties, chemical composition, aroma characteristics, and sensory quality of tobacco leaves (Luo et al., 2022; Wang et al., 2022). Moistening is a prerequisite for threshing and redrying because it increases leaf moisture and temperature, thereby improving leaf elasticity and reducing mechanical damage. In industrial production, the moisture content and temperature of tobacco leaves after moistening are typically controlled at 19–21% and 65–75°C, respectively. Previous studies have demonstrated that steam-water mixed moistening significantly reduces polyphenol and pigment contents while increasing the abundance of aroma compounds compared with steam moistening alone (Ming-ha, 2016). Lian et al. (2020) reported that hot-air moistening facilitated non-enzymatic browning reactions and reduced undesirable odors and pungent sensations. The primary objective of redrying is to reduce moisture content to an appropriate level while eliminating undesirable green and miscellaneous odors (Chida, Sone, & Tamura, 2004). Heat and mass transfer during redrying, together with precise temperature control, play important roles in preserving aroma compounds and maintaining tobacco quality (JIAN, Yang, Wang, & Wang, 2006). Considering physical properties, sensory characteristics, and aroma composition, redrying temperatures are generally maintained between 80 and 90°C (Jin et al., 2016). Qu et al. (2015) found that aroma compounds were released during threshing and redrying, and that elevated redrying temperatures resulted in greater losses of volatile compounds in middle-position leaves than in upper-position leaves. In addition, redrying has been shown to reduce the pH of tobacco leaves (Jin et al., 2016). Although hot-air moistening, threshing and pneumatic separation, and redrying significantly affect chemical composition, aroma characteristics, and sensory quality, previous studies have primarily focused on physical properties and conventional chemical parameters (Hua YiKun et al., 2012; Xu et al., 2025). For example, Long et al. (2018) reported that low-temperature drying reduced nicotine content while improving moisture retention and color uniformity. However, these conventional indicators provide limited information on the dynamic transformation of metabolites and the mechanisms underlying aroma development during tobacco processing. Therefore, developing advanced analytical approaches with broader chemical coverage is essential.

Existing studies on threshing and redrying have mainly examined physical indices, such as leaf fragmentation and residual lamina attached to stems, as well as conventional chemical parameters, including total sugar, reducing sugar, nicotine, and total nitrogen. These studies provided valuable information regarding processing quality evaluation. However, they have largely remained descriptive and have not fully elucidated the metabolic mechanisms associated with quality formation during individual processing stages. In particular, the dynamic metabolic alterations induced by different processing operations and their relationships with aroma development, taste characteristics, and other quality attributes remain poorly understood. With advances in analytical technologies, metabolomics has emerged as a powerful high-throughput tool for comprehensively profiling metabolites with diverse physicochemical properties and is widely used in studies of tobacco quality formation (Banožić et al., 2020). Using LC–MS/MS-based metabolomics, Wei et al. (2024) explored metabolite transformations in tobacco leaves subjected to different curing conditions. A total of 845 metabolites were identified, among which flavonoids, alkaloids, terpenoids, amino acids, and organic acids exhibited substantial

variations during curing. These findings demonstrated that curing temperature and intensity significantly influence tobacco metabolic profiles and that differential metabolites may serve as biomarkers for evaluating processing status and quality development. In addition to non-volatile metabolites, changes in volatile compounds are also closely associated with tobacco aroma formation. Fan et al. (2023) combined headspace solid-phase microextraction–gas chromatography–mass spectrometry (HS-SPME–GC–MS) with multivariate statistical analysis to characterize volatile compound changes during cigar tobacco fermentation. Among the 613 detected volatile metabolites, 263 exhibited significant variations during fermentation. Terpenes, esters, aldehydes, and other aroma-related compounds underwent extensive transformation, involving pathways associated with monoterpene, sesquiterpene, and triterpene biosynthesis as well as amino acid degradation. Zhu et al. (2024) integrated headspace GC–IMS, multivariate statistical analysis, and sensory evaluation to characterize flavor differences among cigar tobacco leaves from different geographical origins in China. Several volatile compounds, including 1,8-pinene, 3-methyl-3-buten-1-ol, and 2,3-dimethyl-5-ethylpyrazine, were identified as potential markers for distinguishing flavor profiles of tobacco leaves from different production regions.

Despite these advances, comprehensive investigations into metabolite remodeling during industrial threshing and redrying remain limited. This study integrated UPLC–Q-TOF/MS and GC–IMS to systematically profile non-volatile metabolites and volatile compounds in tobacco leaves collected at four processing stages: before hot-air moistening, after hot-air moistening, after threshing and pneumatic separation, and after redrying. Orthogonal partial least-squares discriminant analysis (OPLS–DA) was employed to identify stage-specific differential metabolites and aroma-related compounds. This study aimed to elucidate the metabolic responses associated with individual industrial processing stages and their contributions to aroma development and tobacco quality formation, thereby providing a theoretical foundation for process optimization and quality-oriented control.

2. Materials and Methods

2.1 Materials and chemicals

Flue-cured tobacco leaves of the cultivar Zhongyan 100 were obtained from Sanmenxia, Henan Province, China, in 2025. The leaves underwent a standard industrial threshing and redrying process (Fig. 1). Samples were collected at four representative processing stages: before hot-air moistening (group A), after hot-air moistening (group B), after threshing and pneumatic separation (group C), and after redrying (group D). For each stage, three biological replicates were collected and stored at – 80°C until analysis.

Chemical reagents (Merck, Darmstadt, Germany), including methanol, formic acid, and acetonitrile, were of chromatographic grade (> 99.9%). Ultrapure water (Watsons Water, China) was used to prepare all aqueous solutions.

2.2 UPLC–Q-TOF/MS analysis

Non-volatile metabolites were extracted according to the method described by Yang et al. (2017) with minor modifications. Tobacco samples were vacuum freeze-dried using a Scientz-100F freeze dryer and ground into a fine powder at 30 Hz for 1.5 min using an MM 400 mill (Retsch, Germany). Approximately 25 mg of powdered sample was extracted with 1,000 μ L of 70% methanol containing an internal standard. For samples with a mass below 25 mg, the extraction solvent was adjusted proportionally according to sample weight. The internal standard stock solution was prepared by dissolving 1 mg of the standard in 1 mL of 70% aqueous methanol to obtain a concentration of 1,000 μ g/mL, followed by dilution with 70% aqueous methanol to prepare a working solution of 250 μ g/mL. After extraction, 500 μ L of petroleum ether was added to each sample. The mixture was vortexed for 5 min and centrifuged at 12,000 rpm for 10 min at 4°C after phase separation. The entire lower phase was collected, filtered through a 0.22 μ m polytetrafluoroethylene membrane filter, transferred into a glass insert placed in an amber autosampler vial, and stored at – 20°C until LC–MS analysis.

Chromatographic separation was performed using an ExionLC™ AD ultra-performance liquid chromatography system equipped with an Agilent SB-C18 column (1.8 μ m, 2.1 $\times$ 100 mm). The mobile phases consisted of ultrapure water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The gradient program was as follows: 5% B at 0 min; increased linearly to 95% B over 9 min; maintained at 95% B for 1 min; decreased to 5% B from 10.0 to 11.1 min; and equilibrated at 5% B until 14 min. The flow rate was set at 0.35 mL/min, the column temperature was maintained at 40°C, and the injection volume was 2 μ L.

The electrospray ionization source temperature was maintained at 500°C. Ion spray voltages were set at 5,500 V in positive-ion mode and – 4,500 V in negative-ion mode. The ion source gas I, ion source gas II, and curtain gas were set at 50, 60, and 25 psi, respectively. Collision-induced dissociation was performed under high-energy conditions using nitrogen as the collision gas. The declustering potential and collision energy were optimized for individual ion transitions.

Metabolites were annotated against the MetWare Database based on MS/MS spectral information. Isotopic peaks, redundant signals generated by K^+ , Na^+ , and NH_4^+ adducts, and fragment ions derived from higher-molecular-weight compounds were excluded. Metabolite quantification was performed using multiple-reaction monitoring. Peak areas were integrated for all detected metabolites, and signal normalization was conducted for the same metabolite across different samples to improve quantitative accuracy and reproducibility (Fraga et al., 2010).

2.3 GC–IMS Analysis

For GC–IMS analysis, 0.5 g of each tobacco sample was placed into a 20 mL headspace vial, followed by the addition of 10 μ L of 2-octanol solution (100 ppm) as an internal standard. Samples were incubated at 80°C for 15 min before injection, and each sample was analyzed in triplicate. The incubation speed was set at 500 rpm, the injection volume was 200 μ L, and splitless injection was employed. The syringe and injection-port temperatures were maintained at 105 and 80°C, respectively. The chromatographic column temperature was set at 80°C. High-purity nitrogen ($\geq 99.999\%$) was used as the carrier gas. The

carrier-gas flow rate was initially maintained at 2.0 mL/min for 2 min, increased linearly to 10.0 mL/min over 8 min, further increased to 100.0 mL/min over 10 min, and maintained for 20 min. The total chromatographic run time was 40 min. Ionization was achieved using a tritium (^3H) source. The drift tube was 53 mm in length and operated at an electric field strength of 500 V/cm and a temperature of 45°C. The drift gas flow rate was maintained at 75.0 mL/min. Data were acquired in positive-ion mode. According to Zhang et al. (2025), odor activity values (OAVs) were calculated to evaluate the contribution of individual volatile compounds to the overall aroma characteristics:

$$\text{OAV} = \frac{C_i}{T_i}$$

Where C_i represents the concentration of volatile compound i in the sample (mg/kg), and T_i denotes its odor threshold in water (mg/kg).

2.4 Data processing and statistical analysis

Three-dimensional topographic plots, two-dimensional difference plots, and gallery fingerprint plots of volatile compounds were generated using the Reporter and Gallery Plot modules in VOCal software. Principal component analysis (PCA) and OPLS-DA were conducted using SIMCA-P software (version 11.5; Umetrics, Umeå, Sweden). For non-volatile metabolites, differential metabolites were identified according to the criteria of fold change (FC) ≥ 1.5 , $P < 0.05$, and variable importance in projection (VIP) ≥ 1.5 . For volatile compounds, VIP > 1.5 and $P < 0.05$ based on one-way analysis of variance (ANOVA) were considered significantly different. Heatmaps, differential metabolite analyses, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the MetWare Cloud platform.

3. Results and discussion

3.1 UHPLC-Q-Exactive/MS Analysis

3.1.1 Global profiling of non-volatile metabolites

A total of 568 non-volatile metabolites were identified across tobacco leaf samples collected during threshing and redrying (Fig. 2A). These metabolites were classified into 12 major groups, including 154 terpenoids (27%), 85 lipids (15%), 79 alkaloids (14%), 58 amino acids and derivatives (10%), 55 phenolic acids (10%), 46 flavonoids (8%), 24 lignans and coumarins (4%), 22 others (4%), 22 nucleotides and derivatives (4%), 18 organic acids (3%), 3 quinones (1%), and 2 steroids ($< 1\%$). As shown in Fig. 2B, tobacco leaves before hot-air moistening (group A) exhibited the highest overall abundance of non-volatile metabolites. Following hot-air moistening (group B), the overall abundance decreased markedly and then remained relatively stable after threshing and pneumatic separation (group C) and after redrying (group D). These results suggest that hot-air moistening induced substantial alterations in

metabolite accumulation patterns, resulting in an overall trend characterized by an initial decline followed by stabilization.

PCA was performed based on the 568 identified non-volatile metabolites. As shown in Fig. 2C, principal components 1 and 2 explained 53.10% and 27.13% of the total variance, respectively. The four groups were clearly separated in the PCA score plot, with group A showing the greatest separation from the other groups. These results demonstrate that the non-volatile metabolite profiles were substantially remodeled throughout threshing and redrying. Among the processing operations, hot-air moistening exerted the strongest influence on metabolite composition, followed by redrying. Based on their overall variation trends across the four stages, the 12 metabolite classes were categorized into three groups. The first group included alkaloids, terpenoids, flavonoids, lignans and coumarins, and other metabolites, which generally decreased during processing. However, alkaloids, terpenoids, and miscellaneous compounds showed slight increases after threshing and pneumatic separation. The second group consisted of amino acids and derivatives, lipids, phenolic acids, organic acids, steroids, and quinones, which generally increased throughout processing, although the increases in lipids and phenolic acids were relatively limited. The third group comprised nucleotides and derivatives, which showed an initial decrease followed by an increase, suggesting stage-dependent changes in tobacco metabolic accumulation.

3.1.2 Differences analysis of non-volatile metabolites based on OPLS–DA

To further investigate variations in non-volatile metabolite profiles across processing stages, supervised OPLS–DA was performed to maximize discrimination between sample groups (Sun et al., 2025).

Clear separation was observed in all pairwise comparisons (Fig. 3). The model parameters were as follows: A versus B, $Q^2 = 0.999$, $R^2X = 0.891$, and $R^2Y = 1.000$; A versus C, $Q^2 = 0.999$, $R^2X = 0.902$, and $R^2Y = 1.000$; A versus D, $Q^2 = 0.998$, $R^2X = 0.963$, and $R^2Y = 1.000$; B versus C, $Q^2 = 0.990$, $R^2X = 0.804$, and $R^2Y = 1.000$; B versus D, $Q^2 = 0.998$, $R^2X = 0.892$, and $R^2Y = 1.000$; and C versus D, $Q^2 = 0.996$, $R^2X = 0.839$, and $R^2Y = 1.000$. All models showed Q^2 values greater than 0.9, and permutation validation confirmed their robustness and reliability for identifying differential metabolites.

Using $FC \geq 1.5$, $P < 0.05$, and $VIP \geq 1.5$ as screening criteria, 89 key differential metabolites were identified (Fig. 4A and B), including 23 terpenoids, 14 alkaloids, 13 flavonoids, 9 lignans and coumarins, 8 amino acids and derivatives, 7 lipids, 4 organic acids, 4 nucleotides and derivatives, 3 phenolic acids, 3 others, and 1 quinone. In pairwise comparisons, "upregulated" and "downregulated" indicate significantly higher and lower relative abundances in the later processing group, respectively (Figs. 4C-E and 5D; Table S1). Compared with group A, groups B, C, and D exhibited pronounced alternations in alkaloids, lipids, terpenoids, and flavonoids, indicating continuous remodeling of these major metabolite classes throughout processing. The comparison between groups A and B predominantly reflected metabolic

changes induced by hot-air moistening, whereas those between group A and groups C or D represented the cumulative effects of successive processing operations.

Between groups A and B, 20 metabolites were significantly upregulated, including methyltestosterone, L-ornithine, pachypodol, 5,6-DHET, N-(8-hydroxyoctyl) nornicotine, and N-decanoylnornicotine. These differential metabolites were mainly associated with alkaloids, terpenoids, and lipids. In contrast, 18 metabolites were significantly downregulated, including cyclic AMP, 3-ethyl-7-hydroxyphthalide, 3-hydroxy-3-methylglutarate, dendroside G, and malonyl fraxin. These metabolites were primarily associated with organic acids, phenolic acids, and flavonoids. Taken together, these findings indicate that hot-air moistening induced substantial remodeling of nitrogen-containing compounds, lipids, and phenolic metabolites. Among these, alkaloids exhibited the most pronounced changes. In particular, the increased abundance of nicotine-related derivatives, such as N-(8-hydroxyoctyl) nornicotine and N-decanoylnornicotine, suggests that nicotine-associated metabolic pathways were modified during moistening. Nicotine and nornicotine derivatives contribute to tobacco pungency and physiological strength and may also serve as precursors for subsequent aroma-related transformations. Their accumulation changes may be associated with increased enzymatic activity under elevated moisture and temperature conditions during moistening (LU et al., 2020). Terpenoids are important precursors of tobacco aroma compounds and can generate aroma-active volatiles, such as β -ionone and β -damascenone, through thermal degradation. Moreover, alterations in lipid-related metabolites may contribute to the generation of aldehydes and ketones through lipid oxidation. Therefore, the increased abundance of terpenoid- and lipid-related metabolites during hot-air moistening may provide precursor pools for subsequent aroma development during thermal processing (Chen et al., 2025).

The comparison between groups B and C revealed that differential metabolites were predominantly downregulated during threshing and pneumatic separation. Only five metabolites, including isoschaftoside, delphinidin 3-(6-p-coumaroyl) glucoside, lysophosphatidylcholine 16:1, O-feruloyl-7-hydroxycoumarin, and protoaescigenin, were significantly upregulated. In contrast, 16 metabolites were significantly downregulated, including terrestimine, acetamiprid, bis(caffeoyl)spermidine, vitexin 7-O-glucoside, cis-5'-(2-oxopropyl)-nicotine, and limocitrol 3-glucoside. These findings indicate that mechanical processing caused extensive redistribution and alteration of specific metabolites. Alkaloids showed the most substantial changes during threshing and pneumatic separation. The decreased abundance of several nicotine-related compounds may be associated with mechanical disruption of leaf tissues, which could facilitate their redistribution, degradation, or transformation. Several flavonoid glycosides, including vitexin 7-O-glucoside and limocitrol 3-glucoside, also decreased significantly, whereas some flavonoids, such as isoschaftoside and delphinidin 3-(6-p-coumaroyl) glucoside, increased. Given the antioxidant properties of flavonoids, these alterations may reflect a response to mechanical stress and oxidative changes induced during threshing and pneumatic separation (Budiati et al., 2026).

The comparison between groups C and D identified 19 significantly upregulated metabolites and six downregulated metabolites, indicating extensive remodeling of non-volatile metabolite profiles during

redrying. Terpenoids exhibited the most pronounced variations, with some compounds increasing while others decreasing, suggesting that high-temperature treatment may simultaneously promote degradation, transformation, and release of certain bound forms of terpenoid-related compounds. Phenolic compounds, including catechin gallate and glucosyringic acid, also increased significantly. Under high-temperature conditions, these compounds may undergo oxidation, polymerization, and interactions with sugar degradation products and Maillard reaction intermediates, thereby contributing to changes in tobacco color and flavor characteristics (Liu et al., 2022).

Overall, the non-volatile metabolome exhibited distinct stage-dependent variations during industrial threshing and redrying. Hot-air moistening primarily affected alkaloid-, lipid-, and terpenoid-related metabolites and contributed to the accumulation of potential aroma precursors. Threshing and pneumatic separation were associated with broad decreases in alkaloids, flavonoids, and phenolamides, suggesting alterations in metabolites related to mechanical stress and oxidative regulation. Redrying further promoted the transformation of terpenoids, phenolic compounds, lignans, and other secondary metabolites, thereby contributing to aroma development and the formation of final tobacco quality characteristics.

3.1.3 Metabolic pathway enrichment analysis

KEGG enrichment analysis was performed to investigate the biological pathways associated with differential metabolites during hot-air moistening, threshing and pneumatic separation, and redrying. Overall, the differential metabolites were mainly associated with amino acid metabolism, secondary metabolite biosynthesis, and antioxidant-related pathways.

The differential metabolites between groups A and B were significantly enriched in Metabolic pathways, Biosynthesis of amino acids, 2-Oxocarboxylic acid metabolism, Arginine biosynthesis, Arginine and proline metabolism, and Alanine, aspartate and glutamate metabolism. In addition, secondary metabolic pathways, including Biosynthesis of secondary metabolites, Biosynthesis of plant secondary metabolites, and Biosynthesis of phenylpropanoids, were also enriched. The strong enrichment of amino acid-related pathways indicates that hot-air moistening substantially affected the accumulation and transformation of nitrogen-containing metabolites in tobacco leaves (Forde & Lea, 2007). The 2-oxocarboxylic acid metabolic pathway provides precursors associated with alkaloid biosynthesis (Zhou et al., 2024). The increased moisture content and temperature during moistening may facilitate metabolic adjustments in tobacco leaves, resulting in changes in amino acid composition. These altered amino acid pools may influence Maillard reaction-related precursor availability during subsequent high-temperature processing. Furthermore, the enrichment of pathways including Biosynthesis of phenylpropanoids and Biosynthesis of plant secondary metabolites during the moistening stage indicates that hot-air moistening influenced not only primary metabolism but also secondary metabolic processes associated with tobacco quality. Phenylpropanoid metabolism contributes to the production of phenolic compounds, lignin-related metabolites, and aroma-related substances, which may affect the sensory characteristics of tobacco products.

Differential metabolites between groups B and C were mainly enriched in Aminobenzoate degradation, Anthocyanin biosynthesis, Glutathione metabolism, ABC transporters, Arginine and proline metabolism, 2-Oxocarboxylic acid metabolism, Biosynthesis of plant secondary metabolites, and Metabolic pathways. Among these pathways, Glutathione metabolism exhibited pronounced enrichment. Mechanical collision and tissue disruption during threshing and pneumatic separation may disturb cellular integrity and promote the accumulation of reactive oxygen species (ROS), thereby activating the glutathione antioxidant system to maintain cellular redox homeostasis (Hasanuzzaman, Nahar, Anee, & Fujita, 2017). This suggests that redox-related metabolic processes may participate in the response of tobacco leaves to mechanical stress. Thus, Glutathione metabolism may serve as a metabolic indicator associated with oxidative regulation during mechanical processing. In addition, metabolites related to Anthocyanin biosynthesis were specifically enriched at this stage. Anthocyanins are flavonoid compounds whose accumulation is often associated with mechanical injury and oxidative stress responses in plants. Although mature tobacco leaves generally contain relatively low levels of anthocyanins, the enrichment of this pathway suggests alterations in the phenylpropanoid-flavonoid metabolic network. Such changes may contribute to antioxidant regulation, modification of phenolic compound composition, and subsequent effects on tobacco quality formation (Fresquet-Corrales et al., 2017).

Differential metabolites between groups C and D were primarily enriched in pathways related to Biosynthesis of secondary metabolites, Biosynthesis of various plant secondary metabolites, Glutathione metabolism, Biosynthesis of alkaloids derived from ornithine, lysine, and nicotinic acid, Arginine biosynthesis, Arginine and proline metabolism, 2-Oxocarboxylic acid metabolism, Biosynthesis of amino acids, and Metabolic pathways. The highly enriched Biosynthesis of secondary metabolites and Biosynthesis of various plant secondary metabolites pathways indicates that numerous secondary metabolites underwent degradation, release, or reaccumulation during redrying. These changes are closely associated with aroma formation, reduced pungency, and improved sensory quality. Collectively, differential metabolites identified at all three processing stages were consistently enriched in general metabolic pathways (Liu et al., 2024), demonstrating that threshing and redrying induced systematic remodeling of the tobacco metabolic network. Amino acid-related pathways, including Biosynthesis of amino acids, 2-Oxocarboxylic acid metabolism, Arginine biosynthesis, and Arginine and proline metabolism, were repeatedly enriched throughout the processing stages. In contrast, Glutathione metabolism and Biosynthesis of secondary metabolites pathways became increasingly prominent during the later stages. These findings reveal a continuous regulatory sequence involving amino acid metabolic remodeling, antioxidant defense, and secondary metabolite reprogramming. Hot-air moistening primarily stimulated metabolic activation and aroma precursor accumulation, whereas threshing and pneumatic separation triggered oxidative and defense responses associated with mechanical damage. Redrying further promoted the transformation of amino acids and secondary metabolites, ultimately contributing to aroma development and the final tobacco quality.

3.2 GC–IMS detection of volatile compounds

3.2.1 HS–GC–IMS topographic plots

GC–IMS was employed to characterize changes in volatile compound profiles during threshing and redrying. The topographic plots of volatile compounds at different processing stages are shown in Fig. 6A. To facilitate comparison, the spectrum of group A served as the reference, and the spectra of groups B, C, and D were subtracted from the reference spectrum to generate difference plots (Fig. 6B). In these plots, a white background indicates no difference in volatile abundance between the target sample and group A, whereas red and blue signals indicate higher and lower abundances, respectively. Most signals were distributed within retention times of 100–600 s and drift times of 1.0–1.8 s. Numerous red and blue signals were observed in groups B, C, and D, with the greatest differences detected in group D. These results demonstrate substantial variations in both the composition and relative abundance of volatile compounds throughout processing, particularly during redrying. Such dynamic transformations collectively contributed to the development of the characteristic flavor profile of flue-cured tobacco.

A total of 102 characteristic peaks corresponding to 77 volatile compounds were identified, including 19 aldehydes, 17 alcohols, 11 ketones, 9 esters, 8 heterocyclic compounds, 5 terpenoids, 4 acids, 3 sulfur-containing compounds, and 1 aromatic hydrocarbon. Several volatile compounds generated multiple characteristic peaks, possibly due to their high proton affinity or the formation of dimeric and trimeric ions within the drift tube (Brendel, Rohn, & Weller, 2021). Examples included 1-penten-3-one, (E)-2-butenal, 1-hexanal, and (E)-2-methyl-2-butenal. Fingerprint analysis was subsequently performed to visualize stage-dependent variations in volatile compound distribution (Fig. 6C). Group A was characterized by relatively high abundances of butyl 2-propenoate, butyl acetate, butyl butanoate, butanol, diethyl disulfide, 2-ethyl hexanol, 3-methyl-1-pentanol, 4-methyl-2-pentanone, cyclohexanone, 1-nonanal, (E)-2-methyl-2-butenal, heptanol, 2-butanol, 1-hexanol, β -pinene, (+)-aromadendrene, 1-pentanol-4-methyl, 5-methyl furfural, (Z)-2-methylpent-2-enal, 3-methylbutyric acid, 1-pentanol, and 3-methyl-2-butenal. In group B, compounds such as 3-penten-2-one, hexyl acetate, 2-furanmethanol, 1-octen-3-one, 2-acetylfuran, 2,4-dimethylthiazole, diisopropyl disulfide, ethyl octanoate, 2-methyl propanoic acid, γ -butyrolactone, hydroxyacetone, 1-hexanal, propanoic acid, (E)-2-hexenal, and 2-methyl-2-butenal showed relatively high abundances. Group C was distinguished by the enrichment of α -tolualdehyde and 3-octen-2-one. Group D exhibited elevated levels of 1-penten-3-ol, (Z)-2-pentenol, 2-methyl-2-hepten-6-one, isobutyl acetate, 2-butanone, 3-methyl-3-buten-1-ol, 2-methyl-1,3-dithiolane, 2-pentyl furan, and isoamyl acetate.

Following hot-air moistening, the relative abundances of several esters and alcohols decreased, whereas those of aldehydes, ketones, heterocyclic compounds, and sulfur-containing compounds increased. The simultaneous increases in temperature and moisture may promote lipid oxidation, Maillard reactions, and other thermally induced transformations, thereby facilitating the formation and accumulation of volatile compounds associated with green, caramel-like, nutty, and roasted aromas and driving the transition from the aroma of unprocessed tobacco to a more distinct flavor profile (Koiwai & Kisaki, 1979; Zhou et al., 2024).

In contrast, threshing and pneumatic separation induced relatively minor changes in volatile composition. Only a limited number of compounds, such as α -tolualdehyde and 3-octen-2-one, accumulated during this stage. This indicates that threshing and pneumatic separation primarily function as physical separation processes with a limited impact on aroma composition. Consequently, the aroma profile remained relatively stable during this stage, consistent with previous observations (2025).

Redrying further promoted the accumulation of ketones, alcohols, heterocyclic compounds, sulfur-containing compounds, and esters. Prolonged thermal treatment likely accelerated the generation, transformation, and release of volatile compounds. Ketones and heterocyclic compounds can contribute citrus-like, caramel-like, roasted, and nutty notes, thereby increasing aroma diversity (Guan et al., 2022; Shen et al., 2023). Collectively, these results indicate that hot-air moistening represents a critical stage for the transformation of volatile aroma compounds, whereas threshing and pneumatic separation exert comparatively limited effects. Redrying further facilitates the accumulation and remodeling of aroma-active compounds, ultimately generating a richer aroma profile.

3.2.2 Differential analysis of volatile compounds based on OPLS–DA

OPLS–DA was performed to further characterize differences in the volatile compound profiles of tobacco leaves collected at different processing stages. The OPLS–DA score plot showed clear separation among the four groups, with biological replicates within each group exhibiting tight clustering (Fig. 7A). These results indicate that the volatile compound composition was substantially altered during threshing and redrying. The cumulative values of R^2X , R^2Y , and Q^2 for the model were 0.969, 0.980, and 0.919, respectively, demonstrating satisfactory explanatory and predictive performance. Model reliability was further assessed using 200 permutation tests (Fig. 7B). The intercepts of the R^2 and Q^2 regression lines were 0.537 and -1.400 , respectively. The negative Q^2 intercept and the lower Q^2 values obtained from permutation models compared with the original model indicated that the model was stable and not overfitted.

Differential volatile compounds were initially screened based on $VIP > 1.5$ and subsequently validated using one-way ANOVA with $P < 0.05$ as the significance threshold. OAV analysis was then applied to evaluate the potential contribution of 18 differential volatile compounds to the overall aroma and to identify key stage-specific aroma compounds (Fig. 7C and Table S2) (Grosch, 2001). Among the identified differential volatile compounds, butyl 2-propenoate exhibited the highest OAV before moistening (525.56), which was significantly higher than those observed at subsequent processing stages ($p < 0.001$). This finding suggests that butyl 2-propenoate may represent an important contributor to the aroma characteristics of untreated tobacco leaves. Butyl 2-propenoate has been reported to possess fruity and pungent odor characteristics and may contribute to fruity aroma notes in tobacco leaves (Jia et al., 2025). During subsequent processing, its OAV decreased rapidly to 17.79–38.77,

representing a reduction of more than 90%. This decline indicates that its contribution to aroma formation was substantially reduced after processing. The decrease may be attributable to ester volatilization, degradation, or transformation during thermal treatment, which may contribute to changes in aroma characteristics from fresh and fruity notes toward sweeter and more roasted attributes (Wang et al., 2025).

The OAVs of (E)-2-hexenal, 2-methyl-2-hepten-6-one, (E)-2-pentenal, hydroxyacetone, 3-methyl-1-butanol, and pentanal remained above 1 throughout all processing stages, indicating that these compounds likely contributed significantly to the overall aroma profile (Ferreira, López, & Cacho, 2000). Specifically, (E)-2-hexenal is associated with green, banana-like, and fatty notes; 2-methyl-2-hepten-6-one contributes citrus-like and fruity odor characteristics; (E)-2-pentenal is associated with potato- and pea-like odors; hydroxyacetone contributes caramel-like and fresh notes; 3-methyl-1-butanol is associated with banana-like and sweet aromas; and pentanal imparts green, grassy, slightly banana-like, and pungent characteristics. In addition, carvone maintained an OAV above 1 throughout processing, suggesting a continuous contribution of mint-like aroma characteristics. Benzaldehyde, which is associated with bitter almond, cherry-like, and nutty aromas, may further contribute to sweet and nutty aroma attributes. Together, these key volatile compounds contributed to the development of fruity, green, grassy, caramel-like, nutty, and roasted aroma characteristics during threshing and redrying, ultimately shaping the aroma profile of the processed tobacco strips.

4. Conclusion

An integrated UPLC–Q-TOF/MS and GC–IMS approach was applied to systematically characterize metabolic and volatile compound changes in tobacco leaves during industrial threshing and redrying. A total of 568 non-volatile metabolites and 77 volatile compounds were identified. Among the processing stages, hot-air moistening represented the initial and most influential stage driving metabolic remodeling and volatile compound transformation. Although the total relative abundance of non-volatile metabolites decreased markedly, amino acid, lipid, and terpenoid metabolism was substantially regulated, providing potential precursors for subsequent aroma formation. KEGG pathway analysis further indicated that hot-air moistening mainly affected amino acid metabolism and secondary metabolite biosynthesis. Meanwhile, the volatile profile underwent pronounced changes, characterized by reductions in esters and certain alcohols and increases in aldehydes, ketones, and heterocyclic compounds, resulting in a transition from green and grassy notes toward caramel-like, sweet, and roasted aromas. Threshing and pneumatic separation primarily involved physical separation and had a relatively limited impact on the overall metabolite composition and aroma profile. Nevertheless, mechanical damage during this stage appeared to induce oxidative stress responses, as indicated by the enrichment of antioxidant-related pathways, particularly glutathione metabolism. Redrying represented a key stage for aroma maturation and enrichment, promoting the transformation of terpenoids, phenolic compounds, lignans, and other secondary metabolites, as well as heat-induced reactions associated with aroma generation. Consequently, ketones, heterocyclic compounds, sulfur-containing compounds, and certain esters accumulated, contributing to caramel-like, nutty, roasted, and sweet aroma attributes and enhancing

aroma complexity and harmony. Overall, hot-air moistening, threshing and pneumatic separation, and redrying constituted sequential and complementary processing stages that collectively determined the final quality of tobacco strips. This study was limited to tobacco leaves processed under a single set of industrial conditions. Meanwhile, the effects of individual process parameters, including moistening temperature, leaf moisture content, threshing intensity, and redrying temperature, were not systematically evaluated. Future studies integrating process optimization with metabolomic and sensory analyses are needed to establish predictive relationships between processing conditions, metabolic changes, and tobacco quality, thereby supporting precision processing and quality-oriented control during industrial threshing and redrying.

Declarations

Ethics declarations

Not applicable.

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare no conflicts of interest.

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Authors' contributions

Yangyang Yu: Investigation and Writing – original draft; Rongqian Shi and Xiangzhen Liu: Resources, Formal analysis; Pengyu Yang and Yangyang Niu: Methodology, Investigation; Mengyao Sun and Qi Wang: Conceptualization, Data curation. Yongfeng Yang and Hongli Chen: Supervision and Writing – review & editing. All authors read and approved the final manuscript.

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Data availability

The data reporting the findings of this study are contained in this article; further queries could be made to the corresponding author(s).

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Figures

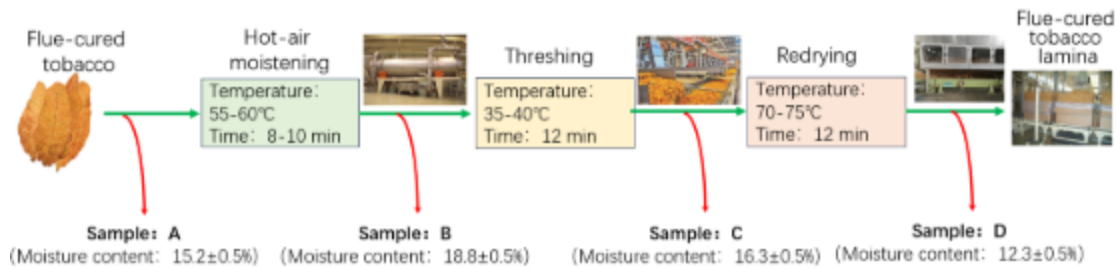


Figure 1

Industrial threshing and redrying process for flue-cured tobacco leaves.

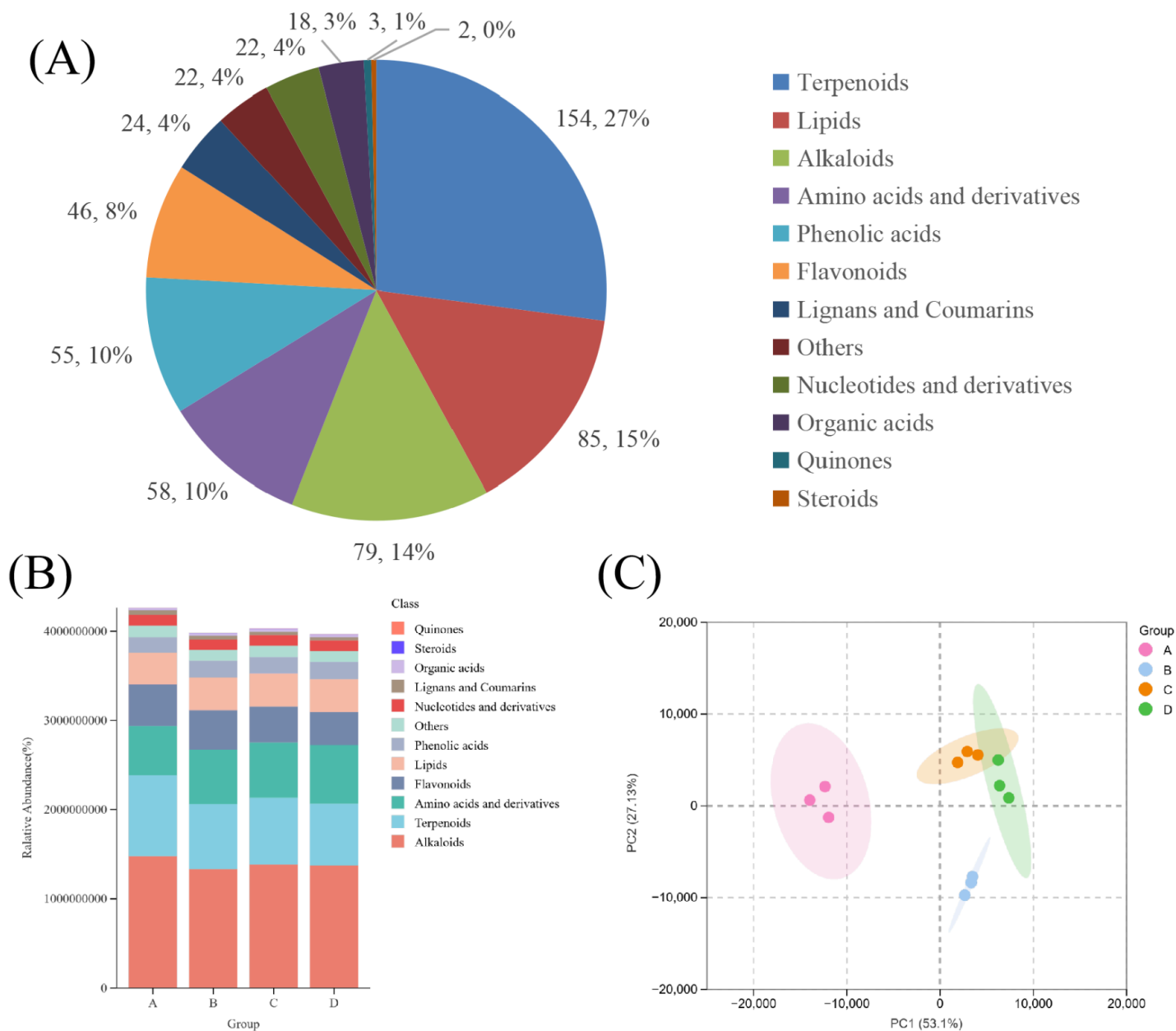


Figure 2

Classification of non-volatile metabolites (A), stacked bar chart of relative metabolite abundance (B), and PCA score plot (C) of tobacco leaf samples collected at different processing stages.

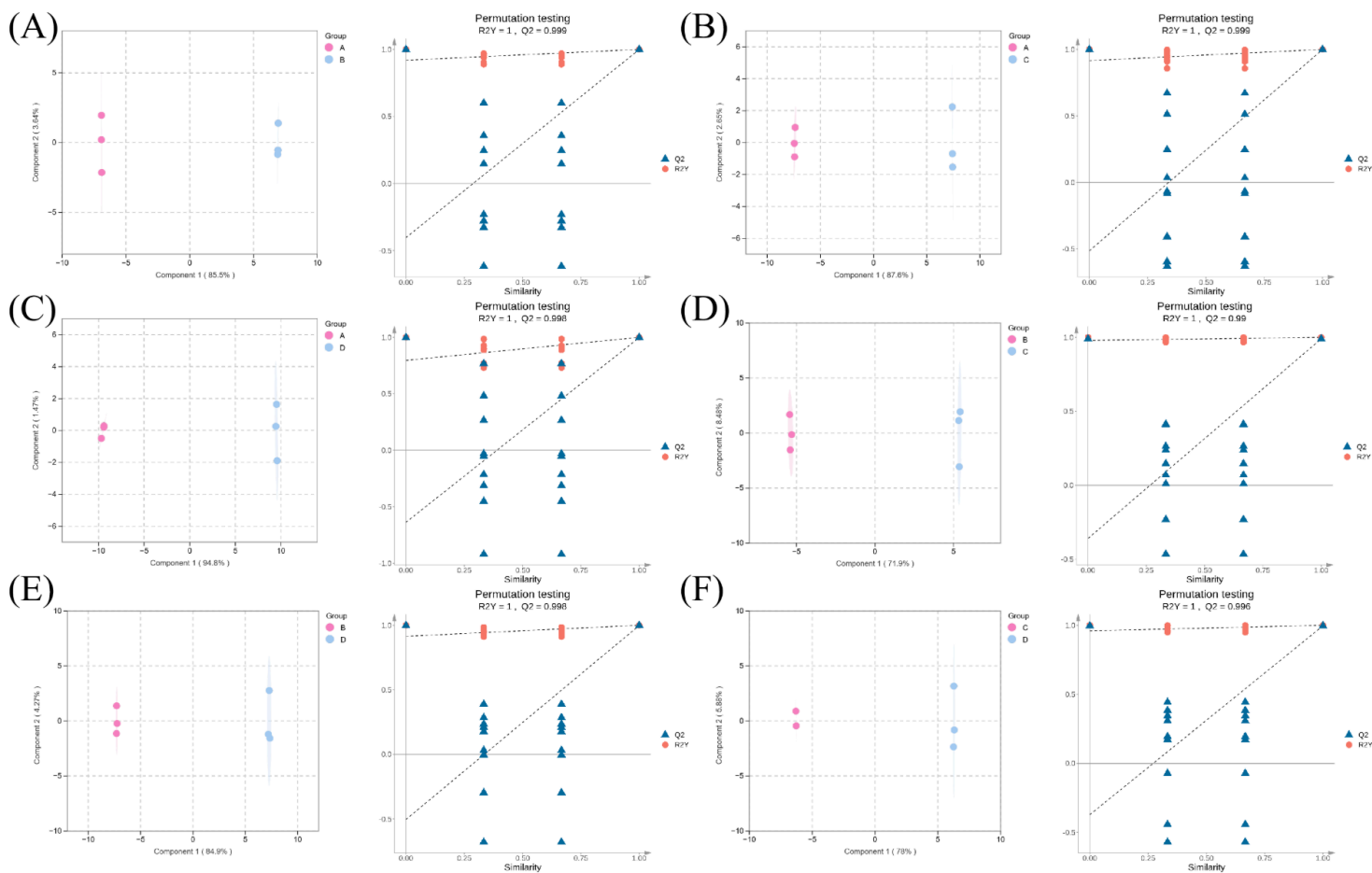


Figure 3

Pairwise OPLS-DA score plots and permutation tests of non-volatile metabolites in tobacco leaf samples at different processing stages: A versus B (A), A versus C (B), A versus D (C), B versus C (D), B versus D (E), and C versus D (F).

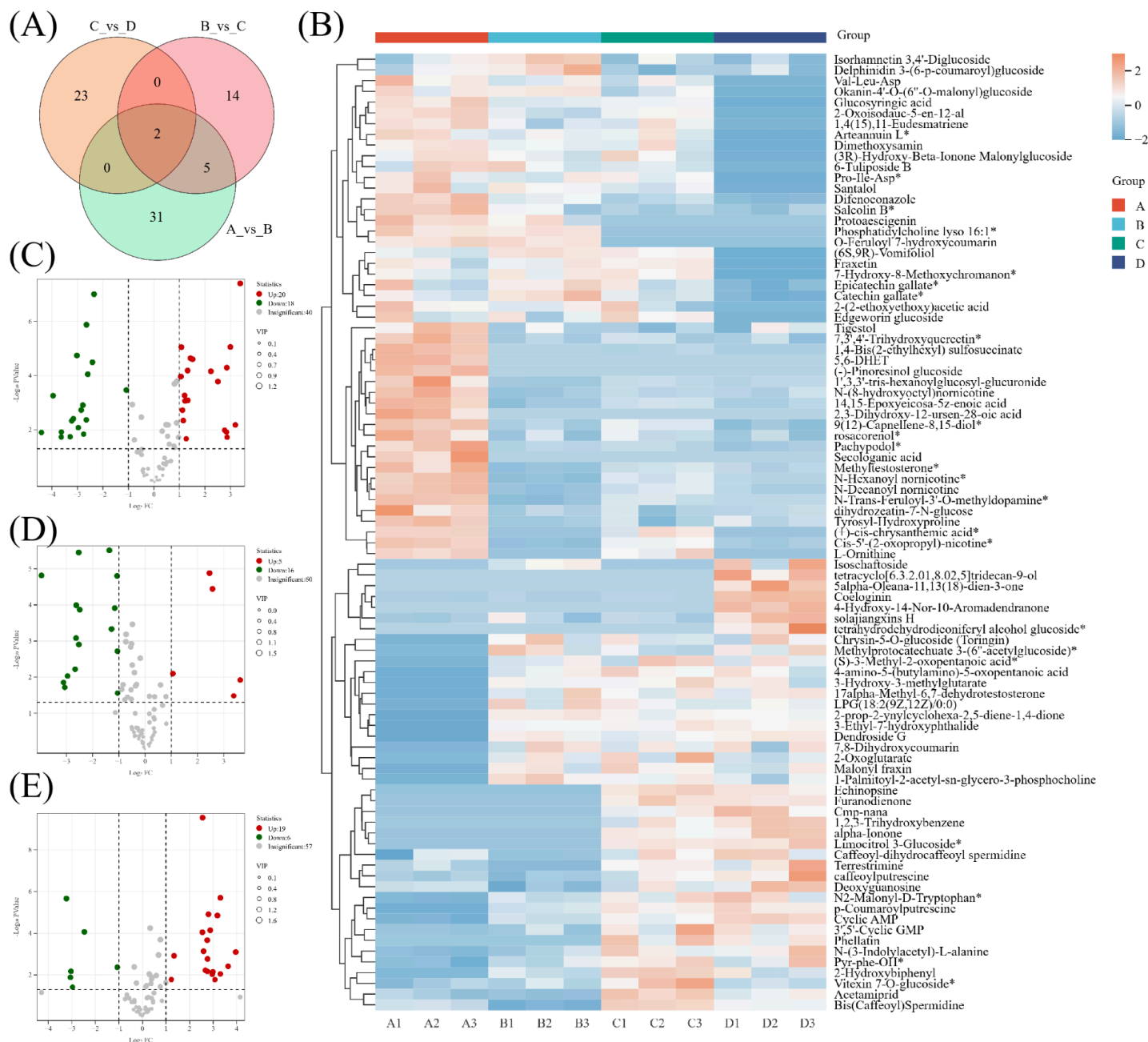


Figure 4

Venn diagram (A), heatmap (B), and volcano plots of differential non-volatile metabolites in tobacco leaves from the pairwise comparisons: A versus B (C), B versus C (D), and C versus D (E).

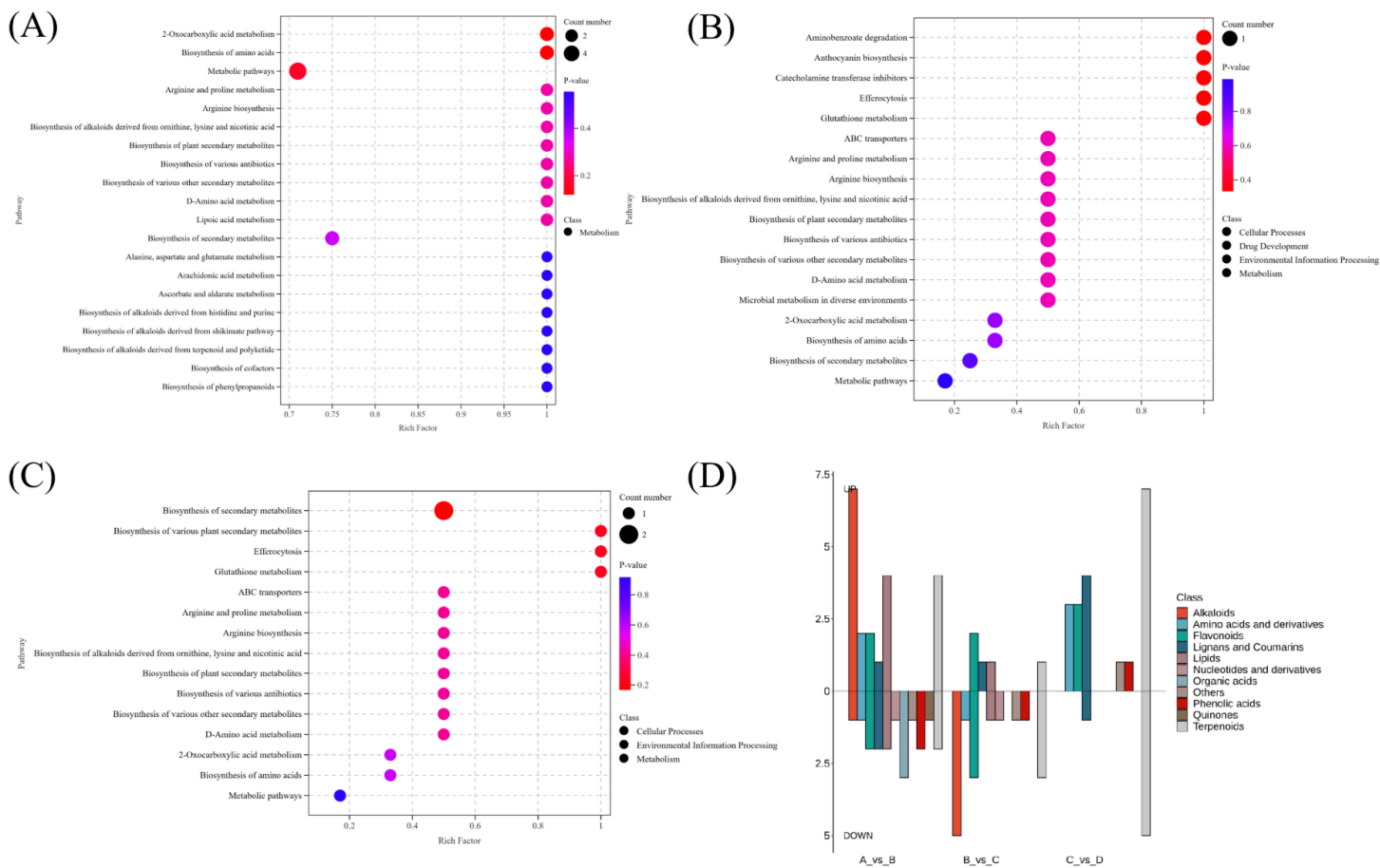


Figure 5

KEGG pathway enrichment analysis of differential non-volatile metabolites: A versus B (A), B versus C (B), and C versus D (C), along with a mirrored bar chart showing the classification of differential metabolites (D).

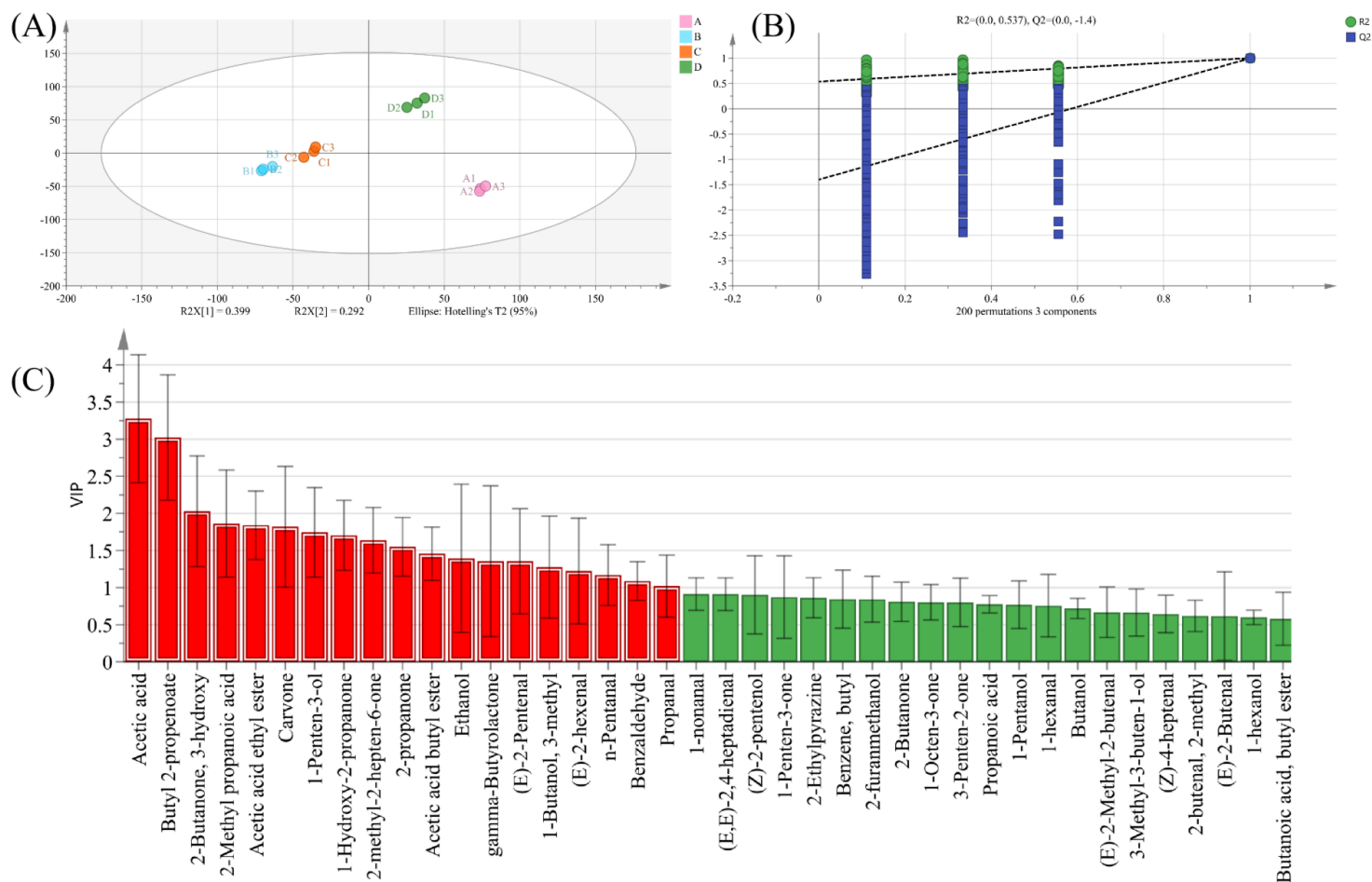


Figure 7

OPLS-DA score plot (A), permutation test (B), and VIP score plot (C) of volatile compounds in tobacco leaf samples collected at different processing stages.

Supplementary Files

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