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Integration of GC-MS, ROAV, and chemometrics to characterize key differential volatile compounds in wild and cultivated blueberries from Northeast China

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ABSTRACT

The flavor of blueberries influences consumers' purchasing behavior and satisfaction, holding significant research value for enhancing market competitiveness. In this study, we analyzed the volatile metabolites of Wild blueberry (Wb) and Cultivated blueberry (Cb) in Northeast China (NC) using headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS), identifying 618 volatile organic compounds (VOCs). Through multivariate statistical analysis, 140 volatile differential compounds were detected between Wb and Cb, including *trans*-alpha-Bergamotene; 2-ButenoicAcid,2-methyl-; Benzoic acid,2-hydroxy-, ethylester; Benzoic acid, ethyl ester, above 4 VOCs were unique to Wb; while 28 VOCs, such as Carotol and 1-Hexanol, were unique to Cb. KEGG enrichment analysis indicated that α-Linolenic acid metabolism and Inflammatory mediator regulation of TRP channels are the key pathways for volatile differential substance formation. By employing relative odor activity value (ROAV) and chemometrics, 131 key aroma-presenting substances were identified. Among them, β-Ionone, 2(5H)-Furanone, 5-ethyl-3-hydroxy-4-methyl- with sweet and fruity aroma were the most crucial. Following comprehensive, in-depth analysis by HS-SPME-GC-MS and ROAV, Carotol and 1-Hexanol were found to be useful as biomarker compounds for distinguishing Wb and Cb. This study provides a theoretical basis for distinguishing the aroma characteristics of Wb and Cb in NC, the processing adaptability of different varieties of blueberries.

1. Introduction

Blueberry (Ericaceae *Vaccinium*), a nutrient-rich small-berry fruit, has been extensively studied for its health benefits (Wang et al., 2023). Substantial research evidence indicates that regular consumption of fresh blueberries and their derived products contributes significantly to human health improvement (Tobar Bolaños et al., 2021). The global blueberry industry has witnessed remarkable development, particularly in China, according to the report of the International Blueberry Organization (2024), which has maintained its position as the world leader in both cultivation area and production volume for four consecutive years. Among China's blueberry production regions, the Northeast stands out as the primary cultivation base and as a region rich in wild resources, thanks to its ideal environmental conditions. This strategic advantage enables Northeast China (NC) to supply premium fresh blueberries and a diverse range of value-added products, including juices, jams, and wines, to both domestic and international markets (Liu et al., 2022).

Flavor, as a crucial sensory attribute of blueberries, serves as a pivotal determinant in both the quality evaluation and gustatory experience of fresh berries while simultaneously exerting substantial influence on the ultimate quality and market viability of processed blueberry products (Gilbert et al., 2014). Significant divergence exists in flavor profiles between wild blueberry (Wb) and cultivated blueberry (Cb). While cultivated varieties, through artificial selection and optimization, typically exhibit more uniform flavor characteristics aligned with

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Abbreviations

HS-SPME-GC-MS	Headspace solid-phase microextraction gas chromatography-mass spectrometry
ROAV	Relative odor activity value
rOAVs	Relative odor activity values
Wb	Wild blueberry
Cb	Cultivated blueberry
VOCs	Volatile organic compounds
PCA	Principal component analysis
OPLS-DA	Orthogonal partial least squares-discriminant analysis
HCA	Hierarchical cluster analysis
FC	Fold change
QC	Quality control
NC	Northeast china
VIP	Variable importance projection

mainstream consumer preferences, Wb often attracts consumers seeking distinctive taste experiences and unique flavor profiles. Notably, wild species represent invaluable germplasm resources for modern cultivar breeding (Long et al., 2024). Comparative studies have demonstrated that lowbush Wb possesses a more pronounced and complex aromatic profile than their highbush-cultivated counterparts (Forney et al., 2022). Furthermore, the specific aroma requirements for raw materials vary significantly across different processed blueberry products in the market, necessitating comprehensive investigation into the flavor characteristics of both Wb and Cb.

Blueberry aroma primarily derives from volatile organic compounds, including terpenoids, esters, and aldehydes (Ferrão et al., 2022), with variations in their composition and concentration serving as fundamental determinants of inter-cultivar aroma differences. Identifying and analyzing key differential volatile compounds in Wb and Cb are essential for elucidating the underlying factors contributing to their distinct flavor profiles. However, the specific flavor components and their biosynthetic pathways responsible for these differences remain insufficiently characterized. This knowledge gap impedes the molecular-level optimization of blueberry flavor regulation and consequently restricts the full development of the blueberry market.

The headspace solid-phase microextraction (HS-SPME) technique, renowned for its operational simplicity, high sensitivity, rapid analysis, cost-effectiveness, and environmental friendliness, has gained widespread application in volatile compound analysis (Hao et al., 2023). When combined with HS-SPME and relative odor activity value (ROAV) analysis, it enables comprehensive characterization of volatile flavor profiles in various fruits, as demonstrated in studies of South Fruit Pear and Grape (Liu et al., 2023; Warner et al., 2023). Furthermore, metabolomics, a crucial component of systems biology, facilitates the simultaneous detection of numerous endogenous metabolites and has been extensively applied in food science research (Dai et al., 2017; Zhang et al., 2024).

This study pioneers a systematic investigation of volatile metabolome differences between Wb and Cb in NC, employing an integrated HS-SPME-GC-MS and non-targeted metabolomics approach. Combining ROAV analysis with chemometric methods, we aim to identify characteristic flavor compounds and elucidate their biosynthetic pathways through flavoromics and metabolomics integration. This study not only provides a scientific basis for the effective identification of blueberry varieties, but also establishes a reliable metabolomics assay for the quality control and adulteration identification of blueberries and their processed products.

2. Materials and methods

2.1. Samples and chemicals

The blueberry varieties utilized in this study comprised Wb and three cultivated varieties: Bluecrop (Blu), Reka (Rek), and Northland (Nor). Fig. 1a show where to pick the four blueberry varieties. The fruits were collected at peak ripeness, and only those exhibiting uniform size, color, and maturity, free from mechanical damage, were selected for the experiment. Immediately after harvest, the fruits were transported to the laboratory on dry ice and stored in a −80 °C freezer for preservation. The experimental design divided the four blueberry varieties into four distinct groups, each subjected to experimental analysis with six replicates per group. Sodium chloride (NaCl), of analytical purity, was procured from Thermo Fisher Scientific (Massachusetts, USA). Hexane and the standard compound (3-hexanone), both of chromatographic purity, were obtained from Merck (Darmstadt, Germany). The standard solution was prepared using hexane as the solvent and stored at −20 °C to maintain stability and integrity for subsequent analytical procedures.

2.2. Optimization of the HS-SPME technique

500 mg (1 mL) of the powder was transferred immediately to a 20 mL headspace vial (Agilent, Palo Alto, CA, USA) containing a NaCl-saturated solution to inhibit any enzyme reaction. The vials were sealed using crimp-top caps with TFE-silicone headspace septa (Agilent). At the time of SPME analysis, each vial was placed at 60 °C for 5 min, then a 120 μm DVB/CWR/PDMS fibre (Agilent) was exposed to the sample's headspace for 15 min at 60 °C.

2.3. Analysis of volatiles using GC-MS

After sampling, desorption of the VOCs from the fibre coating was carried out in the injection port of the GC apparatus (Model 8890; Agilent) at 250 °C for 5 min in the splitless mode. The identification and quantification of VOCs were carried out using an Agilent Model 8890 GC and a 7000D mass spectrometer (Agilent) equipped with a 30 m × 0.25 mm × 0.25 μm DB-5MS (5 % phenyl-polymethylsiloxane) capillary column. Helium was used as the carrier gas at a 1.2 mL/min linear velocity. The injector temperature was kept at 250 °C. The oven temperature was programmed from 40 °C (3.5 min), increasing at 10 °C/min to 100 °C, at 7 °C/min to 180 °C, at 25 °C/min to 280 °C, and held for 5 min. Mass spectra were recorded in electron impact ionization mode at 70 eV. The quadrupole mass detector, ion source, and transfer line temperatures were set, at 150, 230, and 280 °C. The MS was selected, and the ion monitoring mode was used to identify and quantify analytes.

2.4. Calculation of rOAVs

Relative odor activity values (rOAVs) are a widely used method for identifying the key flavor compounds in food by integrating their sensory thresholds. This approach helps determine individual VOCs' contribution to a food product's overall aroma profile. To evaluate the impact of different VOCs on blueberry aroma, rOAVs were calculated using the compounds' sensory thresholds (Yang et al., 2022). In this study, the relative concentration of each volatile compound was determined by multiplying the concentration of the internal standard by the ratio of the peak area of the volatile compound to the peak area of the internal standard (Zhu et al., 2021):

$$rOAV_i = \frac{C_i}{T_i}$$

where $rOAV_i$ is the relative odor activity value of compound i, C_i is the relative amount of compound (μg/g or μg/mL), and T_i is the threshold value of compound (μg/g or μg/mL).

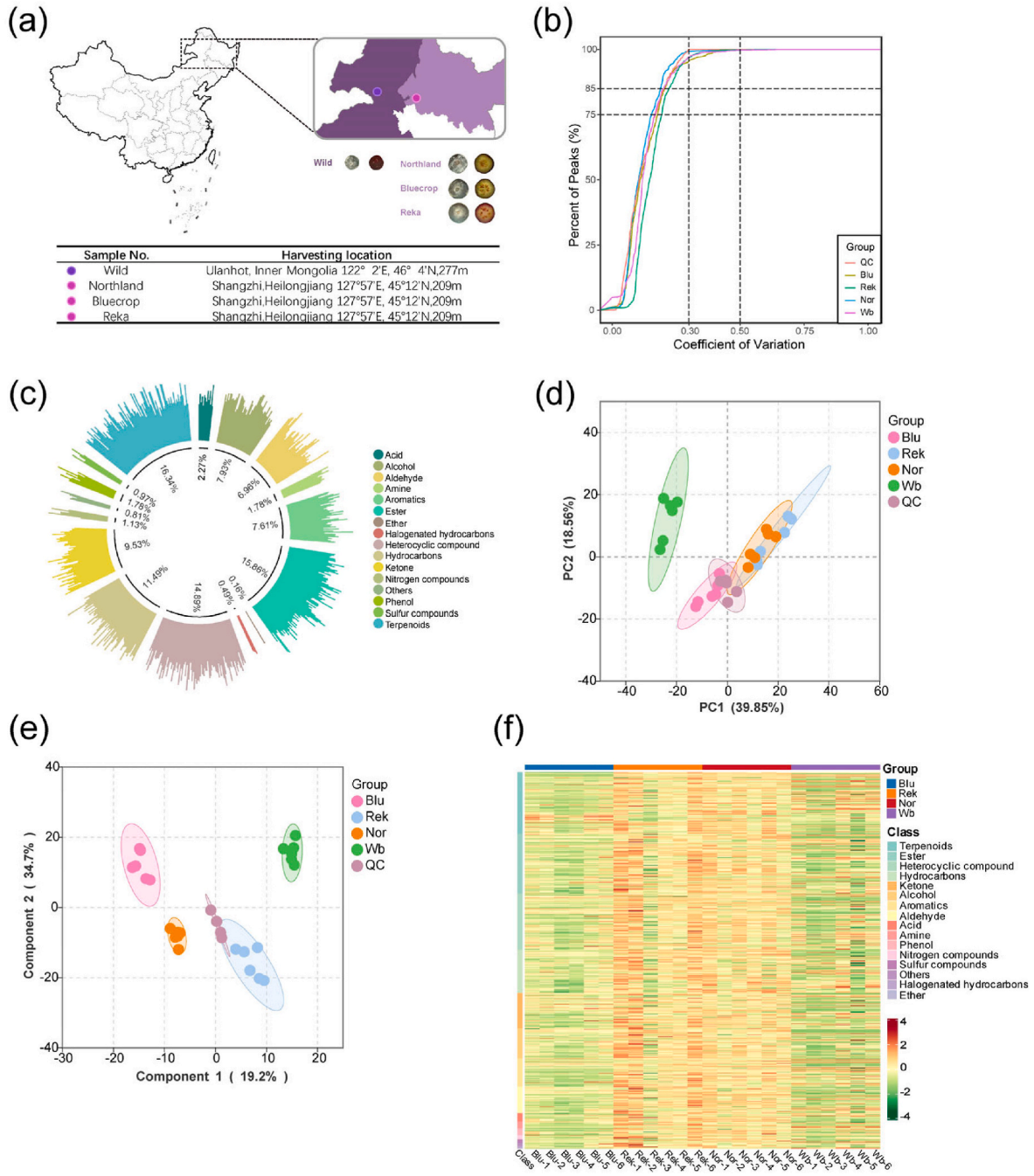

Fig. 1. Metabolite analysis of Wb and Cb.
(a) Geographical distribution diagram of 4 blueberry varieties from China. (b) CV plot. (c) Pie chart of classes and percentage of compounds identified in all samples. (d) PCA plot. (e) OPLS-DA diagram. (f) Cluster analysis heat map.

2.5. Multivariate statistical analysis

All measurements were performed six times, and additional data analysis and image processing were performed using Microsoft Excel 2016 (Microsoft, USA) and GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, USA). HS-SPME-GC-MS data were processed using Statistical R software, the Metabo Analyst R package, the Complex Heatmap package for Principal Component Analysis (PCA), Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) modeling, and hierarchical cluster analysis (HCA), and univariate statistical analyses including hypothesis testing and fold change (FC) analysis. Metabolite annotation was based on the KEGG compound database, and pathway mapping was performed through the KEGG pathway database.

3. Results and discussion

3.1. Overall metabolome analysis of various blueberries

3.1.1. Volatile metabolite profiling of blueberry varieties

VOCs in four blueberry cultivars (Wb, Blu, Rek, and Nor) were systematically analyzed using HS-SPME-GC-MS. As shown in Fig. 1b, the coefficient of variation (CV) reflects the degree of dispersion of the data. The proportion of quality control (QC) samples and all experimental groups with a CV value less than 0.3 is higher than 85 %, indicating that the experimental data are very stable. Six hundred and eighteen VOCs were identified, with 610, 613, 611, and 588 compounds detected in Blu, Rek, Nor, and Wb, respectively. The analysis revealed 16 significant

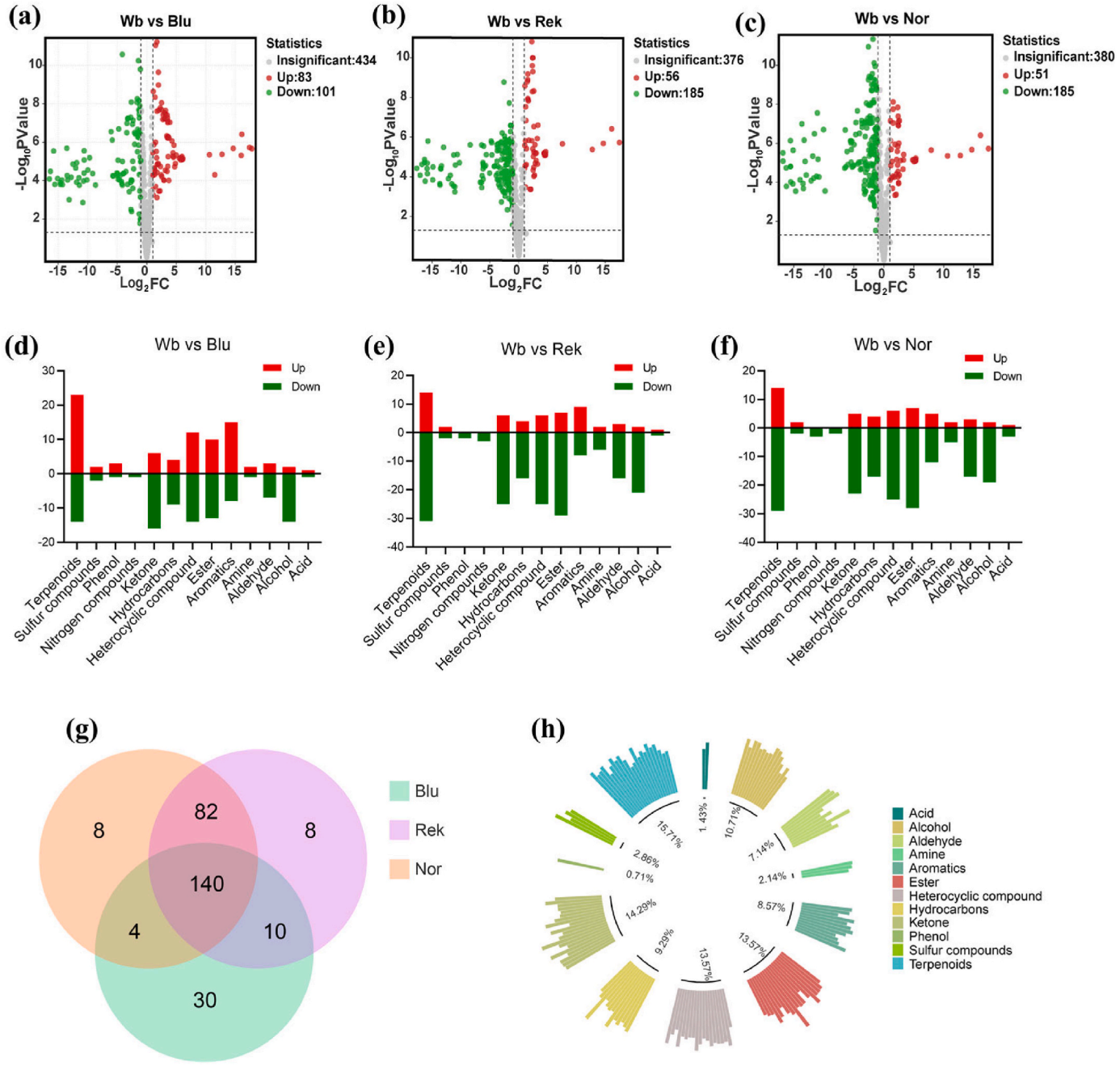

Fig. 2. Identification and analysis of differential VOCs between Cb and Wb in NC.
(a–c) volcano plots of differential metabolites of three comparison groups. (d–f) Different metabolites in three comparison groups. (g) Venn plots of differential metabolites among three comparison groups. (h) Pie chart of classes and percentage of compounds identified in all differential metabolites.

classes of volatile metabolites (Fig. 1c), among which terpenoids (16.34 %), esters (15.86 %), heterocyclic compounds (14.89 %), and carbohydrates (11.49 %) accounted for more than 10 % of the total. Terpenoids and esters were the most abundant volatile metabolites in Wb and Cb, playing a crucial role in fruit aroma. Terpenoids, the largest group of natural compounds, significantly influence blueberry aroma due to their low odor threshold and pleasant scent. Esters, formed through the interaction of free fatty acids and alcohols derived from lipid oxidation, contribute notably to the fruity, sweet aroma of blueberries, particularly short-chain fatty acid esters (Qian et al., 2021).

3.1.2. *Multivariate statistical analysis of blueberry VOCs*

This study analyzed the differences in VOCs among blueberry varieties using PCA and OPLS-DA based on the peak areas of volatile compounds in the experimental and QC groups. The PCA score plot (Fig. 1d) revealed that the top two principal components accounted for 58.41 % of the total variance, which showed that the first principal component (PC1) and the second principal component (PC2) explained 39.85 % and 18.56 % of the principal components. The sample groups were compactly distributed, with a clear separation between groups, and the QC group was centrally located, indicating the reliability and stability of the experimental results. The PCA plot demonstrated that the three cultivated varieties (Blu, Rek, and Nor) exhibited tight clustering, suggesting high similarity in their volatile compound profiles. In contrast, the Wb group deviated significantly from the cultivated varieties, particularly along PC1, highlighting distinct differences in their volatile metabolite composition.

To further validate these differences, as shown in Fig. 1e, the OPLS-

Table 1
32 key differential VOCs between Wb and Cb.

Class I	Compounds	Molecular weight	Formula	CAS	Odor	Log_2FC			Kegg ID
						Wb/ Blu	Wb/ Rek	Wb/ Nor	
Terpenoids	Carotol	222.20	$C_{15}H_{26}O$	465-28-1	pleasant, mild	−15.13	−15.84	−15.52	C09628
	trans-alpha-Bergamotene	204.19	$C_{15}H_{24}$	13474-59-4	woody, tea	14.67	14.67	14.67	C20811
	4,8-Methanoazulen-9-ol,decahydro-2,2,4,8-tetramethyl-stereoisomer	222.20	$C_{15}H_{26}O$	4586-22-5	woody, spicy, earthy	−11.89	−12.19	−12.14	–
	Caryophyllene oxide	220.18	$C_{15}H_{24}O$	1139-30-6	sweet, woody, spicy	−11.42	−11.61	−11.69	C16908
Ketone	2-Hexanone	100.09	$C_6H_{12}O$	591-78-6	fruity	−16.52	−16.77	−16.82	–
	3-Hexanone,2,2-dimethyl-	128.12	$C_8H_{16}O$	5405-79-8	–	−14.66	−16.32	−15.57	–
	1,1'-(1,4-phenylene)bis-Ethanone	162.07	$C_{10}H_{10}O_2$	1009-61-6	–	−9.17	−13.37	−13.30	–
	3-Hexanone	100.09	$C_6H_{12}O$	589-38-8	sweet, fruity, grape	−12.80	−13.02	−13.13	–
	2-Cyclopenten-1-one,2-pentyl-	152.12	$C_{10}H_{16}O$	25564-22-1	woody, iso jasmone	−10.62	−13.47	−12.96	–
	5-Hepten-2-one,4,6-dimethyl-	140.12	$C_9H_{16}O$	31162-48-8	–	−9.68	−10.26	−9.81	–
Heterocyclic compound	5-Methyloxazolidine-	87.07	C_4H_9NO	58328-22-6	–	−16.33	−17.66	−16.84	–
	Fomepizole	82.05	$C_4H_6N_2$	7554-65-6	–	−15.60	−17.08	−16.26	C07837
	1-(Furan-2-yl)-2-methylpentan-1-one	166.10	$C_{10}H_{14}O_2$	1248070-62-3	–	−13.42	−13.62	−13.42	–
	Pyrazine, trimethyl-	122.08	$C_7H_{10}N_2$	14667-55-1	nut skin, earthy	−10.14	−11.05	−11.10	–
	2,2′-Isopropylidenebis(5-methylfuran)	204.12	$C_{13}H_{16}O_2$	59212-75-8	–	−12.03	−10.97	−10.98	–
Aromatics	Benzene,1,4-diethyl-	134.11	$C_{10}H_{14}$	105-05-5	–	−11.87	−13.52	−13.68	–
	Benzene,nitroso-	107.04	C_6H_5NO	586-96-9	–	−10.61	−11.25	−11.57	C06876
	alpha-Methylstyrene	118.08	C_9H_{10}	98-83-9	–	−9.99	−10.64	−10.05	–
Alcohol	2-Hexen-1-ol,(*E*)-	100.09	$C_6H_{12}O$	928-95-0	green, leafy, fruity,	−14.66	−16.34	−15.55	–
	1-Hexanol	102.10	$C_6H_{14}O$	111-27-3	fruity, alcoholic, sweet	−14.28	−15.89	−15.17	C00854
	2-Buten-1-ol,2-ethyl-4-(2,2,3-trimethyl-3-cyclopenten-1-yl)-	208.18	$C_{14}H_{24}O$	28219-61-6	woody, sandalwood	−10.72	−11.30	−11.24	–
Phenol	Phenol,2-(1,1-dimethylethyl)-	150.10	$C_{10}H_{14}O$	88-18-6	–	−13.49	−13.66	−13.50	–
Amine	Cyclohexyl Amine	99.11	$C_6H_{13}N$	108-91-8	–	−14.40	−15.87	−15.13	C00571
Sulfurcompounds	Allyl Sulfurcompounds	116.07	$C_6H_{12}S$	27817-67-0	green	−13.16	−14.86	−13.98	–
Acid	2-ButenoicAcid,2-methyl-	100.05	$C_5H_8O_2$	13201-46-2	–	12.75	12.75	12.75	–
Ester	Benzoicacid,2-hydroxy-,ethylester	166.06	$C_9H_{10}O_3$	118-61-6	pepperminty	17.45	17.45	17.45	–
	2-Butenoicacid,3-methyl-,methyl ester	114.07	$C_6H_{10}O_2$	924-50-5	–	−15.80	−16.21	−16.29	–
	Benzoic acid, ethyl ester	150.07	$C_9H_{10}O_2$	93-89-0	sweet, fruity	16.12	16.12	16.12	C01839
	3-Hexen-1-ol,acetate,(*E*)-	142.10	$C_8H_{14}O_2$	3681-82-1	fruity, green	−13.55	−15.75	−15.56	–
	p-Menth-8-en-3-ol,acetate	196.15	$C_{12}H_{20}O_2$	89-49-6	minty, leafy	−12.52	−14.91	−14.70	–
	E−2-Hexenylbenzoate-	204.12	$C_{13}H_{16}O_2$	76841-70-8	–	−11.51	−11.84	−11.86	–
	Hexanoicacid,3-hexenylester	198.16	$C_{12}H_{22}O_2$	84434-19-5	–	−10.60	−11.05	−10.81	–

DA model enhanced the separation between groups, with the Wb group showing a clear trend of separation from the Blu, Rek, and Nor. This analysis confirmed significant differences in volatile metabolite profiles between wild and cultivated blueberries, which may be attributed to genetic factors (e.g., differential expression of terpene synthesis genes) and environmental conditions (e.g., variations in light, temperature, and soil nutrients) (Long et al., 2024). These findings provide valuable insights into the mechanisms underlying flavor differences in blueberries and support future variety improvement efforts.

Additionally, HCA was performed on all identified volatile metabolites. Fig. 1f further distinguished the volatile profiles of the four blueberry groups, with Wb showing the most significant differences from the Cb. This aligns with the PCA and OPLS-DA findings. Notably, statistically significant differences in VOCs were observed across all categories between Wb and Cb. The dynamic changes in the types and concentrations of these VOCs are key factors contributing to the distinct aroma characteristics of blueberry fruit (Sater et al., 2020).

3.2. Analysis of differential metabolites identification between Wb and Cb

To further elucidate the differences, this study screened differential volatile metabolites in Cb (Blu, Rek, and Nor) and Wb using the criteria of the Variable importance projection (VIP) > 1, $|Log_2FC| > 1$, and $P < 0.05$. The expression levels of these metabolites were visualized using volcano plots (Fig. 2a–c). The analysis revealed 83, 56, and 51 up-regulated metabolites and 101, 185, and 185 down-regulated metabolites in the comparisons of Wb with Blu, Rek, and Nor, respectively. The trends in the up-or down-regulation of different metabolite categories are illustrated in Fig. 2d–f. The results indicated that significantly more metabolites were down-regulated than up-regulated, particularly among flavor-related compounds such as terpenoids, esters, and ketones, which exhibited a pronounced down-regulation trend. This suggests that cultivated blueberries possess a richer diversity of volatile metabolites, implying that the accumulation and enrichment of these metabolites are more favorable under cultivated conditions compared to the natural growing state. Similar findings have been reported in other studies,

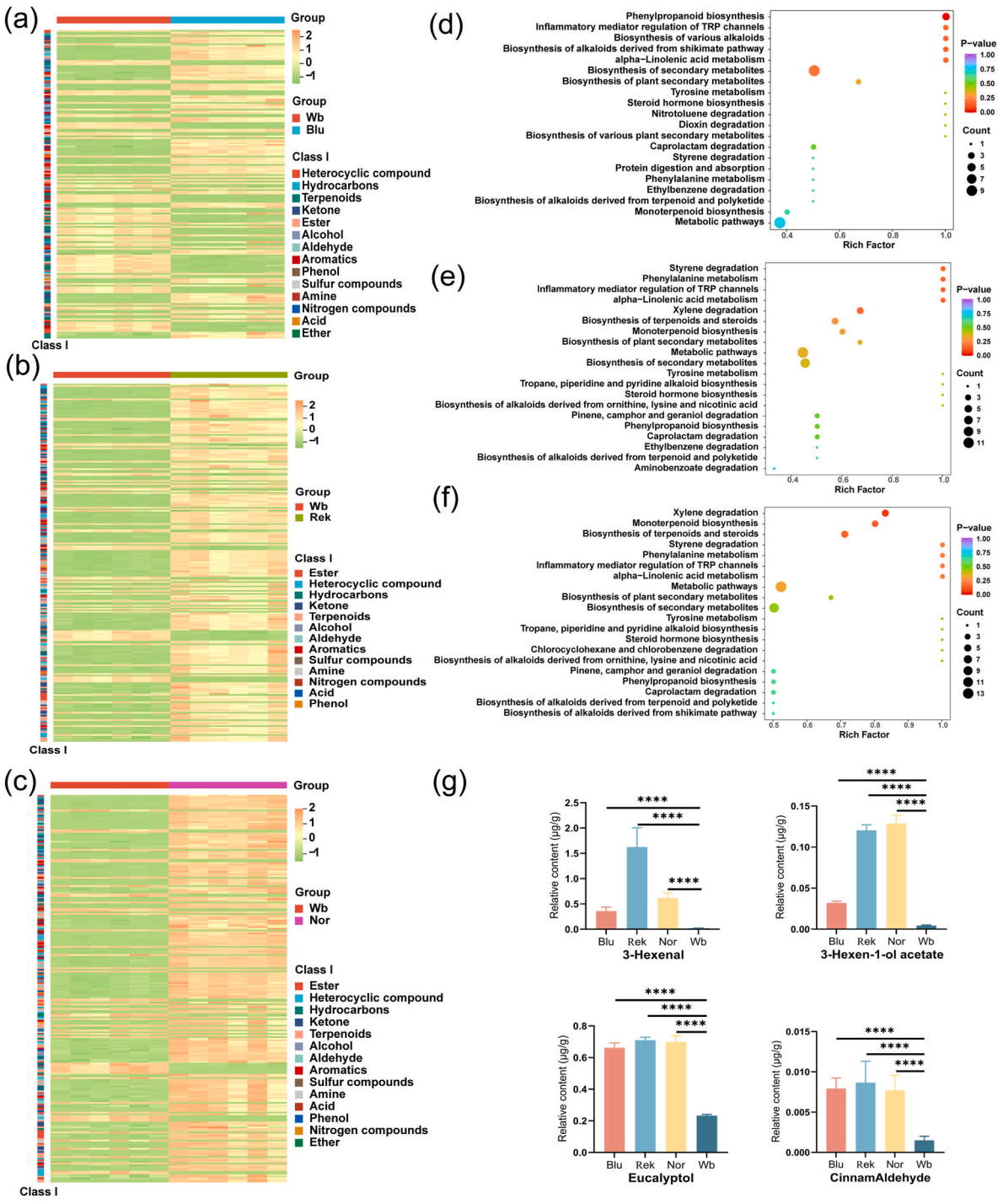

Fig. 3. Differentially expressed key pathways and metabolites identified between Cb and Wb in NC.
(a–c) Clustering heatmaps of differential metabolites for Wb vs. Blu, Wb vs. Rek, and Wb vs. Nor. (d–f) KEGG enrichment analysis of differential volatile metabolites in three comparison groups. (g) Key differential compounds involved in key metabolic pathways.

where cultivated currants demonstrated a greater variety and higher content of volatile compounds compared to their wild counterparts (Bazalar Pereda, Nazareno, & Viturro, 2023).

Fig. 2g identified 140 key differential metabolites between Wb and Cb, with detailed metabolite information provided in Table S1. Among these metabolites, terpenoids (15.71 %), ketones (14.29 %), esters (13.57 %), and aldehydes (7.14 %) constituted the most significant proportions (Fig. 2h). Terpenoids, known for their role in enhancing aroma perception (Dudareva et al., 2013), were found in higher diversity and abundance in cultivated blueberries. Specifically, citronellal (sweet flavor) and eucalyptol (minty flavor) were significantly more abundant in cultivated varieties. This may result from long-term artificial selection and breeding, which likely optimized or enhanced key enzymes in the terpenoids synthesis pathway (e.g., terpenoids synthase TPS), promoting the accumulation of specific terpenoids compounds. Additionally, controlled environmental conditions and optimized light and temperature during cultivation further facilitated terpenoids synthesis and accumulation (Hu et al., 2024), making terpenoids a key factor in the distinct aroma profiles of wild and cultivated blueberries. Similar studies have also argued this point of view, for example, Fan

et al. found that field-grown ginseng roots are more favorable for terpenoids accumulation due to the fact that they receive more nutrients during growth than those in the mountains (Fan et al., 2019).Suruchi Gupta et al. explored the synthesis of terpenoids in wild and cultivated psylliums by using both genetic and transcriptomic perspectives. to investigate the differences in terpenoid synthesis pathways in wild and cultivated psyllium (Suruchi, Ravail, Arti, GulzarA, & SurrinderK, 2022). Ketones, such as 2-hexanone, 3-hexanone, and 2,2-dimethyl, were also more abundant in cultivated blueberries, enriching the flavor complexity of blueberries and their processed products (George et al., 2023). Esters, contributing to the "fruity" aroma, played a crucial role in blueberry flavor (Du & Rouseff, 2014). For instance, 2-butenoic acid ethyl ester (*E*)- enhances sweetness in berries like strawberries and blueberries (Gu et al., 2022). Aldehydes, particularly six-carbon aldehydes such as 2-hexenal (*E*)-, hexanal, and 3-hexenal (*Z*)-, 4-heptenal imparted typical "fresh grassy, earthy, and green" flavors to blueberries, with higher levels observed in cultivated varieties (Farneti et al., 2017). Hexanal, which can be reduced to alcohols or oxidized to hexanoic acid, is primarily detected in unripe fruits and contributes to a fresh, fruity aroma (Oliveira et al., 2006). The higher abundance of 3-hexenal (*Z*)- and 2-hexenal (*E*)- in cultivated blueberries further underscores the role of cultivation in enhancing flavor compound accumulation.

Among the 140 differential metabolites identified, Table 1 show the 32 key volatile metabolites exhibited highly statistically significant differences unique to Cb or Wb. These metabolites primarily comprised esters (7), ketones (6), and heterocyclic compounds (5). Notably, Benzoic acid, 2-hydroxy-, ethyl ester, Benzoic acid, ethyl ester, *trans*-.alpha.-Bergamotene, and 2-Butenoic Acid, 2-methyl-, were present only in Wb. Fig. S1 visualizes 32 key differential VOCs in Wb and Cb in their relative amounts. Benzoic acid, 2-hydroxy-, ethyl ester has a light minty aroma; Benzoic acid, ethyl ester is the predominant volatile flavor compound in kiwifruit (Li, Yuan, et al., 2023), ripe toffee fruit (Song et al., 2023), with an intense fruity flavor and sweetness; *trans*-.alpha. Bergamotene, an uncommon class of terpenoids, was identified as the significant flavor compound in wild chrysanthemum (Gao et al., 2024). 2-Butenoic acid and 2-methyl-, although a key volatile compound in wild blueberries, were not prominent in their aroma profile. In addition, 28 volatile compounds such as Carotol, 2-Hexanone, 2-Butenoic acid, 3-methyl-, methyl ester, 2-Hexen-1-ol, (*E*)-, and 1-Hexanol were present in only three Cbs. Elwira Sieniawska et al. showed that Carotol is the prominent flavor-presenting substance in essential oils (Sieniawska et al., 2016). 2-Hexanone is an important flavor compound in brown rice (Sun et al., 2022). 2-Butenoic acid, 3-methyl-, methyl ester was present at high levels and low thresholds in all three Cbs, whereas it was almost absent in Wb. 2-Hexen-1-ol, (*E*)- has an intense unripe fruit or grass-like odor, sometimes described as fresh green leafy fruit and unripe banana. 1-Hexanol, with its fruity flavor, is the main flavor compound in many fruits, such as apples and blueberries (Yang et al., 2021).

Taken together, the flavor of Wb is usually more distinctively wild-fruity because they have not been artificially selected and cultivated. Cb managed artificially can accumulate more aroma components, producing a more intense fruit flavor. In addition, this research plays an important role in selecting Wb and Cb. The typical flavor profiles of different varieties allow us to accurately select blueberries suitable for use as ingredients in different processed products. Specifically, Cb is preferred for fresh eating due to its high sugar and nutritional content, while Wb has a complex flavor profile and is more suitable for products that seek a rich level of flavor, such as fruit wines.

3.3. KEGG functional annotation and enrichment of differential metabolites

In this study, we visualized the expression of differential volatile compounds in three groups of blueberries, respectively (Fig. 3a–c). Next, the focus is on analyzing the metabolic pathways involved in the synthesis of these differentially volatile compounds. The formation mechanism of volatile flavor compounds in blueberries and other fruits is closely linked to their accumulation during ripening. These compounds typically originate from high molecular weight precursors in the growing environment or within fruit cells, which are transformed through enzymatic catalysis (Yu et al., 2020). We conducted KEGG pathway enrichment analysis to explore the pathways underlying differential metabolite formation in Wb, Blu, Rek, and Nor and visualized the results using bubble plots (Fig. 3d–f). The analysis revealed that the key differential VOCs in the comparisons of Wb vs. Blu, Wb vs. Rek, and Wb vs. Nor were primarily associated with two major pathways: "Inflammatory mediator regulation of TRP channels" and "alpha-Linolenic acid metabolism." The alpha-linolenic acid metabolism pathway is particularly significant, as it generates linolenic acid, a precursor for several flavor compounds such as 1-octen-3-ol and 3-octane (Wu et al., 2021). These precursors undergo hydrogen peroxide formation and cleavage processes, ultimately converting into compounds with distinct flavor profiles. This pathway plays a crucial role in shaping the aroma characteristics of blueberries. The consistent involvement of these pathways across comparisons highlights their importance in the metabolic divergence between Wb and Cb, providing insights into the biochemical basis of their flavor differences.

It is apparent from Fig. 3e–f that there is a high degree of concordance between Wb and Rek and Nor in terms of the major metabolic pathways enriched for differential metabolites, Styrene degradation, Phenylalanine, Xylene degradation, Monoterpenoid biosynthesis, Biosynthesis of terpenoids and steroids. These shared pathways suggest that many of the same differential compounds may exist between wild blueberries and the Rek and Nor cultivars, a finding that is consistent with our previous findings obtained by other methods. Deep mining of metabolomics data revealed that the synthesis of key volatile compounds in blueberry fruit is closely linked to two core metabolic pathways: Inflammatory mediator regulation of TRP channels and alpha-linolenic acid metabolism. Four compounds, eucalyptol, (*E*)-cinnamaldehyde, 3-Hexenal and 3-Hexen-1-ol acetate, were particularly prominent. They play a key role in blueberry flavor formation. They are also core varietal distinguishing markers rigorously selected from various pathways associated with 32 previously identified differential metabolites. Notably, the "inflammatory mediator regulation of TRP channels" pathway produces indole derivatives via tryptophan metabolism, and these compounds synergize with (*E*)-cinnamaldehyde produced by phenylalanine metabolism to form the spicy-floral complex that characterizes Wb. Fig. 3g reflect the differences in the relative contents of these four key differential compounds in the four blueberry varieties through visual data presentation, further validating our conclusions based on metabolomics data analysis. Therefore, from the perspective of key metabolic pathways, these four differential compounds can also be used as key compounds to differentiate Wb and Cb in northeastern China. By pinpointing these key differential metabolites and their synthetic pathways, we can more effectively guide blueberry breeding practices to produce new varieties that better meet market demands and consumer preferences.

3.4. ROAV analysis of the key aroma components

Volatile metabolites are critical contributors to blueberry flavor, although not all volatile metabolites directly influence flavor production (Li, Yuan, et al., 2023). To precisely identify the primary volatile compounds responsible for blueberry flavor, we integrated HS-SPME-GC-MS results with OAVs. Key flavor compounds were identified based on the rOAVs, where rOAVs >1 directly influences the sample's overall flavor profile. Subsequently, the aroma properties of these compounds were confirmed using odor descriptions documented in previous literature (Huang et al., 2022). One hundred and thirty-one key flavor compounds with rOAVs >1 were identified across the four blueberry varieties, forming the foundation of their distinctive flavor profiles. Among these,

Table 2
The relative odor activity values (rOAVs) of the most important aroma-active compounds in the blueberries (rOAVs >10).

Class I	Compounds	Formula	Odor Description	Threshold	rOAVs			
					Blu	Rek	Nor	Wb
Terpenoids	*β*-Ionone	$C_{13}H_{20}O$	floral, woody, sweet, fruity, berry	7.00E-06	******	******	******	******
	Cyclohexanone, 5-methyl-2-(1-methylethyl)-	$C_{10}H_{18}O$	minty	4.70E-03	****	****	****	****
	Eucalyptol	$C_{10}H_{18}O$	eucalyptus, herbal, medicinal	1.50E-02	**	**	**	**
	2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (*E*)-	$C_{13}H_{18}O$	apple, rose, honey, tobacco, sweet	1.50E-03	**	**	**	**
	Limonene	$C_{10}H_{16}$	citrus, herbal, terpene, camphor	1.00E-02	**	**	**	**
	3-Buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo [4.1.0]hept-1-yl)-	$C_{13}H_{20}O_2$	fruity, sweet, berry, woody, violet	1.00E-01	**	**	**	**
	β-Myrcene	$C_{10}H_{16}$	musty, balsamic, spice	1.50E-02	**	**	**	*
	β-Phellandrene	$C_{10}H_{16}$	terpenic, herbal	3.60E-02	**	**	**	*
	Carotol	$C_{15}H_{26}O$	pleasent, mild	8.00E-03	**	**	**	–
Ketone	1-Octen-3-one	$C_8H_{14}O$	mushroom	5.00E-06	*****	*****	*****	*****
	3-Hexanone, 2,2-dimethyl-	$C_8H_{16}O$	–	4.10E-04	***	***	***	–
	5-Methyl-(*E*)-2-hepten-4-one	$C_8H_{14}O$	hazelnut, nutty	5.00E-05	**	**	**	**
	5,9-Undecadien-2-one, 6,10-dimethyl-, (*E*)-	$C_{13}H_{22}O$	fresh, green, fruity, rose, woody	1.00E-02	**	**	**	*
	2-Butanone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	$C_{13}H_{22}O$	earthy, woody, dry, amber	3.60E-03	**	**	**	**
	3,5-Octadien-2-one, (*E,E*)-	$C_8H_{12}O$	fruity, green, grassy	5.00E-04	**	**	**	**
	Ethanone, 1-(2-aminophenyl)-	C_8H_9NO	grape, sweet	2.70E-04	**	**	**	**
Heterocyclic compound	2(5H)-Furanone, 5-ethyl-3-hydroxy-4-methyl-	$C_7H_{10}O_3$	sweet, fruity, sugar, nutty, praline	2.00E-06	*******	*******	*******	*******
	2-Thiophenemethanethiol	$C_5H_6S_2$	roasted, coffee, fishy	4.00E-05	*****	*****	*****	*****
	Furaneol	$C_6H_8O_3$	sweet, cotton, strawberry, sugar	1.00E-03	*****	*****	*****	*****
	Pyrazine, 2-methoxy-3-(2-methyl propyl)-	$C_9H_{14}N_2O$	green bell pepper, pea, galbanum	2.00E-06	****	****	****	*****
	Ethanone, 1-(2-thienyl)-	C_6H_6OS	sulfury, nutty, hazelnut, walnut	1.00E-03	***	***	***	***
	2(5H)-Furanone, 5-ethyl-	$C_6H_8O_2$	spice	9.70E-03	**	**	**	**
	Ethanone, 1-(4,5-dihydro-2-thiazolyl)-	C_5H_7NOS	potato, toasted, bread, nutty	1.00E-03	**	**	**	**
	Furan, 2-pentyl-	$C_9H_{14}O$	fruity, green, earthy, vegetable	6.00E-03	**	**	**	**
	2H-Pyran-2-one, tetrahydro-6-methyl-	$C_6H_{10}O_2$	creamy, fruity, coconut	2.68E-02	**	**	**	*
Aromatics	4a(2H)-Naphthalenol,octahydro-4,8a-dimethyl-,(4.alpha.,4a.alpha.,8a.beta.)-	$C_{12}H_{22}O$	fresh, musty, earthy, soil	2.10E-04	***	***	***	**
	Styrene	C_8H_8	penetrating, balsamic	3.60E-03	**	**	**	*
	Naphthalene	$C_{10}H_8$	pungent, dry, tarry	5.00E-02	**	**	**	**
	Benzene, 1-methyl-4-nitro-	$C_7H_7NO_2$	–	3.00E-03	**	**	**	**
	Benzene, 1,4-dichloro-	$C_6H_4Cl_2$	–	3.00E-02	**	**	**	**
Alcohol	*trans*,*cis*-2,6-Nonadien-1-ol	$C_9H_{16}O$	green, cucumber, violet, leafy	1.00E-03	****	****	****	****
	1-Nonanol	$C_9H_{20}O$	fresh, rose, orange	5.30E-03	***	***	***	***
	3,6-Nonadien-1-ol, (E,Z)-	$C_9H_{16}O$	green, cucumber, fruity, watermelon	3.00E-03	**	**	**	**
	3-Mercaptohexanol	$C_6H_{14}OS$	sulfury, fruity, tropical	6.00E-05	**	**	**	**
	4-Phenyl-2-butanol	$C_{10}H_{14}O$	floral, peony, foliage, sweet	4.30E-03	**	**	**	**
Phenol	2-methoxy-Phenol	$C_7H_8O_2$	nutty	1.60E-03	***	****	****	***
	p-Cresol	C_7H_8O	phenol, narcissus, mimosa	2.40E-04	***	***	***	***
	Phenol, 2-methyl-	C_7H_8O	phenol	3.90E-03	**	**	**	**
Sulfur compounds	3-Cyclohexene-1-methanethiol, *α.,.α.*,4-trimethyl-	$C_{10}H_{18}S$	sulfury, aromatic, grapefruit	2.00E-08	******	*******	*******	********
	Benzenemethanethiol	C_7H_8S	sulfury, garlic, minty, coffee	3.50E-06	******	******	******	*****
	Dimethyl triSulfur compounds	$C_2H_6S_3$	sulfury, savory	8.00E-06	****	****	****	****
Ester	3-Mercapto-3-methylbutyl formate (ester)	$C_6H_{12}O_2S$	sulfury, catty, caramel, onion	2.00E-06	*****	*****	*****	*****
	3-Mercaptohexyl acetate	$C_8H_{16}O_2S$	sulfury, grapefruit, fruity	2.00E-05	****	****	****	****
	Benzoic acid, methyl ester	$C_8H_8O_2$	phenol, wintergreen, floral	5.20E-04	***	****	****	***
	Propanoic acid, 2-methyl-, heptyl ester	$C_{11}H_{22}O_2$	sweet, green, fruity, cherry, apricot	1.20E-02	***	***	***	***
	n-Valeric acid cis-3-hexenyl ester	$C_{11}H_{20}O_2$	green, fruity, kiwi, unripe, banana	6.00E-02	**	**	**	**
	Acetic acid, cyclohexyl ester	$C_8H_{14}O_2$	fruity, sweet, musty, ethereal	1.60E-03	**	**	**	**
	2-Propenoic acid, butyl ester	$C_7H_{12}O_2$	–	2.90E-03	**	**	**	**

(*continued on next page*)

Table 2 (*continued*)

Class I	Compounds	Formula	Odor Description	Threshold	rOAVs			
					Blu	Rek	Nor	Wb
	Heptanoic acid, ethyl ester	$C_9H_{18}O_2$	fruity, pineapple, cognac, wine	2.00E-03	**	**	**	**
Aldehyde	2-Nonenal, (*E*)-	$C_9H_{16}O$	fatty, green, cucumber, aldehydic	8.00E-05	*****	*****	*****	*****
	BenzAldehyde, 4-methoxy-	$C_8H_8O_2$	sweet, powdery, mimosa, floral	2.00E-04	****	****	****	****
	5-Heptenal, 2,6-dimethyl-	$C_9H_{16}O$	fresh, melon, sweet, green	1.60E-02	****	****	****	****
	6-Nonenal, (*Z*)-	$C_9H_{16}O$	green, cucumber, melon, leafy	1.40E-04	***	****	****	***
	2-Hexenal, (*E*)-	$C_6H_{10}O$	green, grassy	3.10E-03	***	****	****	**
	Hexanal	$C_6H_{12}O$	grassy, green, leafy, vinegar	5.00E-03	***	***	***	*
	2,4-Nonadienal, (*E,E*)-	$C_9H_{14}O$	melon, green, cucumber, fruity	1.60E-04	***	***	***	**
	3-Hexenal, (*Z*)-	$C_6H_{10}O$	Green, grassy, weedy, fruity, apple	4.00E-03	**	***	***	*
	Par Aldehyde	$C_6H_{12}O_3$	sweet, aromatic	2.00E-02	**	**	**	**
	(*E*)-2-Octenal	$C_8H_{14}O$	fresh, cucumber, green	3.00E-03	**	**	**	**
	Benzeneacet Aldehyde	C_8H_8O	floral, honey, rose, cherry	6.30E-03	**	**	**	*
Nitrogen compounds	Dodecanenitrile	$C_{12}H_{23}N$	citrus, orange, peel, metallic	9.00E-05	***	***	***	***

a. The range of rOAVs of metabolites is expressed as the amount of "*".
b. "********" indicates that $1 \times 10^7 \leq$ rOAVs $< 1 \times 10^8$; "*******" indicates that $1 \times 10^6 \leq$ rOAVs $< 1 \times 10^7$; "******" indicates that $1 \times 10^5 \leq$ rOAVs $< 1 \times 10^6$; "*****" indicates that $1 \times 10^4 \leq$ rOAVs $(< 1 \times 10^5$; "****" indicates that $1 \times 10^3 \leq$ rOAVs $< 1 \times 10^4$; "***" indicates that $1 \times 10^2 \leq$ rOAVs $< 1 \times 10^3$; "**" indicates that $1 \times 10^1 \leq$ rOAVs $< 1 \times 10^2$; "*" indicates that $0 \leq$ rOAVs < 10.

61 compounds with rOAVs >10 were particularly significant contributors to blueberry flavor in Table 2; the five most impactful volatile compounds were *β*-ionone, 2(5H)-furanone, 5-ethyl-3--hydroxy-4-methyl-, 3-cyclohexene-1-methanethiol, *α*,*α*,4-trimethyl-, 3-mercapto-3-methylbutyl formate, and 2-thiophene methanethiol. Intense fruity and sweet aromas characterize these compounds and are present in relatively high concentrations in blueberries, playing a decisive role in shaping their overall flavor profile. Notably, 3-cyclohexene-1-methanethiol, *α*,*α*,4-trimethyl-, which exhibits a typical fruity aroma, was found in significantly higher concentrations in Wb than in Cb. This compound was more than twenty times more abundant in Wb, highlighting its importance in the unique flavor formation of Wb. These findings provide valuable insights into the biochemical basis of flavor differences between Wb and Cb.

Furthermore, we identified several key flavor compounds exclusively present in Cb, such as carotol and 3-hexanone, 2,2-dimethyl-, which contribute to their distinct flavor profile compared to wild blueberries. Additionally, certain compounds were found at significantly higher concentrations in cultivated blueberries, including *β*-myrcene, 5,9-undecadien-2-one, 6,10-dimethyl-, (*E*)-, hexanal, 3-hexenal, (*Z*)-, 2H-pyran-2-one, benzeneacet aldehyde, tetrahydro-6-methyl-, and styrene. Each of these compounds exhibits unique aroma characteristics: *β*-myrcene is known for its aromatic properties (Song et al., 2024), 5,9-undecadien-2-one, 6,10-dimethyl-, (*E*)- for its milky aroma (Hao et al., 2023), hexanal as a key flavor compound in lowbush blueberries, and 3-hexenal, (*Z*)- for its grassy aroma (Forney et al., 2022). These compositional differences likely play a pivotal role in the pronounced flavor distinctions between Wb and Cb.

3.5. Systematic analysis of VOCs in blueberries

To more intuitively reveal the differential characteristics of the contents of the 282 differential metabolites in the four blueberry species, we employed K-means cluster analysis to systematically analyze the relative contents of the differential metabolites after standardization, according to which the resulting K-means cluster diagram (Fig. 4a) demonstrated the grouping of metabolites with similar changing trends, specifically, the 282 differential metabolites mainly showed six different trends. Among them, subclasses 4 and 6 exhibited the same trend with a total of 129 metabolites with low levels in Blu and Wb, which is consistent with the results of the PCA analysis mentioned previously, but this is not the topic to be explored in this paper. Of interest is that 63 differential metabolites in subclass 3 had significantly higher relative levels in Wb than the other three Cbs (Table S2), and more than 50 % of these metabolites were terpenoids and esters. In contrast, 57 differential metabolites in subclass 5 were present at lower levels in Wb than in Cb (Table S3). It is worth noting that although K-means clustering analysis has provided us with an initial classification framework, the trends of metabolite variation among different subclasses are not completely independent. Indeed, the metabolic network is a highly complex and interconnected system, and changes in one metabolite often affect the levels of multiple other metabolites. Therefore, in subsequent studies, we need to employ more sophisticated bioinformatics methods and experimental validation tools to deeply reveal the interactions and regulatory mechanisms among these differential metabolites.

Sensory analysis of differential metabolites can assist in the examination of sensory flavor profiles in the samples and further clarify the sensory differences between the flavor compounds of Wb and Cb. In this study, we exhaustively identified the differential metabolites in the comparison group of Wb and Cb based on stringent screening criteria, and systematically sorted out the sensory flavor profiles annotated by them. To further analyze the volatile flavor compounds associated with these flavor profiles in detail, the top 10 flavor compounds with the highest VIP values under each flavor category were plotted on a flavor wheel in this study (Fig. 4c), and the specific compound information is shown in Table S4 for subsequent research and validation. These compounds are categorized according to the class to which they belong as shown in Fig. 4b. Of these, the largest percentage of Terpenoids, Ketone, Ester. To visualize the distribution of these flavor profiles, the top 10 sensory flavors with the highest number of annotations were selected and a radar diagram was plotted accordingly (Fig. 4d). Through this radar plot, we can observe the major differences in flavor profiles between wild blueberries and cultivated blueberries, specifically: sweet (25), fruity (23), green (23), herbal (18), and woody (17). And differential compounds present between Wb and Cb visualized by flavor attribute word clouds (Fig. 4e). In order to further investigate the

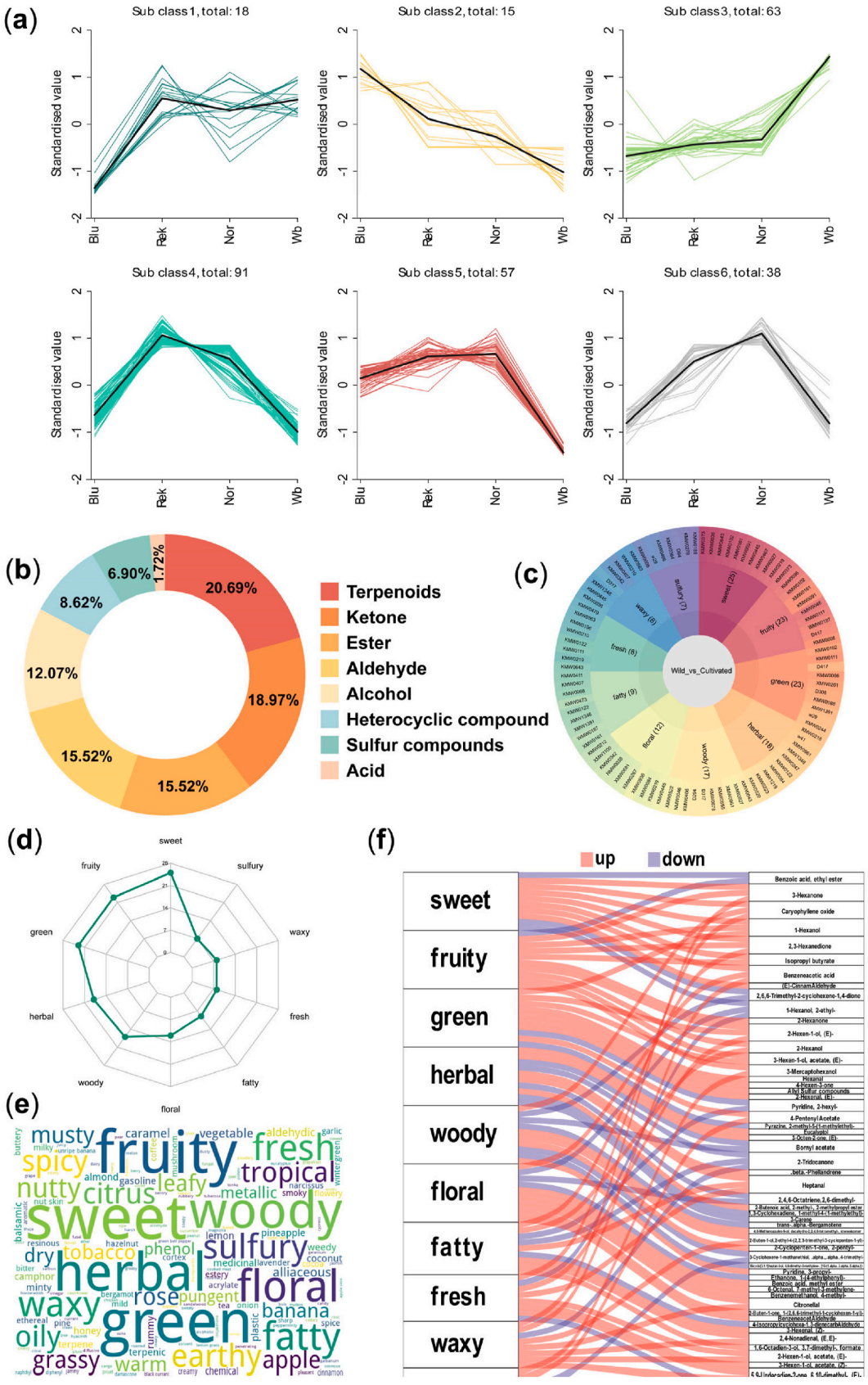

Fig. 4. Systematic analysis of differential metabolites between Wb and Cb.
(a) K-means plot of differential metabolites. (b) Pie chart for categorization of differential metabolites. (c) Flavor wheel of differential metabolites. (d) Radar chart of key differentiated substance flavors. (e) Flavor word cloud of differential metabolites. (f) Sankey diagrams between aroma and key differential volatile compounds in Wb and Cb.

correlation between flavor sensory characteristics and volatile organic compounds. In this study, the highest number of annotations were selected for Sankey diagrams for the differential metabolites identified based on the screening criteria and annotated to the sensory flavor characteristics in each differential comparison group (Fig. 4f). The left column represents the sensory flavor profile, the right column represents the differential metabolites, and the red flow line indicates the flow of up-regulated differential metabolites and the blue flow line indicates the flow of down-regulated differential metabolites. The height of the boxes in the left column depends on the number of differential metabolites displayed in the right column, with the higher the number the higher the box.

After synthesizing and comparing the results of previous research on flavor variability between Wb and Cb, we found some interesting phenomena. Of the 10 key flavor compounds that makeup sweetness, except benzoic acid, ethyl ester, and 2,6,6-trimethyl-2-cyclohexene-1,4-dione, the concentrations of the remaining 8 compounds were significantly higher in Cb than in Wb. These findings suggest that Cb exhibit a more pronounced sweetness, which may be attributed to specific agricultural practices and optimized environmental conditions during cultivation. Notably, among the ten core compounds contributing to fruit flavor, Cb demonstrated significantly higher concentrations compared to Wb. This further underscores the advantage of Cb in delivering a richer and more intense fruity flavor profile, enhancing their overall sensory appeal. In conclusion, the analysis of VOCs reveals that Cb possesses a more complex and appealing flavor profile, characterized by greater sweetness and fruitiness. The use of a flavor wheel provides an intuitive visualization of the key marker compounds that differentiate the flavor profiles of Wb and Cb.

4. Conclusion

In summary, this study comprehensively analyzed the volatile compounds of four blueberry varieties from NC using HS-SPME-GC/MS, identifying a total of 618 VOCs. Terpenoids, esters, and heterocyclic compounds were the most abundant classes of volatile metabolites. Multivariate statistical analysis revealed significant differences in VOCs between Wb and Cb, with cultivated varieties exhibiting higher diversity and abundance of terpenoids, ketones, and esters. Notably, the levels of key flavor compounds, such as citronellal and eucalyptol, were significantly elevated in cultivated blueberries. KEGG pathway analysis indicated that “Inflammatory mediator regulation of TRP channels” and “alpha-linolenic acid metabolism” were the key pathways associated with the formation of differential metabolites. Furthermore, ROAV analysis identified 131 key volatile aroma compounds contributing to blueberry flavor, with cultivated varieties exhibiting more pronounced sweetness and fruity aroma. Carotol and 1-hexanol were identified as potential biomarker compounds for distinguishing the aroma profiles of wild and cultivated blueberries from NC. These findings provide valuable insights into the mechanisms underlying flavor differences in blueberries and offer a scientific foundation for future variety improvement efforts.

CRediT authorship contribution statement

Xu Xie: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Yujie Nie:** Writing – review & editing, Writing – original draft, Software, Formal analysis. **Yuehua Wang:** Writing – review & editing, Methodology. **Bosu Wen:** Writing – review & editing, Methodology, Formal analysis. **Junzhe Shang:** Methodology. **Xin Guo:** Software. **Jinlong Tian:** Methodology. **Yumeng Wang:** Methodology. **Yiming Zhang:** Methodology. **Siyuan Li:** Methodology. **Yue Li:** Methodology. **Bowen Hu:** Methodology. **Baoru Yang:** Software, Methodology. **Ying Zhou:** Software, Methodology. **Mingqian Wang:** Software, Methodology. **Bin Li:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2025.117950.

Data availability

Data will be made available on request.

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