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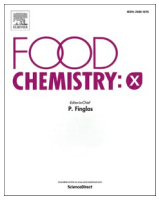

HS–SPME combined with GC–MS and GC–O for characterization of key aroma-active compounds in fruity and grassy peppers (*Capsicum chinense* Jacq.)

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ABSTRACT

Pepper (*Capsicum* spp.) is highly popular due to its unique flavor. However, there was limited research on the primary volatiles that influence the different flavors of fresh peppers. In this study, peppers with three aroma compound types denoted as "grassy," "fruity," and "no special aroma" (control) were analyzed using sensory evaluation combined with gas chromatography–mass spectrometry (GC–MS) and gas chromatography–olfactometry (GC–O). Altogether, 393 volatiles were identified by GC–MS, and the main volatiles in peppers (*C. chinense* Jacq.) were esters and terpenoids. GC–O and relative odor activity value analysis revealed that 2-isobutyl-3-methoxypyrazine had a highly bitter, spicy aroma intensity in all peppers. Hexanal and *trans*-2-hexenal were the main aroma-active compounds in grassy peppers. In addition, citronellal was determined to be a crucial aroma-active compound in fruity peppers. This study offers a theoretical foundation for guiding the growth of the pepper processing industry and breeding.

1. Introduction

Pepper is a commonly grown vegetable crop that is consumed worldwide (Kim et al., 2014). Peppers are extensively utilized across food, medical, and industrial production due to their distinctive spicy flavor, unique aroma, and biologically active substances (Ye et al., 2020). These applications include products such as fermented pepper condiments, hot sauces (Niu et al., 2020) and bean paste (Zhang et al., 2020). Consequently, there is a growing focus on pepper production, with the global output reaching approximately 36.1 million tons in 2020 (Li et al., 2023; Xu et al., 2020).

The aroma of peppers plays a crucial role in determining their quality, whether they are used fresh, processed into fermented products, or incorporated as ingredients in other foods. The genetic diversity among pepper varieties leads to variations in the types and concentrations of volatile compounds, which directly influence the aroma profiles of both fresh peppers and their derived products, thereby affecting consumer preferences (Korkmaz et al., 2017; Rodríguez-Burruezo et al., 2010). However, current research on pepper flavor primarily focuses on products, including the optimization of processing techniques, selection of fermentation microorganisms, and product development, while neglecting the study of fresh pepper flavors (López-Salas et al., 2022; Magalhães et al., 2021). This study was performed to investigate the aroma characteristics and key aroma compounds of different fresh pepper varieties, thereby providing a theoretical foundation for the breeding of improved pepper varieties.

Volatiles extraction by headspace solid–phase microextraction (HS–SPME) is currently a frequently utilized and efficient technique for the extraction of volatiles (Chen et al., 2019). Gas chromatography–mass spectrometry (GC–MS) serves to separate volatiles in complex systems and effectively characterize and quantify these components. It is commonly combined with odor activity value (OAV) analysis to pinpoint the specific volatiles that influence the overall aroma (Nuzzi et al., 2008). However, high OAV volatiles may not always display strong odor properties, thus making it challenging to determine the degree to which the specific aromatic compounds contribute to total fragrance through GC–MS analysis. Gas chromatography–olfactometry (GC–O) combines human olfactometry and GC–MS to enable odor-

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trained panelists to assess the strength and characteristics of individual aromatic compounds (Tatsu et al., 2020; Wang et al., 2021). It is frequently utilized in food aroma investigations (Song & Liu, 2018). GC–O combined with the direct intensity method can identify the impact degree of each aromatic compound on the overall scent. The higher the intensity, the larger its role in the overall aroma. Therefore, combining GC–MS and GC–O analysis can effectively identify volatiles in samples. For example, Xiao et al. analyzed the volatiles within Chinese traditional preserved peppers using HS–SPME/GC–MS and GC–O (Xiao et al., 2010). Ma et al. determined the essential aroma-active compounds in premium Dianhong tea by combining GC–MS and GC–O through sensory–oriented flavor profiling (Ma et al., 2022).

In this study, we utilized *C. chinense* Jacq. as the experimental material to conduct a sensory evaluation and screen three pepper varieties with the following aroma types: "grassy," "fruity," and "no special aroma" (control). Key compounds accountable for the grassy and fruity aromas in peppers were identified using HS–SPME coupled with GC–MS and GC–O. The flavor characteristics of the grassy, fruity, and control peppers were then explored. The current study sought to analyze the impact of diverse kinds of flavor quality on peppers, laying the foundation for future research on pepper flavor characteristics.

2. Materials and methods

2.1. Sample preparation and treatment

All ripe pepper fruits ("grassy peppers," "fruity peppers," and "control peppers") were planted in the greenhouse at Hunan Agricultural University (Changsha, China). The pepper fruits were promptly frozen in liquid nitrogen after harvesting and kept in a − 80 °C refrigerator for the analysis of aroma compounds. Each sample set consisted of three replicates that were harvested with three uniformly sized peppers.

2.2. Chemicals

Authentic standards 3-hexanone, ethyl decanoate, hexanal, *trans*-2-hexenal, citronellol, citronellal, and β-ionone were purchased from Macklin (Shanghai, China). NaCl was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). n-Alkanes (C5-C10) and n-Alkanes (C10-C25) were purchased from ANPEL Laboratory Technologies Co., Ltd. (Shanghai, China) and used to calculate the retention indices (RIs).

2.3. Sensory evaluation

The sensory assessment team consisted of 10 trained members (five males and five females, aged 23 to 45). The participants gave their consent to take part in the sensory study and use their information. Each panel member scored the samples based on the following aroma descriptors: fruity, floral, sweet, grassy, and peppery, and using a scale from 0 to 5 for the intensity (0 = none or no perceptible intensity, 1 = extremely weak intensity, 2 = weak intensity, 3 = moderate intensity, 4 = high intensity, and 5 = extremely high intensity). All data are expressed as averages (Yin et al., 2023).

2.4. Volatiles extraction by headspace–solidphase microextraction

For the HS–SPME analysis, 0.5 g of pepper powder was taken and immediately transferred into a headspace vial with 5 mL NaCl-saturated solution (Agilent, Palo Alto, CA, USA). Following this, 10 μL of internal standard (3-hexanone) was added. The vials were closed with crimp-top closures fitted with TFE-silicone headspace septa (Agilent). For the SPME analysis, each vial was maintained at 60 °C for 5 min, and then a 120 μm DVB/CWR/PDMS fiber (Agilent) was exposed to the sample's headspace for 15 min at 60 °C.

2.5. Volatiles detection by GC–MS

An 8890 gas chromatograph and 7000D mass spectrometer (Agilent, USA) equipped with a 30 m × 0.25 mm × 0.25 μm DB-5MS (Agilent J&W Scientific, Folsom, CA, USA) capillary column were employed for identifying and quantifying volatiles. Helium was employed as the carrier gas at a constant flow rate of 1.2 mL/min. Temperature at the injector was maintained at 250 °C, and the detector temperature was set to 280 °C. The oven temperature was initially set to 40 °C for 3.5 min, then increased to 100 °C at 10 °C/min, followed by 180 °C at 7 °C/min, and finally to 280 °C at 25 °C/min, where it was maintained for 5 min.

The volatiles were determined by matching the mass spectra with the MWGC or NIST database system libraries and the retention index (RI) (Metware Biotechnology Co., Ltd., Wuhan, China). The content of volatiles was determined by comparing their peak areas and the peak area of the internal standard.

2.6. Relative odor activity value (rOAV) calculation

The rOAV of the detected compounds was derived by dividing the relative concentration of each compound by its odor threshold in water. The odor threshold in water was obtained from the literature (Guo et al., 2021). The rOAV can identify key flavor compounds and when combined with sensory thresholds, it can highlight the contribution of each aroma compound to the overall flavor of the sample. In general, rOAV $\geq$1 indicates that the aroma compound has a direct contribution to the sample flavor (Huang et al., 2022).

2.7. GC–O analysis

The GC–MS analytical procedure followed Yin et al. (Yin et al., 2023). The gas chromatography temperature was set at 50 °C for 2 min, increased to 95 °C at 5 °C/min and held for 1 min, then increased to 152 °C at 1.5 °C/min and held for 1 min, and finally increased to 250 °C at 15 °C/min and held for 5 min. No shunt injection was applied as the injection method.

The aromatic active compounds in the pepper samples were analyzed using a sniffing detection port (ODP4, Germany). The effluents were divided into a sniffing port and MS detector at a 1:1 volume ratio. The transfer line temperature of the GC–O sniffing port was 230 °C and the temperature of the GC–O sniffing port was 180 °C. Sniffing analysis was performed by three experienced sniffers and each sniffing test was repeated three times (Yin et al., 2023). The intensity of the aroma attributes was scored using a scale from 0 to 5 (described in Section 2.3).

2.8. Data analysis of volatiles

Bar charts were plotted using Excel 2019 (Microsoft Corp., Redmond, WA). Cluster heatmaps, unsupervised principal component analysis (PCA), and radar plots were created using the Metware Cloud platform (https://cloud.metware.cn). The differential metabolites were measured with variable importance in the projection (VIP) >1 and absolute Log_2FC ($|Log_2FC| > 1.0$).

3. Results and discussion

3.1. Sensory evaluation of pepper aroma

Aroma, which is defined by the composition and contents of volatiles, is a major determinant for appraising fruit quality and typically directs consumer selections (de Araujo et al., 2020). An aroma sensory evaluation was used to explore and identify the odor types of 14 pepper varieties. As shown in Fig. 1 and Table S1, among the 14 pepper varieties, the aroma types were described as fruity, floral, sweet, grassy, and peppery, with varying intensities. All samples had a strong pepper flavor, which is the inherent taste of peppers. Three varieties scored the

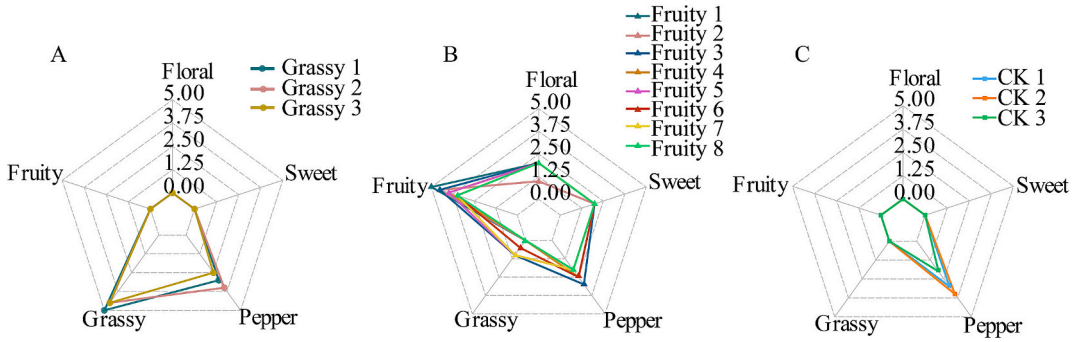

Fig. 1. The Radar Graph: the sensory evaluation of "grassy," "fruity," and "no special aroma" (control) peppers.

highest for the grassy aroma attributes, denoted as "Grassy1," "Grassy2," and "Grassy3," respectively. The eight varieties with the highest fruity aroma attributes were denoted as "Fruity1," "Fruity2," "Fruity3," "Fruity4," "Fruity5," "Fruity6," "Fruity7," and "Fruity8," respectively. Three varieties exhibiting only a green pepper flavor were denoted as "CK1," "CK2," and "CK3," respectively (Table S1 and Fig. 1). Peppers with distinctive aromas have recently gained popularity. The three grassy pepper varieties and eight fruity pepper varieties mentioned above are excellent candidates for breeding high-quality aromatic peppers. In addition, the three pepper varieties with a characteristic pepper aroma serve as excellent controls for studying aroma formation. These 14 pepper varieties possess typical aroma characteristics and can be utilized in subsequent experiments.

3.2. *HS–SPME/GC–MS analysis of volatiles in peppers*

A total of 393 volatiles were detected in the 14 peppers using HS–SPME/GC–MS (Fig. S1). The Total Ion Chromatogram (TIC) of the 14 samples from HS–SPME/GC–MS analysis can be found in Fig. S2. In the PCA score plot, the fruity peppers showed a clear separation from the grassy peppers and control varieties, indicating substantial differences in volatile compounds between the fruity peppers and the other two groups. Notably, the grassy peppers and control varieties did not show a distinct separation in the score plot, instead clustering together, suggesting that the differences in aroma quality between the grassy peppers and control varieties are determined by only a small number of characteristic volatile compounds (Fig. 2A and B).

The detected volatiles were divided into the following 15 categories: esters, terpenoids, ketones, alcohols, aldehydes, and others (Fig. 2C). Terpenoids were the most numerous, with 102 species, accounting for 22.95 % of the total volatilization. These were followed by esters (81), heterocyclic compounds (45), hydrocarbons (33), alcohols (32), aldehydes (29), and ketones (27), with proportions of 20.61 %, 11.45 %, 8.4 %, 8.14 %, 7.38 %, and 6.87 %, respectively, occupying over 90 % of the aggregate volatile volume. The remaining 44 volatiles were aromatics (14), amines (8), acids (8), phenols (4), nitrogen compounds (4), ethers (2), halogenated hydrocarbons (2), and others (2), accounting for 3.56 %, 2.04 %, 2.04 %, 1.02 %, 1.02 %, 0.51 %, 0.51 %, and 0.51 %, respectively (Fig. 2C). Previous research has identified esters and terpenoids as the main volatiles in pepper fruits (Murakami et al., 2019). Alcohols, aldehydes, ketones, and acids are small clusters of aromatic compounds (Huang et al., 2022). Our results are consistent with previous research, supporting our conclusion strongly. Studies have shown that mature phenolic and lipid derivatives, higher alkanes, and sesquiterpenes in sweet peppers formed the primary determinants of genotypic differences, providing a theoretical basis for improving flavor through breeding (Eggink et al., 2012).

As shown in Fig. 2D, The total relative content of volatiles varied

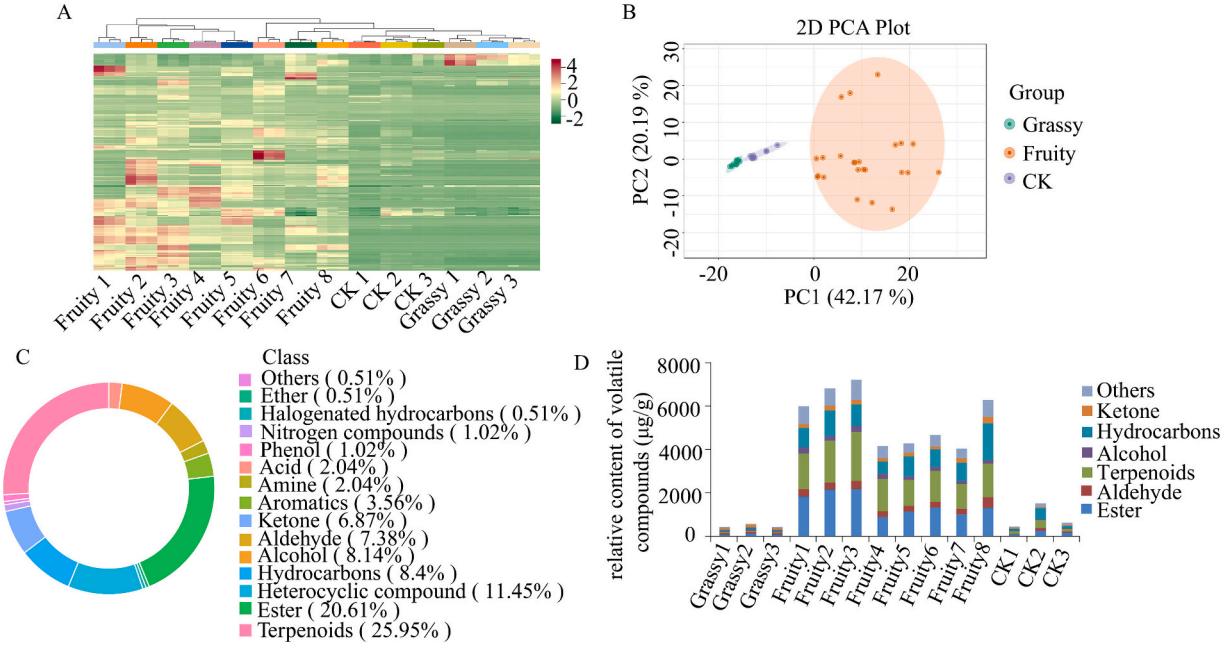

Fig. 2. Volatile compound detection and analysis from 14 varieties of pepper. (A) Cluster heatmap analysis of volatile compounds. (B) PCA score plot of different pepper flesh using all volatile compounds. (C) Categories of volatile compounds. (D) Relative content of volatile compounds quantified to 3-hexanone concentration.

Table 1
The volatile compounds with rOAV values greater than one in pepper flesh produced from different flavor varieties.

Compounds	Categories	CAS	Odor[ψ]	Threshold[#] (μg/L)	rOAV												
					Grassy1	Grassy2	Grassy3	Fruity1	Fruity2	Fruity3	Fruity4	Fruity5	Fruity6	Fruity7	Fruity8	CK1	CK2	CK3
The following was the key aroma compound in all peppers.																		
2-Isobutyl-3-methoxypyrazine	Heterocyclic compound	24683-00-9	green bell pepper, pea, galbanum	0.002	1292081.3 ±34755	630917.3 ±87172.2	852118.5 ±96751.2	792982.1 ±132741.9	292538.8 ±7901.6	329629.1 ±45846.0	97377.1 ±3238.0	269034.4 ±26055.4	86043.7 ±2243.9	677091.0 ±93313.7	630283.9 ±48545.8	263843.3 ±9846.9	430519.9 ±61235.0	794257.7 ±34122.8
The followings were significantly higher in grassy peppers than in CK.																		
trans-2-Hexenal	Aldehyde	6728-26-3	green, grassy	3.1	7323.3 ±254.7	17233.6 ±2524.1	9306.6 ±491.0	2392.6 ±481.6	2885.8 ±935.9	5482.2 ±244.4	2844.9 ±257.5	7116.1 ±1779.6	4218.1 ±215.0	9687.4 ±1826.2	4845.6 ±340.8	2427.9 ±567.9	2793.4 ±501.0	4324.3 ±2109.9
Hexanal	Aldehyde	66-25-1	grassy, green, leafy, vinegar	5.0	3539.4 ±246.2	3556.5 ±437.9	2221.8 ±210.4	722.9 ±96.4	554.0 ±123.6	1472.0 ±113.6	868.1 ±74.2	2038.2 ±417.4	798.5 ±161.2	1131.6 ±296.5	1444.0 ±256.7	769.0 ±107.3	723.8 ±163.9	1499.6 ±289.3
cis-3-Hexenal	Aldehyde	6789-80-6	green, fatty, grassy, fruity	4.0	298.2 ±7.5	555.9 ±54.2	269.6 ±32.1	139.3 ±16.6	93.4 ±19.8	194.4 ±9.1	105.7 ±8.1	208.3 ±35.5	134.8 ±17.9	232.3 ±38.6	133.5 ±26.7	125.7 ±15.3	91.9 ±11.8	159.0 ±44.9
The followings were significantly higher in fruity peppers than in CK.																		
β-Ionone	Terpenoids	14901-07-6	floral, sweet, fruity, berry	0.007	455884.0 ±83760.6	272918.7 ±68050.7	166869.5 ±34474.5	6122450.9 ±1381100.7	4533920 ±159174	4428012.3 ±544955	2091939 ±107573	1846747 ±247033	3343552 ±68714	1620894 ±356879	8446808 ±1571085	201212.3 ±27873.4	1403643.4 ±339390.4	738455 ±280604
β-Damascenone	Terpenoids	23726-93-4	apple, rose, honey, sweet	1.5	1237.5 ±175.8	920.2 ±239.5	307.8 ±96.5	180973.3 ±19108.6	215419.7 ±15521.3	209387.1 ±18026.9	95359.4 ±1354.5	157749.2 ±9251.9	86812.7 ±2980.0	77843.3 ±10688.8	67989.7 ±9452.5	880.2 ±34.8	1964.4 ±626.3	1233.1 ±161.1
(S)-Citronellol	Terpenoids	106-22-9	floral, rose, lime	40.0	1.9 ±0.5	1.8 ±0.6	2.2 ±0.8	448.3 ±49.6	539.2 ±33.3	660.2 ±71.8	218.7 ±6.2	145.9 ±10.3	367.9 ±7.1	223.6 ±36.0	316.4 ±42.0	29.8 ±3.5	84.2 ±14.6	65.3 ±2.9
cis-Citral	Terpenoids	106-26-3	sweet, citral, lemon, peel	1000.0	0.4 ±0.1	0.3 ±0.1	0.4 ±0.2	34.0 ±3.6	48.2 ±2.9	51.7 ±6.0	16.9 ±0.6	21.4 ±1.7	34.5 ±1.0	28.8 ±4.2	37.6 ±4.7	2.6 ±0.3	9.3 ±1.8	7.2 ±0.5
Citronellal	Terpenoids	106-23-0	sweet, dry, floral, aldehydic, citrus	60.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	210.6 ±26.0	38.4 ±1.1	50.5 ±5.7	23.3 ±1.0	64.3 ±7.1	57.6 ±2.0	14.5 ±1.7	21.5 ±3.1	0.6 ±0.1	1.5 ±0.3	1.3 ±0.2
(R)-citronellol	Terpenoids	1117-61-9	citronella oil, rose, leafy, oily,	40.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	2267.4 ±281.5	265.5 ±6.0	918.8 ±78.3	667.4 ±26.4	712.6 ±87.3	2050.7 ±79.8	385.8 ±63.4	29.5 ±5.2	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
Bornyl acetate	Terpenoids	76-49-3	woody, pine, herbal, spice	75.0	0.9 ±0.7	5.0 ±1.7	0.3 ±0.1	3606.6 ±446.2	2291.4 ±188.2	4150.3 ±466.2	4776.8 ±123.1	755.9 ±50.4	576.1 ±21.4	439.4 ±66.3	491.5 ±80.9	2.6 ±0.2	10.6 ±2.9	2.5 ±0.4
cis,trans-α-Farnesene	Terpenoids	26560-14-5	——	87.0	5.1 ±0.6	2.6 ±0.4	4.3 ±1.0	61.5 ±10.0	115.9 ±7.8	87.9 ±7.0	146.0 ±2.7	47.7 ±1.5	98.8 ±5.0	33.5 ±4.2	47.3 ±8.5	1.8 ±0.3	12.0 ±2.4	7.8 ±1.7
2-Methylbutyl 2-methylbutanoate	Ester	2445-78-5	sweet, fruity, estery, berry, green, apple	75.0	9.5 ±0.8	5.8 ±1.3	5.3 ±0.9	332.8 ±41.3	860.3 ±40.5	281.7 ±28.6	41.4 ±1.2	474.7 ±39.1	189.6 ±1.8	605.2 ±83.4	117.6 ±17.3	6.6 ±0.4	12.5 ±1.6	9.5 ±0.8
Sotolone	Ester	28664-35-9	extremely sweet, sugar, coffee	11.0	2.9 ±1.1	16.2 ±5.0	2.2 ±0.5	497.5 ±56.0	323.0 ±9.0	523.5 ±57.2	713.0 ±17.4	193.0 ±14.3	293.8 ±10.3	210.7 ±26.2	753.2 ±111.6	15.2 ±1.4	117.7 ±15.6	3.5 ±0.3
Octyl butyrate	Ester	110-39-4	fresh, waxy, fruity, green	250.0	2.3 ±0.3	1.4 ±0.4	0.7 ±0.1	33.6 ±3.8	24.5 ±1.9	20.2 ±1.6	25.6 ±0.2	16.5 ±0.8	13.8 ±0.7	6.4 ±1.0	9.7 ±1.6	3.0 ±0.2	2.2 ±0.5	1.3 ±0.2
HeptyI isobutyrate	Ester	2349-13-5	sweet, green, fruity, floral,	12.0	12.8 ±1.6	16.4 ±5.3	10.3 ±2.2	1000.9 ±122.6	692.9 ±49.8	968.8 ±110.8	1661.5 ±47.9	235.3 ±16.6	328.9 ±10.4	352.6 ±48.9	252.5 ±34.1	17.4 ±0.4	50.4 ±7.8	25.9 ±0.9
cis-3-Hexenyl hexanoate	Ester	31501-11-8	fruity, green, waxy, pear, tropical, grassy	781.0	0.2 ±0.0	0.2 ±0.1	0.1 ±0.0	130.6 ±15.0	100.0 ±9.1	139.9 ±13.6	70.1 ±0.9	31.6 ±1.8	49.7 ±2.1	23.3 ±3.6	20.4 ±3.3	0.1 ±0.0	0.2 ±0.1	0.3 ±0.0
Octyl Isobutyrate	Ester	109-15-9	green, waxy, fruity, creamy	6.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	1788.7 ±219.5	831.9 ±66.7	1715.4 ±173.9	3040.7 ±45.5	270.9 ±18.7	785.3 ±25.5	170.4 ±25.9	28.1 ±4.6	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
trans-6-Nonenal	Aldehyde	2277-20-5	——	0.022	2340.4 ±485.1	18967.0 ±6193.4	2264.0 ±266.6	510852.2 ±55190.2	340301.4 ±11045.3	520688.1 ±60574.8	693828.5 ±15194.1	206202.2 ±13664.3	307209 ±10084	226991.0 ±29654.0	765564.5 ±113902	16984.6 ±910.2	130316.8 ±17294.4	3514.7 ±187.0

(*continued on next page*)

Table 1 (*continued*)

Compounds	Categories	CAS	Odor[Ψ]	Threshold[#] (μg/L	rOAV													
					Grassy1	Grassy2	Grassy3	Fruity1	Fruity2	Fruity3	Fruity4	Fruity5	Fruity6	Fruity7	Fruity8	CK1	CK2	CK3
Nonanal	Aldehyde	124-19-6	aldehyde, citrus, orange peel	1.0	417.3 ±38.2	332.0 ±53.8	317.7 ±77.0	6866.7 ±831.3	15064.1 ±737.9	5077.1 ±563.1	1306.7 ±41.2	8212.6 ±659.0	3532.5 ±52.2	11138.9 ±1544.4	2357.2 ±318.3	280.9 ±5.8	453.7 ±65.8	380.2 ±40.7
trans-4-Nonenal	Aldehyde	2277-16-9	fruity	2.2	92.3 ±8.6	71.7 ±9.6	70.3 ±4.5	1170.2 ±158.8	3192.8 ±173.9	1000.4 ±113.1	254.0 ±15.8	1833.4 ±145.3	774.4 ±8.0	2162.1 ±325.1	506.1 ±74.2	56.6 ±0.4	101.9 ±11.9	84.8 ±11.8
trans-4-Decenal	Aldehyde	65405-70-1	fresh, aldehydic, orange,	25.0	61.3 ±8.5	135.2 ±41.0	71.6 ±19.3	4449.3 ±483.0	3894.4 ±251.9	5093.5 ±546.0	3185.3 ±73.7	1462.7 ±105.5	4279.7 ±107.7	2781.7 ±420.0	7570.9 ±1103.2	337.7 ±27.9	1504.0 ±220.8	262.0 ±15.9
cis-2-Nonenal	Aldehyde	60784-31-8	green, waxy, cucumber	4.5	1.1 ±0.7	5.1 ±1.1	16.7 ±2.8	886.6 ±103.0	896.2 ±35.6	1294.4 ±136.0	1097.4 ±35.6	194.3 ±16.4	535.4 ±18.0	383.5 ±70.4	584.9 ±82.7	24.8 ±2.5	81.9 ±13.3	8.2 ±1.1
2,4-Undecadienal	Aldehyde	30361-29-6	spicy, citrus	1.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	7033.5 ±1142.6	7174.1 ±557.6	10899.6 ±1063.4	2816.6 ±59.8	1545.9 ±118.0	5052.2 ±259.5	2012.6 ±336.3	802.6 ±181.7	65.4 ±6.0	56.8 ±14.8	56.5 ±5.8
2,4-Decadienal	Aldehyde	25152-83-4	fried, fatty, geranium,	0.07	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	440467.0 ±52886.2	282280.6 ±23697.1	513041.9 ±57436.8	581145.8 ±15711.1	94048.5 ±6451.5	70953.8 ±2675.3	54966.8 ±8374.3	57355.5 ±9593.9	399.0 ±33.2	2878.0 ±500.4	1396.4 ±128.1
trans,cis-2,6-Nonadienal	Aldehyde	557-48-2	green, cucumber, green	0.01	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	1246545.4 ±154816.8	381174.1 ±19421.6	302745.2 ±33900.2	139831.2 ±5685.9	385700.7 ±42993.5	345395 ±11734.	86463.2 ±10979.5	129736.4 ±18623.5	3753.3 ±439.8	8829.3 ±1696.8	2250.2 ±361.9
trans,cis 3,6-Nonadienol	Alcohol	56805-23-3	cucumber, green pepper, fruity, watermelon	3.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	4635.5 ±574.0	1806.1 ±54.1	1235.9 ±147.0	491.3 ±24.5	1446.0 ±140.9	1242.4 ±47.1	307.9 ±40.4	480.0 ±78.2	55.0 ±2.8	79.8 ±15.5	45.3 ±8.5
5-Undecanol	Alcohol	37493-70-2	——	410.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	161.0 ±19.4	105.3 ±8.7	191.8 ±21.1	216.7 ±5.8	35.4 ±2.5	29.0 ±1.0	21.2 ±3.2	35.2 ±5.7	0.4 ±0.0	3.8 ±0.8	1.8 ±0.1
Hotrienol	Alcohol	20053-88-7	sweet, tropical, ocimene, fennel	110.0	2.1 ±0.2	1.5 ±0.3	1.2 ±0.2	50.9 ±6.2	147.5 ±6.8	46.7 ±5.2	6.6 ±0.2	81.7 ±6.5	33.1 ±0.4	97.1 ±13.7	21.2 ±3.4	1.3 ±0.1	2.8 ±0.3	1.9 ±0.2
Skatole	Heterocyclic compound	83-34-1	animalic, indole, civet	0.41	106.2 ±5.1	127.1 ±13.6	99.3 ±7.8	912.0 ±92.8	1251.6 ±79.6	1911.5 ±210.8	567.1 ±23.1	1231.4 ±63.1	939.5 ±46.5	1162.5 ±140.7	811.4 ±141.6	407.6 ±11.4	98.8 ±11.6	118.8 ±14.3
trans-Whiskey lactone	Heterocyclic compound	55013-32-6	sweet, spicy, coconut, vanilla	35.0	0.8 ±0.2	0.5 ±0.1	2.5 ±0.4	169.8 ±19.2	202.9 ±17.5	153.3 ±17.4	95.8 ±2.0	138.1 ±9.3	89.9 ±2.8	149.1 ±22.4	69.1 ±10.6	1.0 ±0.1	5.0 ±1.4	2.9 ±0.3
Quinoline	Heterocyclic compound	91-22-5	musty, tobacco, rubbery,	710.0	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0	15.1 ±1.8	23.4 ±1.6	23.5 ±2.6	7.1 ±0.3	8.3 ±0.7	14.8 ±0.5	12.6 ±1.9	17.4 ±2.3	0.8 ±0.1	3.2 ±0.6	2.5 ±0.1
2-Acetylthiazoline	Heterocyclic compound	29926-41-8	earthy corn, bread, nutty	1.0	28.4 ±5.5	18.0 ±5.1	15.1 ±3.6	1090.6 ±119.6	3390.3 ±184.3	1017.8 ±109.1	129.7 ±5.1	1833.7 ±164.4	737.4 ±12.6	2166.1 ±294.5	440.3 ±66.2	19.2 ±4.8	39.0 ±4.3	26.2 ±2.2
Geosmin	Aromatics	19700-21-1	fresh, musty	0.21	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	23341.8 ±2877.2	13836.5 ±1057.6	19209.1 ±2095.4	11552.1 ±459.2	3134.0 ±196.4	10816.3 ±489.0	3132.7 ±524.2	427.5 ±65.8	117.3 ±7.3	170.1 ±15.6	221.2 ±22.0
Dodecanenitrile	Nitrogen compounds	2437-25-4	citrus, orange, peel, spicy	0.09	959.8 ±100.2	801.2 ±65.7	778.2 ±165.5	21751.5 ±3532.4	36577.1 ±1794.2	29132.7 ±2110.8	53851.9 ±1631.7	25146.4 ±326.8	29888.1 ±1459.8	6157.2 ±861.1	15896.9 ±2908.4	681.3 ±66.8	3180.3 ±767.9	1475.1 ±391.0

"——", no odor description information was found in the literature and database.

Data are "means" ± "standard deviation".

[Ψ] Odor description found in the literature with database (http://www.perflavory.com/search.php; http://www.odour.org.uk/odour/index.html; http://foodflavorlab.cn/#/home; http://www.thegoodscentscompany.com).

[#] All the odor thresholds were obtained from Guo et al., 2021.

Table 2
GC–O identification results of pepper flesh produced from different flavor varieties.

RT	Compounds	CAS	Odor[Ψ]	Grassy1	Grassy2	Grassy3	Fruity1	Fruity2	Fruity3	Fruity4	Fruity5	Fruity6	Fruity7	Fruity8	CK1	CK2	CK3
6.2	Hexanal	66–25-1	Green, Grass	5 ± 0	4 ± 0	5 ± 0	2 ± 0	3 ± 1	2 ± 0	3 ± 0	3 ± 0.5	3 ± 1	1 ± 0	3 ± 0	3 ± 0.5	3 ± 0.5	3 ± 0
7.5	*trans*-2-Hexenal	6728-26-3	Green, Grass	4 ± 0.5	5 ± 0	4 ± 0.5	2 ± 0	2 ± 0	1 ± 0	2 ± 0	2 ± 0	1 ± 0	1 ± 0	1 ± 0	2 ± 0	1 ± 0	2 ± 0
11.29	6-Methyl-5-heptene-2-one	110–93-0	Rancid, Mushroom	3 ± 0	3 ± 0	3 ± 0	–	–	1 ± 0	–	1 ± 0	1 ± 0	1 ± 0	–	–	–	–
11.36	2-Pentyl furan	3777-69-3	Musty, Green, Pea	2 ± 0	2 ± 0	2 ± 0	–	–	–	–	–	–	–	–	1 ± 0	1 ± 0	–
13.1	Benzeneacetaldehyde	122–78-1	Green, Spicy	2 ± 0	2 ± 0	2 ± 0	–	1 ± 0	–	–	–	–	–	1 ± 0	2 ± 0	2 ± 0	2 ± 0
15.6	Linalool	78–70-6	Floral	1 ± 0	–	2 ± 0	–	–	–	–	–	–	–	–	1 ± 0	2 ± 0	1 ± 0
15.7	Isopentyl isovalerate	659–70-1	Sweet, Fruity	–	–	–	2 ± 0	1 ± 0	2 ± 0	1 ± 0		1 ± 0	3 ± 0	–	–	–	–
17.3	Pentyl pentanoate	2173-56-0	Fruity	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	–	–	–	–	1 ± 0	–	1 ± 0	–
17.98	Citronellal	106–23-0	Fruity, Floral, Sweet,	–	–	–	4 ± 0	5 ± 0	4 ± 0	4 ± 0	4 ± 0	5 ± 0	5 ± 0	4 ± 0	–	–	–
18.12	*trans*-2-Nonenal	18,829–56-6	cucumber	2 ± 0	2 ± 0	2 ± 1	3 ± 0	5 ± 1	5 ± 1	4 ± 0	4 ± 0	3 ± 0	3 ± 0	3 ± 0	2 ± 0	3 ± 0	3 ± 0
19.44	2-Isobutyl-3-methoxypyrazine	24,683–00-9	Bitter, Pungent,	4 ± 0	4 ± 0	3 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0	3 ± 0	4 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0
21.8	Citronellol	106–22-9	Fruity, Honey	–	–	–	–	2 ± 0	1 ± 0	1 ± 0	–	2 ± 0	1 ± 0	2 ± 0	–	–	–
32.3	6-Methyl-4-heptenyl 2-meyhylbutanoate	1,215,128–05-4	Fruity, Floral	–	–	2 ± 0	3 ± 0	2 ± 0	3 ± 0	2 ± 0	2 ± 0	1 ± 0	3 ± 0	2 ± 0	1 ± 0	–	–
33.25	6-Methyl-4-heptenyl 3-meyhylbutanoate	1,215,128–06-5	Fruity, Floral	–	–	3 ± 0	2 ± 0	3 ± 0	3 ± 0	3 ± 0	2 ± 0	3 ± 0	3 ± 0	1 ± 0	2 ± 0	3 ± 0	3 ± 0
35.7	α-Ionone	127–41-3	Floral	–	1 ± 0	–	4 ± 0	3 ± 0	3 ± 0	2 ± 0	2 ± 0	2 ± 0	2 ± 0	4 ± 0	1 ± 0	2 ± 0	1 ± 0
39.56	β-Ionone	14,901–07-6	Floral	2 ± 0	3 ± 0	2 ± 0	3 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	5 ± 1	5 ± 0	4 ± 0	3 ± 0	3 ± 0	3 ± 0
44.5	Unknown		Waxy, Metallic	–	–	–	2 ± 0	3 ± 0	2 ± 0	1 ± 0	–	–	–	3 ± 0	–	–	–
53.4	4-Methylpentyl 8-methylnon-6-enoate	1,215,128–18-9	Waxy, Metallic	–	2 ± 0	–	–	1 ± 0	2 ± 0	1 ± 0	–	2 ± 0	1 ± 0	–	–	–	–
54.83	cis-9-Hexadecenal	56,219–04-6	Sweet, Fruity	–	–	2 ± 0	3 ± 0	3 ± 0	2 ± 0	1 ± 0	2 ± 0	1 ± 0	1 ± 0	2 ± 0	–	4 ± 0	–

"——", no odor description information was found in the literature and database.
Data are "means" ± "standard deviation".

among different scented varieties. Notably, the fruity peppers displayed a significantly higher relative content of volatiles compared to the control group. In addition, we also studied the contents of different classes of volatiles. Within these volatiles, esters are typically high in all varieties, subsequent to terpenoids, hydrocarbons, aldehydes, alcohols make up at least 70 % of the volatiles, amines, acids, halogenateds, hydrocarbons, nitrogen compounds, aromatics, phenols, ethers are low in content. All of them were below 100.0 μg/g. It is noteworthy that the content of volatiles in grassy varieties was the lowest, and the content of volatiles in each category was below 100.0 μg/g, and the content of terpenoids, esters, hydrocarbons, aldehydes was lower than that in the control group. According to the content and quantity of volatiles, this study found that the main types of volatiles in peppers (*C. chinense* Jacq.) were esters, terpenoids, hydrocarbons, aldehydes and alcohols.

3.3. rOAV analysis of volatiles in peppers

We analyzed 82 compounds with an rOAV ≥1 (Table S1), including esters (19), terpenoids (17), aldehydes (17), alcohols (7), ketones (5), and others (17). A comparative analysis of the rOAV values for different flavored peppers identified 35 volatiles that may significantly contribute to the aroma of peppers (Table 1). Among them, 2-isobutyl-3-methoxypyrazine had a high rOAV in all samples and contributed significantly to the overall scent profile of peppers. In addition, three key aromatic compounds in grassy peppers, namely *trans*-2-hexenal, hexanal, and *cis*-3-hexenal, contribute to the grassy aroma (Rodríguez-Burruezo et al., 2010; Ul Hassan et al., 2015).

Terpenoids are the most abundant secondary metabolites in plants and play an important role in determining the characteristic aroma and flavor of vegetables and fruits (Kelebek & Selli, 2011). In this study, β-ionone, β-damascenone, citronellol, citronellal, and *cis*-citral exhibited markedly higher rOAV values in the fruity peppers compared to the grassy and control peppers. Scholars have shown that β-ionone and β-damascenone are deisoprene (C13) produced by carotenoid (C40) catalyzed by carotenoid cleavage dioxygenase, with β-ionone exhibiting a violet fragrance and β-damascenone having a floral and honey aroma (Fracassetti et al., 2020). Citronellol, chiefly located in plants of the Rosaceae family, is known due to its pleasant floral scent combined with antibacterial, antioxidant, calming, pain-relieving, and mood-enhancing properties (Santos et al., 2019). Citronellal, an oxidized derivative of citronellol, features a powerful lemon, citronella, and rose aroma and is widely utilized as a key flavoring ingredient (Xu et al., 2024).

Esters are major aromatic compounds in peppers. They possess sweet or fruity flavors and can refine the flavor by lessening the impact of offensive odors (Aubert & Milhet, 2007). In this research, the rOAV of 2-methylbutyl 2-methylbutanoate, heptyi isobutyrate, sotolone, octyl butyrate, cis-3-hexenyl hexanoate, and octyl isobutyrate in fruity peppers were higher than those in other varieties. This suggested that they may be crucial in the creation of fruity peppers flavor.

Saturated and unsaturated volatile C6 and C9 aldehydes and alcohols are key elements in the distinctive flavors of fruits, vegetables, and green leaves (Schwab et al., 2008). These compounds can retain the fresh, green, and fruity aromas of fruits and are primarily produced by the oxidative degradation of linoleic and linolenic acids (Xu et al., 2020). In this study, *trans*-6-nonenal, nonanal, *trans*-4-nonenal, *cis*-2-nonenal, *trans,cis*-2,6-nonadienal, and *trans,cis*-3,6-nonadienol, which belong to this class of compounds, were identified in the samples. Their rOAV was significantly higher in fruity peppers than in grassy and the control samples, indicating that they may be the main volatiles in fruity peppers (Buttery et al., 1969).

3.4. GC–O analysis of volatiles in peppers

The TIC of the 14 samples from GC–O analysis can be found in

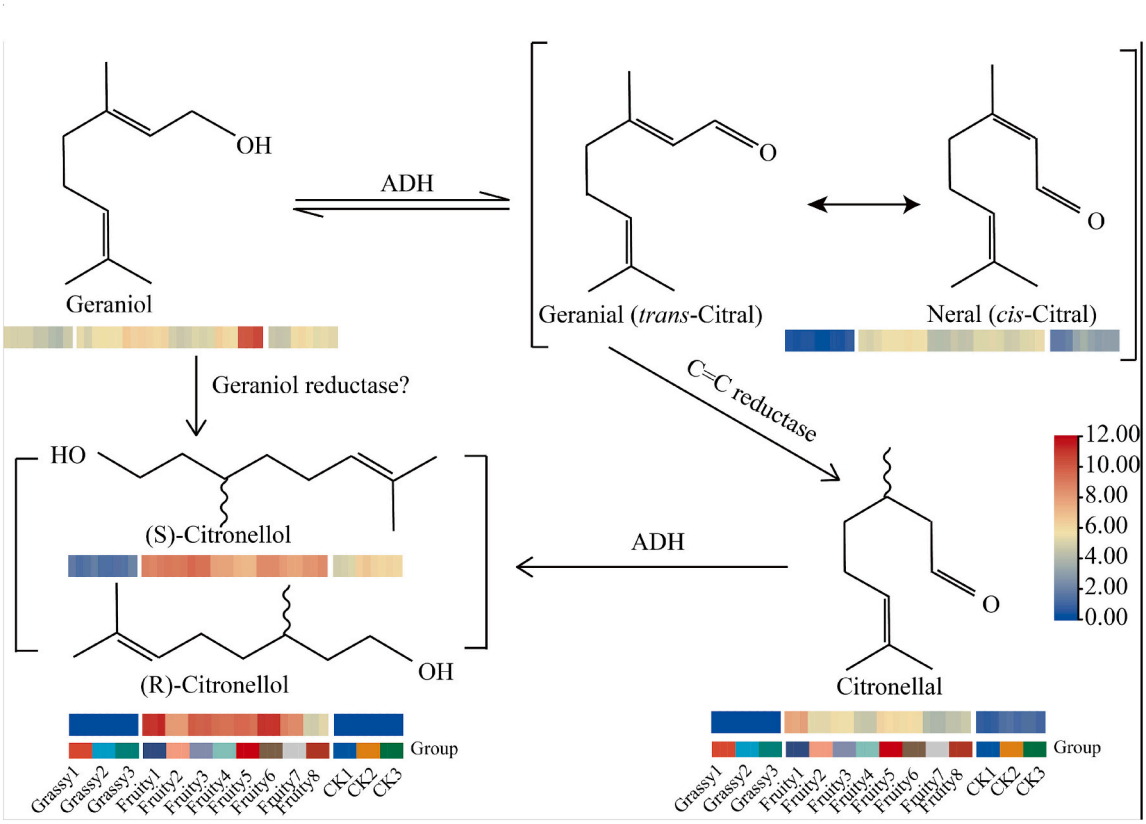

Fig. 3. Synthesis pathway of citronellal and citronellol. The clustering heat map shows the rOAV values of different varieties. The question mark indicates that this step lacks enzyme characterization.

Fig. S3. 19 key aroma compounds were identified using GC–O analysis, including 18 compounds and one unknown compound of target aromatic compounds was obtained using mass spectrometry and RI (Table 2). Among them, 2-isobutyl-3-methoxypyrazine exhibited a bitter, spicy flavor profile and contributed to the robust aroma found in all pepper varieties. Furthermore, hexanal and *trans*-2-hexenal, which possess a grassy and green odor, exhibited varying intensities of aromatic odor in all peppers. However, the aroma intensity of grassy varieties was significantly higher than that of other varieties, ranging from 3.5 to 5. The aroma intensity of 6-methyl-5-heptene-2-one and 2-pentyl furan was higher in grassy varieties compared to other varieties, but with an intensity of only around 2. Therefore, compared to hexanal and *trans*-2-hexenal, they may contribute minimally to the aroma profile of grassy peppers. In addition, citronellal exhibited a high aroma intensity in fruity pepper varieties, yet it was not detected in the other varieties. Thus, citronellal was considered the primary aromatic compound that distinguished fruity peppers from other varieties. Furthermore, *trans*-2-nonenal and β-ionone presented high-intensity aromas in all the pepper. More specifically, 2-nonenal had a strong cucumber scent, while β-ionone was characterized by a floral aroma. The results reveal that although they were important aromatic compounds, *trans*-2-nonenal and β-ionone made a limited contribution to the overall aroma type of peppers.

3.5. Identification of key aroma-active compounds in peppers using rOAV and GC–O analysis

Combining rOAV and GC–O analysis can effectively and rapidly screen key volatiles (Yin et al., 2023). Previous research has identified that 2-isobutyl-3-methoxypyrazine can influence the flavor of sweet peppers (Lo Scalzo et al., 2020). In this study, GC–O and rOAV analysis indicated that 2-isobutyl-3-methoxypyrazine had a high aroma activity in all materials. Therefore, we identified it as one of the key aroma-active compounds in peppers. Although the rOAV of 2-isobutyl-3-methoxypyrazine varied between samples, it cannot be used to distinguish different varieties.

rOAV analysis indicated that hexanal, *trans*-2-hexenal, and *cis*-3-hexenal were crucial differential volatiles in grassy peppers compared with fruity and control peppers. The GC–O results revealed that hexanal and *trans*-2-hexenal possess strong grassy and green odors, with a higher aroma intensity in grassy peppers compared to the other varieties, while *cis*-3-hexenal was not detected. This may be because the rOAV was calculated based on relative quantitative results, while the true OAV is small. Therefore, *cis*-3-hexenal may contribute negligibly or not at all to the overall fruit aroma. This indicates that hexanal and *trans*-2-hexenal are key volatiles in grassy peppers. Scholars have shown that these compounds are primarily responsible for the characteristic “green odor” of green leaf volatiles (GLVs) (Ameye et al., 2018). Moreover, these compounds can enhance peppers' resistance to stress, for example by repelling/attracting herbivores and their predators, activating and initiating plant defenses, activating genes related to abiotic stress, and exhibiting antibacterial and antifungal properties. Thus, it is of great significance to explore the regulatory mechanism of the synthesis of hexanal and *trans*-2-hexenal. Research indicated that *C. chinense* Jacq. and *C. frutescens* L. exhibited fruity and exotic aromas, were found to contain high levels of certain esters and ionones, while having minimal amounts of green and vegetable volatiles (Rodríguez-Burruezo et al., 2010). However, in this study, the higher contribution of hexanal and *trans*-2-hexenal in grassy peppers led to their characteristic green and grassy aroma. The results showed that these three varieties of peppers can provide good germplasm resources for cross-breeding.

Previous studies rely solely on GC–MS identification and differential analysis without verification through GC–O analysis, which limits the final results (Magalhães et al., 2021). Previous analyses using GC and GC/MS detected limonene, β-phellandrene, citronellal, and geranyl acetate as the major volatiles in Japanese peppers (*Zanthoxylum piperitum* DC.). (Yamasaki et al., 2022). However, the absence of GC–O verification for their key flavor attributes and the confirmation of whether they are the main volatiles in fruity peppers. In this study, combining the GC–O and rOAV analysis highlighted citronellal as a key volatile for distinguishing fruity peppers from other peppers. This is the first time that citronellal has been identified as an important volatile in fruity peppers (*C. chinense* Jacq.), providing a fruity aroma. Geraniol is oxidized to geranial in a reversible reaction under the catalysis of alcohol dehydrogenase 3 (ADH3). Neral and geranial are the two isomers of citral. Geranial is selectively reduced by geraniol reductase at the conjugated double bond between C2 and C3, yielding citronellal. Finally, under the action of ADH3, the C═O double bond in citronellal is reduced to form citronellol. On the other hand, geraniol can be directly reduced to citronellol (Xu et al., 2017). There was no significant difference in geraniol among different cultivars. However, the levels of neral, citronellal, and citronellol were significantly higher in the fruity peppers than in other varieties. Geranial was not detected, possibly because geranial occurs with other co-eluting compounds, resulting in its masking. Since neral and geranial are interconvertible, geranial might also be significantly higher in the fruity peppers than in other varieties. The lack of difference in geraniol might be due to its partial direct reduction to citronellol, which results in a balanced level across the different varieties (Fig. 3). However, the GC–O analysis did not detect geraniol and citral, possibly due to their relatively low OAV. Interestingly, citronellal exhibited an extremely high intensity in the fruity peppers, while it was not detected in the other varieties, indicating the significant contribution of citronellal to fruity peppers. This lays the foundation for research on the primary volatiles in fruity peppers.

4. Conclusions

In this study, the key aroma compounds of various aromatic types of peppers (specifically, three grassy peppers, eight fruity peppers, and three green pepper flavor peppers) were identified through sensory evaluation combined with HS–SPME/GC–MS, rOAV and GC–O. A total of 393 volatiles were identified across all pepper samples using HS–SPME/GC–MS, primarily including terpenoids, esters, hydrocarbons, aldehydes, and alcohols. The analysis of relative content revealed that fruity peppers possess the highest total volatile compound content, followed by peppers with green pepper flavor and grassy peppers. Through rOAV and GC–O analysis, 82 aroma compounds with rOAV $\geq$1 and 19 olfactorily identifiable aroma compounds were determined as contributors to the overall aroma quality of peppers. Notably, 2-isobutyl-3-methoxypyrazine was identified as the key compound responsible for the spicy aroma of peppers, while hexanal and trans-2-hexenal were crucial for the grassy aroma, and citronellal was pivotal for the fruity aroma. The findings of this study can advance the theoretical understanding of pepper aroma chemistry and provide insights for cross-breeding and improving the quality of germplasm resources in terms of aromatic compounds. Future work will focus on elucidating the regulatory mechanisms of hexanal, *trans*-2-hexenal, and citronellal involved in the formation of pepper flavor.

Ethical statement

The authors guarantee that the described work has been conducted in compliance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for human experimentation.

The national laws do not require ethical approval for sensory evaluation. There are no human ethics committees or formal documentation procedures available for sensory evaluation.

The authors affirm the utilization of the suitable protocol to safeguard the rights and privacy of all participants, including no coercion to participate, full disclosure of study requirements and risks, verbal consent of participants, no release of participant data without their knowledge, and the ability to withdraw from the study at any time.

Ethics approval

The study did not involve any human or animal testing.

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CRediT authorship contribution statement

Qingyun Shan: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Yu Wan:** Visualization, Investigation, Data curation. **Jude Liang:** Investigation, Data curation. **Wanjuan He:** Investigation. **Jing Zeng:** Investigation. **Wenhui Liang:** Investigation. **Siwei Xiong:** Investigation. **Meiling Zhang:** Investigation. **Bing Wang:** Investigation. **Xuexiao Zou:** Project administration, Funding acquisition. **Cheng Xiong:** Writing – review & editing, Methodology. **Feng Liu:** Resources, Project administration, Funding acquisition.

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.

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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.2024.101944.

Data availability

Data will be made available on request.

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