Characterization of volatile compounds and microstructure in different tissues of ‘Eureka’ lemon (Citrus limon)

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**ABSTRACT**

Lemon fruits have a non-uniform histological structure, and are characterized by abundant volatile compounds. However, the distribution of volatile compounds in different tissues remain unknown. Herein, electronic nose (E-nose) and headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS) were employed to qualitatively and quantitatively analyze the volatile compound content in different tissues of fresh lemon fruits, while their microstructures were observed using several microscopy methods. The numbers of volatile compounds detected in the flavedo, albedo, juice vesicles, and segment wall were 41, 23, 29, and 19, respectively. The volatile compounds detected in the four tissues were mainly alkenes, alcohols, aldehydes, esters, and ketones, with a significant descending order in the total content \((p < .05)\). The measurements of E-nose and HS-SPME-GC-MS both showed that the highest total content of volatile compounds was in the flavedo, followed by albedo, juice vesicles, and segment wall \((p < .05)\). Furthermore, oil sacs and oil droplets were only observed in the flavedo by microstructure observation, where more essential oils (mainly volatile compounds) were distributed. The microstructure observation results also verified the measurement results of the E-nose and HS-SPME-GC-MS. These findings provide basic data for sensory evaluation and the comprehensive processing and utilization of lemon fruits.

**Introduction**

Lemon (Citrus limon) is one of the most popular fruits around the world because of its sweet taste and nutritional value.\(^{[1,2]}\) Currently, China has progressed to become the third global producer of lemon fruit. The annual production capacity in China was approximately \(2.71 \times 10^6\) tons in 2019, ranking behind only India and Mexico (FAO, 2019). The whole fruit of lemon can be eaten fresh or processed into dried lemon slices and lemon powders.\(^{[3]}\) Lemon fruits are rich in nutrients and health-promoting compounds, such as essential oils, vitamins, amino acids, and phenolics.\(^{[4–6]}\) Lemon essential oil is a natural and safe fragrance and has high nutritional and medicinal value, which can improve the circulatory system, enhance immunity, and prevent diseases such as depression, anxiety, and...
neurological disorders.\cite{7,8} Essential oils are important components of the flavor quality of lemon fruits. In lemon fruits, 85%–99% (m/m) of the entire essential oils are composed of volatile flavor compounds, such as monoterpenes, sesquiterpenes, aldehydes, monoterpen alcohol, and monoterpen esters.\cite{9,10,11}

Volatile flavor compounds in lemon fruits and processed lemon products have been investigated in the recent years. Lota et al.\cite{12} analyzed the chemical composition of volatile flavor compounds in different varieties of lemon peels by capillary gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS),\cite{13} C nuclear magnetic resonance (NMR), and principal component analysis (PCA), and concluded that lemon peel oils can be distinguished by three major chemotypes: limonene, limonene/β-pinene/γ-terpinene, and limonene/linalyl acetate/linalool. Allegrone et al.\cite{13} identified 35 volatile compounds from the fresh juices of Italian lemons by headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC–MS), which mainly consisted of mono- and sesquiterpene hydrocarbons and oxygenated molecules (aldehydes, monoterpen alcohol, and monoterpen esters). Mehl et al.\cite{10} investigated the differentiation of volatile compounds in cold-pressed lemon essential oil using various analytical techniques to identify and classify the geographic origin and production process of cold-pressed lemon oil. Njorge et al.\cite{14} and Sawamura et al.\cite{15} analyzed the chemical composition of the volatile component of lemon peel essential oil using capillary GC, GC–MS, and HS-SPME-GC–MS.

Headspace solid-phase microextraction (HS-SPME) is a new type extraction method for volatile flavor compounds in food and has been widely used by researchers in recent years.\cite{16,17} It is easy to perform and can be combined with GC and liquid chromatography. It can be used for the analysis of volatile compounds from all kinds of materials such as gas, liquid, solid, and other samples. The electronic nose (E-nose) is a simple and cost-effective analysis tool for volatile flavor compounds, which can identify a mixture of volatile organic samples as a whole, without the need to identify individual chemical substances in the sample mixture.\cite{18} GC–MS can detect the contents of individual compounds. The GC–MS analysis results can aid in the interpretation of the analysis results of the electronic nose. The combination of E-nose and GC–MS can accurately measure the volatile compounds in the sample from both the macro and micro levels.\cite{18,19,20,21} Above all, it can be found that the test methods for volatile compounds in lemon fruits are available and feasible, and the current research reports on the volatile flavor compounds of lemon fruits have mainly focused on the analysis of lemon peel. The main components of volatile compounds in lemon fruits are monoterpenes, sesquiterpenes, alcohols, aldehydes, esters, ketones, and acids, which can vary depending on the variety and the origin of lemon, methods of extraction, and detection of volatile compounds, among other factors.

In China, lemons are mainly produced in the provinces of Sichuan, Chongqing, Yunnan, Hainan, and other places. Anyue city of Sichuan is known as the ‘Lemon Hometown of China,’ accounting for more than 80% of the national planting area and production in China.\cite{22} Eureka lemon is one of the main commercial fruits in the international market, and is widely planted in China because of its perfect cultivation behavior and quality characteristics.\cite{23} Lemon fruit has a non-uniform histological structure that is mainly composed of flavedo, albedo, juice vesicles, and segment walls.\cite{24} There are obvious differences in the material composition and structure of the different lemon fruit tissues. However, the distribution characteristics of volatile flavor compounds in different tissues of lemon fruits have not been reported.

In this study, the Eureka lemon from Anyue City, Sichuan Province, China, was taken as the research object. Two different methods, E-nose and HS-SPME-GC–MS, were employed to detect the volatile flavor compounds of flavedo, albedo, juice vesicles, and the segment wall in lemon fruits. Meanwhile, the microstructure of the four tissues was observed to achieve a more comprehensive understanding of the distribution characteristics of volatile flavor compounds in lemon fruit. This study is expected to provide basic data for sensory evaluation and the comprehensive processing and utilization of lemon fruits.
Materials and methods

Sample preparation

Fresh lemon fruits (Eureka, Anyue, Sichuan, China) were purchased from a local orchard. Approximately 20 lemon fruits of uniform size (shaped as an approximate ellipse, with major axis diameter of 70 ± 5 mm and minor axis diameter of 60 ± 5 mm), with bright color, and damage-free were randomly selected as the experimental materials. These were cleaned with tap water and part of the head and tail were removed. The fruits were cut into round slices (10 mm in thickness) along their equator circles. The four tissues of flavedo, albedo, juice vesicles, and segment wall (Figure 1) were separated from the lemon slices as samples for E-nose, HS-SPME-GC-MS, and microstructure observation.

Volatile compounds analysis by E-nose

E-nose analysis was conducted using a commercial PEN3 device (Airsense GmbH, Schwerin, Germany), attached to the software platform for PCA. The PEN3 E-nose was equipped with 10 metal oxide semiconductors (W1C, W5S, W3C, W6S, W5C, W1S, W1W, W2S, W2W, and W3S). Each type of sensor element corresponds to a certain difference in the type of the main sensitive substance, and the sensors are listed in Table 1.

The test sample (2 g) was weighed from the prepared tissues of flavedo, albedo, juice vesicles, and segment wall. The test sample (2 g) was added to 10 ml distilled water, and broken with a homogenizer (FA25, FLUKO, Shanghai Fluke Fluid Machinery Manufacturing Co., Ltd., China) at a rotation speed of 9000 rpm for 2 min, and then diluted with distilled water to 20 mL of constant volume. Five milliliters of the distilled solution sample was placed in a 15 mL headspace bottle, containing a magnetic rotor. After sealing with a polytetrafluoroethylene septum, the headspace bottle with the solution sample was stirred for 30 min with a magnetic heating stirrer (PC-620D, Corning Company, NY, USA) at a rotation speed of 400 rpm and 50°C. The sample headspace gas in the bottle was then

Figure 1. Schematic of main tissues in lemon fruit.
pumped into the sensor array at a constant rate of 300 mL/min, and the measurement lasted 400 s. The characteristics of the volatile compounds in each sample are described by the response values corresponding to the 10 sensors.

**Volatile compounds analysis by HS-SPME-GC-MS**

Volatile compounds in the lemon tissues were extracted and detected using the HS-SPME-GC–MS methods published by Zhang et al., as with slight modifications. HS-SPME-GC-MS measurements were conducted with three replicate samples for each tissue of the flavedo, albedo, juice vesicles, and segment wall. Generally, the extraction method was as follows: the 15 mL headspace bottle with 5 mL diluted solution sample was prepared according to the same method used for E-nose analysis, but with a further 2 µL of cyclohexanone added to the solution sample as an internal standard in the quantitative analysis of GC-MS; then, the sample bottle was put on the heating platform of the magnetic heating stirrer to equilibrate for 30 min at a rotate stirring speed 400 r/min and 50°C, during which the extraction of volatile compounds was conducted by inserting the preconditioned (activated at 260°C for 20 min) SPME fibers (50/30 µm divinylbenzene carboxene-poly (dimethylsiloxane), DVB/CAR/PDMS, Supelco, Bellefonte, PA, USA) into the head space of the bottle.

At the end of the extraction, the fiber was desorbed into the injection port of the GC at 250°C for 5 min, and the data were collected simultaneously. All analyses were performed on an Agilent 7890 B GC system coupled with a quadrupole mass filter for mass spectrometric detection (Agilent Technologies, Santa Clara, CA). Volatile compounds were separated on an HP-5 ms capillary column (30 m × 0.25 mm × 0.25 µm), and the custom temperature program was set as follows: the initial oven temperature was 50°C, held for 2 min, ramped at 10°C/min to 230°C, then held for 3 min. Helium carrier gas was used at a flow rate of 1 mL/min. The injection port temperature was 250°C, and the injection mode was manual. Mass spectrometric detection parameters were set as follows: ionization method EI, electron energy 70 eV, ion source temperature 230°C, MS quadrupole temperature 150°C, mass scanning range mass-to-charge ratio (m/z) 30–450, scanning rate 5.27 times/s.

Qualitative identification of the volatile compounds was as follows: qualitative ion fragments acquired from MS were analyzed with the mass spectral database NIST Library Search program, and hypothetical compounds with match scores > 70% were selected as the possible components of unknown compounds, a series of n-alkanes (C5–C20) were used as the standard to perform the GC-MS measurement using the same procedure used in the sample measurement, the retention index (RI) of the compounds in the sample was calculated by its retention time, and the corresponding components were determined based on the RI of compounds provided by the mass spectral database and some relevant literature.

Quantitative analysis of the volatile compounds was carried out according to the peak area of each compound and the peak area of the internal standard substance (cyclohexanone) in the total ion current spectrum. The content of each component was calculated according to Eq. (1):

**Table 1. Sensors used and their main application in PEN3 system.**

| Sensor serial number | Sensor model | Performance description for sensitive substances |
|----------------------|--------------|--------------------------------------------------|
| 1                    | W1C          | Aromatic, benzene                               |
| 2                    | W5C          | Broadrange, sensitive to nitrogen oxides         |
| 3                    | W3C          | Sensitive to aromatic and ammonia                |
| 4                    | W6S          | Mainly selective to hydrides                    |
| 5                    | W5S          | Aromatic components of short-chain alkanes       |
| 6                    | W1S          | Sensitive to methyl compounds                    |
| 7                    | W1W          | Sensitive to sulfides                           |
| 8                    | W2S          | Sensitive to alcohols, aldehydes and ketones     |
| 9                    | W2W          | Aromatic                                         |
| 10                   | W3S          | Sensitive to long-chain alkanes                  |
\[ c_i = \frac{s_i \times m_0}{s_0 \times m_d} \]  

where \( c_i \) is the content of the volatile compound (mg/g), \( s_i \) is the peak area of the volatile compound \((m^2)\), \( m_0 \) is the mass of the internal standard substance added (mg), \( s_0 \) is the peak area of the internal standard substance added \((m^2)\), and \( m_d \) is the mass of the tissue sample added (g).

**Microstructure observation of the four tissues of lemon fruits**

The four fresh prepared tissues were formed into slices samples with dimensions of 10 mm \( \times \) 10 mm \( \times \) 2 mm for flavedo, 10 mm \( \times \) 10 mm \( \times \) 5 mm for albedo, 10 mm \( \times \) 10 mm \( \times \) 10 mm for juice vesicles, and 10 mm \( \times \) 10 mm \( \times \) 1 mm for the segment wall, which were used for the microstructure observations below.

**Microstructure analysis by light microscopy**

Light microscopy (LM) was conducted according to the method described by Ignat et al.,\(^{[26]}\) with slight modifications. The prepared slice samples were fixed in a formalin-acetic acid-alcohol solution (FAA, 90% ethanol, 5% acetic acid, and 5% formalin, v/v/v) for 24 h at 4°C. The fixed samples were successively placed in 30%, 50%, 70%, 90%, and 100% ethanol, with a gradient elution for 1 h at each ethanol concentration. The samples were then processed using an automatic histoprocessor to embed the tissue in paraffin, which was cut into 5 μm paraffin tissue slices with a tissue slicer. The paraffin tissue slices were baked to remove paraffin, stained with Safranin O/Fast Green, and finally sealed in glass slides for optical microscopy observation.

**Microstructure analysis by scanning electron microscopy**

Scanning electron microscopy (SEM) observations were carried out according to the method described by Pietrysiak et al.,\(^{[27]}\) with slight modifications. The slice samples of the four tissues were fixed in a 2.5% glutaraldehyde solution (v/v) for 24 h at 4°C, then soaked three times with phosphate buffer (0.1 M, pH 7.0) for 15 min each time, to remove the glutaraldehyde solution. The samples were then fixed with 1% osmic acid solution (v/v) for 2 h, and soaked three times with phosphate buffer (0.1 M, pH 7.0) for 15 min each time, to remove the osmic acid solution. The fixed samples were successively placed in 30%, 50%, 70%, 80%, 90%, and 95% ethanol, with gradient elution for 15 min at each ethanol concentration, and were then placed in 100% ethanol for 20 min, 2 times. The samples were then placed in a mixture of ethanol and isoamyl acetate at a volume ratio of 1:1 for 30 min, and then treated with pure isoamyl acetate for 1 h. The treated samples were attached to the sample holder using carbon conductive glue and gold-sputtered with an ion sputtering instrument (CPD-030, BAL-TEC Company, Liechtenstein). The prepared samples were observed on the surface using a SEM (SU8010, Hitachi Company, Tokyo, Japan), at an accelerating voltage of 3 kV. Typical SEM images of the four tissues were taken for microstructural analysis.

**Microstructure analysis by transmission electron microscopy**

Transmission electron microscopy (TEM) observations were carried out according to the method described by Zhou et al.,\(^{[28]}\) with slight modifications. The prepared slice samples were subjected to the same treatments as those in the SEM observation until the gradient elution step of ethanol. The slice samples were then embedded in Spur resin and polymerized for 8 h at 20°C, then pruned and cut into thin slices (70–90 mm in thickness) using an ultramicrotome (LKB-8800, LKB Products Co., Ltd., Sweden). Finally, the thin slices were double-stained using uranyl acetate and lead citrate and observed using a TEM (JEM-1400, JEOL Inc., Tokyo, Japan). Typical TEM images of the four tissues were taken for microstructural analysis.
**Microstructure observation by Confocal laser scanning microscopy**

Confocal laser scanning microscopy (CLSM) observations were conducted according to the method described by Dhital et al., [29] with minor modifications. Nile Red (N3013 Sigma) and Calcofluor White (18909 Sigma) were used as the fluorochromes for the observation of the four tissues using a CLSM (ZX-05, Ultra View VOX, PE, USA). The prepared slice samples were further formed into thin slices (thickness: 1 mm). These thin slices from the four tissues were placed on a petri dish and stained with 50 mL of 1 mg/mL Calcofluor White and 50 mL of 10% NaOH (m/m) for 3 min, and then with 50 μL of 1 mg/mL Nile Red in ethanol for 3 min. The samples were then observed by CLSM using a dual track at 405 nm for Calcofluor White and 555 nm for Nile Red. Typical CLSM images of the four tissues were taken at 20× magnification for microstructural analysis.

**Statistical data analysis**

E-nose data were subjected to PCA using Winmuster software of the E-nose system. The volatile compound content data in the GM-MS table are expressed as mean values with corresponding standard deviations. Analysis of variance was performed using SPSS (version 19.0; IBM, Chicago, IL, USA). Statistical comparisons were performed using the Tukey-Kramer test (p < .05). Hierarchical cluster and heat map analyses were performed using the Permut Matrix (Version 1.9.3).

**Results and discussion**

**Identification of volatile compounds in various tissues of lemon fruits by electronic nose**

The PEN3 electronic nose simulates the human olfactory system and contains 10 metal oxide sensors, including W1C (aromatic, benzene), W5S (nitrogen oxides), W3C (ammonia and aromatic), W6S (hydrides), W5C (short-chain alkanes), W1S (methyl compounds), W1W (sulfides), W2S (alcohols, aldehydes, and ketones), W2W (aromatic), and W3S (long-chain alkanes). Volatile compounds can be identified by the sensor response radar diagram. Because each sensor is selectively sensitive to the corresponding volatile compounds. [30]

The radar diagram of the sensor response values for the four tissues of lemon fruit is shown in Figure 2, in which each straight line represents the variation in the relative resistivity of the sensor. Significant differences in volatile compounds of the four tissues were found. In the flavedo, sensors W2W, W1C, W5S, and W2S presented relatively higher response values than other sensors, indicating that volatile compounds of flavedo are mainly involved in aromatic compounds, nitrogen oxides, alcohols, aldehydes, and ketones. Higher response values were observed in the W2W (aromatic) and W1C (aromatic, benzene) sensors for albedo, W2W (aromatic), W1C (aromatic, benzene) and W2S (alcohols, aldehydes and ketones) for juice vesicles, and W1C (aromatic, benzene) and W2S (alcohols, aldehydes and ketones) for the segment wall, respectively. In general, it can be concluded from the profiles of response values for the four tissues that flavedo contained the highest content of volatile compounds, followed by albedo, juice vesicles, and segment wall.

PCA was carried out on the E-nose response values of the four tissues, which can intuitively distinguish their differences in volatile compounds in different tissues of lemon fruits. The largest contribution rate and the most important factor were obtained from PCA. As shown in Figure 3, the contribution rates of the principal components PC1 and PC2 were 86.28% and 12.02%, respectively, and the cumulative contribution rate was 98.30%, indicating that PC1 and PC2 can reflect the information of the original data for the volatile compounds in lemon fruit tissues. Based on the comprehensive results of PC1 and PC2 in Figure 3, the samples of the four tissues of lemon fruits presented no overlapping area and maintained a certain distance between each other, indicating that there were certain differences in the volatile compounds in the four tissues of lemon fruits.
Determination and analysis of volatile compounds in various tissues of lemon fruits by GC-MS

Overall composition of volatile compounds: The composition and content of volatile compounds in the four tissues of lemon fruits detected by GC-MS are shown in Table 2. With regard to the number of volatile compounds, a total of 43 volatile compounds were detected in the four tissues of fresh lemon fruits, of which 16 were commonly owned components for the four tissues. The volatile components detected in the flavedo, albedo, juice vesicles, and segment wall were 41, 23, 29, and 19, respectively. Some unique volatile components with low content were detected in the tissues. The unique components in flavedo included β-Ocimene (0.18 mg·g⁻¹), α-Caryophyllene (0.16 mg·g⁻¹), γ-Bisabolene...
(0.20 mg·g⁻¹), 1-Octanol (0.04 mg·g⁻¹), L-Borneol (0.04 mg·g⁻¹), (R)-(−)-Citronellal (0.16 mg·g⁻¹), Undecanal (0.12 mg·g⁻¹), Citronellyl acetate (0.09 mg·g⁻¹), Camphor (0.07 mg·g⁻¹), and Elixene (0.19 mg·g⁻¹). The unique components in juice vesicles included 3,5,5-Trimethylhexanal (0.03 mg·g⁻¹). Segment wall was found to contain a unique component cis-2-Hexen-1-ol (0.02 mg·g⁻¹).

The top three volatile compounds in terms of content for every tissue were D-Limonene, Citral and γ-Terpinene, respectively, as shown in Figure 4. The contents of the three volatile compounds in the same tissue all presented significant differences, with D-Limonene accounting for 32.41%, 47.40%, 40.38%, and 41.83% of the total content of volatile compounds in the tissues of flavedo, albedo, juice vesicles, and segment wall, respectively, presenting an absolute dominant position. As far as the contents of D-Limonene in the four tissues were concerned, the content in flavedo was the highest, followed by those in albedo or juice vesicles, and third in the segment wall. The same differences were observed in the contents of Citral and γ-Terpinene in the four tissues.

As shown in Table 3, the volatile compounds detected in the four tissues were mainly involved in alkenes, alcohols, aldehydes, esters, and ketones, which were ranked in descending order of total content. There were significant differences in the total contents of all compound categories (p < .05), in which alkenes accounted for the highest content of volatile compounds, and ketones accounted for the lowest content. In terms of the total contents of volatile compounds, flavedo was detected at the highest value, 43.32 mg·g⁻¹, followed by albedo (5.78 mg·g⁻¹), juice vesicles (4.68 mg·g⁻¹) and segment wall (2.51 mg·g⁻¹), showing significant differences (p < .05). The total contents of volatile compounds in the four tissues detected by GCMS were consistent with the results of the profiles of sensor response values detected by E-nose.

**Heat map and clustering analysis of volatile compounds in different tissues**

Based on the data in Table 2, heat map and clustering analysis were carried out to display the differences in the main volatile compounds for the four tissues in lemon fruits, in which the three replicate samples for each tissue were included, as shown in Figure 5. The samples were grouped based on their similarity or closeness. It was found that volatile compounds behaved in a similar manner in the 12 samples of the four tissues that fell into seven main clusters, according to the vertical dendrogram. According to the horizontal dendrogram, three replicate samples in the flavedo can be viewed as an independent main cluster, while the other samples in albedo, segment wall, and juice vesicles can be classified as another main-cluster. Moreover, samples in the albedo and segment wall behaved in a similar manner and could be classified as one sub-cluster, and samples in juice vesicles were divided into another sub-cluster. Based on the colors in the heat map, it can be seen that the content of volatile compounds in the flavedo were much higher than those in the other tissues.

**Various types of volatile compounds in different tissues:** Alkenes accounted for the highest ratio of volatile compounds in lemon fruits, which has been confirmed by other researchers.[2,10,12] In this study, as shown in Table 2 and Table 3, the numbers of alkenes detected in flavedo, albedo, juice vesicles and segment wall were 21, 13, 12, and 9, respectively, while their contents were 27.36 mg·g⁻¹, 3.72 mg·g⁻¹, 2.69 mg·g⁻¹ and 1.60 mg·g⁻¹, respectively. The main alkenes detected in the four tissues were D-Limonene, β-Pinene and γ-Terpinene. Although alkenes were detected at much higher concentrations than other types of volatile compounds, their contribution to lemon aroma quality was smaller than that of oxygenated terpenoids.[25]

Aldehydes are viewed as one of the most important class of volatile compounds contributing to the quality of the lemon aroma. In this experiment, aldehydes were observed with the highest content among the oxygenated terpenoids in lemon fruits. The numbers of aldehydes detected in flavedo, albedo, juice vesicles and segment wall were 7, 3, 6, and 2, respectively, and their contents were 10.62 mg·g⁻¹, 1.04 mg·g⁻¹, 0.84 mg·g⁻¹ and 0.38 mg·g⁻¹, respectively. Citral and Nonanal were the most common volatile compounds in all four tissues, and the content of Citral was much higher than that of the other aldehydes.
Table 2. Analysis of the main volatile compounds in different tissues of lemon fruit. Retention index<sup>a</sup> is achieved from the experiments. Retention index<sup>b</sup> is reported in the literature.<sup>c</sup> ND, not detected. Error represents the standard deviation in n = 3 samples. Different letters in the same index indicate significant differences at p = .05 by Tukey-Kramer test.

| Compound category | Compound name | Retention time/min | Retention index<sup>a</sup> | Retention index<sup>b</sup> | Match score/% |
|-------------------|---------------|---------------------|----------------------------|-----------------------------|---------------|
| Alkenes           | α-Phellandrene| 9.148               | 810                        | 876                         | 95            |
|                   | α-Pinene      | 9.460               | 824                        | 881                         | 96            |
|                   | β-Phellandrene| 11.373              | 905                        | 964                         | 93            |
|                   | β-Pinene      | 11.599              | 911                        | 923                         | 93            |
|                   | β-Myrcene     | 12.283              | 932                        | 947                         | 95            |
|                   | α-Terpinepine | 13.596              | 941                        | 966                         | 95            |
|                   | p-Cymene      | 14.046              | 981                        | 1027                        | 94            |
|                   | D-Limonene    | 14.444              | 993                        | 986                         | 92            |
|                   | β-Ocimene     | 14.680              | 1013                       | 991                         | 97            |
|                   | γ-Terpinepine | 15.796              | 1027                       | 1014                        | 96            |
|                   | Terpinolene   | 17.008              | 1059                       | 1037                        | 96            |
|                   | β-Caryophyllene| 32.686           | 1413                       | 1348                        | 96            |
|                   | Aromadendrene | 33.392              | 1432                       | 1445                        | 90            |
|                   | α-Caryophyllene| 34.132          | 1449                       | 1381                        | 89            |
|                   | Valencene     | 35.737              | 1487                       | 1499                        | 94            |
|                   | β-Farnesene   | 36.386              | 1504                       | 1458                        | 87            |
|                   | α-Bergamotene | 33.283              | 1429                       | 1417                        | 91            |
|                   | (S)-β-Bisabolene| 34.889        | 1451                       | 1439                        | 90            |
|                   | Eixene        | 35.860              | 1491                       | 1431                        | 91            |
|                   | γ-Bisabolene  | 37.584              | 1538                       | 1536                        | 91            |
| Alcohols          | 1-Octanol     | 16.416              | 1044                       | 1072                        | 98            |
|                   | Linalool      | 17.809              | 1078                       | 1052                        | 98            |
|                   | (S)-cis-Verbenol| 20.862         | 1147                       | 1130                        | 89            |
|                   | L-Borneol     | 21.264              | 1156                       | 1176                        | 98            |
|                   | 4-Terpinenol  | 21.716              | 1166                       | 1163                        | 94            |
|                   | α-Terpinene   | 22.457              | 1183                       | 1175                        | 94            |
|                   | Nerol         | 23.946              | 1216                       | 1210                        | 92            |
|                   | Citronellol   | 24.338              | 1220                       | 1227                        | 96            |
|                   | cis-2-Hexen-1-ol| 13.436        | 967                        | 1004                        | 78            |
| Aldehydes         | Geraniol      | 25.218              | 1244                       | 1253                        | 95            |
|                   | Octanal       | 12.969              | 952                        | 957                         | 97            |
|                   | Nonanal       | 18.040              | 1083                       | 1084                        | 98            |
|                   | Citronellal   | 20.467              | 1137                       | 1154                        | 96            |
|                   | Decanal       | 23.13                | 1196                       | 1202                        | 96            |
|                   | Citral        | 20.028              | 1231                       | 1241                        | 94            |
|                   | Undecanal     | 27.881              | 1302                       | 1310                        | 96            |
|                   | (S)-(-)-Perillaldehyde| 26.267        | 1266                       | 1280                        | 92            |
|                   | 3,5,5-Trimethylhexanal| 13.431        | 967                        | 956                         | 79            |

(Continued)
Table 2. (Continued).

| Compound category | Compound name    | Retention time/min | Retention index(\textsuperscript{a}) | Retention index(\textsuperscript{b}) | Match score/% | Content of the volatile compounds/(mg·g\textsuperscript{-1}) |
|-------------------|------------------|--------------------|--------------------------------------|--------------------------------------|---------------|----------------------------------------------------------|
|                   |                  |                    |                                      |                                      |               | **Flavedo** | **Albedo** | **Juice vesicles** | **Segment wall** |
| Esters            | Citronellyl acetate | 29.715             | 1299                                  | 1293                                  | 90            | 0.09 ± 0.04\textsuperscript{a} | ND | ND | ND |
|                   | Neryl acetate    | 30.123             | 1355                                  | 1360                                  | 97            | 1.42 ± 0.37\textsuperscript{a} | 0.26 ± 0.03\textsuperscript{b} | 0.14 ± 0.03\textsuperscript{c} | 0.11 ± 0.02\textsuperscript{c} |
|                   | Geranyl acetate  | 30.975             | 1374                                  | 1379                                  | 97            | 0.86 ± 0.22\textsuperscript{a} | 0.11 ± 0.00\textsuperscript{b} | 0.09 ± 0.02\textsuperscript{b} | 0.06 ± 0.01\textsuperscript{c} |
| Ketones           | Carvone          | 24.781             | 1234                                  | 1248                                  | 92            | 0.07 ± 0.04\textsuperscript{a} | ND | 0.02 ± 0.01\textsuperscript{b} | ND |
|                   | Camphor          | 19.968             | 1127                                  | 1151                                  | 90            | 0.07 ± 0.03\textsuperscript{a} | ND | ND | ND |
| Total             |                  |                    |                                      |                                      |               | 43.32 ± 4.23\textsuperscript{b} | 5.78 ± 0.18\textsuperscript{b} | 4.68 ± 0.36\textsuperscript{c} | 2.51 ± 0.17\textsuperscript{d} |
Alcohols are viewed as another important component of lemon fruit flavors, whose formation is mostly related to the esterase in the fruit. Alcohols act as solvents or carriers for the synthetization of aroma components. The numbers of alcohols detected in flavedo, albedo, juice vesicles and segment wall were 9, 5, 7, and 6, respectively, and their contents were 3.08 mg·g⁻¹, 0.65 mg·g⁻¹, 0.90 mg·g⁻¹ and 0.36 mg·g⁻¹, respectively. The detected alcohols included Octanol, Linalool, (S)-cis-Verbenaol, 4-Terpineol, α-Terpineol.

Esters are one of the main sources of fragrance in fruits which can reflect the quality of the fruit aroma to a certain extent. The numbers of esters detected in the flavedo, albedo, juice vesicles and segment wall were 3, 3, 2, and 3, respectively, and their contents were 2.37 mg·g⁻¹, 0.40 mg·g⁻¹, 0.23 mg·g⁻¹ and 0.19 mg·g⁻¹, respectively. The ester content of is relatively low. Neryl acetate and Geranyl acetate accounted for the fruity and rose fragrance of lemon fruits. The ketones detected in the four tissues of lemon fruits included Carvone and Camphor, whose contents are very low compared to others.
**Observation on the microstructure of the four tissues of lemon**

**Microstructure observation by LM:** Optical microscopy images of the four tissues of lemon fruit are shown in Figure 6. Before the LM observation, the sliced samples of different tissues were stained with safranin O/fast green to visualize the cell structure, which can make lignified cell walls red and cellulose cell walls green. Oil sacs were observed beneath the epidermis of the flavedo, which presented the shape of an ellipse. Oil sacs of the flavedo originate from the cell division of globular/elliptical cell clusters of young meristem cells under the epidermis. The size of the central oil cells increased significantly after the growth and coalescence of one or more vacuoles. These cells then begin to undergo cleavage and lysis, thereby forming a secretory cavity and further expanding, and finally, the structure and composition of oil sacs are formed in the flavedo.\(^{[33]}\) It is well known that most essential oils in lemon fruit are distributed in the oil sacs of the flavedo.\(^{[34–36]}\) Moreover, 85%–99% (m/m) of the essential oils in lemon fruit are composed of volatile compounds.\(^{[10,36]}\) It has been verified by other
researchers that the content of essential oils in the flavedo of lemon fruit accumulates with the development of oil sacs, and the oil sacs contain the highest essential oil when the fruit is nearly ripe.\(^\text{[37–39]}\)

In contrast to the flavedo, albedo was observed with larger cells and arranged loosely. Red and green cell walls were observed in both the flavedo and albedo, indicating the presence of lignified and cellulose cell walls. Most of the cell walls in the flavedo and albedo are composed of cellulose, while the lignified cell walls mostly appeared in the epidermal cells of the flavedo and vascular bundle of albedo.

Juice vesicles are mainly composed of water and soluble solids, which are stained with light colors and observed with larger cells. The cells of the segment wall presented a long strip shape and were arranged extremely densely. Due to the fact that among the four tissues, only flavedo was observed to contain oil sacs, it can be inferred that more volatile compounds were distributed in the tissue of flavedo than in other tissues, which was consistent with the results of the E-nose and GC-MS analyses.

**Microstructure observation by SEM and TEM:** The SEM images of the four tissues in lemon fruits are shown in Figure 7, in which we can observe similar cell morphologies, as shown in Figure 6. Among the four images with \(\times100\) magnification (magnification \(\times30\) for flavedo), only flavedo was observed with the morphology of oil sacs, showing a concave oval structure in Figure 7 (a1). Under the same magnification \(\times600\) for the four tissues, a porous microstructure appeared in the flavedo with the smallest-sized apertures (Figure 7 (a2)), in albedo with the middle-sized apertures (Figure 7 (b2)), the largest-sized apertures in juice vesicles (Figure 7 (c2)), and the surface of the segment wall presented with regular stripes.

TEM images of the four tissues in lemon fruits are shown in Figure 8. Oil drips were observed around the cell wall of the flavedo, but no oil drips were observed in other tissues. Lemon essential oils in the flavedo are present in the cells in the form of oil droplets, and their main components are volatile compounds.

**Microstructure observation by CLSM:** The CLSM images of the four tissues are shown in Figure 9. CLSM can visualize plant tissues with oil and cellulose components by staining heat-stable dyes of Nile Red and Calcofluor White.\(^\text{[29,40]}\) Nile Red is widely used as a fluorescent dye to visualize oil components in food matrices,\(^\text{[41,42]}\) appearing orange-red or red color under CLSM. Calcofluor White is a nonspecific fluorescent dye that can bind to cellulose in the cell walls of plant tissues, presenting a blue color under CLSM.
Figure 7. Scanning electron microscopy images of the four tissues in lemon fruit (×30 for a1, ×100 for b1, c1 and d1; ×600 for a2, b2, c2 and d2). a, a1, a2: flaved; b, b1, b2: albedo; c, c1, c2: juice vesicles; d, d1, d2: segment wall; E: epidermis; O: oil sacs.
Figure 8. Transmission electron microscopy images of the four tissues in lemon fruit (×2000). a: flavedo; b: albedo; c: juice vesicles; d: segment wall; OD: oil drips.

Figure 9. Confocal laser scanning microscopy images of the four tissues in lemon fruit (×20). a, a1, a2, a3: flavedo; b: albedo; c: juice vesicles; d: segment wall.

Figure 9 (a1-a3) show the microstructure of the flavedo tissue. Figure 9 (a2,a3) present a structural view of the oil sacs and vascular bundles in the flavedo tissue, respectively. Furthermore, a larger red area was observed near the outer surface of the flavedo tissue (Figure 9 (a1)), in the oil sacs (Figure 9 (a2)), and the vascular bundles (Figure 9 (a3)), indicating a higher essential oil content. Compared with the flavedo, a smaller red area was observed in the albedo (Figure 9(b)), juice vesicles (Figure 9(c)), and segment walls (Figure 9(d)). Subsequently, it could be inferred from the microstructure observation by CLSM that essential oils (mainly volatile compounds) were mainly distributed in the flavedo tissue, which generally confirmed the results of volatile compounds measured by E-nose and GC-MS.

Conclusion

The volatile compounds in the four tissues of fresh lemon fruits (flavedo, albedo, juice vesicles and segment wall) were qualitatively and quantitatively analyzed using the E-nose and HS-SPME-GC-MS methods. The microstructures of the four tissues were further observed by LM, SEM, TEM, and
The results showed that 41, 23, 29, and 19 volatile compounds were detected in the flavedo, albedo, juice vesicles, and segment wall, respectively. The detected volatile compounds were mainly alkenes, alcohols, aldehydes, esters, and ketones, with a significant descending order in the total content ($p < .05$). The highest total content of volatile compounds was found in the flavedo, followed by albedo, juice vesicles, and segment wall ($p < .05$). Oil sacs and oil droplets were only observed in the flavedo tissue by microstructure observation, and more essential oils (mainly volatile compounds) were distributed in the flavedo tissue; these were consistent with the results of the E-nose and GC-MS analyses.

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**Disclosure statement**

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