Dynamic Changes on Floral Aroma Composition of the Three Species from *Tilia* at Different Flowering Stages

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Abstract: The floral aroma, sometimes known as an incorporeal gift of flowers, is one of the primary ornamental features of plants. Flowers of genus *Tilia* are fragrant and have great value for development and utilization. In this study, for the first time, headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) combined with chemometrics were used to analyze the dynamic variations of scent components of the three species from *Tilia* at different flowering stages. 47 aroma compounds were preliminarily identified, including terpenes, alcohols, ethers, esters, aldehydes, heterocyclics and alkanes. The UpSet diagram displayed great variations in the amount of aroma components at various flowering stages for each species. Partial least squares discriminant analysis (PLS-DA) indicated the proximity of aroma composition characteristics and the significant components that can distinguish the three species from one another. Variable importance projection values (VIP) along with the Kruskal-Wallis non-parametric analysis were performed to identify 9 crucial aroma components, such as α-Farnesene, D-Limonene, Germacrene D, Linalool, etc. In the end, we discovered that, in sharp contrast to *T. miqueliana* Maxim., *Tilia cordata* Mill. may have a closely related phylogenetic relationship with *Tilia tomentosa* Moench. by evaluating the aroma similarity rates.

Keywords: *Tilia*; floral aroma; headspace solid-phase microextraction (HS-SPME); gas chromatography-mass spectrometry (GC-MS); chemometrics

1. Introduction

The aroma released by plant organs is constituted of secondary metabolites which are a combination of volatile chemicals with a very low molecular weight [1]. Floral fragrance, one of a plant’s most significant ornamental characteristics, is especially crucial in influencing pollinator behavior and controlling flowering plant reproduction [2–4]. The various volatile substances released by plants during their growth and development result in characteristic fragrances [5] that attract specific insects for pollination [6]. Bees can use floral cues to hunt for and identify food sources [7], and the mass emission of floral material may encourage frequent activity of the primary pollinators [8]. In addition, researches demonstrated that floral fragrances can also have a synergistic effect on the visual presentation of flowers [9]. For instance, an orchid blossom can imitate the appearance and scent of a species of female bees, causing the males to attempt mating and resulting in pollination [9,10].

The floral aroma is closely associated with human life as well. Moreover, specific receptors in the human olfactory system may detect volatile fragrant substances, which can then produce pleasurable sensations [11]. Currently, more than 1700 volatile aroma substances have been identified in plants [12]. They are mainly classified as aldehydes, ketones, alcohols, esters, terpenes, alkanes, acids, ethers and aromatic compounds [13].
The range of scent compounds in various plants can reach tens or even hundreds, as evidenced by the 39 fragrant components found in *Rosa odorata* var. erubescens petals [14] and 89 fragrant compounds found in the entire flowers of *Camellia japonica* ‘Kramer’s Supreme’ [15]. Utilizing extraction from plants and artificial synthesis, floral substances have been widely used in spices, essential oils, cosmetics, food additives, air fresheners and other fields.

Trees of genus *Tilia* (Tiliaceae family) are fragrant, deciduous and have an abundance of flowers, making them good attractive trees for landscaping [16]. The dried inflorescences of *Tilia cordata* Mill. are processed into flower tea in Europe that can effectively relieve colds and coughs brought on by colds [17]. Volatile organic compounds (VOCs) in its essential oil such as α-pinene, γ-terpinene and linalool can be used for alleviating anxiety [18]. Studies on volatile organic compounds (VOCs) from *Tilia* species have recently concentrated on essential oils of inflorescences, bracts and leaves [19,20], but researches comparing the floral composition of different species in *Tilia* are scarce.

In this study, flowers of *Tilia cordata* Mill., *Tilia tomentosa* Moench. and *Tilia miqueliana* Maxim. in different periods were selected as experimental materials, which are adaptable and growing well in eastern and northern China. They deserve more promotion because they are superior nectar and fragrance producing plants. Therefore, headspace-solid phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) combined with chemometrics were used to explore the composition and dynamic change of floral aroma, which might also provide a scientific basis for in-depth research and utilization for the flowers of *Tilia*, as well as having crucial implications for plant phylogeny development.

2. Materials and Methods
2.1. Materials and Chemicals

Flowers of *T. cordata*, *T. miqueliana* and *T. tomentosa* were collected in Guangzhuang, Zhuzhen, Lihue District, Nanjing (32.51° N, 118.71° E), on 27 May, 29 May and 1 June 2021 between 09:00 and 10:00. For each species, three excellent strains of similar growth and profuse flowering were selected, and inflorescences of various flowering stages were picked from the upper, middle and lower regions of the canopy in the north, south, east and west directions, packed in self-sealing polybags, placed in ice cases for temporary storage and rapidly brought back to the laboratory. According to the degree of flower opening, the flowers were divided into bud stage (a), half-open stage (b) and blooming stage (c) (Figure 1). The basic situation of the selected materials can be shown in Table 1. The C7-C40 (HJ894-2017) standards were purchased from Tan-Mo technology Co., Ltd. Changzhou, China.

![Figure 1. Flowers of the three species from Tilia at different flowering stages: bud stage (a), half-open stage (b), and blooming stage (c).](image-url)
Table 1. Basic situation of plant materials selected from the three species.

| Species      | Abbreviation | Blooming Stages (mm) | Flower Diameter (mm) | Collection Date |
|--------------|--------------|----------------------|----------------------|-----------------|
| T. cordata   | TC           | 10–12                | 27 May 2021          |
| T. miqueliana| TM           | 11–13                | 29 May 2021          |
| T. tomentosa | TT           | 12–14                | 1 June 2021          |

2.2. Detection Methods

Randomly selected flower samples at various stages were weighed to a mass of around 0.300 g, placed in a 20 mL headspace glass vial with a screw-top, sealed and capped, then heated to 45 °C in an electric thermostatic water bath for 30 min. Subsequently, an aged 65 µm PDMS/DVB SPME fiber inserted into the vial through a PTFE spacer was placed 1 cm above the flowers samples and extracted in the headspace for 26 min. Three independent replicates were performed for each stage of each sample.

After extraction, the fiber was withdrawn and inserted into the GC inlet for desorption (3 min, 240 °C), and volatile components of the samples were analyzed using the Thermo Fisher Scientific Trace ISQ GC-MS system [21]. A DB-5MS flexible quartz capillary column (30 m × 0.25 mm × 0.25 µm) was used; the carrier gas was high purity helium He (purity > 99.999%) at a flow rate of 1 mL/min [22]; the ramp-up procedure was 60 °C for 1 min, 10 °C/min to 120 °C, 5 °C/min to 160 °C for 2 min, and 10 °C/min to 240 °C for 1 min. The mass spectrometer was set to ionization mode EI, ionization energy 70 eV, mass scan ranged 33-450 amu and solvent delay time was 0.5 min. The operating system was Xcalibur software (v. 2.2, Thermo Fisher Scientific, Waltham, MA, USA) [21].

2.3. Identification and Quantification of Aroma Components

Aroma components were identified by retention index (RI) and mass spectrum compared with the reference standards obtained from the NIST Chemistry WebBook (https://webbook.nist.gov/chemistry/), accessed on 15 August 2021. When reference standards were no available, identification was taken by comparing mass spectra with those in the NIST Chemistry WebBook and RI published in the literatures. Calculation of RIs according to the retention times of C7-C40 alkanes separated under the GC-MS conditions mentioned above, utilizing the formula below (Equation (1)). The relative content (%) of aroma compounds in the sample was calculated by normalization of the total ion peak area in the chromatography [23]. The average of the three measurements was taken as the relative content of each compound [24].

\[
RI = 100n + \frac{100(t_x - t_n)}{t_{n+1} - t_n}
\]  

(1)

where \(t_x\) is the retention time (min) for compound \(x\) to be detected; \(t_n\) is the retention time (min) of \(n\)-alkanes \(C_n\); \(t_{n+1}\) is the retention time (min) of \(n\)-alkanes \(C_{n+1}\); \(n\) is the number of carbon atoms in an unknown compound.

2.4. Calculation of Aroma Similarity Rates

We referred to the cosine similarity method [25] to calculate the aroma similarity rates of the three species from Tilia at different flowering stages, using the relative content (%) of aroma components (Equation (2)).

\[
S(A, B) = \cos \theta = \frac{\sum_{i=1}^{n} a_i b_i}{\sqrt{\sum_{i=1}^{n} a_i^2} \sqrt{\sum_{i=1}^{n} b_i^2}}
\]  

(2)
where $a_i$ and $b_i$ are the relative content of each aroma component in samples A and B.

2.5. Statistical Analysis

Excel (Microsoft Office Standard 2019, Microsoft Corporation, Redmond, WA, USA) was used to calculate the relative content (%) and standard deviation of each aroma component; UpSet diagrams were performed by using the OmicShare tools, a free online platform for data analysis (https://www.omicshare.com/tools), accessed on 5 June 2022; SPSS Statistics 26.0 software (IBM, Armonk, NY, USA) was used for the Kruskal–Wallis non-parametric test; SIMCA 14.1.0.2047 software (Umetrics, Umeå, Sweden) was used for the PLS-DA model, drawing score scatter plot and loading scatter plot, calculation of VIP value and permutation test; Matlab R2021a software (Math Works Corporation, Natick, MA, USA) was used for calculating the aroma similarity rates; aroma characteristics were collected from ‘The Good Scents’ company network database (www.thegoodscentscompany.com), accessed on 5 January 2022. OriginPro 2021 software (OriginLab Corporation, Northampton, MA, USA) was applied to draw heat map.

3. Results

3.1. Dynamic Changes of Floral Aroma at Different Stages

47 aroma compounds were preliminarily identified in the three species of Tilia at different flowering stages, including terpenes, alcohols, ethers, esters, aldehydes, heterocyclic and alkanes, 4 compounds remained uncertainly labelled as unknown (Table 2). At different stages, there were 19, 20 and 18 aroma substances from T. cordata; 14, 16 and 15 from T. tomentosa; 12, 13 and 15 from T. miqueliana, respectively. Figure 2 demonstrated that the three species had a wide range of floral aroma compound quantities, and the relative content of terpenes were dominating in the floral aroma components.

![Figure 2](image-url)
| Retention Time | Compounds Name | CAS Number | RI | TC-a | TC-b | TC-c | TT-a | TT-b | TT-c | TM-a | TM-b | TM-c |
|----------------|----------------|------------|----|------|------|------|------|------|------|------|------|------|
| 5.96           | (1R)-2, 6, 6-Trimethylbicyclo[3.1.1]hept-2-ene | 77857-70-8 | 948 | -    | -    | -    | 2.41 ± 0.64 | 2.85 ± 0.14 | 4.04 ± 0.02 | -    | -    | -    |
| 6.73           | β-Myrcene     | 123-35-3  | 906 | -    | -    | -    | -    | 2.90 ± 0.12 | 4.40 ± 0.51 | -    | -    | -    |
| 7.02           | α-Phellandrene | 99-83-2   | 1014 | -    | -    | -    | -    | 0.32 ± 0.01 | -    | -    | -    |
| 7.39           | D-Limonene    | 5989-27-5 | 1038 | -    | -    | -    | 19.88 ± 0.50 | 19.04 ± 0.49 | 22.55 ± 0.43 | 0.21 ± 0.01 | 0.31 ± 0.06 | -    |
| 7.43           | trans-β-Ocimene | 3779-61-1 | 1040 | 0.88 ± 0.05 | 1.03 ± 0.02 | 0.96 ± 0.06 | -    | -    | -    | -    | -    | -    |
| 7.62           | β- Ocimene    | 12077-91-3 | 1052 | 29.47 ± 0.76 | 34.44 ± 1.67 | 36.90 ± 2.61 | 32.62 ± 1.43 | 32.44 ± 1.92 | 32.68 ± 1.13 | -    | -    | -    |
| 7.85           | γ-Terpene     | 99-85-4   | 1067 | -    | -    | -    | 4.80 ± 0.23 | 4.97 ± 0.38 | 5.53 ± 0.03 | -    | -    | -    |
| 8.36           | Cyclohexene, 1-methyl-4-(1-methylcyclohexene)- | 586-62-9 | 1099 | -    | -    | -    | 12.22 ± 0.30 | 12.35 ± 0.33 | 11.39 ± 0.20 | -    | -    | -    |
| 9.00           | 2, 4, 6-Octatriene, 2, 6-dimethyl-α-Cubebene | 17699-14-8 | 1370 | 0.43 ± 0.02 | 0.34 ± 0.02 | 0.34 ± 0.08 | -    | -    | -    | -    | -    | -    |
| 13.40          | Aromadendrene | 109119-91-7 | 1409 | 1.03 ± 0.01 | 0.82 ± 0.05 | 0.85 ± 0.14 | -    | -    | -    | -    | -    | -    |
| 15.07          | β-Copaene     | 18232-44-3 | 1346 | 4.39 ± 0.12 | 3.02 ± 0.43 | 2.54 ± 0.34 | -    | -    | -    | -    | -    | -    |
| 15.64          | (E)-β-Farnesane | 18794-84-8 | 1459 | -    | -    | -    | 0.09 ± 0.01 | 0.11 ± 0.01 | 0.16 ± 0.01 | -    | -    | -    |
| 16.12          | cis-Murola-4(15),5-diene | 157477-72-0 | 1447 | 0.63 ± 0.01 | 0.56 ± 0.03 | 0.50 ± 0.12 | -    | -    | -    | -    | -    | -    |
| 16.40          | γ-Murolene    | 30021-74-5 | 1491 | 1.52 ± 0.03 | 1.25 ± 0.10 | 1.11 ± 0.09 | -    | -    | -    | -    | -    | -    |
| 16.57          | 1, 3, 6, 10-Dodecatetraene, 3, 7, 11-trimethyl, (Z,E)-Germacrene D | 23986-74-5 | 1501 | 22.30 ± 0.08 | 18.67 ± 1.47 | 16.83 ± 0.91 | 0.48 ± 0.05 | 0.66 ± 0.04 | 0.90 ± 0.03 | -    | -    | -    |
| 16.96          | α-Farnesene   | 502-61-4  | 1514 | -    | -    | -    | 0.75 ± 0.07 | 0.93 ± 0.01 | 0.95 ± 0.02 | 58.63 ± 1.48 | 59.35 ± 1.03 | 60.37 ± 1.26 |
| 17.43          | Naphthalene, 1,2,3,4-tetrahydro-7-methyl-4-methylenel-1-(1-methylcyclohexene), (1α,4α,β,8αα)-Naphthalene | 39029-41-9 | 1533 | 1.81 ± 0.02 | 1.59 ± 0.05 | 1.45 ± 0.24 | -    | -    | -    | -    | -    | -    |
| 17.59          | 1,2,3,5,6,8-hexahydro-4,7-dimethyl-1-(1-methyl ethyl), (15-cis)-Naphthalene | 403-76-1 | 1539 | 3.07 ± 0.04 | 2.69 ± 0.06 | 2.44 ± 0.41 | -    | -    | -    | -    | -    | -    |
| 18.00          | 1,2,3,5,6,8-hexahydro-4,7-dimethyl-1-(1-methylcyclohexene), (15-cis,1α,4α,β,8αα)-Alcohol | 24406-05-1 | 1556 | 0.66 ± 0.01 | 0.59 ± 0.02 | 0.54 ± 0.10 | -    | -    | -    | -    | -    | -    |
| 8.05           | Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylcyclohexene), (1α,2β,5α)- | 15537-55-0 | 1079 | -    | -    | -    | 0.33 ± 0.03 | 0.36 ± 0.02 | -    | -    | -    | -    |
| 8.52           | Linanol       | 78-70-6   | 1109 | 14.74 ± 0.45 | 18.73 ± 1.16 | 21.30 ± 0.50 | -    | -    | -    | -    | -    | -    |
| 8.91           | Phenylethyl Alcohol | 60-12-8 | 1180 | -    | -    | -    | 7.43 ± 0.80 | 7.17 ± 0.52 | 5.70 ± 0.20 | 0.68 ± 0.11 | 1.59 ± 0.19 | 1.77 ± 0.29 |
| 10.03          | 4-methyl-1-(1-methylcyclohexene), (R)-Lilac alcohol (isomer III) | 20126-76-5 | 1204 | -    | -    | -    | 0.35 ± 0.01 | 0.32 ± 0.01 | 0.37 ± 0.02 | -    | -    | -    |
| 10.42          | Lilac alcohol (isomer IV) | - | 1228 | 0.64 ± 0.02 | 0.40 ± 0.06 | -    | -    | -    | -    | -    | -    |
| 10.62          | Lilac alcohol D | - | 1241 | 0.88 ± 0.07 | 0.46 ± 0.10 | -    | -    | -    | -    | -    | -    |
| 10.91          | Lilac alcohol E | - | 1259 | -    | -    | -    | -    | -    | -    | 0.10 ± 0.02 | -    | -    |
| 9.85           | Benzoic acid, ethyl ester | 93-89-0 | 1208 | -    | -    | -    | 0.62 ± 0.09 | 0.94 ± 0.07 | 0.70 ± 0.04 | -    | -    | -    |

**Table 2.** Identification of aroma components and relative contents in samples of the three species of *Tilia* (means ± SD).
## Table 2. Cont.

| Retention Time | Compounds Name | CAS Number | RI     | Relative Content (%) |
|----------------|----------------|------------|--------|-----------------------|
| 11.48          | Acetic acid, 2-phenylethyl ester | 103-45-7   | 1295   | 0.44 ± 0.07 0.50 ± 0.06 0.16 ± 0.04 0.29 ± 0.10 0.61 ± 0.06 |
| 22.34          | Benzyl Benzoate | 120-51-4   | 1744   | 0.86 ± 0.21 0.52 ± 0.10 - - - |
| 7.72           | Benzenecelaldehyde | 122-78-1   | 1088   | 5.48 ± 1.45 6.41 ± 1.20 5.40 ± 0.53 |
| 9.43           | Lilac aldehyde (isomer II) | -         | 1166   | 5.48 ± 1.45 6.41 ± 1.20 5.40 ± 0.53 |
| 7.32           | Benzene, 1-methoxy-4-methyl- | 104-93-8   | 1056   | 0.25 ± 0.08 0.39 ± 0.02 0.43 ± 0.03 |
| 8.29           | Benzene, (2-methoxyethyl)- | 3558-60-9  | 1102   | 0.13 ± 0.02 0.19 ± 0.03 0.22 ± 0.01 |
| 9.96           | Benzene, (2-methoxyethenyl)- | 4747-15-3  | 1216   | 0.13 ± 0.02 0.19 ± 0.03 0.22 ± 0.01 |
| 10.36          | Estragole | 140-67-0   | 1225   | 3.96 ± 0.43 3.87 ± 0.14 2.32 ± 0.39 |
| 13.69          | Phenol, | 97-54-1    | 1434   | 1.14 ± 0.14 2.20 ± 0.18 1.00 ± 0.08 |
| 14.55          | Methylmethoxyl | 93-15-2    | 1443   | - - - - 3.71 ± 2.42 7.00 ± 0.74 10.26 ± 0.95 |
| 18.28          | Benzene, 1,2,3-trimethoxy-5-(2-propenyl)- | 487-11-6  | 1609   | - - - - 0.60 ± 0.21 0.76 ± 0.08 0.66 ± 0.09 |
| 12.17          | Tridecane | 629-50-5   | 1300   | - - - - 0.19 ± 0.18 - |
| 8.35           | trans-Linalool oxide (furanoid) | 34995-77-2 | 1098   | 1.55 ± 0.04 1.04 ± 0.09 0.87 ± 0.14 - - - |
| 9.91           | (3R,6S)-2,2,6-Trimethyl-6-vinyltetrahydro-2H-pyran-3-ol | 39028-58-5 | 1196   | 0.51 ± 0.04 0.34 ± 0.03 0.29 ± 0.05 - - - |
| 11.78          | unknown 1 | -         | 1301   | - - - - 0.09 ± 0.01 - |
| 15.67          | unknown 2 | -         | 1461   | 1.18 ± 0.03 0.86 ± 0.08 0.81 ± 0.09 - - - |
| 17.00          | unknown 3 | -         | 1515   | 1.27 ± 0.01 0.95 ± 0.09 0.80 ± 0.15 - - - |
| 19.14          | unknown 4 | -         | 1603   | - - - - 0.59 ± 0.03 0.62 ± 0.02 0.69 ± 0.01 |

**Note:** RT—Retention time; RI—Retention index.
The primary aroma components released from *T. cordata* flowers are β-Ocimene, Germacrene D and Linalool. With the gradual blossoming, the relative content of β-Ocimene increased from 29.47% to 34.44% and lastly to 36.90% at different flowering stages; Linalool was 14.74%, 18.73% and 21.30%. Both β-Ocimene and Linalool showed an increasing trend. The relative content of Germacrene D, on the other hand, kept on diminishing from 22.30% to 18.67% and 16.83%.

β-Ocimene, D-Limonene, and Cyclohexene, 1-methyl-4-(1-methylethylidene)- were predominant fragrant substances of *T. tomentose*. However, β-Ocimene and Cyclohexene,1-methyl-4-(1-methylethylidene)- did not vary significantly at the three flowering stages. The relative content of D-Limonene increased marginally, containing 19.88% and 19.04% at the bud and half-open stages, respectively, and going up to 22.55% during the blooming period.

The dominating aroma substance of *T. miqueliana* was α-Farnesene, with an inconspicuous increase in relative content at the three flowering stages, containing 58.63%, 59.35% and 60.37%, respectively.

The amount of fragrance compounds present in the three species at various flowering phases varied greatly, as shown by Table 2 and Figure 2. Additionally, some aroma components were only available during special periods.

At different flowering phases, *T. cordata* had 17 aroma substances in common at the three flowering stages; 2 exclusive substances appeared in both the bud and the half-open stages, and only 1 particular component in the half-open stage and the blooming stage. *T. tomentosa* contained 12 aroma substances in common across the three flowering stages, the half-open stage shared 2 unique components with the bud stage and the blooming stage, respectively, while α-phellandrene only popped up in the blooming stage. *T. miqueliana* possessed 11 fragrant compounds that appeared in all three flowering stages and 1 particular component in both the bud stage and the half-open stage; the blooming stage, however, exhibited 4 distinct aroma compounds, such as unknown 1, Lilac alcohol D, Lilac aldehyde (isomer II) and Benzene, 1-methoxy-4-methyl-. Moreover, Tridecane was detected at the half-open stage from *T. miqueliana*, which has been proven to be one of the ingredients of plant waxes that covers not only leaves but also other plant organs like flowers and fruits [26]. Thus, it is possible to assume that wax layers are present on the floral surface of *T. miqueliana*.

*T. cordata* owned the largest number of volatile aroma components among the three species, and it shared 2 aroma components with *T. tomentosa*: β-Ocimene and Germacrene D, while only sharing Lilac aldehyde (isomer II) with *T. miqueliana*. In addition, 4 aroma compounds including D-Limonene, α-farnesene, Phenylethyl Alcohol, and Acetic acid, 2-phenylethyl ester were discovered in both *T. miqueliana* and *T. tomentosa*, but their relative contents in each were different. Considering β-Ocimene, Germacrene D, D-Limonene and α-farnesene all exhibited high relative percentages among the three species, it may be inferred that they are the typical scent chemicals in flowers of *Tilia*.

### 3.2. Chemometric Analysis of Floral Aroma Components

Partial least squares discriminant analysis (PLS-DA) is a multivariate statistical analysis method for discriminant analysis [27] that enhances group differences by using information about class members provided by the auxiliary matrix in the form of codes [28]. In order to ascertain whether there were variations in the quantities of floral scent components in the three species of *Tilia*, a PLS-DA model was employed in this study to interpret the data collected by GC-MS. In all, 9 groups of samples with positive contribution rates were used as independent variables in the mathematical model. After fitting, the PLS-DA model contained 2 principal components (PC1 = 0.599 and PC2 = 0.366), and the fitting parameters $R^2_X = 0.965$ and $Q^2 = 0.997$, with a cumulative explanatory degree of 96.5%, can comprehensively reflect the original information of floral fragrance for the three species of *Tilia*, which has good predictability.

The scent features of each species at various flowering phases were similar in the score scatter plot (Figure 3a) with good aggregation, and the data points of the three species were
distributed within a 95% confidence interval. Samples of *T. miqueliana* were separated in the t1 direction, *T. tomentosa* and *T. cordata* were separated in the t2 direction. In the loading scatter plot (Figure 3b), the contributions of aroma components to PC1 and PC2 raised as the distance from the coordinate axis' origin increased and vice versa. Great contributions from β-Ocimene (P7) and Germacrene D (P37) offered positively significant contribution to PC1 (0.495 and 0.213, respectively), while Linalool (P14) was negatively distributed in PC2 (−0.392).

Overfitting is a well-known issue with the PLS-DA model, thus a permutation test was used to validate the model’s accuracy (Figure 4). The three species included in the model were subjected to a permutation test, with 200 replacements. The results were shown as follows: $R^2 = (0.0, 0.209)$ and $Q^2 = (0.0, 0.237)$ of *T. cordata*; $R^2 = (0.0, 0.2)$ and $Q^2 = (0.0, −0.29)$ of *T. miqueliana*; $R^2 = (0.0, 0.192)$, $Q^2 = (0.0, −0.328)$ of *T. tomentosa*. In general, the intercept values of prediction points of the fragrant compositions for the three species were all lower than the original model, indicating that the PLS-DA model was not over-fitting and the analysis results were reliable.

![Figure 3. PLS-DA model for aroma components at different stages of the three species. (a) Score scatter plot; (b) Loading scatter plot.](image)

![Figure 4. Permutation test of PLS-DA model. (a) *T. cordata*; (b) *T. miqueliana*; (c) *T. tomentosa*.](image)
3.3. Determination of Crucial Components

The contribution of different variables to the overall classification can be explained by the variable importance for the projection (VIP) [29]. The more notable the difference in variables across groups, the higher the VIP value is. When the VIP value is higher than 1.00, the corresponding variable can be defined as the key variable of the discriminant model. To make the analysis more accurate, a Kruskal–Wallis nonparametric test was used to further examine the compounds with VIP greater than 1.00, along with the P-values less than 0.05 as the selection criteria, and 9 crucial components were eventually identified (Table 3), which were consistent with the results of the PLS-DA loading scatter plot, indicating statistical variations of the three species. Moreover, the olfactory properties of the 9 crucial components are varied, primarily manifesting as citrus, woody, floral.

| No. | Components Name                      | VIP   | p-Value | Aroma Characteristics       |
|-----|--------------------------------------|-------|---------|-----------------------------|
| 1   | α-Farnesene                          | 3.22  | 0.02    | Citrus, herbal, lavender    |
| 2   | D-Limonene                           | 2.32  | 0.03    | Citrus, orange              |
| 3   | Germacrene D                         | 2.16  | 0.02    | Woody, spice                |
| 4   | Linalool                             | 2.13  | 0.02    | Citrus, woody, floral       |
| 5   | Cyclohexene, 1-methyl-4-(1-methylethylidene)- | 1.78  | 0.02    | Citrus, woody               |
| 6   | Phenylethyl Alcohol                  | 1.30  | 0.02    | floral, rose                |
| 7   | γ-Terpinene                          | 1.16  | 0.02    | Oily, woody, lemon          |
| 8   | Methyleugenol                        | 1.05  | 0.02    | Spicy, cinnamon, clove      |
| 9   | Benzeneacetaldehyde                  | 1.00  | 0.02    | Honey, floral, cocoa        |

VIP: variable importance in projection.

A heat map was utilized for the analysis to visualize the distribution of the 9 crucial components at the different flowering stages of the three species, as shown in Figure 5. The three species could be totally separated from one another by the 9 crucial components, which had varying distributions and high abundances.

Figure 5. Heat map of content distribution about 9 crucial components in the three species from Tilia.

3.4. Aroma Similarity Rates

The statistics between T. cordata and T. tomentosa in terms of aroma similarity rates were higher than 0.570 in the three flowering stages, which means that they might have a very close phylogenetic relationship (Table 4). In contrast, the aroma similarity rates between T. miqueliana and T. cordata or T. tomentosa revealed that they were basically dissimilar. Nevertheless, the aroma similarity rates of each species at various flowering stages were
all greater than 0.970, suggesting that the overall olfactory characteristics fluctuated a little. It may be deduced that the primary floral scent constituents of the three species were synthesized during the bud stage, with only a minor amount of decomposition and transformation occurring during the half-open stage and the blooming stage.

Table 4. Aroma similarity rates between the three species of *Tilia* at different flowering stages.

|        | TC-a | TC-b | TC-c | TT-a | TT-b | TT-c | TM-a | TM-b | TM-c |
|--------|------|------|------|------|------|------|------|------|------|
| TC-a   | 1.000| 0.987| 0.972| 0.582| 0.586| 0.572| 0.000| 0.000| 0.000|
| TC-b   | 1.000| 0.997| 0.626| 0.629| 0.613| 0.000| 0.000| 0.000| 0.000|
| TC-c   | 1.000| 0.636| 0.640| 0.623| 0.000| 0.000| 0.000| 0.000| 0.000|
| TT-a   | 1.000| 0.997| 0.990| 0.990| 0.022| 0.025| 0.023| 0.027| 0.027|
| TT-b   | 1.000| 0.994| 0.026| 0.026| 0.026| 0.026| 0.099| 0.994| 0.994|
| TT-c   | 1.000| 0.990| 0.026| 0.028| 0.028| 0.028| 0.998| 0.998| 1.000|
| TM-a   | 1.000| 0.997| 0.990| 0.990| 0.022| 0.025| 0.023| 0.027| 0.027|
| TM-b   | 1.000| 0.994| 0.026| 0.026| 0.026| 0.026| 0.998| 0.998| 1.000|
| TM-c   | 1.000| 0.990| 0.026| 0.028| 0.028| 0.028| 0.998| 0.998| 1.000|

Note: The closer clustering relationship is, the aroma similarity rate draws nearer to 1.000.

4. Discussion

The fundamental parenchyma of the floral organ or specialized glandular epidermal cells are usually the places plant fragrance is produced [30]. The formation and accumulation of flower scent are influenced by a variety of elements, but mostly depend on genetic effect. However, the release of VOCs from floral organs does not hinge on the volatilization of its components, but on the selective release of these components into the membrane structure of the synthesis zone by specific carriers [31,32]. The primary floral aroma components in *T. cordata* and *T. tomentosa* flowers in this study vary markedly from the VOCs of their inflorescence essential oils in previous research [17,18]. The synthesis and emission of VOCs may be affected by different genotypes and stand conditions together. Studies have also found that the main components of floral volatiles vary greatly even among species of proximal origin [12], and that the primary components of flower volatiles are irregular even within the same genus [33].

Moreover, volatile substances frequently undergo dynamic changes throughout flowers’ blossom. Variables were found in the quantity and relative content of fragrance compounds in the three species of *Tilia* at various flowering periods, which coincided with the analysis of volatile components at different flowering stages in other fragrant plants, such as *Hosta* [34], *Polianthes tuberosa* [35], *Prunus mume* [36], and *Osmanthus fragrans* [37]. According to the volatile components, which are controlled by the actions of related biosyntheses within the flowers, reactions like dehydrogenation, deoxygenation, catalysis and degradation are carried out from opening to decay in order to accumulate or generate new compounds to take part in body metabolism [38]. This ultimately results in variations in categories and relative contents of the volatile components released over time.

The three species from *Tilia* were all in the blooming stage when the peak period of fragrance emission as seen by the results. Previous research on floral aroma production indicates that some plants, such as *Acacia Cyclops* was in the bud stage [39]; however, *Osmanthus fragrans* ‘Boye Jingui’ was in their early bloom stage [40]. As a result, the floral aroma of different plants has varying compositions and release patterns, which could have an impact on the pollinators’ attraction to certain plants [41]. Environmental influences and the internal biological clock both have an impact on the pattern of scent emission [42]. In the diurnal variation of the full blooming Stage of *Dendrobium Chrysotoxum*, the release volume of α-pinene and β-Ocimene reached a peak at 11:00 and 14:00, respectively [43]. Under various light intensity treatments, there were noticeable variations in the content and concentration of fragrance from *Oncidium* Sharry Baby ‘Sweet Fragrance’ [44]. Additionally, high temperature inhibited the blooming and aroma emission of *Jasminum sambac* Ait, while high humidity and a plentiful water supply can encourage fragrance production [45].
Several components of the 9 key floral scent components determined in this research can not only participate in plant growth and development, environmental response and other physiological processes, but also have biological activities. For instance, α-Farnesene plays an important role in plant defense [46] and can be used as a precursor for modern biofuel farnesane production [47]. D-Limonene can be utilized as a synergist for hygienic pesticides [48] and as a raw ingredient for artificial neroli and lemon oils, both of which have phlegmatic and anticancer properties [49]. Linalool contains antibacterial, anti-inflammatory and anti-infective properties and is frequently utilized in the synthesis of essential oils [50]. Escherichia coli and Bacillus subtilis are significantly inhibited by the antimicrobial activity of Germacrene D [51]. They provide a variety of fragrant qualities that are beneficial to human mood and health. It is obvious that the three species of *Tilia* are highly valuable in terms of commerce, environment, and medicine. Consequently, based on the results of this research, we recommend more investigation into the metabolic pathways, regulatory genes, associated enzymatic activities of floral components and subcellular localization of *Tilia*.

5. Conclusions

In this study, HS-SPME/GC-MS combined with chemometrics were used to analyze the aroma components for the three species of *Tilia* at different flowering stages. The majority of the scent compounds were detectable at each stage, however, several fragrance components were only present at certain intervals. Phylogeny and coevolutionary history have an impact on the variation in volatile components found in flowers. The three species belonging to the same genus show different degrees of similarity to each other regarding aroma. It’s also intriguing that, in sharp contrast to *T. miqueliana*, *T. cordata* and *T. tomentosa* have a tighter relationship, implying that the three species have diverse proximity of ecological origins.

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