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Study on essential oils from four species of Zhishi with gas chromatography–mass spectrometry

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Abstract

Background: Citrus fruits are widely used as food and or for medicinal purposes, and they contain a host of active substances that contribute to health. The immature fruits of Citrus sinensis Osbeck and its cultivars (CS), C. junos Sieb. ex Tanaka (CJ), C. aurantium L. and its cultivars (CA) and Poncirus trifoliate Raf. (PT) are the most commonly used medicinal herbs in Traditional Chinese Medicine, called Zhishi. And their mature fruits can be used as food.

Results: In this study, the essential oils of four different Zhishi species were extracted by steam distillation and detected using gas chromatography–mass spectrometry (GC-MS). A total of 39 volatiles from the four species were tentatively identified. The limonene was the most abundant amongst the four species. Principal component analysis (PCA) of essential oils showed a clear separation of volatiles among CS, CJ and PT. However, CA could not be separated from these three species. Additionally, the volatiles accounting for the variations among the widely separated species were characterized through their corresponding loading weight.

Conclusion: Sesquiterpenes were identified as characteristic markers for PT. The content of some monoterpenes could be as taxonomic markers between CS and CJ. This work is of great importance for the evaluation and authentication of Zhishi samples through essential oils.

Keywords: Citrus fruits, Essential oils, GC-MS, PCA, Zhishi

Background

Citrus genus is the most important fruit tree crop in the world, with an annual production of approximately 102 million tons [1]. Citrus fruits are used in the food, cosmetic and pharmaceutical industry. They are consumed fresh and processed, as juices, jam, jellies, molasses, etc. The fresh fruits are important starting materials for juice production [2]. Especially, chemical industry extracts from Citrus bioactive compounds like flavonoids, vitamins, dietary fiber and essential oils, etc. have beneficial effects on human health. The essential oils of Citrus fruits are widely used as flavoring in foods, perfumes and pharmaceutical formulations due to their functional properties like antimicrobial, antifungal, as well as analgesics, cough suppressants, and expectorants for eliminating phlegm [3-8]. Citrus oils extraction methods include steam distillation [9], cold pressing [10] and static headspace solid-phase microextraction [11].

The immature fruits of CA, CS, CJ and PT can be used as Zhishi, one of the primary traditional Chinese medicinal plants in China [12,13]. The herbs from different species and cultivation locations are widely used in clinical applications. Such herbs have been used to treat stuffiness, intestinal fullness sensation and distending pain, diarrhea and dysentery in addition to the retention and preservation of food [14]. However, the efficacy of these species of Zhishi for treating digestive disturbances varies due to differences in the types and quantities of the chemical substances they contain [15,16]. Our previous studies based on four flavanone compounds demonstrate that compounds from different species of Zhishi, such as CA, CS and CJ, can be distinguished by established differential modes of acquisition [17]. In addition to flavanones, essential oils are an important type of pharmacodynamic substance in Zhishi. Therefore, it is
Important to elucidate the differences between volatile substances among different species. Numerous studies [18-23] on the essential oils of Zhishi have been conducted using GC and GC-MS. However, because there is still a lack of information regarding the essential oils of different species of Zhishi, a comparative study is necessary.

Therefore, in this study, 75 authentic Zhishi samples from four species of Citrus fruit (CA, CS, CJ and PT) were collected. The essential oils were obtained by using steam distillation, and then were separated and identified by GC-MS. In total, 39 volatiles were tentatively identified and quantified, and among them were 26 monoterpenes, 8 sesquiterpenes, and 5 esters, aliphatic alcohols and aliphatic aldehydes. The details were shown in the Additional file 1. Then, a multivariate statistical analysis method was employed to elucidate the variation of volatile substances among different species of Zhishi. This is the first report on comparison study of essential oils from these four Zhishi species.

Results and discussion
Quantification of the volatile components
In total, 39 compounds were tentatively identified, constituting approximately 85.14-100% of the entire volatile concentration (Table 1). Each compound was identified by matching its retention characteristics and MS fragmentation patterns against standards and library spectra. The results are expressed as relative weight percentages calculated from peak areas. Volatiles were categorized into five chemical groups consisting of monoterpenes, sesquiterpenes, esters, aliphatic alcohols and aliphatic aldehydes. For all of the four Citrus species, the total content of essential oils primarily consisted of 26 monoterpenes, which accounted for 52.17-100% of the entire volatile concentration. The other four chemical groups – sesquiterpenes, esters, aliphatic alcohols and aliphatic aldehydes – contained in CA, CS and CJ had minor contributions to the entire volatile concentration accounting for only 0–2.28%. Sesquiterpenes were present in the oils analyzed in the greatest amount (3.8-34.28%) in the PT species compared to the other three species. This result is not surprising as sesquiterpenes hydrocarbons are important in the characteristic aroma of many kinds of Citrus fruits [24]. Alpha and beta-pinene are chemically unstable bicyclic terpenes due to a strained four membered ring. Thus, they are found at low levels and evenly distributed in all the four species.

GC-MS analyses of the four different species of Zhishi samples
In the 35 samples of CA, monoterpenes produced the highest percentage of volatiles (84.67–99.61%). Among the volatiles, the most predominant was limonene (27–82.84%, Mean 63.47%), followed by gamma-terpinene (0.25–32.31%, Mean 12.01%), beta-linalool (0–26.15%, Mean 9.10%), beta-pinene (0.4-17.01%, Mean 2.59%), beta-cis-ocimene (0–9.99%, Mean 2.21%) and (+)-sabinene (0.1-8.26%, Mean 1.77%). Meanwhile, the mean values of volatiles such as alpha-pinene, beta-myrcene, p-menth-1,4(8)-diene, omega-cymene, (–)-terpinene-4-ol and alpha-terpineol were higher than 0.5%, with an average percentage of total volatile concentration of 1.25%, 1.05%, 0.66%, 0.63%, 0.61% and 0.60%, respectively. Other chemicals, such as germacrene D, alpha-methyl-alpha-[4-methyl-3-pentenyl]-oxiranemethanol and n-octanal, belonging to sesquiterpenes, aliphatic alcohols and aliphatic aldehydes chemical groups, were minor volatile components in CA species, averaging 0.26%, 0.35% and 0.33% of the total percentage, respectively.

For CS species, monoterpenes were also the highest concentration of the volatile, averaging 85.3-100% of the total percentage. Limonene was the most abundant monoterpenone in this species accounting for 72.75-89.35%, with an average value at 82.25%. Additionally, (+)-sabinene and beta-linalool were recorded at a relatively lower proportion, 3.61-12.82% and 1.34-9.21%, respectively. Other compounds within the monoterpenes group, such as beta-myrcene, gamma-terpinene, (–)-terpinene-4-ol, beta and alpha-pinene, also occurred at a higher proportion, the mean amounts were 1.14%, 0.91%, 0.73%, 0.62% and 0.50%, respectively. Compounds of other chemical groups had a minor amount, and the mean values were lower than 0.1%.

For CJ species, the total amounts of monoterpenes compounds ranged from 89.31-99.81%. The most two abundant essentials, limonene and gamma-terpinene accounted for 35.53-60.60% (Mean 46.47%) and 21.71-46.74% (Mean 34.16), respectively. Other monoterpenes, such as beta-linalool, beta-pinene, alpha-pinene, p-menth-1,4(8)-diene, omega-cymene, alpha-thujene, beta-myrcene, (+)-4-carene, alpha-terpineol, p-cymen-2-ol and (+)-sabinene, each had average amounts higher than 0.5% at 3.53%, 2.80%, 2.61%, 1.63%, 1.51%, 1.12%, 0.94%, 0.88%, 0.60%, 0.55% and 0.54%, respectively. Except for germacrene D, which had an average value at 0.15% and belongs to the sesquiterpenes, compounds of other chemical groups were less than 0.1%.

Interestingly, in the PT species, sesquiterpenes (3.80-34.28%) were an important chemical group of character impact volatiles because of the relatively high percentage in the overall volatile composition compared to the other three species. In addition, it serves a characteristic aroma for many types of Citrus fruits. Among these sesquiterpenes, the most predominant was caryophyllen (Mean 5.32%), followed by gamma-elemene (Mean 3.81%), beta-farnesene (Mean 2.80%) and germacrene D (Mean 1.83%). Limonene and beta-myrcene were the major monoterpenes in this species as the average value accounted for up to 48.14% and 18.29%, respectively.
| No. | Name                     | RI   | CA-1 ~ CA-35 Relative concentration Mean | CJ-1 ~ CJ-15 Relative concentration Mean | CS-1 ~ CS-21 Relative concentration Mean | PT-1 ~ PT-4 Relative concentration Mean |
|-----|--------------------------|------|------------------------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| 1   | α-Thujene                | 943  | 0.1-1.5%                                 | 0.4%                                     | 0.35-1.71%                               | 1.12%                                    |
| 2   | α-Pinene                 | 948  | 0.28-2.97%                               | 1.25%                                    | 0.88-3.97%                               | 2.61%                                    |
| 3   | (+)-Sabinene             | 979  | 0.1-8.26%                                | 1.77%                                    | 0-2.21%                                  | 0.54%                                    |
| 4   | β-Pinene                 | 982  | 0.4-17.01%                               | 2.59%                                    | 1.64-3.96%                               | 2.80%                                    |
| 5   | β-Myrcene                | 992  | 0.2-7.22%                                | 1.05%                                    | 0.55-1.55%                               | 0.94%                                    |
| 6   | α-Phellandrene           | 1004 | 0-0.42%                                  | 0.05%                                    | 0.04-0.37%                               | 0.09%                                    |
| 7   | (+)-4-Carene             | 1015 | 0-0.88%                                  | 0.33%                                    | 0.54-1.23%                               | 0.88%                                    |
| 8   | o-Cymene                 | 1022 | 0-5.86%                                  | 0.63%                                    | 0.38-4.96%                               | 1.51%                                    |
| 9   | Limonene                 | 1026 | 27.82-84.8%                              | 63.47%                                   | 35.53-60.60%                             | 46.47%                                   |
| 10  | β-trans-Ocimene          | 1033 | 0-0.66%                                  | 0.06%                                    | 0-0.57%                                  | 0.06%                                    |
| 11  | β-cis-Ocimene            | 1042 | 0-9.99%                                  | 2.21%                                    | 0-0.89%                                  | 0.38%                                    |
| 12  | γ-Terpinene              | 1052 | 0.25-32.31%                              | 12.01%                                   | 21.71-46.74%                             | 34.16%                                   |
| 13  | cis-β-Terpineol          | 1060 | 0-0.52%                                  | 0.06%                                    | 0-0.13%                                  | 0.05%                                    |
| 14  | p-Mentha-1,4(8)-diene    | 1081 | 0.07-1.79%                               | 0.66%                                    | 0.95-2.33%                               | 1.63%                                    |
| 15  | β-Linalool               | 1089 | 0-26.15%                                 | 9.10%                                    | 0.73-7.95%                               | 3.53%                                    |
| 16  | trans-1-methyl-4-(1-methylethyl)-2-Cyclohexen-1-ol | 1112 | 0-1.1%                                  | 0.04%                                    | 0-0.13%                                  | 0.05%                                    |
| 17  | cis-1-methyl-4-(1-methylethyl)-2-Cyclohexen-1-ol | 1129 | 0-0.07%                                  | \                                        | \                                        | \                                        |
| 18  | β-Citronellal            | 1141 | 0-0.2%                                   | 0.04%                                    | 0-0.05%                                  | 0.01%                                    |
| 19  | (-)-Terpinen-4-ol        | 1164 | 0-3.23%                                  | 0.61%                                    | 0.21-1.02%                               | 0.48%                                    |
| 20  | α-Terpinol               | 1176 | 0-1.93%                                  | 0.60%                                    | 0.30-0.85%                               | 0.60%                                    |
| 21  | cis-Carveol              | 1202 | 0-0.95%                                  | 0.03%                                    | \                                        | \                                        |
| 22  | β-Citronellol            | 1210 | 0-0.48%                                  | 0.06%                                    | 0-0.04%                                  | 0-0.03%                                  |
| 23  | β-Citral                 | 1224 | 0-0.72%                                  | 0.06%                                    | 0-0.04%                                  | 0-0.21%                                  |
| 24  | trans-Geraniol           | 1236 | 0-1.16%                                  | 0.05%                                    | \                                        | \                                        |
| 25  | α-Citral                 | 1251 | 0-0.35%                                  | 0.04%                                    | 0-0.03%                                  | 0.01%                                    |
| 26  | p-Cymen-2-ol             | 1269 | 0-1.29%                                  | 0.11%                                    | 0-1.23%                                  | 0.55%                                    |
|     | Monoterpenes (Total)     |     | 84.67-99.61%                             | 96.88%                                   | 89.31-99.81%                             | 98.42%                                   |
|     |                          |     | 85.30-100%                               | 99.48%                                   | 52.17-96.06%                             | 80.80%                                   |
| 27  | n-Octanal                | 1002 | 0-0.87%                                  | 0.33%                                    | 0-0.27%                                  | 0.09%                                    |
| 28  | n-Decanal                | 1191 | 0-0.52%                                  | 0.06%                                    | 0-0.07%                                  | 0.02%                                    |
|     | Aliphatic Aldehydes (Total) |     | 0-1.39%                                  | 0.39%                                    | 0-0.38%                                  | 0.09%                                    |
| 29  | 1-Octanol                | 1064 | 0-0.21%                                  | 0.02%                                    | 0-0.04%                                  | 0-0.03%                                  |
| 30  | α-Methyl-α-[4-methyl-3-pentenyl] oxiranemethanol | 1065 | 0.04-1.26%                             | 0.35%                                    | 0-0.38%                                  | 0.11%                                    |
|     | Aliphatic Alcohols (Total) |     | 0.04-1.26%                             | 0.37%                                    | 0-0.38%                                  | 0.11%                                    |
| 31  | Nerol acetate            | 1334 | 0-0.17%                                  | 0.03%                                    | \                                        | \                                        |
| 32  | Esters (Total)           |     | 0-0.17%                                  | 0.03%                                    | \                                        | \                                        |
| 33  | β-Elemene                | 1362 | 0-0.21%                                  | 0.01%                                    | \                                        | \                                        |
| 34  | Caryophyllene            | 1387 | \                                        | \                                        | \                                        | \                                        |
| 35  | β-Farnesene              | 1418 | \                                        | 0-0.15%                                  | 0.03%                                    |
| 36  | Germacrene D             | 1442 | 0-0.99%                                  | 0.26%                                    | 0-0.55%                                  | 0.15%                                    |
|     | 4-isopropylidene-1-vinyl-α-Menth-8-ene | 1452 | 0-0.08%                                  | 0.01%                                    | 0-0.05%                                  | 0.01%                                    |

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Principal component analysis
The variability of essential oils in the four *Citrus* species of Zhishi shown in Table 1 was not anticipated. Principal component analysis (PCA) was employed to examine this large data set without having to assign classifications before analysis. PCA is an unsupervised clustering method that requires little prior knowledge of the data set and acts to reduce the dimensionality of multivariate data without losing important information. Par-scaled (scaled to square root of standard deviation) mathematical methods were performed to pre-treat the data set resulting from the volatiles of different *Citrus* species. The multivariate statistical analysis methodology helps to identify inherent patterns in the data in an unbiased manner and highlights the similarities and differences amongst samples. This methodology also helps identify those essential oils that are the most representative within the entire data set.

PCA score plot
As shown in Figure 1, each coordinate represents a sample, and the separation of the four different species of Zhishi samples was observed in the PCA scores plot. A two-component PCA model cumulatively accounted for 54.5% of the total variance. The CA species could not be separated from the other three species; however, according to our previous studies [17], CS, CJ and CA could be differentiated from one other by choosing flavanones as marker compounds. The high degree of clustering and minimal overlap of CS, CJ and PT suggest that these three species have volatile profiles that are unique to each cultivar. These three species, CS, CJ, and PT, were further analyzed by PCA. We observed three widely separated clusters in Figure 2 consisting of CS, CJ and PT. The PT samples were clearly separated from CS and CJ by principal component 2 (PC2), whereas the CJ and CS samples were clearly separated by principal component 1 (PC1). The results confirmed that CS, CJ and PT were different regarding the levels and occurrence of these essential oils. However, the volatiles in CA could not be distinguished from the other three species.

PCA loading plot
The corresponding PCA loading plot was utilized to identify the differential volatiles accountable for the separation among CS, CJ and PT. Although some information of low

| Table 1 Identifications of Zhishi volatiles and their relative total ion current peak area from four different species (Continued) |
|---|---|---|---|---|---|---|---|
| 37 | (Z,E)-α-Farnesene | 1462 | 0-0.05% | \ | 0-0.08% | 0.01% | 0-0.03% | \ | 0-1.31% | 0.33% |
| 38 | γ-Elemene | 1493 | 0-0.19% | 0.01% | \ | \ | \ | 0.01-0.25% | 0.01% | \ | \ |
| 39 | α-Sinensal | 1567 | \ | \ | \ | \ | \ | 0.20% | 0.71% | \ | \ |
| | Sesquiterpenes (Total) | \ | 0-1.32% | 0.29% | 0-0.75% | 0.20% | 0-0.71% | 0.01% | 3.8-34.28% | 14.44% |

Listed values are the percentage of the total peak area for each species. ‘\’ Not detectable. The detailed data of the 75 samples from the four species is listed in the Additional file 1.

Figure 1 PCA scores plot (PC1 vs. PC2) of the four species of Zhishi samples: 35 samples from CA, 15 samples from CJ, 21 samples from CS and 4 samples from PT. (CA = (red star) CJ = (green triangle), CS = (black diamond) and PT = (blue circle)).

Figure 2 PCA scores plot (PC1 vs. PC2) of the three species of Zhishi samples: 15 samples from CJ, 21 samples from CS and 4 samples from PT. (CJ = (red star), CS = (green triangle) and PT = (black diamond)).
intensity might be lost due to Par scaling, the loading plot clearly facilitated the profiling of the volatiles in our experiments. The volatiles that accounted for maximum variance in the data set are given more weight or loading. As shown in Figure 3, the variation within PC2 in the loading plot was due to the marker volatiles that accounted for the differences between PT and the others as observed in the scores plot. Analogously, the distribution in PC1 of the loadings plot demonstrated the variation of CS and CJ, respectively. For example, sesquiterpenes, such as germacrene D (0.34 on PC2), caryophyllen (0.33 on PC2), β-myrcene (0.32 on PC2), β-elemene (0.31 on PC2) and α-farnesene (0.27 on PC2) were the most differentiating volatiles, which played an important role in differentiating PT from the CS and CJ. Meanwhile, in PC1, monoterpene compounds, such as p-mentha-1, 4(8)-diene (loading of 0.29), limonene (0.28), (+)-4-carene (0.28), β-pinene (0.28), γ-terpinene (0.28), α-thujene (0.27), p-cymen-2-ol (0.26) and α-pinene (0.26), appeared to be of some relevance to the observed variability between CS and CJ.

**Discussion**

Literature reveals that limonene and γ-terpinene were the major components of the *Citrus* oils obtained by steam distillation and cold pressing [23]. The essential oils of four different species of Zhishi samples have been well studied and the major chemical groups are similar to the previous studies [21-23]. Limonene as a major constituent in several citrus oils (orange, lemon, mandarin, lime and grapefruit) is identified as the most predominant compound in the four species of Zhishi samples. The chemical compositions and their content of the essential oils are varied in different species. Especially, sesquiterpenes were present in the oils analyzed in the greatest amount in the PT species compared to the other three species. The contents of some monoterpene compounds such as p-mentha-1,4(8)-diene, limonene, (+)-4-carene, β-pinene, γ-terpinene, α-thujene, p-cymen-2-ol and α-pinene could be used as taxonomic markers between CS and CJ. The volatiles in CA could not be distinguished from the other three species. This may be because that CA samples distributed in a wide regions in China (Jiangxi, Hunan, Sichuan and Zhejiang). In the future, the active constituents contained in Zhishi samples from different regions need to be further investigated. Considering that all of the samples were collected in May or June under the same conditions impact of the growth stage factors on essential oil composition variability has been excluded.

**Experimental**

**Plant material**

All of the 75 Zhishi samples were collected from the main Zhishi-producing provinces of China: Jiangxi, Sichuan, Zhejiang, Fujian and Guizhou in May or June during a three-year period. For each of the 75 Zhishi samples, the species, local name, collection location, year of collection and growing environment are listed in Table 2. The diameters of collected Zhishi samples were limited to 0.5 cm ~ 2.5 cm, which is consistent with the requirements of the Chinese Pharmacopoeia (Chinese Pharmacopoeia, 2010). Professors Ge Fei, Yan Zhuyun, Zhang Yungui, Xu Jianguo and Ke Fuzhi identified them as genuine samples of *C. aurantium* L. and its cultivars (CA), *C. sinensis* Osbeck and its cultivars (CS), *C. junos* Sieb. ex Tanaka (CJ) and Poncirus trifoliata Raf. (PT). The dried specimens (marked as CA-1 ~ CA-35, CS-1 ~ CS-21, CJ-1 ~ CJ-15 and PT-1 ~ PT-4) were deposited at the Institute of Basic Theory, China Academy of Chinese Medical Sciences, Beijing, P. R. China. These four PT samples were wild growing.

**Extraction of essential oils**

Fresh, immature fruits of Zhishi were cut and dried in a manner similar to that described in the Pharmacopoeia of China [14]. The dried fruits were soaked in water for 2 hours and steam-distilled in a cleveunger-type apparatus for 8 hours. This procedure was repeated 6 times. The oils were then dried over anhydrous sodium sulfate, diluted by acetone, and stored at 4–6°C before analysis.

**GC–MS analysis**

GC–MS analysis was performed on GCMS-QP2010 Plus (Shimadzu, Kyoto) equipped with a capillary column (Rxi-5 ms, 30 m × 0.25 mm, 0.25 μm). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Oven
temperature was varied from 50°C (1 min held) to 140°C (1 min held) at 5°C/min, and then from 140°C to 200°C (3 min held) at 10°C/min. The injector and interface temperatures were held at 250°C. Mass spectra in the electron impact mode (EI-MS) were generated at 70 eV. The ion source temperature was held at 250°C. The scanning time was 0.5 sec over a range of m/z 45–450. An oil sample of 1 μL was injected in the split mode injection (split ratio, 60:1).

**Table 2** The origins of the 75 Zhishi samples collected

| No. | Species | Local name | Location and collection time | Growing environment |
|-----|---------|------------|------------------------------|---------------------|
| CA-1 ~ 3 | CA cv Xiucheng | Xiucheng | Xingan, Jiangxi; 2011 | Plain (N 27° E 115°; Alt.20 ~ 30 m) |
| CA-4 ~ 5 | CA × P. trifoliata | Citrange | Yuanjiang, Hunan; 2010 | Plain (N 29° E 112°; Alt.30 ~ 40 m) |
| CA-6 ~ 9 | CA | Sour orange | Jiangjin, Sichuan; 2010-2012 | Hillsides (N 29° E 106°; Alt.229 m) |
| CA-10 | CA cv Daidai | Daidai | Jiangjin, Sichuan; 2012 | Field margins (N 29° E 106°; Alt.200 ~ 230 m) |
| CA-11 ~ 19 | CA cv Xiucheng | Xiucheng | Xingan, Jiangxi; 2012 | Plain (N 27° E 115°; Alt.20 ~ 30 m) |
| CA-20 ~ 24 | CA cv Xiucheng | Xiucheng | Zhangshu, Jiangxi; 2012 | Plain (N 27° E 115°; Alt.20 ~ 30 m) |
| CA-25 | CA cv Jizicheng | Jizicheng | Zhangshu, Jiangxi; 2012 | Plain (N 27° E 115°; Alt.20 ~ 30 m) |
| CA-26 | CA cv Daidai | Daidai | Huangyan, Zhejiang; 2012 | Hillsides (N 28° E 121°; Alt.45 m) |
| CA-27 | CA cv Morocco sour orange | Morocco sour orange | Huangyan, Zhejiang; 2012 | Hillsides (N 28° E 121°; Alt.45 m) |
| CA-28 ~ 35 | CA cv Xiucheng | Xiucheng | Xingan, Jiangxi; 2013 | Plain (N 27° E 115°; Alt.20 ~ 30 m) |
| CJ-1~3 | CJ | Xiangcheng | Xingan, Jiangxi; 2011 | Hillsides (N 27° E 115°; Alt.50 ~ 60 m) |
| CJ-4 | CJ | Tuanye|Xiangcheng | Jiangjin, Sichuan; 2012 | Field margins (N 29° E 106°; Alt.200 ~ 230 m) |
| CJ-5 ~ 10 | CJ | Xiangcheng | Xingan, Jiangxi; 2012 | Hillsides (N 27° E 115°; Alt.50 ~ 60 m) |
| CJ-11 ~ 15 | CJ | Xiangcheng | Xingan, Jiangxi; 2013 | Hillsides (N 27° E 115°; Alt.50 ~ 60 m) |
| CS-1 | CS cv Jin Cheng | Jin Cheng | Qinglong, Guizhou; 2011 | Hillsides (N 25° E 105°; Alt.1200 ~ 1300 m) |
| CS-2 | CS | Navel Orange | Xingan, Jiangxi; 2011 | Hillsides (N 27° E 115°; Alt.50 ~ 60 m) |
| CS-3 | CS cv Jin Cheng | Jin Cheng | Qinglong, Guizhou; 2011 | Hillsides (N 25° E 105°; Alt.1200 ~ 1300 m) |
| CS-4 | CS cv Navel orange | Navel orange | Qinglong, Guizhou; 2011 | Hillsides (N 25° E 105°; Alt.1200 ~ 1300 m) |
| CS-5 | CS cv Blood orange | Blood orange | Qinglong, Guizhou; 2011 | Hillsides (N 25° E 105°; Alt.1200 ~ 1300 m) |
| CS-6 | CS cv Valencia | Valencia | Qinglong, Guizhou; 2011 | Hillsides (N 25° E 105°; Alt.1200 ~ 1300 m) |
| CS-7 | CS cv Blood orange | Blood orange | Jiangjin, Sichuan; 2012 | Hillsides (N 29° E 106°; Alt.238 m) |
| CS-8 | CS cv Valencia | Valencia | Jiangjin, Sichuan; 2012 | Hillsides (N 29° E 106°; Alt.238 m) |
| CS-9 | CS cv Navel orange | Navel orange | Jiangjin, Sichuan; 2012 | Hillsides (N 29° E 106°; Alt.238 m) |
| CS-10 | CS cv Peng an 100 | Peng an 100 | Jiangjin, Sichuan; 2012 | Hillsides (N 29° E 106°; Alt.238 m) |
| CS-11 ~ 14 | CS cv Liu Ben Cheng | Liu Ben Cheng | Huangyan, Zhejiang; 2012 | Plain (N 28° E 121°; Alt.7 m) |
| CS-15 | CS cv Hamlin | Hamlin | Huangyan, Zhejiang; 2012 | Plain (N 28° E 121°; Alt.7 m) |
| CS-16 | CS cv Jin Cheng | Jin Cheng | Huangyan, Zhejiang; 2012 | Hillsides (N 28° E 121°; Alt.40 ~ 60 m) |
| CS-17 | CS cv Navel orange | Navel orange | Huangyan, Zhejiang; 2012 | Hillsides (N 28° E 121°; Alt.40 ~ 60 m) |
| CS-18 ~ 21 | CS cv Delta Valencia | Delta Valencia | Huangyan, Zhejiang; 2012 | Hillsides (N 28° E 121°; Alt.40 ~ 60 m) |

**Poncirus trifoliate Raf.**

| PT-1 | PT | Trifoliate orange | Jiangjin, Sichuan; 2012 | Field margins (N 29° E 106°; Alt.200 ~ 230 m) |
| PT-2 | PT | Trifoliate orange | Huangyan, Zhejiang; 2012 | Hillsides (N 28° E 121°; Alt.45 m) |
| PT-3 ~ 4 | PT | Trifoliate orange | Huangyan, Zhejiang; 2012 | Hillsides (N 28° E 121°; Alt.45 m) |

**Identification and quantitative determination**

The volatile components were tentatively identified based on linear retention index (RI) and by the comparison of mass spectra with MS data of reference compounds. The linear retention indices were determined for all constituents by using a homologous series of n-alkanes (C₈–C₂₀). The components were identified by comparison of their mass spectra with those of the NIST05 and NIST05S mass spectral library and further
confirmed by comparison with the literature values [19,25]. The relative proportions of the essential oil constituents were expressed as percentages obtained by peak area normalization with all relative response factors being taken as one. The samples were run in triplicate.

Statistical analysis
The GC-MS data of different species of Zhishi samples were analyzed to identify potential discriminant variables. Multivariate statistical analyses, including unsupervised PCA and supervised partial least squares-discriminant analysis (PLS-DA), were performed using the SAS 9.1.3 statistical package (order no. 195557). PCA was used to observe the natural interrelationship among the chemical components for each of the four Citrus species. Analysis of variance was employed to identify those volatiles that would be most differentiating among the four species. Furthermore, the corresponding loading plot was used to interpret the variations among the samples. The critical $p$ value for all analyses in this study was set to 0.05.

Conclusion
GC-MS coupled with multivariate statistical analysis of volatiles from different Citrus species, revealed a diverse volatile distribution among CS, CJ and PT. However, the volatiles contained in CA exhibited less specificity; therefore, they could not be separated from the other three species. The PCA scores plot of CS, CJ and PT appeared as widely distinct clustering. The chemical compounds accountable for the different volatile profiles of the three species are germacrene D, carvophyllen, $\beta$-myrcene, $\beta$-elemene, $\alpha$-farnesene, p-mentha-1,4(8)-diene, limonene, (+)-4-carene, $\beta$-pinene, $\gamma$-terpinene, $\alpha$-thujene, p-cymen-2-ol and $\alpha$-pinene, all of which are significantly up- or down- regulated in the different three species. In short, sesquiterpene compounds could serve as characteristic markers for PT. The content of some monoterpene compounds could be used as taxonomic markers between CS and CJ. This work demonstrates the variations of essential oils among different species of Citrus fruits and is of great significance in the pharmacological and clinical use of Zhishi.

Additional file

**Additional file 1: Table S1.** Identifications of Zhishi volatile in Citrus sinensis Osbeck and its cultivars. **Table S2.** Identifications of Zhishi volatile in Citrus junos Sieb. ex Tanaka. **Table S3.** Identifications of Zhishi volatile in Poncirus trifoliate Raf. **Table S4.** PCA loadings plot scores of all the GC-MS signals of the three species of Zhishi samples: 15 samples from CJ, 21 samples from CJ and 4 samples from PT.
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