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Discrimination and characterization analysis of key flavor compounds in pomegranate from different regions by HS-SPME-GC×GC-TOF-MS and flavoromics

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Abstract: Flavor compounds influence consumer acceptance of pomegranate, so detailed knowledge of flavor compositions and its aroma profile are vital for assessing sensory quality. This study identified and characterized the key flavor compounds and profile in pomegranate from the four production areas, including Panzhihua in Sichuan Province (SL-1), Huaiyuan in Anhui (SL-2), Mengzi in Yunnan (SL-3), and Lintong in Shanxi (SL-4) using HS-SPME-GC×GC-TOF-MS and flavoromics. The results showed that flavor compounds, mainly including alcohols, ketones, esters, and organic heterocycles, was identified in pomegranate (SL) from the four growing regions. PCA and OPLS-DA can effectively distinguish the pomegranate samples from the four regions. Relative odor activity values (ROAV) analysis identified 11 key flavor compounds, including 2,3-butanedione; 2-nonenal, (E)-; Butanal,2-methyl-; furan, 2-pentyl-; heptanal; 1-octen-3-one; limonene; 2-octenal, (E)-; α -terpineol; 1-hexanol; and 2-Propenoic acid, ethyl ester. Among them, 6 key flavor compounds with VIP values greater than 1, such as 2-nonenal, (E)-; furan, 2-pentyl-; limonene; 2-octenal, (E)-; α -terpineol and 1-hexanol, were selected as critical flavor markers to distinguish the aroma of pomegranate samples. The flavor radar charts and Sankey diagram indicated that SL-4 sample has distinct fruity, sweet, fatty, floral and herbal aroma. SL-1 and SL-3 are relatively similar in terms of fruity, green, and fatty aroma, etc. However, SL-3 has a more prominent sweet and waxy aroma than SL-1; SL-1 shows more obvious floral and herbal aroma than SL-3. The floral and herbal aroma of SL-1 were closer to those of SL-4. The SL-2 sample is relatively deficient in these flavor characteristics. Overall, this study provides valuable insights into flavor substance basis and style characteristics differences of pomegranates from different origins, offering scientific purchasing guidance for consumers.

Keywords: Pomegranate; Flavor compounds; HS-SPME-GC×GC-TOF-MS; Relative odor activity values (ROAV); Flavoromics

1. Introduction

Pomegranate (*Punica granatum L.*), also known as An Pomegranate, Danruo, etc., is a plant belonging to the genus *Punica* of the family Lythraceae. It mainly grows in warm climates and is widely distributed in subtropical regions^{1,2}. Its fruit is round, with the outer skin being red or yellow, and contains many translucent seeds, rich in juice³. Nowadays, the cultivation area and consumption level of pomegranate in China have ranked among the top. They are mainly distributed in eight major production areas, including Yecheng in Xinjiang, Lintong in Shaanxi, Zaozhuang in Shandong, Xingyang in Henan, Kaifeng in Henan, Huaiyuan in Anhui, Panzhihua in Sichuan, and Mengzi in Yunnan^{2,4}. Pomegranate is rich in various nutrients such as vitamin C, polyphenols, flavonoids, and anthocyanins, which have a wide range of biological activities including antioxidant⁵, antibacterial⁶, antiviral⁷, antitumor⁸. Pomegranate is an important plant with dual functions of both medicinal and edible value⁴, and is widely used in the fields of food⁹, medicine¹⁰, and health supplements^{11,12}. So far, the quality analysis¹³ and nutritional components^{14,15} of pomegranate fruits have been widely studied. Yu et al. have conducted an investigation on the fruit quality, nutritional components, and antioxidant activities of four varieties of pomegranates in Southern Xinjiang. The results show that there were significant differences in the nutritional components and bioactive components of different pomegranate fruits, and there is a correlation between the active components and antioxidant activity². Li et al. have evaluated the cold resistance of five varieties of pomegranate¹⁶. Wu et al. have studied the genetic diversity of the pomegranate (*Punica granatum L.*) germplasm resources in Shandong and constructed DNA fingerprinting, which providing theoretical basis for scientific classification, variety identification and genetic improvement¹⁷. Deng et al. have reported on effective components of different parts of pomegranate and their significant biological activities, aiming to provide valuable references for the dual medicinal and food applications of pomegranate¹⁸. Despite these elegant reports, further expansion for the systematically analysis of unique flavor characteristics and their differences of pomegranate is in great demand. This

research is beneficial for the choices of consumers and promotes the high-value development of pomegranate resources as well as the design of flavor-oriented products.

The concept of flavoromics was first proposed in 2008, which has played a significant role in promoting the development of the field of food flavor chemistry¹⁹. This technology utilizes non-targeted metabolomics to obtain as much chemical information as possible, and then employs chemometrics methods to link the chemical components with sensory characterization, thereby identifying the compounds that affect flavor perception²⁰. Therefore, flavoromics is based on metabolomics and conducts targeted and comprehensive analysis of all flavor-related metabolites. In recent years, it is possible to conduct a more comprehensive analysis of volatile flavor substances and aroma characteristics in foods, such as fruits²¹, vegetables²², Chinese medicinal herbs²³, seasonings²⁴, wine²⁵ and tea²⁶, by using flavoromics technology and chemometrics statistics methods based on chromatography-mass spectrometry detection technology. The flavor characteristics of fruits are related to consumer acceptance and market preferences²⁷. The flavor and quality of pomegranates are largely determined by their complex volatile flavor components. So far, the protocol for research on the volatile flavor compounds of pomegranates is still relatively limited. Feng et al. have studied the optimization of polysaccharide clarification process for pomegranate juice and its impact on flavor compounds using gas chromatography-mass spectrometry (GC-MS) technology. The results show that aldehydes, esters and alcohols, are the key flavor sources of pomegranate juice, imparting it with characteristics such as fruity, woody, sweet and floral aromas²⁸. In addition, the volatile flavor compounds in pomegranate fruit wine have also been studied. Zhang's group have investigated the migration and change patterns of volatile flavor compounds during the production of pomegranate wine using gas chromatography-ion mobility spectrometry (HS-GC-IMS). The results showed that a total of 36 volatile components were detected and identified in both the pomegranate juice and fruit wine samples. Among them, the pomegranate juice contained aldehyde components such as heptanal, nonanal, and hexanal, which disappeared after fermentation²⁹. Li's group have detected 74 volatile flavor components in the pomegranate wine,

mainly including alcohols, esters, acids and terpenoids, using headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) technique³⁰. It is noteworthy that the detection technology used in the above examples can only detect a limited number of compounds, and it is prone to miss some volatile flavor components³¹⁻³³. Consequently, it is highly desirable to comprehensively reveal the key aroma components of pomegranates by using advanced detection techniques, and comparing the flavor characteristics and differences of pomegranates from different origins.

Headspace solid-phase microextraction (HS-SPME), coupled with comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-TOF-MS) is an emerging chromatographic -mass spectrometry separation technology. This detection technology employs a modulator to connect two fixed-phase chromatographic columns with different separation mechanisms and independent of each other in series, achieving orthogonal separation of components with different boiling points and different polarities³⁴. It has advantages of high throughput, high resolution, and strong analytical capability, and can realize rapid separation and accurate identification of complex sample components³⁵. Nowadays, the GC×GC-TOF-MS detection technology has drawn increasing attention in the analysis of complex food flavors, and has been applied to the analysis of volatile components and flavor characteristics in foods such as seasonings³⁶⁻³⁷, fruits and vegetables³⁸, wines³⁹, tea⁴⁰, and meat products⁴¹. For example, Gao and co-workers have identified and characterized the key flavor compounds in *Zanthoxylum bungeanum* shells and seeds from the seven growing regions of Tianshui city, Gansu province, using GC×GC-TOF-MS and chemometrics³⁷. Fang's group have identified key aroma compounds determination in new Chinese apple cultivars using GC×GC-QTOFMS approach based on sensory-directed flavor decoding. This research will provide a scientific basis for the selection of new superior apple varieties based on flavor quality guidance³⁸. The Wang's group have systematically elucidated the chemical and sensory evolution of Jiangxiangxing Baijiu over 4 to 18 years of aging by integrating sensory evaluation and GC×GC-TOF-MS³⁹. Despite these outstanding achievements, the application of the HS-SPME-GC×GC-TOF-MS detection

technology in the field of food flavor chemistry is still very limited. To our knowledge, the study of key aroma components and flavor characteristics in pomegranate based on the HS-SPME-GC×GC-TOF-MS detection technology is still unexplored.

Inspired by these elegant works and our continuous studies in food flavor chemistry⁴²⁻⁴⁵, this study comprehensively identified and characterized the flavor compound profiles in pomegranate from the four production areas, including Panzhihua in Sichuan Province, Huaiyuan in Anhui, Mengzi in Yunnan, and Lintong in Shanxi, using HS-SPME-GC×GC-TOF-MS and flavoromics. Multivariate statistical analysis (PCA and OPLS-DA) was able to effectively distinguish the pomegranate samples from four different regions. Through the analysis of heat maps and volcano plots of differences substances of pomegranates from different origins, important different volatile compounds between groups can be screened. More importantly, the key flavor differential markers of different pomegranate samples were identified based on the analysis of relative odor activity values (ROAV) and sensory flavor feature radar charts, which is crucial for further evaluating their sensory contribution and flavor characteristics. The analysis results of the sankey diagram can reflect the flavor differences among pomegranate samples. The correlation network diagram between sensory aroma characteristics and flavor compounds were established to elucidate their roles in important sensory characteristics of pomegranate samples. This research aimed to clarify the flavor substance basis and style characteristics differences of pomegranates from different origins, and to provide an effective basis for the identification of pomegranate origins and the choices of consumers.

2. Materials and methods

2.1. Sample source and preparation

The information of the pomegranate samples from the four production areas is shown in Table 1. The fruit samples used in this study, including pomegranates from Panzhihua in Sichuan Province, Huaiyuan in Anhui Province, Mengzi in Yunnan Province, and Lintong in Shanxi Province, were all purchased from regular commercial online stores. The collection and use of

fruit samples are permitted by the commercial stores. Fresh and undamaged pomegranate fruits were selected, and manually removed the peel, the white inner membrane and other impurities to obtain pure pomegranate seeds. The pure pomegranate seeds were placed in a juicer for thorough pressing to obtain crude pomegranate juice. Then, multiple layers of sterile gauze were used to repeatedly filter the crude juice to obtain clear and transparent pomegranate juice. 30 mL of the clear pomegranate juice was immediately stored in 4°C refrigerator to maintain freshness until analysis.

Table 1. The information of the pomegranate sample

Sample number	Production area	Color of pomegranate juice	Taste characteristics
SL-1	Panzhihua in Sichuan	Deep red	High acidity
SL-2	Huaiyuan in Anhui	Pale yellow	Low in sweetness and acidity
SL-3	Mengzi in Yunnan	Pale yellow	High in sweetness
SL-4	Lintong in Shanxi	Red	Balanced sweet and sour ratio

2.2. Experimental methods

2.2.1 HS-SPME conditions

A 0.3 g sample was placed in a 20 mL headspace vial, to which 10 µL of 1 mg/L deuterated hexanol-d13 as internal standard solution was added. Then, the sample was incubated at 80 °C for 10 min. Before extracted sample, the SPME fiber was aged at 270 °C for 10 min. The SPME was then transferred to the incubator at 80 °C for 25 min. After the adsorption, the SPME fiber was desorbed at 250 °C for 5 min in GC injector. After the injection, the SPME fiber was aged at 270 °C for 10 min. 10 µL of 1 mg/L n-alkane was transferred to a 20 mL injection vial for extraction and injection.

2.2.2 GC×GC-TOF-MS instrumental conditions

GC × GC: Analyses were carried out using a LECO Pegasus® 4D instrument (LECO, St. Joseph, MI, USA) consisting of an Agilent 8890A GC (Agilent Technologies, Palo Alto, CA, USA) system equipped with a split/splitless injector, and dual stage cryogenic modulator

(LECO) coupled with TOFMS detector (LECO). A DB-Heavy Wax (30 m × 250 μm I.D., 0.5 μm) (Agilent, USA) was used as first dimension column (¹D) and Rxi-5Sil MS (2.0 m × 150 μm I.D., 0.15 μm) (Restek, USA) was used as a second-dimension column (²D). Carrier gas: He (>99.999%; 1.0 mL/min). The oven temperature of first dimension column was held at 50 °C for 2 min at first, then raised to 230 °C at the rate of 5 °C/min and held for 5 min. The secondary oven temperature was operated at 5 °C higher than the first oven. The temperature of the modulator is always 15 °C higher than that of the second column. Modulation period: 6.0 s. The GC injector temperature: 250 °C.

TOF-MS: Flavor substance was performed on LECO Pegasus BT 4D. Ion source temperature: 250 °C; EI source mode (70 eV); Transfer line temperature: 250 °C; Detector voltage: 1960 V; Mass range: m/z 35–550; The acquisition frequency: 200 spectra/s.

2.2.3 Statistical Analysis

The raw GC×GC-TOFMS data was initially processed using the R software (version 3.3.2) platform. The total matter stacked jade block diagram, total ion flow diagram and venn diagram were generated using Biodeep software. After editing, the data matrix was subsequently imported into the ropls package within the R software platform for multivariate analysis, including PCA and OPLS-DA. The metabolic differences between the two groups were analyzed based on the OPLS-DA score plot. The differential substances were screened according to the variable importance in projection (VIP) values and significant differences ($p < 0.05$). Cluster analysis (CA) was conducted with SPSS Statistics 27.0. Compound identification was performed by referencing the standard spectral libraries of the National Institute of Standards and Technology (NIST) and the Wiley Registry metabolomics database⁴⁶.

2.2.4 Identification and quantification of volatile compounds

A stock solution with 1 mg/L of n-alkanes was prepared in n-hexane and stored at 4°C refrigerator. The retention indices (RI) of the volatile compounds in the sample were calculated using retention times of n-alkanes (C5-C20) and retention time of the target compound based on

the Van den Dool and Kratz equation⁴⁷. Then, the acquired MS fragment ion structures and RI were matched against the NIST20 mass spectral database for identification of volatile compounds. The quantitative analysis of the aroma components was conducted using deuterated hexanol-d13 as the internal standard. The concentrations of target volatile compound were normalized to the concentration of deuterated hexanol-d13. The quantitative formula for the volatile compounds is as follows⁴⁸:

$$C_a = \frac{\frac{A_a}{A_{is}} \times C_{is}}{m}$$

C_a: Compound concentration (mg/g); A_a: Peak area of aroma component; A_{is}: Peak area of internal standard; C_{is}: Concentration of internal standard (mg/L); m: Sample mass (g).

2.2.5 Calculation of ROAV for volatile compounds

The relative odor activity value (ROAV) was employed to evaluate the contribution of volatile component to the overall flavor substances. The calculation formula is as follows:

$$ROAV_A \approx 100 \times \frac{C_A}{C_{stan}} \times \frac{T_{stan}}{T_A} \quad (1)$$

Where: C_A is the relative content (%) of a certain aroma component; C_{stan} is the relative content (%) of the largest contribution component in the aroma system. T_A is the aroma threshold of a certain component. T_{stan} is the aroma threshold of the largest contribution component. 1≤ROAV≤100 is the key aroma component, 0.1≤ROAV<1 is important modifying aroma components, and ROAV<0.1 is potential modifying flavor components⁴⁹.

2.2.6 Flavoromics analysis

The flavor characteristics of these compounds were annotated using the Odor and Flavor DB databases, including flavor names, the minimum and maximum concentration ranges for each flavor, and sensory attributes (e.g., fruity, sweet, floral, green). Sankey plots and radar charts were generated with the ggplot package in R software, while network diagrams were constructed using Cytoscape.

3. Results and discussion

3.1. Flavor compound compositional analysis

In this study, HS-SPME-GC×GC-TOF-MS was used to systematically analyze the volatile compounds of pomegranate from the four production areas. A total of 3187 volatile compounds (VOCs) were identified and classified into ten categories based on their chemical structure, including alcohols (214), aldehydes (37), ketones (170), esters (209), carboxylic acids and their derivatives (52), lipid and lipid-like molecules (280), hydrocarbons (473), organic heterocyclic compounds (354), organic sulfur compounds (29), and benzene ring-type compounds (303) and so on according to the three-dimensional chromatograms (Figure 1). As shown in Figure 2, the number of volatile substances detected in different origin pomegranates vary significantly. The number of volatile substances detected in the SL-1, SL-2, SL-3 and SL-4 samples was 1428, 1233, 1354 and 1600 respectively. The pomegranates from LinTong in Shanxi had the highest number of volatile compounds, followed by those from Panzhihua in Sichuan, Mengzi in Yunnan and Huaiyuan in Anhui. The venn diagram analysis visually presents the compositional differences of volatile compounds in the four pomegranate samples. The diagram shows that 392 volatile compounds were common among all four pomegranate samples, while SL-1, SL-2, SL-3, and SL-4 contained 516, 369, 428, and 548 unique volatile compounds, respectively (Figure 3). The radar chart showing the number and relative content of flavor substances in the four pomegranate samples is shown in Figure 4. The trends of the number of substances of pomegranates from the four growing regions were similar (Figure 4A, See the Table S1 for details). Among them, the substances with a higher number were hydrocarbons, organic heterocyclic compounds, benzene compounds, lipids and lipid molecules, which were followed by alcohols, ketones, and esters. Overall, the SL-4 sample had the highest number of these four types of compounds, followed by SL-1 and SL-3, while SL-2 had the lowest number. The SL-1 sample has the highest content of lipid and lipid-like molecules; the SL-2 sample has the highest content of alcohols and benzene compounds; the SL-3 sample has relatively low contents of

various compounds, which may result in a slightly inferior flavor and texture compared to the other samples; the SL-4 sample has higher contents of alcohols, lipid and lipid-like molecules (Figure 4B, See the Table S2 for details). The other types of compounds for four samples had similar numbers. It is worth noting that although hydrocarbons and lipids and lipid-like molecules were present in high amounts in the four samples, most of them do not possess flavor. Apart from alcohols, compounds containing carbonyl groups, such as ketones, aldehydes, and esters, are the main sources of aroma. Compounds containing carbonyl groups exhibited a mixed flavor of sweet, fruity, floral, green or wine-like aroma.

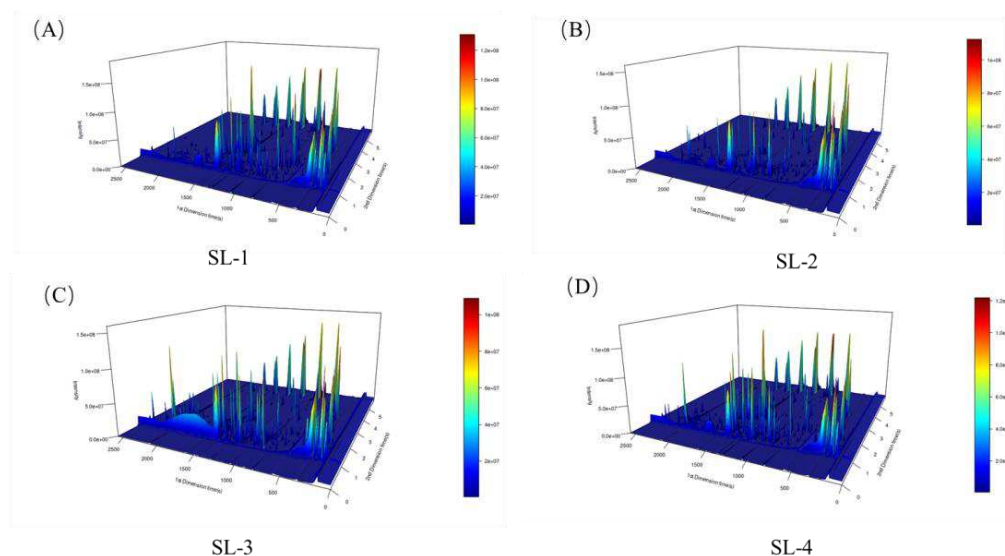


Figure 1. The three-dimensional chromatograms of pomegranate from the four production areas

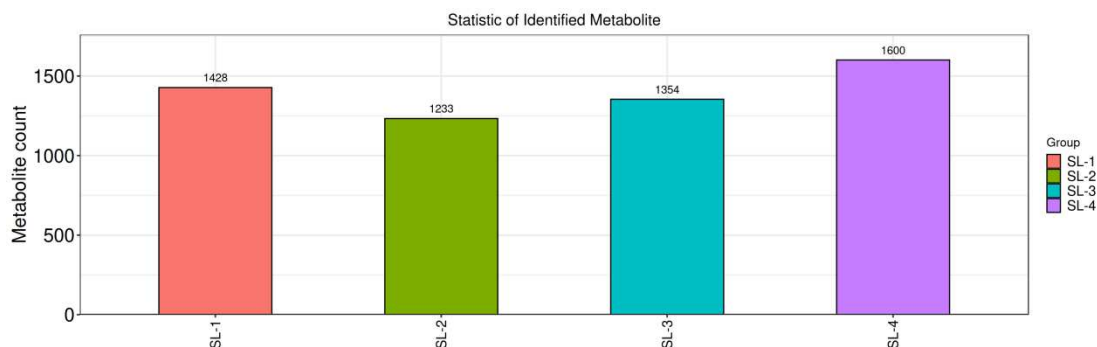


Figure 2. The number of volatile substances of pomegranate from the four production areas

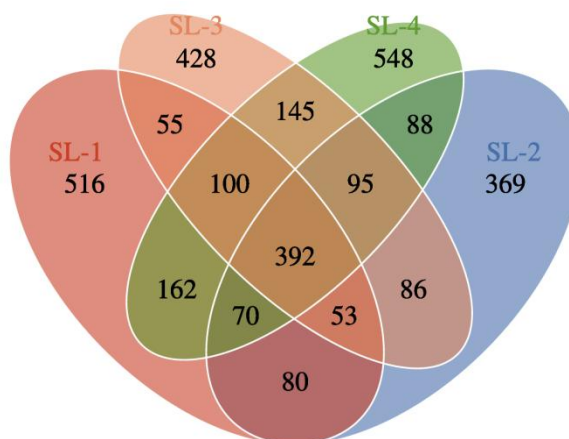


Figure 3. Venn diagram of aroma compounds

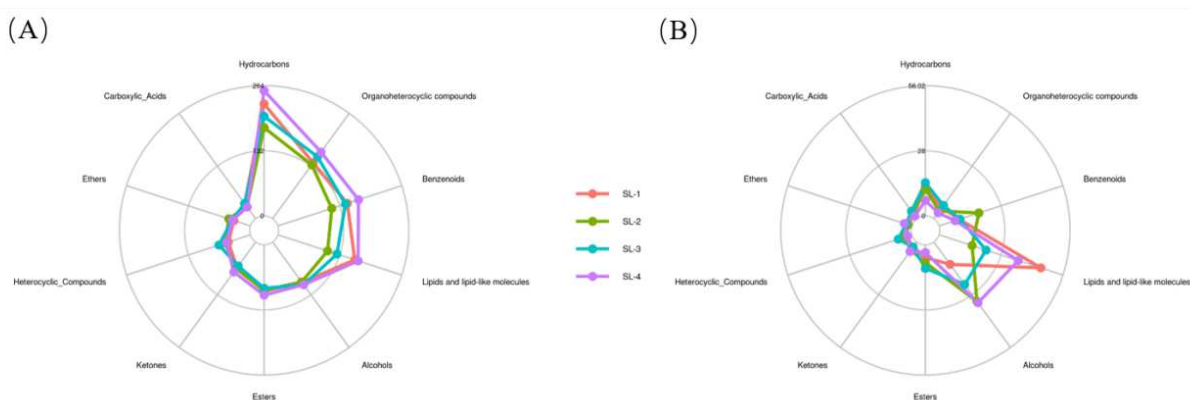


Figure 4. Radar chart of flavor substances of four different origin pomegranates: (A) Based on the number of compounds; (B) Based on the relative content of compounds.

Note: The horizontal axis represents the types of substances, the vertical axis represents the number or content of flavor substances, and the colors indicate different groups.

3.2 Multivariable statistics analysis

3.2.1. Principal component analysis (PCA)

PCA is a detection method based on multivariate statistics. As an unsupervised learning method, the PCA model reflects the original state of metabolomic data, which is conducive to conducting global and descriptive evaluations of sample distributions and improving the accuracy of the model. PCA was used to analyze the differences in flavor compound profile in

pomegranate (SL) from the four production areas growing regions. As shown in Figure 5, there are significant differences among the four samples. The first two principal components (PC1 and PC2) accounted for 21.3% and 20.6% of the differences among SL samples, respectively. These two components together explained 41.9% of the total variance and were distributed in different regions of the score plot. In conclusion, the PCA model did not exhibit overfitting. It can comprehensively and descriptively assess the sample distribution, highlighting natural groupings, trends, and outliers.

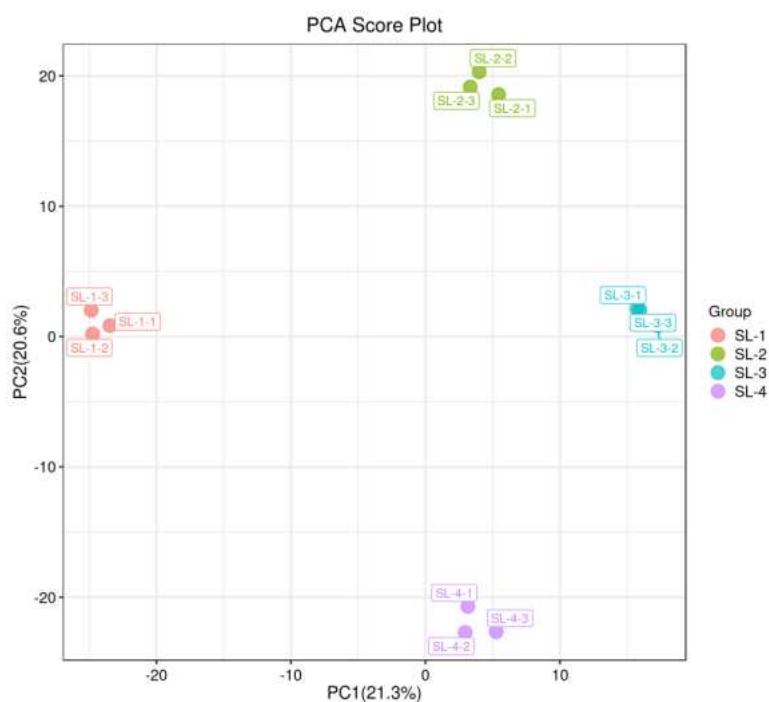


Figure 5. PCA score plots of SL

3.2.2. Orthogonal partial least squares discriminant analysis (OPLS-DA)

OPLS-DA was further employed to establish a correlation model between the volatile compound contents and the sample categories, aiming to better characterize and explain the differences among samples. The statistical results of the OPLS-DA model for the aroma components of pomegranates as shown in Figure 6A. Overall, the three samples from the four production areas of pomegranates were basically clustered in the same region, indicating good

repeatability. Moreover, there were certain differences in the flavor substances of the pomegranate samples from different production areas. The independent variable fitting index R^2X (cum) is 0.342, indicating that the four principal components explain 34.2% of the variation in variable X. The OPLS-DA score plot of SL had R^2Y (cum) and Q^2 (cum) values of 0.999 and 0.868, respectively, indicating a valid model with good predictive ability, which clearly distinguished all the SL samples from the four regions. Both R^2Y and Q^2 exceeded 0.5, indicating that the fitting results of this model were acceptable. Furthermore, Figure 6B presents the permutation test plot. After 200 permutation tests, the intersection of the Q^2 regression line with the vertical axis was less than 0.05, and the true values on the rightmost side of both R^2 and Q^2 were higher than the predicted values on the left, indicating that the model did not exhibit overfitting. Therefore, the OPLS-DA model can be used for the discrimination analysis of aroma components in pomegranate (SL) from the four production areas growing regions.

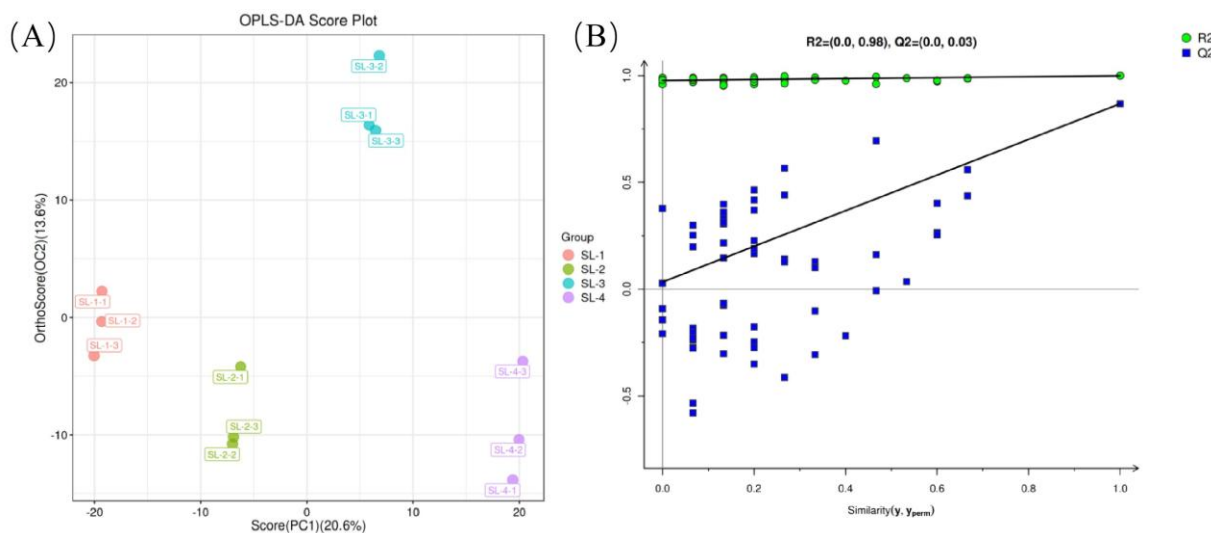


Figure 6. (A) OPLS-DA score plots of SL; (B) OPLS-DA permutation test.

3.3. Analysis of differences in pomegranate samples

3.3.1. Analysis of heatmap of differential substances

To identify the differences among the common substances from the sample substance list, based on the 392 common substances identified. Using the criteria of p value < 0.05 and $VIP > 1$

296 in the t-test or one-way ANOVA test as the screening conditions for relevant differential
 297 substances, a total of 230 differential substances were selected. The 230 substances were
 298 subjected to hierarchical clustering analysis, and the 50 substances with the largest differences
 299 were represented by a heatmap (Figure 7). In the hierarchical clustering heatmap, the redder the
 300 color is, the higher the relative content; the bluer the color is, the lower the relative content. The
 301 samples can be identified based on the differences in the content of compounds among them.
 302 From the comparative analysis of the samples, in sample SL-1, a total of 15 substances with the
 303 highest content include cycloheptane, 1,3,5-tris(methylene)-; 2-(trifluoromethyl)pyridine; (Z)-
 304 ethyl 2-methylbut-2-enoate; 2-isopropylidene-5-methylhex-4-enal; benzene, (methoxymethyl)-;
 305 dodecyl acrylate; 2-Cyclohexen-1-one, 3-methyl-6-(1-methylethenyl)-, (S)- etc. Among them,
 306 benzene, (methoxymethyl)- has fruity, green and floral aroma and is an important component of
 307 the unique aroma of pomegranates. 2-Isopropylidene-5-methylhex-4-enal is a monoterpene
 308 aldehyde compound, providing an important flavor characteristic for the fruity and green aroma
 309 of the pomegranate. In sample SL-2, the contents of 4 substances were relatively high, such as
 310 1H-3a,7-methanoazulene-6-methanol, 2,3,4,7,8,8a-hexahydro-3,8,8-trimethyl-, [3R-
 311 (3a,3abeta,7beta,8aa)]-; a-campholenal; (Z)-undec-6-en-2-one; 3-cyclohexene-1-methanol, 5-
 312 hydroxy-a,a,4-trimethyl-, (1S-trans)-. In sample SL-3, 11 substances such as 1-decene; indole;
 313 nonane; tetradecanal; 1-octanol; tetradecanoic acid, ethyl ester; 1-decanol; octane, 2-methyl-;
 314 pyrazine, trimethyl- had the highest contents. Among them, 1-octanol and 1-decanol have the
 315 characteristics of fatty and floral aroma. In SL-4, 6 substances such as 10-undecenoic acid, ethyl
 316 ester; 2-nonenal, (E)-; 2,4,6-cycloheptatrien-1-one, 2-hydroxy-4-(1-methylethyl)-; pentanedioic
 317 acid, ethyl methyl ester; 2-heptenal, (E)-; 1-hexanol had the highest contents. It is worth noting
 318 that in SL-4, the 2-nonenal (E) and 2-heptenal (E) contribute to the green, fatty and fruity aroma
 319 reminiscent of cucumbers, melons, and citrus fruits. 1-Hexanol offers a sweet, fruity, gentle
 320 floral aroma and a wine-like sensation, making the fruitiness more mellow.

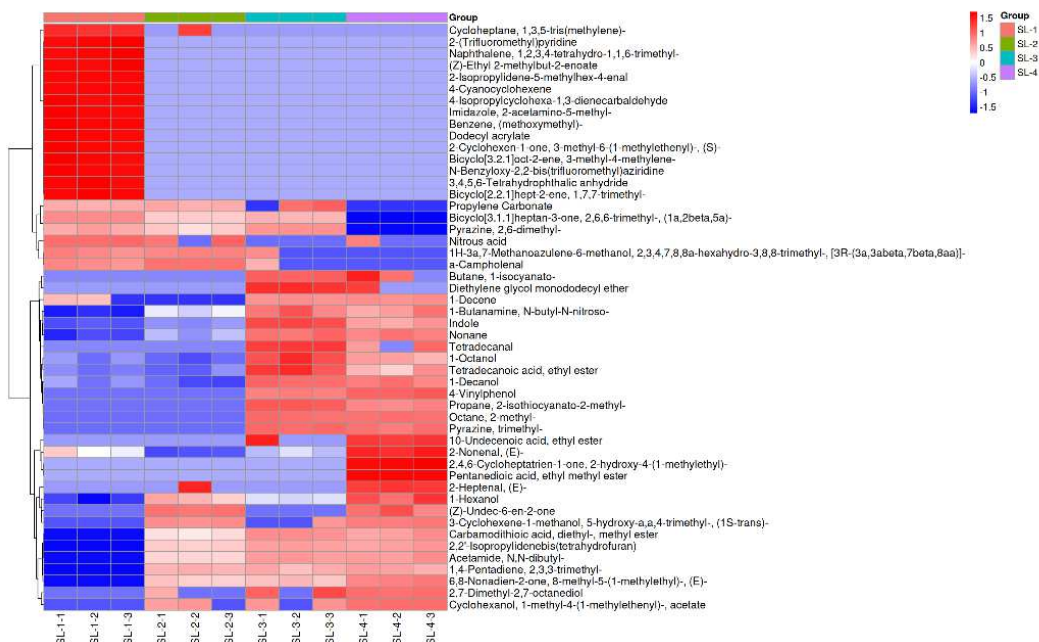


Figure 7. Differential molecular heat map

3.3.2. Analysis of volcano plot of differential substances

The volcano plot can visually display the distribution and change trend of the differential substances between the two groups of samples. As shown in Figure 8, the larger the absolute values of the horizontal and vertical coordinates, the more significant the differential expression of the substance between the two samples. In addition, red dots represent up-regulated substances, blue dots represent down-regulated substances, and gray dots represent substances that do not meet the differential screening conditions. By comparing the SL-1 and SL-2 samples, it can be concluded that [3-(4-methoxyphenyl)-1,2-oxazol-5-yl]methanol; 4-cyanocyclohexene; 2-propenoic acid, 2-cyano-3-(4-fluorophenyl)-, ethyl ester; fenchol can be regarded as different volatile compounds. These compounds can be used as differential markers to distinguish between the SL-1 and SL-2 samples (Figure 8A). In addition, compounds such as indole; 5,9-undecadien-2-one,6,10-dimethyl-,(E)-; 1-propanol, 3-(methylthio)- can be regarded as the different volatile compounds between the SL-2 and SL-3 samples (Figure 8B). The differentiating markers between the SL-3 and SL-4 samples are benzene, 4-ethyl-1,2-dimethoxy-; 3-heptanol, 2-methyl-; phenol; pyrazine, 2,6-dimethyl-; benzothiazole, etc., which can be used to

distinguish these two samples of pomegranates (Figure 8C). Similarly, compounds such as fenchol; 2-isopropylidene-5-methylhex-4-enal; cyclohexanol, 1-methyl-4-(1-methylethenyl)-, acetate can be regarded as the different volatile compounds between the SL-1 and SL-4 samples (Figure 8D). In conclusion, the distribution of the differential substances in each pair of pomegranate samples can be visualized in the volcano plot.

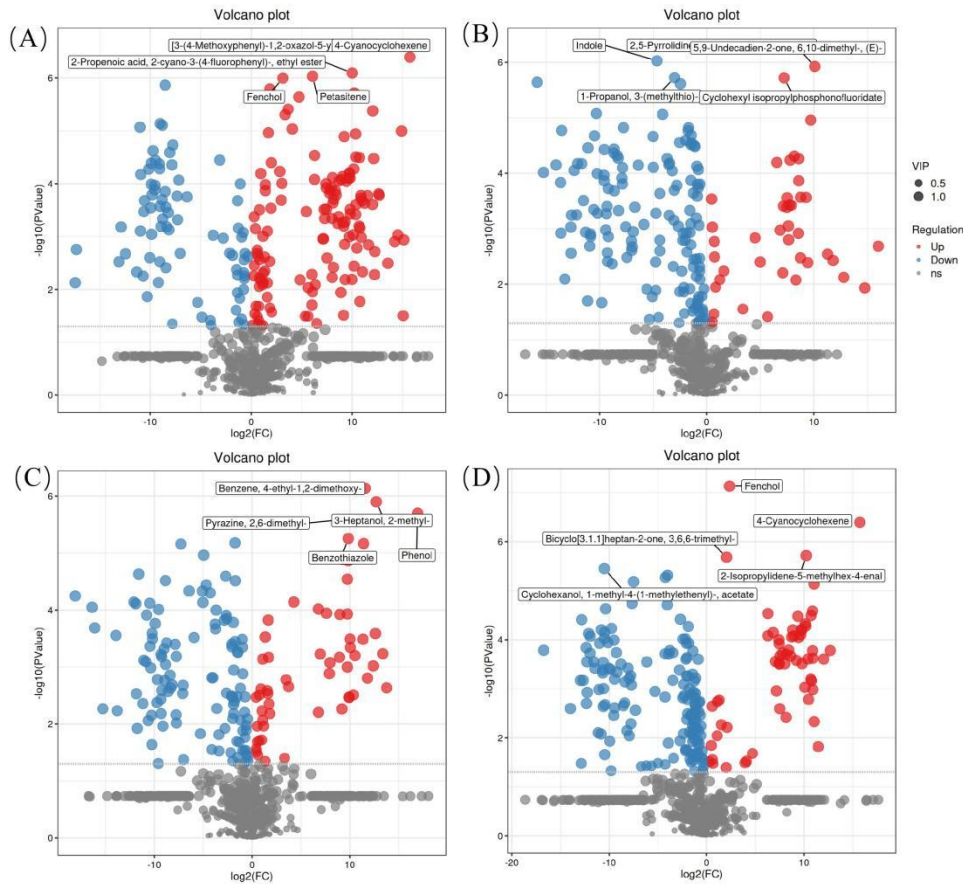


Figure 8. Volcano plot of differential substances: A) SL-1 vs SL-2; B) SL-2 vs SL-3; C) SL-3 vs SL-4; D) SL-1 vs SL-4.

3.4. Analysis of sensory flavor characteristics in pomegranate samples

3.4.1. Analysis of key flavor compounds based on relative odor activity value (ROAV)

Based on the online flavor database and its corresponding odor thresholds, we used the relative odor activity value (ROAV) to evaluate the flavor of the samples and analyzed the differences in flavor characteristics among the four different origin pomegranates. Generally, the

larger the ROAV of the component, the greater the contribution of that substance to the overall flavor of the sample. It is generally believed that the components with $ROAV \geq 1$ are the key flavor compounds of the sample, while the components with $0.1 \leq ROAV < 1$ have significant modifying effects on the overall flavor of the sample. The ROAV values of the substances and their flavor descriptions are shown in Table 2 (See the Table S3 for details). The key flavor components of SL-1 mainly consist of 8 compounds, namely 2,3-butanedione; 2-Nonenal, (E)-; butanal, 2-methyl-; furan, 2-pentyl-; heptanal; 1-octen-3-one; limonene; 2-octenal, (E)-. SL-2 mainly contains 10 key flavor substances, such as 2,3-butanedione; 2-Nonenal, (E)-; butanal, 2-methyl-; furan, 2-pentyl-; heptanal; 1-octen-3-one; trans-2-octenal, (E); α -terpineol; 1-hexanol; 2-propenoic acid, ethyl ester, etc. The key flavor components of SL-3 mainly include 2-nonenal, (E)-; butanal, 2-methyl-; furan, 2-pentyl-; heptanal, 1-octen-3-one, 2-Octenal, (E)-; α -terpineol, 1-hexanol, and 2-propenoic acid ethyl ester, totaling 9 flavor compounds. The key flavor components of SL-4 are 2,3-butanedione; 2-nonenal, (E)-; butanal, 2-methyl-; furan, 2-pentyl-; and heptanal, etc., a total of 5 flavor compounds.

Among the substances with $ROAV \geq 1$, the compounds 2-nonenal (E-); butanal, 2-methyl-; furan, 2-pentyl-; and heptanal are the key flavor substances shared by four pomegranate samples, imparting the typical crisp, juicy, and slightly grassy style characteristics of pomegranates. Among the SL-2 and SL-3 samples, the ROAV values of 2-nonenal; furan, 2-pentyl-; and heptanal were relatively high. Their contribution to the aroma was stronger than that of SL-1 and SL-4. This also indicated that pomegranate fruits of SL-2 and SL-3 might be in the early or middle stage of ripening, not yet reaching the fully ripe and soft state. Thus, they imparted pomegranates with more prominent green and fruity aromas, giving them a refreshing and elegant flavor. Furthermore, 2,3-butanedione is mainly produced during the ripening process of pomegranates, adding a butter-like richness to the fruity aroma of pomegranates. In the samples SL-1, SL-2, and SL-4, especially in SL-1 and SL-4, the ROAV values are relatively high of 2,3-butanedione, which makes these two origin pomegranates more mature and giving them a more

intense fruity sweetness aroma. It is worth noting that limonene is the unique key aroma component in SL-1, which gives it a fruity sweetness with a high acidity. In the SL-2 and SL-3 samples, the ROAV values of α -terpineol, 1-hexanol, and 2-propenoic acid ethyl ester were all greater than 1, while in the SL-1 and SL-4 samples, their ROAV values were between 0.1 and 1. Therefore, these three compounds can also be regarded as the key flavor compounds shared by the SL-2 and SL-3, featuring grassy, floral, and fresh plant juice-like aroma characteristics. This might also be another reason for the more delicate and refreshing flavor of the SL-2 and SL-3 compared to the SL-1 and SL-4.

Furthermore, OPLS-DA was used to analyze the quantitative aroma compounds in different samples, and the variable importance in projection (VIP) values were obtained in Table 2 (see Table S4 for details). Generally, variables with $VIP > 1$ play an important role in distinguishing different samples. As shown in Table 2, there are 6 aroma compounds with VIP values > 1 , namely 2-nonenal, (E)-; furan, 2-pentyl-; limonene; 2-octenal, (E)-; α -terpineol and 1-hexanol. These aroma components can be used as aroma difference markers to distinguish the four different origin pomegranate samples.

Table 2. Analysis of ROAV and VIP of aroma compounds in four different origin pomegranates

Entry	Compounds	CAS	ROAV				Aroma description	VIP value
			SL-1	SL-2	SL-3	SL-4		
1	2,3-Butanedione	431-03-8	66.67	15.15	0	100	pleasant, buttery	0.38
2	2-Nonenal, (E)-	18829-56-6	36.73	44.5	86.49	7.71	fatty, cucumber	1.28
3	Butanal,2-methyl-	96-17-3	29.2	49.14	4.77	1.13	cocoa, almond	0.50
4	Furan, 2-pentyl-	3777-69-3	16.28	78.22	100	6.16	green beans, vegetable	2.08
5	Heptanal	111-71-7	12.92	64.35	90.17	1.72	citrus, fat, green, nut	0.87
6	1-Octen-3-one	4312-99-6	1.63	1.89	2.09	0.31	mushroom-like	0.08
7	Limonene	138-86-3	1.43	0.42	0.5	0.03	pleasant, lemon-like fragrance	1.02
8	2-Octenal, (E)-	2548-87-0	1.10	7.86	9.03	0.47	nuts, green, fatty	2.03

9	a-Terpineol	98-55-5	0.73	1.86	2.45	0.18	wooden, floral	1.58
10	1-Hexanol	111-27-3	0.52	3.83	3.18	0.17	banana, flower, grass, herb	1.79
11	2-Propenoic acid, ethyl ester	140-88-5	0.14	8.84	6.27	0.21	sweet, ester, plastic, alcohol, sharp, ammoniacal	0.94

3.4.2. Analysis of sensory flavor characteristics based on radar chart

Based on the aroma description derived from volatile flavor compounds, the flavor characteristics of pomegranates from different origins were further analyzed. Flavor radar charts were constructed according to the occurrence frequency of flavor compounds. As shown in Figure 9, the overall trend of the aroma characteristics of the four pomegranates samples is relatively consistent, but there are some differences. The four samples of pomegranates from different origins all have aroma characteristics such as fruity, sweet, green, waxy, floral and herbal aroma. Compared with other samples, the SL-4 sample shows more prominent flavor characteristics such as fruity, sweet, waxy, floral and herbal aroma, which is likely related to the 5 key flavor compounds, namely 2,3-butanedione; 2-nonenal (E-); butanal, 2-methyl-; furan, 2-pentyl-; and heptanal. SL-1 and SL-3 have similar aroma characteristics in terms of fruity, green and fatty aroma, but they differ in flavor emphasis. SL-3 has more prominent sweet and waxy aroma, while SL-1 shows more obvious floral and herbal aroma compared to SL-3. The floral and herbal aroma of SL-1 is similar to SL-4. Compared with other samples, the SL-2 sample is relatively deficient in the above flavor dimensions, possibly due to the fewer types or lower content of related flavor compounds. Therefore, in terms of the overall flavor layering and taste coordination, SL-4 and SL-1 may be superior to SL-3 and SL-2. This is consistent with the previous analysis results based on the ROAV of pomegranate samples regarding their maturity and flavor characteristics.

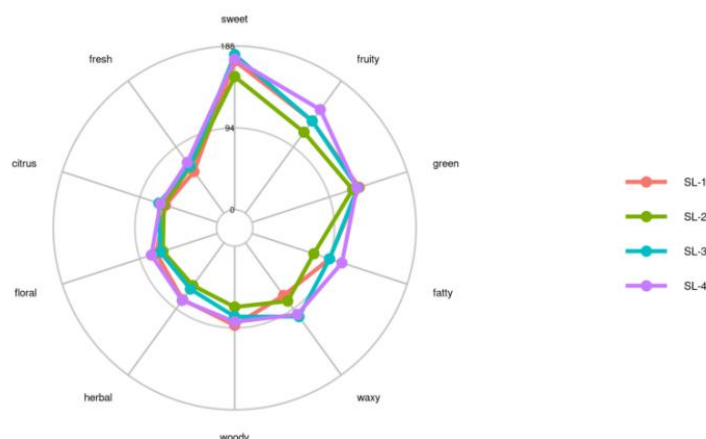
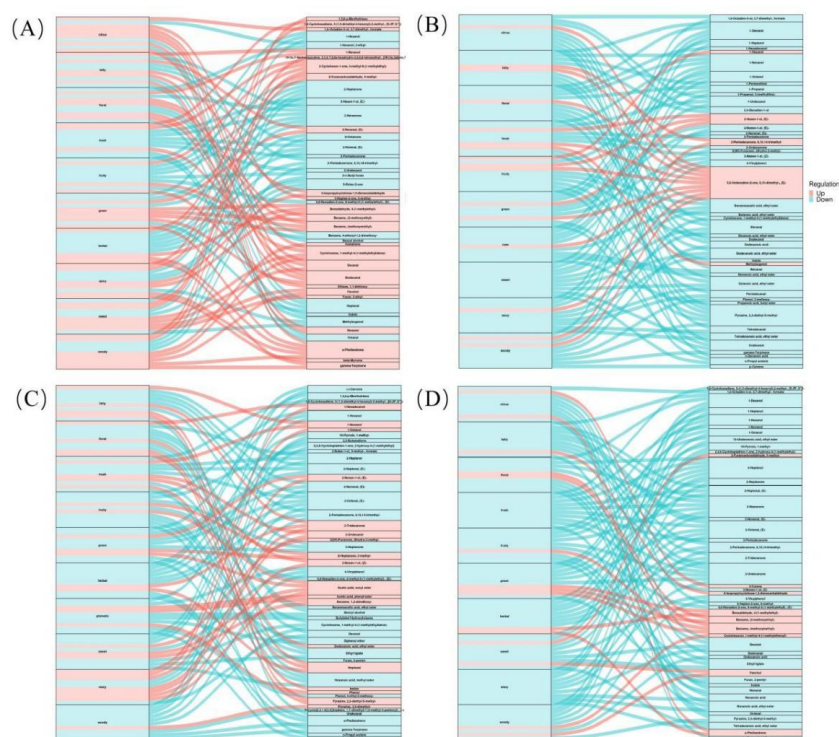


Figure 9. Sensory flavor characteristic analysis radar chart

3.4.3. Analysis of flavor characteristic of differential compounds based on Sankey diagram

The sankey diagram based on the differential flavor compounds and sensory flavor characteristics of the four groups of samples was further drawn. As shown in Figure 10, the left side of the Sankey diagram represents the sensory flavor characteristics, and the right side represents the difference substances. The red arrows represent the flow direction of the up regulated difference substances, and the green arrows represent the flow direction of the down regulated difference substances. The height of the boxes on the left side depends on the number of difference substances shown on the right side. The more the number, the higher the box. The analysis results show that compared with SL-2, the flavor characteristic substances such as citrus, sweet, floral, green, and woody fragrance in SL-1 have shown a significant upward trend. This may be related to compounds such as limonene; decanal; α -phellandrene; fenchol; 2-furancarboxaldehyde, 5-methyl-; benzene, (methoxymethyl)-; benzene (2-methoxyethyl)-; 2-nonenal, (E)-; benzaldehyde 4-(1-methylethyl)-; γ -Terpinene, etc. (Figure 10A). Compared with SL-3, the flavor characteristic substances such as citrus flavor, floral fragrance, fruit aroma, sweet aroma, green aroma, waxy aroma, woody aroma, etc. in SL-2 showed a significant downward trend. The result made SL-3 present more prominent excellent aroma characteristics

430 compared to SL-2. This might be related to compounds such as decanal; 2,4-decadien-1-ol;
 431 indole; dodecanoic acid, ethyl ester; benzeneacetic acid, ethyl ester; 2-nonen-1-ol, (E)-;
 432 tetradecanal; γ -terpinene etc. (Figure 10B). The aroma characteristics of SL-3 and SL-4 are
 433 relatively similar. Both have similar aroma features such as floral, fatty, and green aroma. SL-4
 434 shows more prominent fruity, sweet, and woody aroma compared to SL-3. The SL-3 presents
 435 more prominent waxy aroma than SL-4 (Figure 10C). Compared with SL-4, the flavor
 436 characteristic substances such as citrus, fatty, floral, fruity, sweet, green, waxy, and woody
 437 aroma in SL-1 showed a significant downward trend. This resulted in SL-4 presenting more
 438 prominent excellent aroma characteristics compared to SL-1 (Figure 10D). In conclusion, the
 439 analysis results of the Sankey diagram are largely consistent with those of the previous radar
 440 chart, and can reflect the flavor differences among the four different origin samples of
 441 pomegranates. It also provides a relatively effective theoretical basis for the description of taste
 442 characteristics.



443

Figure 10. Flavor characteristics and flavor substance correlation Sankey diagram: A) SL-1 vs SL-2; B) SL-2 vs SL-3; C) SL-3 vs SL-4; D) SL-1 vs SL-4.

3.4.4. Sensory flavor characteristics and flavor substance correlation network diagram

Using the FlavorDB database, correlation network diagram was constructed between the top 10 sensory flavor characteristics and the flavor substances in SL (Figure 11). As shown in Figure 11, blue circles represent sensory flavor characteristics, and red circles represent flavor compounds. The larger the blue circle, the greater the number of flavor compounds associated with that sensory flavor characteristics, indicating its higher importance. Similarly, the larger the red circle, the greater the number of sensory flavor characteristics linked to that flavor compound, indicating its greater significance. The network correlation diagram indicates that sweet, fruity, green, herbal, fresh, fatty, woody and floral aroma are the important aroma characteristics of different origin pomegranate samples. Among these, sweet and fruity were the most important sensory characteristics, with 27 and 22 flavor compounds respectively associated with each of them. Sweet was linked to 27 compounds including 1-decanol; 1-hexanol; 2,3-hexanedione; 2-heptanone; 2-nonanone and hexanoic acid, methyl ester. Fruity was linked to 22 compounds such as 3,7-dimethyl-, formate; 1-hexanol; benzene, (methoxymethyl)-; benzyl alcohol; butanoic acid, ethyl ester; hexanoic acid, methyl ester and pentanoic acid, ethyl ester. 1-Hexanol was linked to sweet, fruity, green, fresh, floral and citrus. Benzene, (methoxymethyl)- was linked to fruity, green and floral. Hexanoic acid, methyl ester was linked to sweet, fruity, fresh and floral. It is worth noting that this analysis result provides certain theoretical basis for the flavor analysis of the compounds with higher content in the previous heat map Figure 7, such as benzene, (methoxymethyl)-; 1-decanol and 1-hexanol. Overall, this network diagram analysis reveals the complex relationship between sensory flavor characteristics and the flavor compounds in SL. A single sensory characteristic is usually determined by multiple compounds. Some important flavor compounds may be associated with multiple sensory characteristics, indicating that these

compounds have multiple flavor contributions and are crucial for creating flavor layers and complexity.

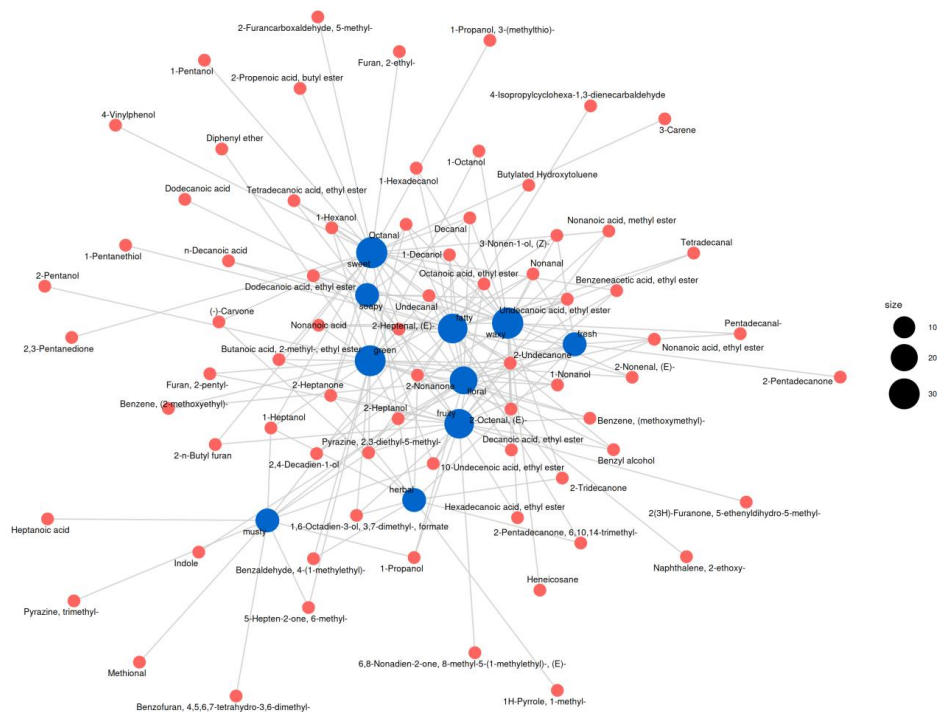


Figure 11. Sensory flavor characteristics and flavor substance correlation network diagram

4. Conclusion

This study employed the HS-SPME-GC×GC-TOF-MS technique to analyze the volatile components of pomegranates from four different origins. The results showed that the flavor compounds in pomegranates mainly include alcohols, ketones, esters and organic heterocyclic compounds. Through the analysis of heatmaps and volcano plots of differences substances of pomegranates from different origins, important different volatile compounds between groups can be screened. These compounds can serve as markers for distinguishing pomegranates from two different origins. Through relative odor activity values (ROAV) and radar chart analysis, the key flavor characteristics of each origin of pomegranates were identified. For example, the SL-4 sample showed the most prominent flavor characteristics in terms of fruity, sweet, fatty, floral,

and herbal aroma, which is likely related to the 5 key flavor compounds such as 2,3-butanedione; 2-nonenal (E)-; butanal, 2-methyl-; furan, 2-pentyl-; heptanal. The four different-origin pomegranates all have aroma characteristics such as sweet, fruity, green, fatty, floral , and herbal aroma. SL-1 has a higher acidity than sweetness, and the taste is a blend of sourness and sweetness. SL-2 has a lower sweetness and acidity, showed a mild and elegant taste. SL-3 has a higher sweetness than SL-2, presenting a refreshing taste. SL-4 has a balanced acid-sweet ratio and a pleasant and suitable taste. This study conducted a differential analysis of volatile flavor compounds of pomegranates from different origins using flavoromics technology, and evaluated the style characteristics, which providing an effective reference basis for preference selection of consumers.

CRedit authorship contribution statement

Xiaoyu Chen: Data curation, Formal analysis, Writing-original draft; **Hao Zhu:** Investigation, Formal analysis; **Zongying Wang:** Investigation, Formal analysis; **Yuchen Yao:** Data curation, Investigation; **Zhe Chen:** Data curation, Formal analysis; **Wenxuan Zhang:** Data curation, Formal analysis; **Xiaobin Li:** Writing-review & editing; **Zhiyong Wu:** Investigation, Methodology; **Mingqin Zhao:** Supervision, Writing-review & editing.

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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506 **Data availability**

507 Data will be made available on request.

508 **Reference:**

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