Short communication

Discrimination of *Citrus reticulata* Blanco and *Citrus reticulata* ‘Chachi’ by gas chromatograph–mass spectrometry based metabolomics approach

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Abstract

*Citri Reticulatae Pericarpium*, mainly including the pericarp of *Citrus reticulata* Blanco and the pericarp of *Citrus reticulata* ‘Chachi’, has been consumed daily as food and dietary supplement for centuries. In this study, GC–MS based metabolomics was employed to compare comprehensively the volatile constituents in *Citrus reticulata* Blanco and *Citrus reticulata* ‘Chachi’. Principal component analysis and orthogonal partial least squares discrimination analysis indicated that samples could be distinguished effectively from one another. Fifteen metabolites were finally identified for use as chemical markers in discrimination of *Citri Reticulatae Pericarpium* samples. The antimicrobial activity against Gram-negative and Gram-positive bacteria of the volatile oil from *Citrus reticulata* Blanco and *Citrus reticulata* ‘Chachi’ was investigated preliminarily.

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1. Introduction

*Citri Reticulatae Pericarpium* (CRP), the sun-dried tangerine pericarp, is consumed daily not only as a food and condiment, but also as a popular dietary supplement (Ho & Kuo, 2014; Manthey, Guthrie, & Grohmann, 2001). CRP includes mainly the pericarp of *Citrus reticulata* Blanco (‘Chenpi’ in Chinese, CP) and *Citrus reticulata* ‘Chachi’ (‘Guangchenpi’ in Chinese, GCP) in China. GCP, particularly planted and harvested in Xinhui County (Guangdong province, China) is traditionally considered to have superior qualities compared with CP (Tan et al., 2015).

Phytochemical studies demonstrate that CRP contains various bioactive constituents, including flavonoids and volatile oils (Qin et al., 2013; Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007; Wang et al., 2008; Yu, Li, Liu, Xu, & Liang, 2009). In our previous study, both the composition and contents of flavonoid compounds in CP and GCP showed significant differences, and GCP samples could be distinguished from CP samples based on flavonoid contents (Liu et al., 2013). Most recent reports focus on the analysis of flavonoids in CRP (Han, Kim, & Lee, 2012; Khan, Abert-Vian, Fabiano-Tixier, Dangles, & Chemat, 2010; Kim et al., 2011; Tistaert et al., 2011; Yi, Yu, Liang, & Zeng, 2008), but the volatile compounds, which also have potent bioactive properties, are neglected (Ellouze et al., 2012; Radulovic et al., 2013). To the best of our knowledge, no previous study has been designed to compare the volatile compounds in GCP and CP. Comparative analysis between CP and GCP volatile compounds will help not only to find chemical markers for these varieties, but also provide scientific evidence for dietary practice.

Metabolomics is a powerful technology for describing the similarities and differences between biological samples by profiling and comparing metabolites in an organism (Leonor & Antonio, 2015). Metabolomics analysis has been established for investigating metabolic differences between diseases (Denkert et al., 2006), food classes and plant species (Yi, Dong, Liu, Yi, & Zhang, 2014) over recent decades. Of various profiling techniques in metabolomics, gas chromatography mass spectrometry (GC–MS), which offers higher resolution, sensitivity and availability of databases, has been applied extensively in natural product discovery and species differentiation (Pasikanti, Ho, & Chan, 2008).

In this study, GC–MS based metabolomics analysis was employed to compare comprehensively the volatile constituents in CP and GCP. Multivariate statistical methods, including principal component analysis (PCA) and orthogonal partial least-squares discrimination analysis (OPLS-DA) were utilized to find chemical markers for discriminating CP and GCP. This study might provide a feasible strategy for authentication of citrus fruits and facilitate better understanding of their different traditional uses.
2. Materials and methods

2.1. Plant materials

In China, CRP is composed of GCP and CP. GCP was harvested in Xinhui County (Guangdong province, China), whilst CP was harvested in other regions throughout China. In this work, twelve batches of GCP (labeled G1-G12) and eleven batches of CP (labeled C1-C11) were harvested in 2014. The other batches of GCP (G13-G15) and CP (C12-C14) were harvested in 2012 and were stored for two years. GCP samples were collected from Jiangmen, Guangdong Province, and CP samples were collected from different regions in China. All of the plant materials were authenticated by Professor E-Hu Liu. The voucher specimens were deposited in the Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China.

2.2. Extraction of volatile oil

The peels were manually removed, sun-dried and stored under dry conditions. Before extraction, the peels were powdered using a mill (Beifang Medical Equipment Manufacture Company, China) and passed through a 24 mesh sieve. Approximately 45 g of CP powder or 15 g of GCP powder were rehydrated with 500 ml of distilled water prior to hydro-distillation for 3 h using a standard extractor. The volatile oil was collected in a sterilized glass vial. Water was removed from the volatile oil using anhydrous sodium sulfate. The extraction yield was calculated in milliliter of oil per 100 g of dried samples. The anhydrous volatile oil was stored at −80 °C in the dark and then dissolved in n-hexane prior to GC–MS analysis.

2.3. GC–MS analysis of volatile oil

GC–MS analysis was performed using an Agilent Technologies 7890B GC equipped with a fused silica capillary column (HP-5MS, 0.25 mm × 30 m, film thickness 0.25 μm) and coupled with a 5977A MS detector (Agilent Technologies, Palo Alto, CA, USA). The initial oven temperature was held at 60 °C initially (maintained for 2 min), which was then increased to 110 °C at a rate of 4 °C/min and finally to 260 °C at a rate of 10 °C/min. The inlet temperature was 260 °C. The carrier gas was helium with a constant flow rate of 1.0 mL/min. The ionization mode was the electron impact at 70 eV. The mass spectra plot was acquired using full scan monitoring mode with a mass scan range of m/z 30–500 and the splitting ratio was 20:1. The samples were diluted 100 times in

Fig. 1. GC–MS typical total ion chromatograms of the CP and GCP samples.
2.4. Antimicrobial activity assays

2.4.1. Microbial strains

The antimicrobial activity was tested using oils against a panel of microorganisms. Four bacteria including 2 g-positive (Staphylococcus aureus and Staphylococcus epidermidis) and 2 g-negative (Escherichia coli and Pseudomonas aeruginosa) were used.

2.4.2. Determination of minimum inhibitory concentration (MIC)

A broth microdilution method was used to determine the MIC. Stock solutions of the volatile oil were prepared in methanol (v/v). No antimicrobial activity was noticed for methanol. Serial dilutions (ranging from 1.25 to 40 μL/mL) of volatile oil were prepared with hot nutrient media to which Tween 80 (0.5%) was added in order to guarantee homogenisation and spread in petri dishes. The dish was left to cool down and to solidify at room temperature for 30 min. The plates were spotted, and then inoculated with 2 μL of bacterial strains (8 × 10^6 cells/mL). Tests were carried out in duplicate. These plates were incubated at 37 °C for 16 h for bacteria. The MIC is defined as the lowest concentration of the volatile oil at which the microorganism does not demonstrate visible growth.

2.5. Data processing, statistical analysis and metabolite identification

The contents of oil are presented as the average value ± standard deviation. Statistical differences between the results were analyzed using paired Student’s T-test between groups.

The GC/MS raw data were extracted and aligned by using an Agilent MassHunter Qualitative Analysis (version B.06.00) and Agilent Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA), respectively. Files in compound exchange format (.cef files) were created for each sample and exported into the MPP for further processing. In the next step, alignment of RT and m/z values was carried out across the sample set using a tolerance window of 0.1 min and 5 ppm, respectively.

The data matrix was mean centered and Pareto scaled with the SIMCA-P 13.0 (Umetrics, Sweden) before the multivariate analysis. Unsupervised principal component analysis (PCA) was selected to obtain the first understanding of the relationships among the data matrix. Then, supervised orthogonal partial least-squares discriminant analysis (OPLS-DA) was used to examine the differences of volatile metabolite of CRP of different geographical origins. The corresponding variable importance in projection (VIP) value was calculated in the OPLS-DA model. The VIP value represents the differences of the variables. Components that played important roles in differentiation were picked out when the VIP value was more than 1.5. The marker compounds were characterized from their mass spectral data using the NIST Mass Spectral Library and confirmed by comparing linear retention indices (LRI) calculated relative to (C8-C20) n-alkanes with LRI database.

3. Results and discussion

3.1. Comparison of global yields of volatile oil in CP and GCP

The volatile oils from the CP and GCP samples were extracted by hydrodistillation. Table 2 shows the global yields of volatile oil in CP and GCP. On average, the total volatile oil content in GCP was significantly higher than CP (p < 0.05). The volatile oil yields from CP were approximately 1.59 ± 0.60%, while oil yields from GCP were fourfold higher (8.14 ± 1.79%). After two years’ storage, the content of volatile oil in CP decreased compared with fresh samples (p < 0.05). However, no significant differences in the oil content were found between fresh and stored GCP samples.

3.2. Comparison of chemical profiling of volatile oil in CP and GCP

In this work, chemical profiling of volatile oil was achieved by GC–MS and typical total ion chromatograms (TIC) of CP and GCP samples are shown in Fig 1. It was observed that the two chromatograms were quite similar, and two peaks were found to be the predominant constituents in both CP and GCP samples. After a library search carried out for target peaks using the NIST/EPA/NIH version 2.0, peak 1 (Rt = 8.5 min) was identified as d-limonene and peak 2 (Rt = 9.4 min) was γ-terpinene. n-limonene was the most abundant volatile compound in all samples (88.4% on average in CP and 75.1% in GCP, respectively) but γ-terpinene was also present in high concentration (4.8% in CP and 13.5% in GCP, respectively). The majority of the volatile compounds identified in CRP samples belonged to monoterpenes and sesquiterpenes. It must also be noted that there were considerable differences in the compositions and responses of minor peaks (Rt 12–24 min) in CP and GCP volatile oil. The variation in chemical compositions of the volatile oils might be attributed to different geographic and environmental conditions.

3.3. Discrimination of CP and GCP based on metabolomics

In order to discriminate CP and GCP, and find potential markers responsible for such classification, multivariate statistical methods, such as PCA and OPLS-DA, were employed.

The GC–MS data from 29 batches of CRP samples after several pretreatment procedures were applied to PCA to visualize grouping trends. As shown in PCA score plot (Fig. 2A), samples could be...
clearly classified as one of two groups corresponding to CP and GCP. The PCA results indicated that CRP samples were indeed different in terms of levels and/or occurrence of components.

To find potential chemical markers that contributed to the differences between CP and GCP, GC–MS data were further analyzed using OPLS-DA. The OPLS-DA loading plot (Fig. 2B) shows that the metabolic profiles of CP and GCP could be separated, indicating the composition of CP and GCP volatile oils were quite different. To select the chemical markers, the ions were further screened based using VIP value. The higher the VIP value for the ions was, the further away it is from the origin.

Consequently, fifteen metabolites with VIP greater than 1.5 were selected as marker metabolites that were responsible for the discrimination of CP and GCP. By comparison of their mass spectra and LRIs with those from NIST library, fifteen markers (Methyl methanthranilate, α-Sinensal, Geranyl acetate, β-Elemen, δ-Elemen, Camphene, Cyclohexane, 2,4-diosopropenyl-1-methyl-1-vinyl, gemacrene B, Thymol, γ-Elemen, Neryl acetate, Camphor, Neryl acetate, Citronellol, Perilla aldehyde, (R)-(+) - elemen and (−)- elemen) were tentatively identified (Golmakani & Moayyedi, 2015; Wang & Liu, 2014; Yang & Kang, 2013). As shown in Table 1, the contents of methyl methanthranilate, α-sinensal and camphene were significantly different in CP and GCP. The PCA results indicated that CRP samples were indeed different in terms of levels and/or occurrence of components.

Additionally, CP and GCP volatile oils showed different MIC values for both Gram-negative and Gram-positive bacteria, however, GCP might exhibit better antimicrobial effects due to the greater oil yield. The difference in yield from GCP and CP might partly explain why GCP have superior qualities.

4. Conclusion

Volatile compounds in CP and GCP were compared using GC–MS based metabolomics. Samples from CP and GCP could be rapidly discriminated from each other, and 15 potential chemical markers could be used to ensure consistent quality of CRP. The volatile oils in CP and GCP showed similar antimicrobial activity, however, the oil yield from GCP was much higher than that from CP, which might partly explain the fact that GCP have superior qualities. This work might provide a feasible strategy for authentication of citrus fruits from different varieties and regions, and facilitate better understanding of their different traditional uses.

Acknowledgements

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Table 1

Details of potential chemical markers.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>CAS number</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Rt</th>
<th>LRI</th>
<th>VIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methyl methanthranilate</td>
<td>85-91-6</td>
<td>C8H11NO2</td>
<td>165</td>
<td>19</td>
<td>1408</td>
<td>2.49</td>
</tr>
<tr>
<td>2</td>
<td>α-Sinensal</td>
<td>17909-77-2</td>
<td>C10H14O</td>
<td>154</td>
<td>12.5</td>
<td>1153</td>
<td>1.70</td>
</tr>
<tr>
<td>3</td>
<td>Geranyl acetate</td>
<td>105-87-3</td>
<td>C11H20O2</td>
<td>196</td>
<td>18.6</td>
<td>1384</td>
<td>2.32</td>
</tr>
<tr>
<td>4</td>
<td>β-Elemen</td>
<td>515-13-9</td>
<td>C11H18O2</td>
<td>196</td>
<td>18.6</td>
<td>1384</td>
<td>2.32</td>
</tr>
<tr>
<td>5</td>
<td>δ-Elemen</td>
<td>20397-84-0</td>
<td>C11H18O2</td>
<td>196</td>
<td>18.6</td>
<td>1384</td>
<td>2.32</td>
</tr>
<tr>
<td>6</td>
<td>Camphene</td>
<td>79-92-3</td>
<td>C10H16</td>
<td>136</td>
<td>6.2</td>
<td>947</td>
<td>2.12</td>
</tr>
<tr>
<td>7</td>
<td>Cyclohexane, 2,4-diosopropenyl-1-methyl-1-vinyl</td>
<td>1108823-68-2</td>
<td>C15H24</td>
<td>204</td>
<td>18.7</td>
<td>1391</td>
<td>2.00</td>
</tr>
<tr>
<td>8</td>
<td>Gemacrene B</td>
<td>15423-57-1</td>
<td>C15H24</td>
<td>204</td>
<td>21.4</td>
<td>1559</td>
<td>1.80</td>
</tr>
<tr>
<td>9</td>
<td>Thymol</td>
<td>89-83-8</td>
<td>C10H16O</td>
<td>136</td>
<td>16.7</td>
<td>1292</td>
<td>1.80</td>
</tr>
<tr>
<td>10</td>
<td>γ-Elemen</td>
<td>29973-99-2</td>
<td>C10H16O</td>
<td>136</td>
<td>16.7</td>
<td>1292</td>
<td>1.80</td>
</tr>
<tr>
<td>11</td>
<td>Neryl acetate</td>
<td>141-12-8</td>
<td>C12H20O2</td>
<td>196</td>
<td>18.2</td>
<td>1365</td>
<td>1.76</td>
</tr>
<tr>
<td>12</td>
<td>Citronellol</td>
<td>105-87-3</td>
<td>C12H20O2</td>
<td>196</td>
<td>18.6</td>
<td>1384</td>
<td>2.32</td>
</tr>
<tr>
<td>13</td>
<td>Perilla aldehyde</td>
<td>2111-75-3</td>
<td>C10H16O</td>
<td>136</td>
<td>16.3</td>
<td>1274</td>
<td>1.68</td>
</tr>
<tr>
<td>14</td>
<td>(R)-(+) - Citronellol</td>
<td>1117-61-9</td>
<td>C10H16O</td>
<td>136</td>
<td>15.1</td>
<td>1228</td>
<td>1.67</td>
</tr>
<tr>
<td>15</td>
<td>(−)-Spathulenol</td>
<td>77171-55-2</td>
<td>C15H24O</td>
<td>220</td>
<td>22.3</td>
<td>1635</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Table 2

Global yields (%) of volatile oil in CP and GCP.

<table>
<thead>
<tr>
<th></th>
<th>All samples</th>
<th>Young samples</th>
<th>Two years stored samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>1.59 ± 0.62</td>
<td>1.86 ± 0.41</td>
<td>0.91 ± 0.50</td>
</tr>
<tr>
<td>GCP</td>
<td>8.14 ± 1.80</td>
<td>8.57 ± 1.57</td>
<td>6.40 ± 1.83</td>
</tr>
</tbody>
</table>

Table 3

Antimicrobial activity of CP and GCP volatile oil.

<table>
<thead>
<tr>
<th>Samples</th>
<th>The MIC (μg/mL) a</th>
<th>Gram-positive bacteria</th>
<th>Gram-negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
<td>S. epidermidis</td>
<td>E. coli</td>
</tr>
<tr>
<td>CP</td>
<td>40</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>GCP</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

a Minimum inhibitory concentration.


