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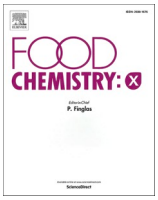

Characterization and comparative analysis of volatile organic compounds in four aromatic wild strawberry species using HS-SPME-GC-MS

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ABSTRACT

Strawberries are valued for their aroma, which is mainly determined by volatile organic compounds (VOCs). Wild strawberries, with broader and more intense VOC profiles, are especially important in breeding programs. Using HS-SPME-GC–MS, 126 VOCs were identified in the ripe fruit of 22 cultivars from four wild strawberry species. Significant interspecies differences were found, with *Fragaria mandshurica* and *F. nilgerrensis* showing several times higher VOC levels than *F. vesca* and *F. viridis*, primarily due to high lactone content (up to 79.71 % of total VOCs). Phylogenetic analysis revealed conserved VOC profiles within species and genotype–aroma correlations. PCA and PLS-DA identified 60 biomarkers explaining 37.07 % of the variance, with 10 biomarkers validated for species identification, achieving 100 % accuracy. Key biomarkers for *F. mandshurica* included furaneol and perillyl acetate, while butanoic acid butyl ester was characteristic of *F. vesca*. This study emphasizes the role of VOCs as biomarkers for species differentiation and their potential in strawberry breeding.

1. Introduction

Strawberry (*Fragaria* spp.) is one of the most valued economic crops globally (Senger et al., 2022). Cultivated strawberry (*Fragaria* × *ananassa*) is renowned for its large fruit, vibrant color, and strong antioxidant properties, akin to those of walnuts, which are beneficial to human health; however, it is often criticized for lacking an intense aroma. (Negri et al., 2015; Sadighara et al., 2016; Wang et al., 2024). Aroma is a complex mixture of various volatile organic compounds (VOCs) that not only endow a unique fragrance but also play crucial roles in the plant's ecological adaptability, such as pollinator attraction and pathogen defense (Dudareva et al., 2013; Pan et al., 2024). Over the past few decades, researchers have identified over 979 VOCs in fresh strawberry (Schwieterman et al., 2014; Ulrich et al., 2007), and have extensively studied the diversity of VOCs between wild and cultivated strawberries (Drawert et al., 1973; Ulrich et al., 1997).

Strawberry, *Fragaria* L. (Rosaceae), encompasses approximately 25 recognized species and exhibits natural ploidy levels ranging from diploid to decaploid (2×, 4×, 5×, 6×, 8×, and 10×) (Qiao et al., 2021). Genotypic variation significantly impacts VOCs composition and flavor perception (Jouquand et al., 2008). Previous studies on the aroma profiles of 16 *F. vesca* accessions and 5 *Fragaria* × *ananassa* cultivars have identified significant differences in the accumulation of various esters, ketones, and terpenoids (Ulrich & Olbricht, 2013). Cultivated strawberries contain higher levels of small esters, such as ethyl hexanoate, methyl butanoate, and methyl hexanoate, compared to wild strawberries. The primary aroma compounds in wild strawberries, such as *F. vesca* and *F. moschata*, include methyl anthranilate (MA), methyl benzoate, and ethyl hexanoate, which distinguish them from cultivated varieties (Negri et al., 2015). The key ester MA is more abundant in *F. vesca*. Furthermore, wild strawberries have higher concentrations of ketones (e.g., 2-pentanone, 2-heptanone, and 2-nonanone) and terpenoids (e.g., myrtenal, myrtenyl acetate, and α-terpineol), except for the monoterpene linalool, which is more prevalent in cultivated strawberries (Ulrich & Olbricht, 2014). Additionally, specific monoterpenes, aldehydes, and esters vary significantly among strawberry species, differentiating varieties (Du et al., 2011). However, current studies often focus on a limited number of strawberry germplasm resources,

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suggesting that characteristic aroma compounds may not be species specific, indicating the necessity for further research.

Wild species have been utilized as breeding resources to expand hereditary variation and enhance cultivated strawberry (Noguchi et al., 1997). A new aromatic strawberry variety was developed by crossing *F.* × *ananassa* (8×) and *F. nilgerrensis* (2×), resulting in a hybrid sharing aromatic components with *F. nilgerrensis* (Noguchi et al., 2002). Therefore, as strawberry breeding techniques advance, there is increasing interest in exploring aroma differences between cultivated and wild strawberries. Wild strawberry species generally have higher VOC levels than cultivated ones. For instance, a comparative analysis of fruit volatiles showed that *F. pentaphylla*, a wild strawberry species, exhibits a richer and more intense aroma profile than cultivated *Fragaria* × *ananassa*. This difference is attributed to the higher concentration and diversity of VOCs, including esters and terpenes, in wild species (Aharoni et al., 2004; Ulrich et al., 2007). These findings underscore the potential of wild strawberries as valuable sources of aromatic compounds for breeding purposes (Cheng et al., 2015; Schwab et al., 2008) and indicate that wild strawberries possess more potential aroma biomarkers that could be used to enhance the aroma characteristics of cultivated strawberries.

Headspace solid-phase microextraction-gas chromatography–mass spectrometry (HS-SPME-GC–MS) is a powerful analytical technique used to detect volatile and semi-volatile compounds in complex samples (Lancioni et al., 2022). It combines solid-phase microextraction (SPME) for sensitive and efficient VOC extraction from the headspace of a sample with gas chromatography (GC) for separation and mass spectrometry (MS) for detailed identification and quantification. This method offers high sensitivity, requires minimal sample preparation, and avoids the use of solvents, making it environmentally friendly (Azari et al., 2024). As it is widely used in food, environmental, and biological sample analysis, HS-SPME-GC–MS is particularly useful for studying VOCs in complex matrices, with applications in flavor and fragrance analysis, environmental monitoring, and health research (Azzi-Achkouty et al., 2017; Sadighara et al., 2023; Zhakupbekova et al., 2019).

Principal component analysis (PCA) is a statistical technique that reduces the complexity of high-dimensional data by transforming it into principal components that determine the primary features of the data. Partial least squares discriminant analysis (PLS-DA) integrates regression and classification to identify and predict categories within datasets with highly correlated variables. These methods have been effectively employed in citrus and peach to analyze the relationships between species and pinpoint key flavor compounds (Xi et al., 2017; Zhang et al., 2019). Moreover, PLS-DA has been applied to identify compounds with differing accumulation among species, and those exhibiting high variable importance in projection (VIP) values have been selected as potential specific biomarkers (Duan et al., 2016).

This study aimed to comprehensively analyze the distribution and diversity of VOCs in 22 wild strawberry varieties using HS-SPME-GC–MS technology. After identifying 126 VOCs across four wild strawberry species, we sought to uncover species-specific aroma biomarkers and evaluate the conservation of aroma profiles both within the same species and across different species. PCA and PLS-DA further distinguished the four wild strawberry species and identified key VOCs associated with their specific aroma characteristics. These findings provide insights into the chemical basis of strawberry aroma and highlight the potential of using VOCs as biomarkers for identifying and classifying strawberry germplasm resources. Moreover, wild strawberry germplasm with desirable aromatic traits can be utilized in strawberry breeding, as well as in various commercial applications, such as strawberry jam production and strawberry essence extraction.

2. Materials and methods

2.1. Plant materials

The 22 wild strawberry cultivars used in this study were grown under outdoor field conditions, and fruit was harvested at the commercial ripening stage from the China National Strawberry Germplasm Resource Nursery at the Jiangsu Academy of Agricultural Sciences (Nanjing, Jiangsu, China). These cultivars comprised eight *F. vesca*, six *F. mandshurica*, four *F. nilgerrensis*, and four *F. viridis* cultivars. The fruit was chopped into small pieces, immediately ground into a powder using liquid nitrogen, and then stored at −80 °C for further analysis.

2.2. Volatile compounds extraction and analysis by HS-SPME-GC–MS

VOCs were extracted and analyzed using HS-SPME-GC–MS, following the method described by Zorrilla-Fontanesi et al. (2012), with minor modifications. For each extraction, 0.1 g of powdered fruit and 2 mL of distilled water (100 °C) were placed in a 20-mL capped vial. Samples were incubated at 70 °C for 15 min, and volatiles were extracted at 70 °C for 30 min using a polydimethylsiloxane (PDMS)-df 100 μm fiber (Supelco, Bellefonte, PA, USA). The fiber was withdrawn and inserted into the heated injector port of the gas chromatograph for desorption at 240 °C for 5 min in split mode. After injection, the fiber was reconditioned in the injector of the GC. Each cultivar was analyzed in triplicate.

A 7890B GC system (Agilent, CA, USA) and a 5977B GC/MS Triple Qual Quadrupole Mass Detector (Agilent, CA, USA) were used for solid-phase microextraction. Volatiles were separated on an HP-5 MS column (250 μm × 30 m, 0.25 μm, Agilent) with helium as the carrier gas at a constant flow rate of 1.6 mL/min. The initial column temperature was maintained at 50 °C for 3 min, then increased at a rate of 4 °C/min to 265 °C, and held for 5 min. The key MS operating parameters included an ion source temperature of 230 °C and an interface temperature of 150 °C. Mass spectra were recorded at 20 scans per second over a mass range of 30–600 amu, with an ionization energy of 70 eV.

2.3. Qualitative and quantitative analysis

VOCs were identified by matching their mass spectra and retention indices (RIs) against the National Institute of Standards and Technology (NIST2014) and the Wiley Registry database. Hydrocarbon standards (C5–C20, Accu Standard, New Haven, USA) were analyzed under the same conditions to determine the RIs of the VOCs, following the method described by Vandendool and Kratz (1963). The contents were represented by their peak areas.

2.4. Statistical analysis

All data were derived from three independent experiments, with concentrations expressed as the mean of three replicates. Statistical analysis was performed using SPSS 24.0 software and Microsoft Excel 2019. Data are presented as the mean ± SD ($n = 3$). An analysis of variance (ANOVA) was employed to compare the volatile content among wild strawberry cultivars. PCA and plotting were conducted using the PCAtools and tidyverse packages in R (v 4.3). Venn diagrams were generated using *E*-Venn (www.ehbio.com).

PLS-DA and VIP value calculations were performed using the mixOmics, ropls, ggforce, and ggplot2 packages. Compounds with VIP values greater than 1.5 were selected as potential biomarkers to distinguish the four wild strawberry species. Differences in aroma content among the species were confirmed using box plots generated with the ggpubr and tidyverse packages in R. ANOVA was conducted using the nortest package in R.

3. Results

3.1. Detection and characterization of volatile components in 22 wild strawberry cultivars

The VOCs in the ripe fruit of 22 cultivars from four wild strawberry species were determined using HS-SPME-GC–MS. In total, 126 VOCs that contributed to aroma formation were identified, primarily consisting of fatty alcohols (13), fatty ketones (11), fatty acids (6), fatty aldehydes (11), fatty esters (28), benzenoids (18), phenylpropenes (6), furanones (4), lactones (8), terpenes (18), and MAs (3) (Table S1). As shown in Fig. 1, further phylogenetic analysis indicated that despite significant differences in aroma composition among strawberry species, the aroma components within the same species remained relatively conserved. Based on the content of volatile substances, we classified these compounds into two major clusters: groups A and B, each comprising three subgroups. Group A formed the core aromatic profile for most strawberry germplasms, with the content of substances decreasing sequentially from subgroup A-1 to A-3. In contrast, group B included volatile substances unique to certain germplasms, aiding in the differentiation of strawberry germplasm resources. Most substances in subgroup B-1 were undetectable or found in very low amounts in *F. nilgerrensis* and *F. viridis*, while most volatile substances in subgroup B-2 accumulated only in *F. nilgerrensis*. Similarly, several substances in subgroup B-3, such as aromatic ether 1, aromatic ether 2, and phenol1, were found in higher amounts in only *F. viridis*. The results indicate that different strawberry species have specific volatile profiles.

3.2. Impact of different types of VOCs on the Total content in 22 wild strawberry cultivars

We calculated the content of each VOC in each sample and observed significant differences in the total volatile content across all samples (Fig. 2). ‘G1’ (*F. nilgerrensis*) had the highest content, which was several hundred times greater than the lowest content in ‘LSCM’ (*F. viridis*). Lactones were identified as the primary contributors to the total volatile content. The total lactone content in *F. mandshurica* significantly differed from that in *F. vesca* and *F. viridis*, as was the case for *F. nilgerrensis* (Fig. S1A). Heatmap analysis of the eight detected lactones revealed that γ-decanolactone, δ-decalactone, and γ-dodecalactone were predominant, particularly in *F. mandshurica* and *F. nilgerrensis*. Clustering further found that ‘J5’ (*F. mandshurica*) was grouped with *F. vesca*, likely due to the absence of γ-6-(*Z*)-dodecenolactone (Fig. S1B). Although they comprised a small proportion of the total content, terpenes showed significant differences between species (Fig. S1C). Despite the diversity of terpenes, myrtenyl acetate and myrtenol were important due to their high levels in *F. mandshurica* and *F. vesca* (Fig. S1D).

Fatty acid derivatives, especially fatty ketones and fatty aldehydes, also contributed significantly to the volatile content. For example, in *F. mandshurica*, except for ‘H3’ and ‘68’, where MAs were the second most abundant after lactones, the other four cultivars had higher levels of fatty ketones. Specifically, ‘0501,’ ‘GS10,’ and ‘J5’ were dominated by 2-undecanone, while ‘DL002’ was dominated by 2-tridecanone (Fig. 2 and Fig. S2A). In *F. vesca*, fatty acid derivatives were primary contributors, with fatty ketones, such as 2-nonanone, being dominant in ‘C2.’ In ‘H4,’ ‘MY10,’ ‘DB,’ ‘MY12,’ and ‘MY27,’ fatty aldehydes, particularly 2-hexenal, were more abundant (Fig. 2 and Fig. S2B).

Although *F. viridis* had a lower total VOC content, it had a relatively high phenylpropene content, especially in three of the four cultivars, excluding ‘LSCM’ (Fig. 2). Chavicol, anethole, and estragole were exclusive to *F. viridis* (Fig. S3 A). Methyl anthranilates were the volatile compounds with the fewest types, with only MA, methyl methylanthranilate, and *N*-acetyl methylanthranilate. Despite their limited diversity, MA was present in all cultivars, with particularly high levels in ‘68,’ ‘H3,’ ‘MY11,’ and ‘LSJ’ (Fig. 2 and Fig. S3B). Similar to MA, furans contained only four volatile compounds: 2-pentylfuran, 3-methyl-2(5H)-furanone, furaneol, and mesifuran. Among these, furaneol was exclusively found in *F. mandshurica* and ‘LSJ’ (*F. vesca*), while other furans varied significantly across cultivars (Fig. 2 and Fig. S3C).

3.3. Volatile constituents in four wild strawberry species

To understand the specificity of VOCs among the four strawberry species, we used a Venn plot, which indicated that most VOCs were detected in at least two species, with 78 volatile compounds common to all four species. Unique compounds were also identified: 2 in *F. mandshurica*, *10 in F. nilgerrensis*, 1 in *F. vesca*, and 1 in *F. viridis* (Fig. 3A). PCA showed that the first principal component explained 37.07 % of the variance, and *F. nilgerrensis* was clearly separated from *F. viridis*, *F. mandshurica*, and *F. vesca* on the PC1 axis. The second principal component explained 25.19 % of the variance, with *F. viridis*, *F. mandshurica*, and *F. vesca* were all separated from each other on the PC2 axis (Fig. 3B). Significant contributors to PC1 were aromatic compounds, fatty acid derivatives, and terpenoids, while PC2 included a more diverse set of compounds, such as furanone, lactone, phenol, terpenoids, aromatic compounds, and fatty acid derivatives (Fig. 3C).

3.4. PLS-DA analysis of volatile Vompounds in four wild strawberry species

PLS-DA was used to identify the potential volatile biomarkers differentiating the strawberry species. Almost, all samples were grouped by species (Fig. 4). In total, 60 potential volatile biomarkers were identified that could be used to distinguish strawberry species (Table 1). For *F. mandshurica*, 19 volatile compounds with VIP values greater than 1.5 were identified as biomarkers differentiating it from other species,

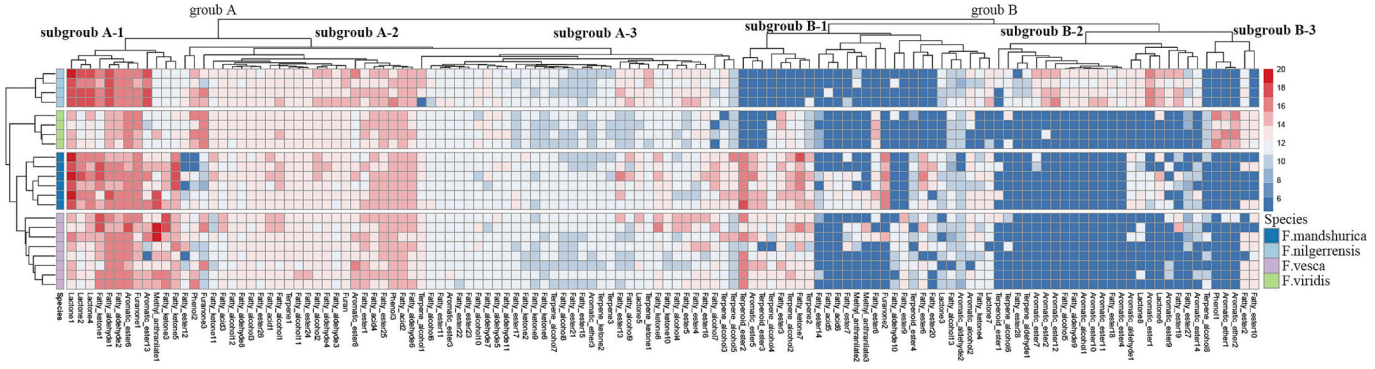

Fig. 1. Heat map and clustering of volatile matter content in 22 wild strawberry cultivars. All values of volatile compounds for each cultivar studies are shown in the heat map, from blue (negative) to red (positive). All cultivars belonging to the same species are marked with the same color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

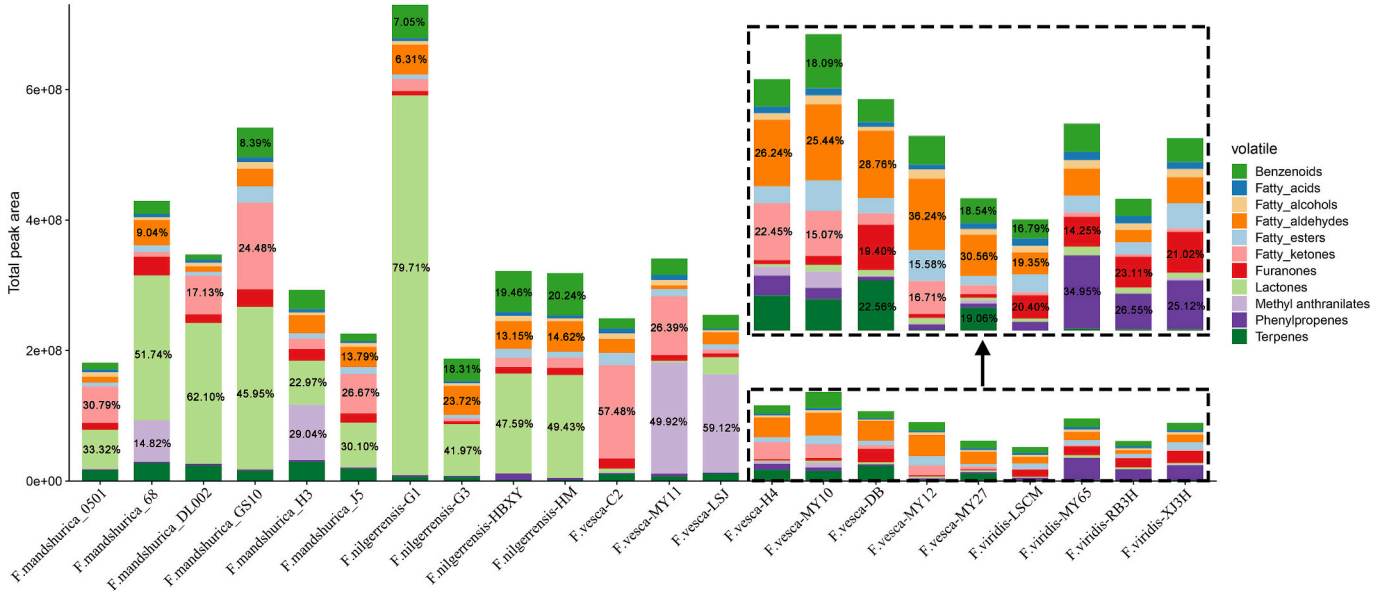

Fig. 2. Stacked chart of the content of each volatile substance in 22 wild strawberry cultivars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

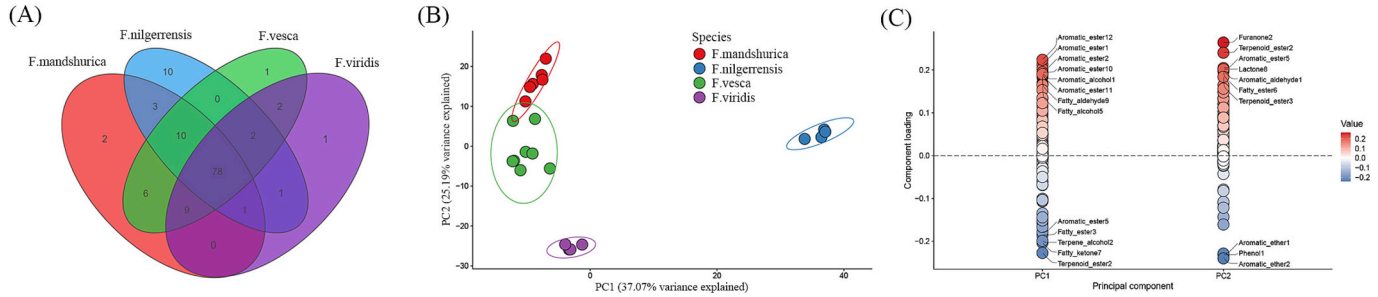

Fig. 3. Volatile compounds in four strawberry species. (A) Venn plot of the volatile compounds in four strawberry species. (B) Principal component analysis (PCA) of the volatile profiles of four strawberry species. (C) Primary volatile compounds contributing to the PCA results.

with furaneol and perillyl acetate being the most prominent (VIP value >2) (Table 1). As shown in Fig. 5A, except for 3-nonen-2-one, 2-undecanol acetate, methyl 3-hydroxydodecanoate, and perillyl acetate, all other potential biomarkers had higher levels in *F. mandshurica* compared to other species, with n-hexadecanoic acid, tetradecanoic acid, and furaneol unique to *F. mandshurica*. For *F. vesca*, PLS-DA identified seven VOCs as candidate biomarkers (Table 1). Although 1-octen-3-ol was the most important compound (VIP value >2), 2-nonanone and butanoic acid butyl ester had higher levels in *F. vesca* compared to other species, with butanoic acid butyl ester exclusively accumulated in *F. vesca* (Fig. 5B). In *F. viridis*, 11 biomarker compounds were selected (Table 1), of which three compounds, namely estragole (2.73), anethole (2.42), and 4-(2-propenyl)-phenol (2.20), had VIP values greater than 2. Six potential markers, namely estragole, anethole, 4-(2-propenyl)-phenol, decanal, eugenol, and mesifuran, were present at higher levels in *F. viridis* than in other species. Additionally, dihydroactinidiolide was undetectable in *F. viridis*, while 4-(2-propenyl)-phenol was exclusively found in *F. viridis* (Fig. 5C). *Fragaria nilgerrensis* had 23 unique biomarkers (Table 1). Comparing the contents of these biomarkers in *F. nilgerrensis* with those in other species revealed that γ-hexadecalactone, decanoic acid ethyl ester, benzaldehyde, and butanoic acid-3-methyl-phenylmethyl ester were present at lower levels in *F. nilgerrensis*, while the levels of other biomarkers were higher. Moreover, nine biomarkers, namely (*Z*)-4-decen-1-ol, (E)-4-decenal, nonanoic acid ethyl ester, benzyl alcohol, benzenepropanoic acid ethyl ester, (E)-2-butyloct-2-enal, hexadecanoic acid phenylmethyl ester, linoleic acid phenylmethyl ester, and 9-octadecenoic acid (Z)-phenylmethyl ester, only accumulated in *F. nilgerrensis* (Fig. 5D).

3.5. Four biomarkers for the identification of four wild strawberry species

We selected specific biomarkers for distinguishing each species: three for *F. mandshurica*, two for *F. vesca*, two for *F. nilgerrensis*, and three for *F. viridis* (Fig. 6). To further validate the reliability of these biomarkers in identifying the four strawberry species, we analyzed 14 additional wild strawberry cultivars, with three from *F. mandshurica*, four from *F. nilgerrensis*, 3 from *F. vesca*, and 4 from *F. viridis*. The results confirmed that these markers effectively differentiated each species, with 2-tridecanone, butanoic acid butyl ester, hexadecanoic acid phenylmethyl ester, and 4-(2-propenyl)-phenol being the most effective for *F. mandshurica* (Fig. S4A), *F. vesca* (Fig. S4B), *F. nilgerrensis* (Fig. S4C), and *F. viridis* (Fig. S4D), respectively. Overall, the selected biomarkers were effective in distinguishing each species.

4. Discussion

4.1. Important volatile organic compounds in wild strawberry germplasm

Strawberries are popular for their distinctive aroma, which is a complex mixture of various VOCs. In this study, 126 VOCs were detected

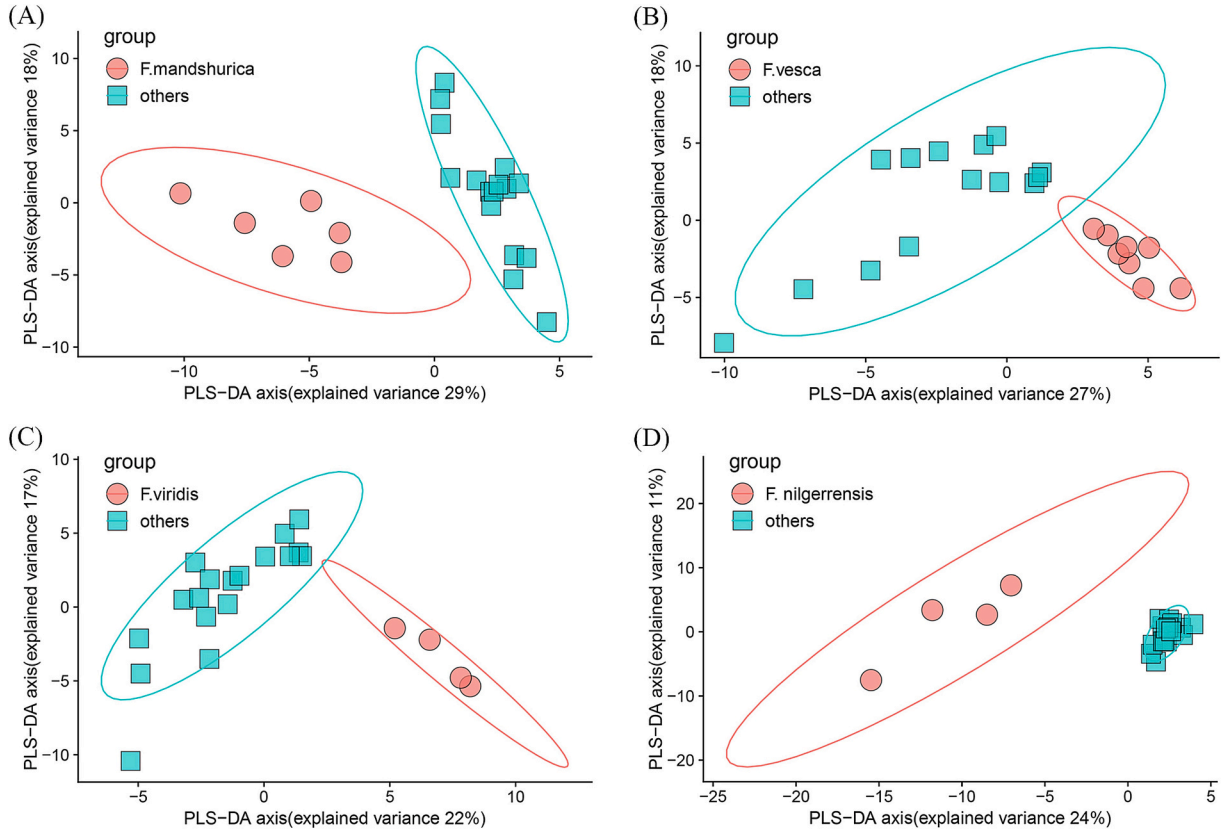

Fig. 4. Partial least squares discriminant analysis (PLS-DA) score plots of four strawberry species. (**A–D**) PLS-DA clearly distinguished *Fragaria* mandshurica (A), *F. vesca* (B), *F. viridis* (C), and F. nilgerrensis (D) from the other three strawberry species using volatile profiles.

Table 1
Potential biomarkers selected in four strawberry species.

ID	Compounds	VIP	*P-value*	ID	Compounds	VIP	*P-value*
F.mandschruica				***F. vesca***			
aroma96	Furaneol	2.10	2.64E-01	aroma2	1-Octen-3-ol	2.32	1.53E-03
aroma112	Perillyl acetate	2.06	9.36E-02	aroma50	Butanoic acid, butyl ester	1.89	1.58E-02
aroma58	Octanoic acid, ethyl ester	1.97	4.46E-02	aroma37	(*E*)-2-Nonenal	1.77	2.69E-02
aroma29	Tetradecanoic acid	1.90	1.34E-01	aroma15	6-methyl-5-hepten-2-one	1.62	5.94E-03
aroma121	Limonene	1.84	1.30E-02	aroma121	Limonene	1.55	1.30E-02
aroma117	(E)-Nerolidol	1.82	9.05E-02	aroma26	Nonanoic acid	1.52	6.60E-03
aroma61	Methyl 3-hydroxydodecanoate	1.81	1.51E-01	aroma16	2-Nonanone	1.50	5.98E-02
aroma18	2-Undecanone	1.81	8.60E-02				
aroma67	Methyl stearate	1.79	1.18E-02	**F. nilgerrensis**			
aroma110	Myrtenyl acetate	1.76	2.40E-01	aroma77	Benzenepropanoic acid, ethyl ester	1.98	1.65E-01
aroma95	3-Methyl-2(5H)-furanone	1.72	2.16E-02	aroma74	Benzoic acid, ethyl ester	1.95	2.13E-01
aroma122	α-Pinene	1.68	6.71E-02	aroma86	Acetic acid, cinnamyl ester	1.93	1.12E-01
aroma20	2-Tridecanone	1.64	8.73E-02	aroma105	γ-Hexadecalactone	1.92	2.02E-01
aroma28	Dodecanoic acid	1.61	1.37E-02	aroma70	Benzyl alcohol	1.92	1.92E-01
aroma30	n-Hexadecanoic acid	1.58	1.20E-01	aroma59	Nonanoic acid, ethyl ester	1.90	1.69E-01
aroma24	cis-1-methylbicyclodecane-2,10-dione	1.58	8.88E-03	aroma39	4-Decenal, (E)-	1.88	2.30E-02
aroma15	6-methyl-5-hepten-2-one	1.53	5.94E-03	aroma5	(Z)-4-Decen-1-ol	1.88	2.26E-02
aroma17	3-Nonen-2-one	1.50	1.64E-01	aroma75	Benzyl Benzoate	1.87	2.02E-01
aroma47	2-Undecanol, acetate	1.50	1.96E-01	aroma106	(E)-2-butyloct-2-enal	1.81	1.35E-01
				aroma72	Benzaldehyde	1.79	1.98E-01
F. viridis				aroma101	γ-Dodecalactone	1.79	7.16E-02
aroma92	Estragole	2.73	2.81E-02	aroma80	2-Ethylhexyl salicylate	1.76	1.65E-01
aroma91	Anethole	2.42	2.64E-02	aroma52	2,2,4-Trimethyl-1,3-pentanediol monoisobutyrate	1.76	3.47E-04
aroma88	Phenol, 4-(2-propenyl)-	2.20	1.75E-02	aroma85	9-Octadecenoic acid (Z)-, phenylmethyl ester	1.72	2.24E-01
aroma38	Decanal	1.98	2.82E-04	aroma83	Hexadecanoic acid, phenylmethyl ester	1.71	1.94E-01
aroma89	Eugenol	1.76	4.97E-02	aroma84	Linoleic acid, phenylmethyl ester	1.69	1.90E-01
aroma49	Butanoic acid, 2-methyl-, methyl ester	1.69	1.54E-02	aroma99	δ-Decalactone	1.69	1.03E-01
aroma31	Hexanal	1.64	2.61E-02	aroma60	Decanoic acid, ethyl ester	1.67	1.67E-01
aroma32	2-Hexenal	1.63	1.86E-02	aroma107	β-Ionone	1.66	4.81E-02
aroma100	Dihydroactinidiolide	1.62	4.92E-02	aroma94	2-Pentylfuran	1.64	2.19E-02
aroma97	Mesifuran	1.59	2.61E-02	aroma82	Butanoic acid, 3-methyl-, phenylmethyl ester	1.62	1.34E-01
aroma19	3-Undecanone	1.51	3.63E-03	aroma104	δ-Tetradecalactone	1.57	1.56E-01

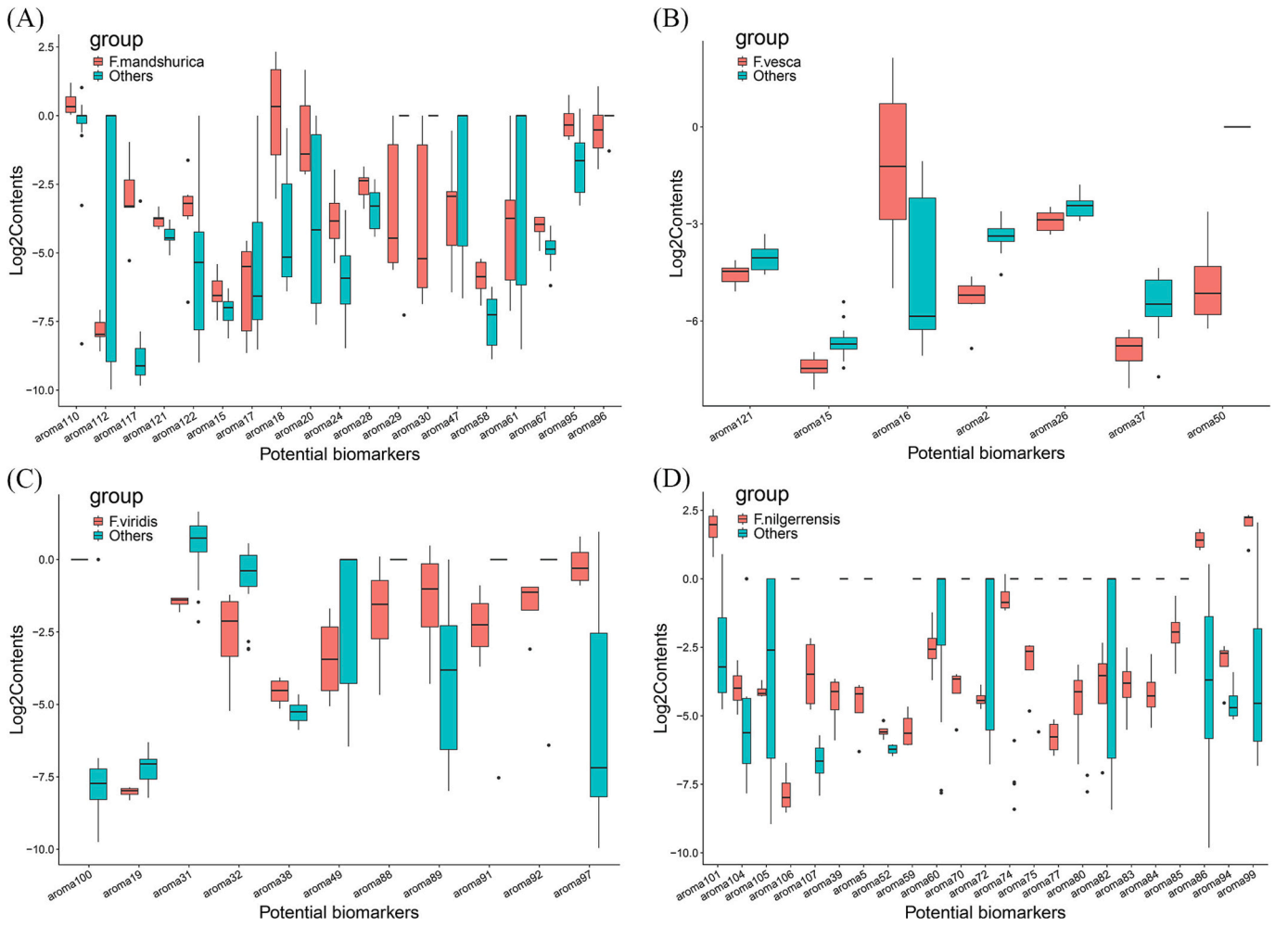

Fig. 5. Boxplots showing the contents of biomarkers in *Fragaria mandshurica* (A), *F. vesca* (B), *F. viridis* (C), and *F. nilgerrensis* (D) and the other strawberry germplasm resources. The biomarkers are listed in Table 1.

from 22 wild strawberry varieties using HS-SPME-GC–MS. A previous study found a similar result, identifying 131 volatile compounds in ripe strawberry fruit of PdT (from *F. moschata*) and RdV (from *F. vesca*) (Negri et al., 2015). Phylogenetic analysis revealed that strawberry varieties of the same species cluster together, indicating a strong correlation between aroma composition and genotype (Fig. 1). The total volatile contents in *F. nilgerrensis* and *F. mandshurica* were significantly higher than those in *F. vesca* and *F. viridis*, aligning with the aroma phenotype of their ripe fruits. Additionally, the contribution of lactone to the total volatile content was much higher in *F. nilgerrensis* and *F. mandshurica* than in *F. vesca* and *F. viridis*. This observation, as shown in Fig. 2, may explain the intense aroma of *F. nilgerrensis* and *F. mandshurica*. In addition to lactones, short-chain fatty acid derivatives, including fatty ketones, fatty aldehydes, and fatty esters, significantly contribute to the total volatile content, especially in *F. vesca*, where they accounted for more than 50 % of aromatic compounds. Regardless of the specific germplasm resource, certain volatile compounds, such as furanones, terpenes, MAs, phenylpropenes, and benzenoids, although generally low in content, significantly affected the aroma composition of wild strawberry species. Our results indicate that each strawberry species has unique volatile compounds, suggesting the potential for identifying strawberry species based on their aromatic compounds.

4.2. Biomarkers for differentiating various strawberry germplasms

Traditionally, wild strawberries have been identified based on morphology. However, some wild strawberry germplasm resources are difficult to distinguish by appearance alone and can be differentiated by the type and content of their aromatic compounds. This study determined the volatile composition of *F. mandshurica*, *F. nilgerrensis*, *F. vesca*, and *F. viridis*, which are known for their strong aromas. The fruit aroma of *F. mandshurica* and *F. vesca* is far more intense and persistent, with heatmap analysis showing similar volatile compositions. PLS-DA revealed that *F. mandshurica* had 19 metabolic biomarkers, significantly more than the seven found in *F. vesca* (Table 1). Higher levels of biomarkers, such as furaneol, myrtenyl acetate, and various lactones, distinguished *F. mandshurica* from *F. viridis*. Additionally, n-hexadecanoic acid, tetradecanoic acid, and furaneol were unique to *F. mandshurica*. These differences in volatile compound levels may explain the distinct aroma of *F. mandshurica* compared to other wild strawberry species and can be used to differentiate *F. mandshurica* from them. A total of 60 metabolic biomarkers were identified to distinguish wild strawberry species. Of these, 10 specific markers were selected to test their differentiation effectiveness, with the results showing very high accuracy. The only challenge was with biomarker '2-nonanone' in *F. vesca*, which had difficulty differentiating between *F. vesca* and *F. mandshurica*. Therefore, using metabolic biomarkers to differentiate wild strawberry germplasm is highly reliable and can serve as a preliminary identification method for wild strawberry classification.

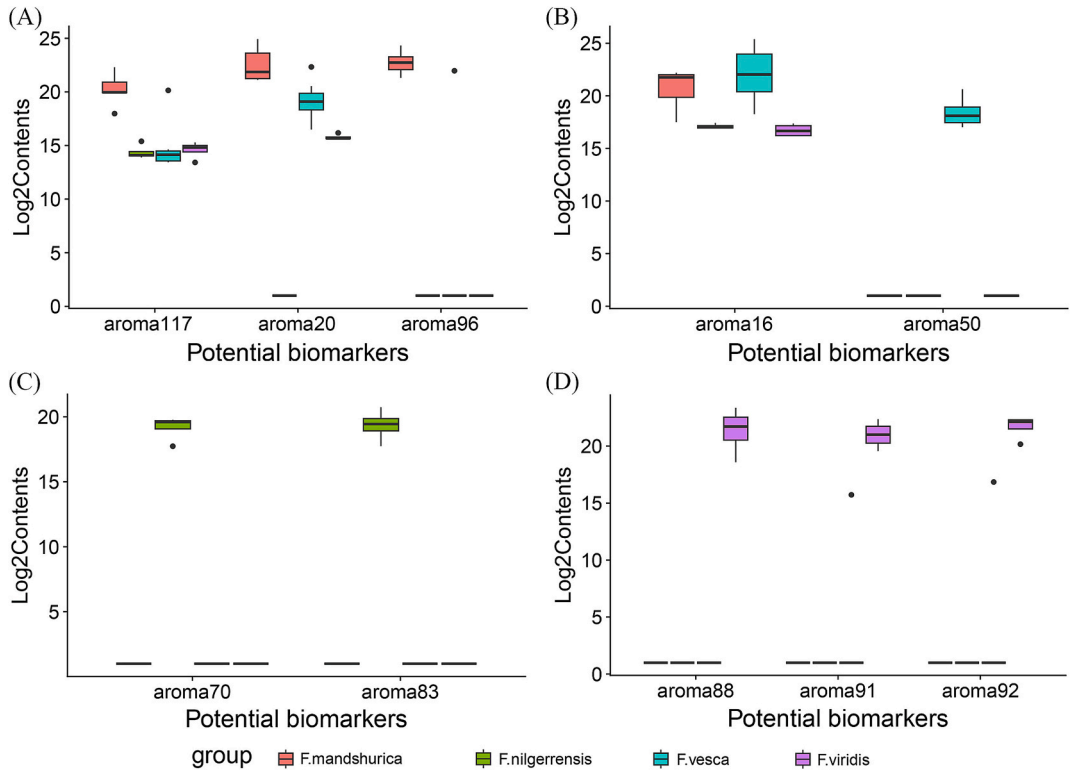

Fig. 6. Boxplots showing the important screened biomarkers in *Fragaria mandshurica* (A), *F. vesca* (B), *F. viridis* (C), and *F. nilgerrensis* (D). The biomarkers are listed in Table 1.

4.3. Biomarkers linked to unique odor profiles in strawberry species

Different volatile compounds impart various sensory characteristics to fruit (Jetti et al., 2007). The diversity of strawberry aroma arises from variations in the types and amounts of volatile compounds present in different strawberry fruit. Previous studies have shown that the main aroma types of strawberry include peachy, pineapple, fruity, floral, and caramel (Sheng et al., 2021). A recent study on the transcriptome and metabolome of *F. nilgerrensis* revealed that lactones, especially δ-decalactone, form the basis of its intense peachy aroma (Wang et al., 2022). Similarly, other studies have confirmed the association of lactones with peachy aroma (Du et al., 2011; Schwab et al., 2008). Zhao et al. (2014) identified two characteristic lactones in *F. nilgerrensis*, with γ-dodecalactone potentially linked to the peachy aroma. Among the 23 potential biomarkers in *F. nilgerrensis*, four are lactones, with δ-decalactone and γ-dodecalactone present in significantly higher amounts. Benzaldehyde contributes to its unique aroma with an almond scent. In *F. viridis*, the primary aromatic compounds include furaneol methyl ether, methyl eugenol, 2-methylbutyric acid, anethole, and isoeugenol, with anethole imparting an anise-like aroma (Aharoni et al., 2004). Our study identified anethole as an important biomarker in *F. viridis*, which abundantly accumulated, and playing a crucial role in its unique aroma profile. Other biomarkers, such as hexanal and 2-hexenal, may contribute to their floral and fruity aromas. The most important biomarker in *F. mandshurica* was furaneol (2,5-dimethyl-4- hydroxy-3(2H)-furanone, DMHF), which, despite its low concentration, has a low odor threshold and contributes to the caramel-like aroma of wild strawberries (Du et al., 2011; Zhang et al., 2018). The potential biomarkers butanoic acid butyl ester and nonanoic acid in *F. vesca* contribute to pineapple and cheesy aromas, respectively (Abouelenein et al., 2023).

4.4. Potential utilization of biomarkers in wild strawberry germplasms

In strawberry breeding, traits such as fruit yield, maturity, and color attract attention from breeders, while aroma is often overlooked. Wild strawberries are rich in volatile compounds and have a strong aroma. However, a survey of 35 cultivated strawberry varieties has shown that even the most aromatic commercial varieties contain fewer than 80 VOCs (Schwieterman et al., 2014). Therefore, in recent years, researchers have begun to focus on the differences in aroma between cultivated and wild strawberries. For instance, Ulrich et al. (2007) compared the volatile compounds of one cultivated strawberry variety and four wild strawberry varieties with distinct aromas and found a higher volatile content in wild strawberries. The nasal impact frequency (NIF) profiles of wild strawberry cultivars are more diverse and intense compared to that of *Fragaria* × *ananassa*. Previous studies have also shown that *Fragaria* × *ananassa* lacks specific compounds found in wild strawberry species, such as myrtenyl acetate, a monoterpene common in wild species but rare in *Fragaria* × *ananassa* due to the absence of its precursor, α-pinene (Aharoni et al., 2004). Moreover, *Fragaria* × *ananassa* only DMHF, with 2,5-dimethyl-4-methoxy-3(2H)-furanone (DMMF) being undetectable (Wang et al., 2018; Wang & Yin, 2018). However, higher levels of DMMF in some *F. mandshurica* varieties may represent valuable breeding material to enhance the DMMF content in cultivated strawberries.

VOCs have garnered significant attention in the food industry for their multifunctional roles as natural additives, including flavor enhancers, fragrance agents, and antimicrobial agents, and for their potential health benefits (Gong et al., 2023). Numerous VOCs, such as limonene, β-cymene, citral, cinnamaldehyde, carvacrol, menthol, and thymol, have been approved by the European Food Safety Authority (EFSA) for safe use in food products (Pellegrini et al., 2018). Recently, research has highlighted the ability of plant-derived aromatic compounds to influence metabolic processes and deliver a wide range of health-promoting effects (Li et al., 2015; Rekha et al., 2013). Linalool and its derivative α-terpineol have shown significant antibacterial efficacy against periodontopathic and cariogenic bacteria, prompting their incorporation into oral care products, such as toothpaste and

mouthwash (Park et al., 2012). Furthermore, linalool has been reported to exhibit anti-inflammatory activity by activating the Nrf2 signaling pathway (Li et al., 2015). As natural constituents of plant-based foods and as food additives, plant VOCs are increasingly recognized as essential elements in the development of future flavor profiles, playing an irreplaceable and growing role in the food industry.

5. Conclusion

This study provides a detailed characterization of VOCs in the ripe fruit of 22 wild strawberry cultivars using HS-SPME-GC–MS and identifies 126 VOCs linked to aroma formation. The results reveal that the composition of aromatic compounds is closely associated with the genotype of each species, with *F. nilgerrensis* and *F. mandshurica* exhibiting significantly higher total volatile contents. Additionally, this study demonstrated that VOCs can serve as reliable biomarkers for differentiating wild strawberry species, identifying 60 potential markers through the application of multivariate statistical techniques, such as PCA and PLS-DA. These findings establish a critical foundation for the use of volatile compounds in the identification and genetic improvement of strawberry species and provide insights that can inform breeding programs aimed at enhancing the flavor profiles of cultivated varieties.

A major strength of the study lies in its comprehensive analysis of strawberry volatiles, which opens up potential applications for flavor improvement in strawberry breeding and the food industry. However, there are notable limitations. This study lacked sensory evaluations to correlate VOCs with the perceived aroma, and the relatively small sample size may limit the generalizability of the findings. Moreover, the focus on ripe fruit excludes VOC variations across growth stages, which may be a significant factor in the overall volatile profile. Although 22 wild strawberry varieties were examined, the findings may not fully represent the diversity within the broader spectrum of wild strawberry species. Additionally, the analytical methods employed are effective, they may not capture all VOCs, especially those present at low concentrations or those with high volatility. Furthermore, the study did not account for environmental factors or post-harvest processing effects on the VOC content, and VOCs were analyzed at a single time point.

Therefore, future research should investigate the variations in VOCs across different stages of strawberry development while expanding the analysis to encompass a broader range of wild strawberry species and cultivars. Additionally, it is essential to explore the biosynthetic pathways of key VOCs to gain deeper insights into the genetic and environmental factors that influence aroma profiles. These findings can be leveraged in breeding programs aimed at developing strawberries with enhanced aromatic qualities. However, it is important to consider the potential risk of overexploiting wild strawberry germplasm resources, which may jeopardize their natural populations and overall biodiversity. Thus, it is imperative to implement sustainable practices and preserve genetic diversity when utilizing wild germplasm resources.

CRediT authorship contribution statement

Linlin Xu: Writing – original draft, Funding acquisition, Data curation. **Wan Liu:** Validation, Formal analysis. **Zhiliang Pan:** Investigation, Data curation. **Fuhua Pang:** Resources. **Yongqi Zhang:** Visualization, Validation. **Jiahui Liang:** Visualization. **Qinglian Wang:** Funding acquisition. **Jing Wang:** Methodology, Formal analysis. **Mizhen Zhao:** Project administration. **Yushan Qiao:** Funding acquisition. **Huazhao Yuan:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

All 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.102092.

Data availability

Data will be made available on request.

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