Analytical Methods

Simultaneous determination of six bioactive flavonoids in Citri Reticulatae Pericarpium by rapid resolution liquid chromatography coupled with triple quadrupole electrospray tandem mass spectrometry

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A rapid resolution liquid chromatography/electrospray ionisation tandem mass spectrometry (RRLC–ESI-MS\textsuperscript{n}) method has been firstly developed and validated for simultaneous determination of six bioactive flavonoids in Citri Reticulatae Pericarpium (CRP). The antiproliferative activities of the six flavonoids in CRP, namely naringin, hesperidin, nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, tangeretin and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, were evaluated and compared by Cell Counting Kit-8 Assay. Quantification was carried out on an Agilent triple quadrupole LC–MS system using multiple reaction monitoring mode. The established method was successfully applied for determination of the six flavonoids in samples collected from different regions in China. Compared with the reported analytical methods, the RRLC–ESI-MS\textsuperscript{n} method is powerful in quantitative analysis of multi-component in terms of time savings and sensitivity. Hierarchical cluster analysis (HCA) was also performed to differentiate and classify the samples based on the contents of the six characteristic flavonoids. The HCA results indicated that Citrus reticulata 'Chachi' samples could be easily distinguished from other CRP samples. The developed RRLC–ESI-MS\textsuperscript{n} method combined with HCA might be utilised as a quality control method for CRP.

1. Introduction

Citri Reticulatae Pericarpium (CRP), the dried ripe pericarp of Citrus reticulata Blanco (the mandarin orange) or its cultivars, is not only consumed as dietary supplement in China or other eastern countries, but also one of the most popular traditional medicinal herbs used in clinical practise for the treatment of indigestion and inflammatory syndromes of the respiratory tract (Chinese Pharmacopoeia Commission, 2010). The inclusion of CRP in the diet provides sugars as a quick source for energy as well as many other substances which may contribute to a person’s well being. Phytochemical and pharmacological studies demonstrated that the major components in CRP are dietary flavonoids, which are generally categorised into two groups, flavanone glycosides (e.g., hesperidin and naringin) and polymethoxylated flavones (e.g., nobiletin and tangeretin). It’s reported that these Citrus flavonoids possess various biological activities, including anticonvulsion (Dimpfel, 2006), anticarcinogenic (Walle, 2007), antimutagenic (Miyazawa, Okuno, Fukuyama, Nakamura, & Kosaka, 1999), anti-inflammatory (Li et al., 2007; Lin et al., 2003) and antioxidative properties (Barreca, Belluco, Caristi, Leuzzi, & Gattuso, 2011; Miyake, 2006). According to the theory of Traditional Chinese Medicine, the dried ripe pericarp of Citrus reticulata ‘Chachi’ mainly produced in Xinhui district of Guangdong Province, is regarded as a genuine CRP species on account of its excellent clinical efficacy. The different efficacy of various CRP species may be correlated with the contents of flavonoids.

In the practise of quality control, it might be acceptable to quantify some bioactive components for herbal medicines. High performance liquid chromatography coupled to mass spectrometry (HPLC/MS) has now been widely accepted to be the predominant tool for qualitative and quantitative analysis of chemical constituents in botanical products (Chai, Li, & Li, 2005; Chen et al., 2006; Lee et al., 2008; Xu et al., 2012). In the past few years, several studies have reported the determination of flavonoids in Citrus herbs by TLC (Wang & Luo, 1989), HPLC-UV (Zheng et al., 2009), HPLC–ECD (Careri, Elviri, Mangia, & Musci, 2000), and CE-ECD (Peng, Liu, & Ye, 2006). Most of them mainly focused on the determination of flavonoids in Citrus fruits or juices of only one or two of Citrus species, however, little is known about the activities of the flavonoids in CRP. In our previous study, we developed an approach utilising LC/MS for the identification and profiling of chemical composition in CRP (Zheng et al., 2013). To the best of our knowledge, no HPLC/MS method has been developed to simultaneously quantify the
bioactive flavonoids in CRP and no chemometric approach has been employed to discriminate Citrus reticulata ‘Chachi’ from other CRP species in China.

To evaluate the quality of CRP effectively and comprehensively and ensure its clinical use, in this paper, we developed and validated an accurate and reliable rapid resolution liquid chromatography/electrospray ionisation tandem mass spectrometry (RRLC–ESI-MSn) method for the simultaneous determination of six bioactive flavonoids (Fig. 1) namely naringin, hesperidin, nobiletin, 3,5,6,7,8,3',4'-heptamethoxyflavone, tangeretin and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone in CRP samples collected from different main Citrus producing areas in China. Hierarchical clustering analysis (HCA) was also performed to evaluate and classify the samples according to the contents of the six flavonoids. The results provided detailed information for the identification of botanical origin and chemotaxonomic investigation of CRP.

2. Materials and methods

2.1. Chemicals and materials

Twelve samples including four different cultivars were collected from different main Citrus producing regions around China in September 2011–November 2012 (Table 1). About 6–8 kg of fresh fruits was collected from each sampling area. Then, the Citrus peels were removed and dried in the sun for about 5–7 days, which were used for the tested sample. The voucher specimens, identified by Prof. Ping Li, have been deposited at the State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, China.

The solvents, HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany), and formic acid with a purity of 96% is of HPLC grade (Tedia, USA). Deionized water (18 MQΩ) was prepared by distilled water through a Milli-Q system (Millipore, Milford, MA, USA). Other reagents and chemicals are of analytical grade. The reference standards of naringin, hesperidin, nobiletin, 3,5,6,7,8,3',4'-heptamethoxyflavone, tangeretin and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone were isolated and purified from CRP by conventional column chromatography coupled with high-speed countercurrent chromatography. Their structures were identified by ESI-MS, 1H NMR, and 13C NMR in comparison with the literature data, and purities were determined to be >98% by high-performance liquid chromatography–diode array detection analysis based on a peak area normalisation method.

2.2. Antiproliferation of six bioactive flavonoids

2.2.1. Cell culture

Human hepatoblastoma cell line (HepG2) and Human lung carcinoma (A549) were obtained from Chinese Academy of Sciences,
and cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640, respectively, supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen). The cells were incubated in a humidified atmosphere of 5% CO2 at 37 °C. Five subsequent cycles of subculturing was done before the cells were incubated with study drugs.

2.2.2. Cell proliferation assay

Cell proliferation was determined by using the Cell Counting Kit-8 Assay, which is based on the conversion of water-soluble tetrazolium, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen). The cells were incubated in a humidified atmosphere of 5% CO2 at 37 °C. Five subsequent cycles of subculturing was done before the cells were incubated with study drugs.

2.3. Preparation of standard solutions

Standard stock solutions of six accurately weighed reference compounds were directly prepared in methanol. Working standard solutions containing each of the six compounds were prepared by diluting the stock solutions with methanol to a series of proper concentrations. An aliquot of 2 μl was injected into LC/MS for analysis.

2.4. CRP sample preparation

The tested samples were cut into smaller pieces and further ground into powder. Every sample powder (0.1 g) was weighed accurately and extracted by ultrasonator with 25 mL methanol for 30 min. After that, the sample was filtered and the volume of solution was set at 25 mL. The extracted solution was centrifuged at 14,000 g for 10 min, and the supernatant was transferred to an autosampler vial for RRLC-ESI-MS5 analysis.

2.5. Analytical system

Chromatographic analysis was performed on an Agilent 1200 Series HPLC system (Agilent Corporation, MA, USA) equipped with a binary pump, micro degasser, an autosampler and a thermostat-ically controlled column apartment. Chromatographic separation was carried out at 30 °C on an Agilent ZorBax SB-C18 column (4.6 × 50 mm, 1.8 μm). The mobile phase consisted of 0.1% formic acid solution (A) and acetonitrile (B) using a gradient elution of 20–80% B at 0–12 min, 80–25% B at 12–13 min, 25–55% B at 13–20 min, 55–85% B at 20–25 min and 85–95% B at 25–30 min. The flow rate was kept at 0.5 mL/min.

All MS experiments were conducted on an Agilent 6410 triple quadrupole mass spectrometer equipped with electrospray ionisation (ESI) interface (Agilent Corporation, MA, USA). Quantification was performed using positive ion multiple reaction monitoring (MRM) mode. The MS analysis was performed under the following conditions:

Table 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>Cultivars</th>
<th>Place of collection</th>
<th>Naringin</th>
<th>Hesperidin</th>
<th>Nobletin</th>
<th>3,5,6,7,8,3′,4′-Heptamethoxy-flavone</th>
<th>Tangeretin</th>
<th>5-Hydroxy-6,7,8,3′,4′-pentamethoxy-flavone</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>C. reticulata 'Erythrosa'</td>
<td>Shimen County, Hunan Province</td>
<td>0.556 ± 0.009</td>
<td>74.236 ± 0.845</td>
<td>1.910 ± 0.015</td>
<td>3.015 ± 0.036</td>
<td>0.821 ± 0.009</td>
<td>0.124 ± 0.002</td>
</tr>
<tr>
<td>S2</td>
<td>C. reticulata 'Unshiu'</td>
<td>Chahé Town, Hubei Province</td>
<td>0.211 ± 0.003</td>
<td>60.540 ± 0.763</td>
<td>2.371 ± 0.021</td>
<td>3.056 ± 0.045</td>
<td>1.112 ± 0.016</td>
<td>0.135 ± 0.003</td>
</tr>
<tr>
<td>S3</td>
<td>C. reticulata 'Unshiu'</td>
<td>Yangshuo County, Guangxi Zhuang Autonomous Region</td>
<td>0.397 ± 0.006</td>
<td>70.232 ± 0.487</td>
<td>1.685 ± 0.016</td>
<td>1.898 ± 0.023</td>
<td>0.840 ± 0.004</td>
<td>0.240 ± 0.005</td>
</tr>
<tr>
<td>S4</td>
<td>C. reticulata 'Subcompressa'</td>
<td>Yunguan Town, Zhejiang Province</td>
<td>0.582 ± 0.010</td>
<td>100.525 ± 1.398</td>
<td>1.905 ± 0.028</td>
<td>4.390 ± 0.048</td>
<td>0.738 ± 0.007</td>
<td>0.239 ± 0.004</td>
</tr>
<tr>
<td>S5</td>
<td>C. reticulata 'Subcompressa'</td>
<td>Huashan Town, Zhejiang Province</td>
<td>0.569 ± 0.007</td>
<td>62.678 ± 0.697</td>
<td>1.359 ± 0.010</td>
<td>2.825 ± 0.027</td>
<td>0.562 ± 0.003</td>
<td>0.183 ± 0.003</td>
</tr>
<tr>
<td>S6</td>
<td>C. reticulata 'Chachi'</td>
<td>Gujin Town, Xinhui District, Guangdong Province</td>
<td>3.409 ± 0.013</td>
<td>62.919 ± 0.543</td>
<td>10.876 ± 0.093</td>
<td>1.752 ± 0.019</td>
<td>7.401 ± 0.054</td>
<td>1.818 ± 0.026</td>
</tr>
<tr>
<td>S7</td>
<td>C. reticulata 'Chachi'</td>
<td>Huicheng Town, Xinhui District, Guangdong Province</td>
<td>2.535 ± 0.026</td>
<td>59.012 ± 0.787</td>
<td>9.448 ± 0.065</td>
<td>1.551 ± 0.013</td>
<td>6.375 ± 0.067</td>
<td>1.627 ± 0.031</td>
</tr>
<tr>
<td>S8</td>
<td>C. reticulata 'Chachi'</td>
<td>Luokeng Town, Xinhui District, Guangdong Province</td>
<td>2.433 ± 0.010</td>
<td>74.973 ± 0.845</td>
<td>14.017 ± 0.120</td>
<td>1.730 ± 0.021</td>
<td>11.548 ± 0.093</td>
<td>2.760 ± 0.039</td>
</tr>
<tr>
<td>S9</td>
<td>C. reticulata 'Chachi'</td>
<td>Daze Town, Xinhui District, Guangdong Province</td>
<td>3.870 ± 0.037</td>
<td>54.075 ± 0.578</td>
<td>7.521 ± 0.013</td>
<td>1.110 ± 0.012</td>
<td>5.571 ± 0.048</td>
<td>1.494 ± 0.025</td>
</tr>
<tr>
<td>S10</td>
<td>C. reticulata 'Chachi'</td>
<td>Yamen Town, Xinhui District, Guangdong Province</td>
<td>4.292 ± 0.040</td>
<td>88.087 ± 1.062</td>
<td>13.321 ± 0.126</td>
<td>1.805 ± 0.023</td>
<td>9.779 ± 0.097</td>
<td>2.169 ± 0.018</td>
</tr>
<tr>
<td>S11</td>
<td>C. reticulata 'Chachi'</td>
<td>Shaoshui Town, Xinhui District, Guangdong Province</td>
<td>3.332 ± 0.035</td>
<td>51.921 ± 0.768</td>
<td>8.951 ± 0.099</td>
<td>1.468 ± 0.016</td>
<td>6.938 ± 0.045</td>
<td>1.750 ± 0.021</td>
</tr>
<tr>
<td>S12</td>
<td>C. reticulata 'Chachi'</td>
<td>Siqian Town, Xinhui District, Guangdong Province</td>
<td>3.673 ± 0.048</td>
<td>50.137 ± 0.301</td>
<td>6.325 ± 0.072</td>
<td>1.026 ± 0.014</td>
<td>5.387 ± 0.058</td>
<td>1.394 ± 0.014</td>
</tr>
</tbody>
</table>

* Data are represented as the mean ± SD.
operation parameters: both the auxiliary and sheath gases were nitrogen with a flow rate of 12 L/min. The dry gas temperature was set at 350 °C, the fragmentor voltage (FV) was 120 V and the nebulizer pressure was set at 45 psi. Full scan data acquisition and dependant scan event data acquisition were performed from m/z 100 to 1000. The collision energy (CE) was adjusted from 30 to 40 V according to the detection of different analytes. The system was controlled by MassHunter software (Agilent Corporation, MA, USA).

2.6. Hierarchical clustering analysis

Hierarchical cluster analysis (HCA) is a statistical method to identify relatively homogeneous groups of cases based on measured characteristics. The hierarchical clustering process can be represented as a tree or dendrogram, where each step in the clustering process is illustrated by a joint of the tree. The contents of the six analytes were defined as six characteristics in the analysis so as to analyse, differentiate and classify the 12 samples. HCA of samples were performed by SPSS software (SPSS 13.0 for Windows, SPSS Inc., USA).

3. Results and discussion

3.1. Antiproliferative activities of six flavonoids from CRP

Table 2 summarises data on the IC50 of the tested flavonoids against A549 and HepG2 cell lines. The data demonstrated the rank order of potency of the flavonoids against the tumour cells was: 5-hydroxy-6,7,8,3'-4'-pentamethoxyflavone > nobiletin > tangeretin > 3,5,6,7,8,3'-4'-heptamethoxyflavone > hesperidin > naringin. Generally, the flavonoid glycosides (hesperidin and naringin) had weaker antiproliferative activity against tumour cell lines than the polymethoxylated flavones. Comparison of the antiproliferative activities of polymethoxylated flavones revealed the importance of hydroxylation. Among the tested polymethoxylated flavones, 5-hydroxy-6,7,8,3'-4'-pentamethoxyflavone showed the highest antiproliferative activity against both A549 and HepG2 cell lines.

3.2. Optimisation of RRLC–ESI-MSn analysis

To obtain good chromatographic behaviour and appropriate ionisation, different mobile phase system (methanol–water, acetone–water, methanol–acid aqueous solution, and acetone–acid aqueous solution) were examined and compared. Finally, acetone–0.1% aqueous formic acid was chosen as the eluting solvent system to give the acceptable separation and desired ionisation within a run time of 13 min.

The chemical structures of six analytes were characterised by comparison with their retention behaviour and MS information of the standard compounds obtained from RRLC–ESI-MSn analysis (Kim, Kim, Park, & Lee, 2012; Wang, Wang, Huang, Tu, & Ni, 2007). The MRM mode afforded by tandem mass spectrometry had great advantage in reducing interference and enhancing sensitivity over the selected ion monitoring (SIM). The six analytes were firstly characterised by MS n scan and MS 5 product ions to ascertain their precursor ions and to select product ions for use in MRM mode, respectively (see Fig. 1). To obtain the richest relative abundance of precursor ions and product ions, the parameters for CE was optimised as it played an important role in parent and product ion responses. The MRM transition were chosen to be m/z 581 → 273 for naringin, m/z 611 → 303 for hesperidin, m/z 403 → 373 for nobiletin, m/z 433 → 403 for 3,5,6,7,8,3'-4'-heptamethoxyflavone, m/z 373 → 343 for tangeretin and m/z 389 → 359 for 5-hydroxy-6,7,8,3'-4'-pentamethoxyflavone. Retention time (RT) and MS information for each analyte including [M+H]+, MS n fragmentation ions, quantitative ions, FV and CE are shown in Table 2, and typical MRM chromatogram of mixed standards and CRP sample are shown in Fig. 2.

3.3. Analytical method validation

3.3.1. Linearity, limits of detection (LOD) and limits of quantification (LOQ)

The calibration curves were plotted with a series of concentrations of standard solutions. Each analyte curve was made at least six levels. Acceptable linear correlation and high sensitivity at these conditions were confirmed by the correlation coefficients (r 2, 0.9974–0.9995). LOD and LOQ expressed by 3- and 10-fold of the ratio of the signal-to-noise (S/N) were also acquired, respectively. Detailed information regarding calibration curves, linear ranges, LOD and LOQ is displayed in Table 3.

3.3.2. Precision, repeatability and stability

The precision of the method was validated by the determination of intra- and inter-day variances. For intra-day test, the samples were analysed for six times within the same day, while for inter-day test, the samples were examined twice per day for three consecutive days. The concentration of each solution was determined by a calibration curve formed at the same day. The intra- or inter-day precisions calculated as relative standard deviation (RSD) were within the range of 0.96–2.57% or 1.26–3.09%.

To confirm the repeatability, six different working solutions prepared from the same CRP sample (S8 was randomly selected) were analysed in parallel by the above-established method. The RSD values of six analytes were within the range from 1.78% to 3.91%, which revealed high repeatability of the method.

Stability of sample solution was tested at room temperature. The RSD values of six analytes were all within 5%, which demonstrated a good stability in methanol solution within the tested period (shown in Supplementary material).

3.3.3. Recovery

Recovery was used to further evaluate the accuracy of the method. Known amounts of each standard solution were mixed with known amounts of CRP samples (S8). Then the samples were extracted and analysed by the above-established method, and triplicate experiments were repeated at each level. The average recoveries were estimated by the formula (1):

\[
\text{recovery(%) = (detection - original amount)/addition} \times \frac{100}{(1)}
\]

The results demonstrated that the mean recovery rates of six analytes varied from 90.23% to 102.61% (RSD ≤ 4.51%) (shown in Supplementary material).

<table>
<thead>
<tr>
<th>Compound</th>
<th>A549</th>
<th>HepG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naringin (1)</td>
<td>&gt;100</td>
<td>99.69 ± 3.26</td>
</tr>
<tr>
<td>Hesperidin (2)</td>
<td>57.03 ± 4.33</td>
<td>93.48 ± 4.18</td>
</tr>
<tr>
<td>Nobiletin (3)</td>
<td>34.99 ± 2.58</td>
<td>37.43 ± 2.52</td>
</tr>
<tr>
<td>3,5,6,7,8,3',4'-Heptamethoxyflavone (4)</td>
<td>65.31 ± 1.11</td>
<td>71.08 ± 1.61</td>
</tr>
<tr>
<td>Tangeretin (5)</td>
<td>51.15 ± 1.66</td>
<td>45.26 ± 2.76</td>
</tr>
<tr>
<td>5-Hydroxy-6,7,8,3',4'-pentamethoxyflavone (6)</td>
<td>36.63 ± 2.14</td>
<td>19.67 ± 1.66</td>
</tr>
</tbody>
</table>

Data are means ± SD from three independent experiments (n = 3).
3.4. Quantitative analysis of samples

3.4.1. Sample analysis

The developed analytical method was subsequently applied to analysis of six bioactive flavonoids in 12 batches of CRP samples collected from different main Citrus producing areas in China. The contents of six analytes were calculated with external standard methods based on the respective calibration curves (Table 1). The proposed RRLC–ESI-MS\(^n\) assay was a successful application in quantification of major flavanone glycosides and polymethoxylated flavones in different CRP samples within a run time of 13 min. Compared with the reported analytical methods, the RRLC–ESI-

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**Table 3**

Retention time (RT), MS data, calibration curves, linear ranges, LOD and LOQ of the six flavonoids.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>[M+H](^+) MS(^n) (m/z)</th>
<th>Quantitative ion (m/z)</th>
<th>FV</th>
<th>CE</th>
<th>Regression equation</th>
<th>R(^2)</th>
<th>Linear range (ng/mL)</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naringin (1)</td>
<td>4.59</td>
<td>581</td>
<td>435([M+H–rhamnosyl](^+)), 273([M+H–rhamnosyl–glucosyl](^+))</td>
<td>273</td>
<td>120</td>
<td>30</td>
<td>y = 9.843x + 2.884</td>
<td>0.9975</td>
<td>137–137,000</td>
<td>9.582</td>
</tr>
<tr>
<td>Hesperidin (2)</td>
<td>4.96</td>
<td>611</td>
<td>465([M+H–rhamnosyl](^+)), 303([M+H–rhamnosyl–glucosyl](^+))</td>
<td>303</td>
<td>120</td>
<td>30</td>
<td>y = 56.723x + 44.202</td>
<td>0.9987</td>
<td>15.84–15,840</td>
<td>1.241</td>
</tr>
<tr>
<td>Nobiletin (3)</td>
<td>8.89</td>
<td>403</td>
<td>388([M+H–CH(_3)](^+)), 373([M+H–CH(_2)O](^+))</td>
<td>373</td>
<td>120</td>
<td>30</td>
<td>y = 8.7144x + 1.3971</td>
<td>0.9995</td>
<td>1.84–1840</td>
<td>0.084</td>
</tr>
<tr>
<td>3,5,6,7,8,3',4'-heptamethoxyflavone (4)</td>
<td>9.37</td>
<td>433</td>
<td>418([M+H–CH(_3)](^+)), 403([M+H–CH(_2)O](^+))</td>
<td>403</td>
<td>120</td>
<td>40</td>
<td>y = 86.427x + 8.6974</td>
<td>0.9974</td>
<td>2.20–2200</td>
<td>0.095</td>
</tr>
<tr>
<td>Tangeretin (5)</td>
<td>9.71</td>
<td>373</td>
<td>358([M+H–CH(_3)](^+)), 343([M+H–CH(_2)O](^+))</td>
<td>343</td>
<td>120</td>
<td>35</td>
<td>y = 24.989x + 21.502</td>
<td>0.9974</td>
<td>1.68–1680</td>
<td>0.076</td>
</tr>
<tr>
<td>5-Hydroxy-6,7,8,3',4'-pentamethoxyflavone (6)</td>
<td>10.63</td>
<td>389</td>
<td>374([M+H–CH(_3)](^+)), 359([M+H–CH(_2)O](^+))</td>
<td>359</td>
<td>120</td>
<td>40</td>
<td>y = 15.826x + 7.4617</td>
<td>0.9992</td>
<td>1.32–6600</td>
<td>0.053</td>
</tr>
</tbody>
</table>

LOD, limits of detection; LOQ, limits of quantification.

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Fig. 2. Multiple reaction monitoring chromatogram of mixed standards (a) and typical extract of Citri Reticulatae Pericarpium sample (b) by RRLC–ESI-MS\(^n\) analysis. naringin (1), hesperidin (2), nobiletin (3), 3,5,6,7,8,3',4'-heptamethoxyflavone (4), tangeretin (5) and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (6).
MSn method is powerful in quantitative analysis of multi-component in terms of time savings and sensitivity (shown in Supplementary material). The data indicated that the content of each flavonoid varied significantly among the different cultivars and regions. Among the cultivars examined, the content of hesperidin was the highest in 12 samples, varying from 50.137 to 100.525 mg/g. For the other flavanone glycoside determined, Citrus reticulata ‘Erythrosa’, Citrus reticulata ‘Unshiu’ and Citrus reticulata ‘Subcompressa’ exhibited much lower content of naringin (0.221–0.582 mg/g), compared to Citrus reticulata ‘Chachi’ produced in Guangdong Province. Apart from flavanone glycosides, the content differences of the four polymethoxylated flavones (nobiletin, 3,5,6,7,8,3’0,4’0–heptamethoxyflavone, tangeretin and 5-hydroxy-6,7,8,3’0,4’0–pentamethoxyflavone) in various cultivars may be more significant. The contents of nobiletin, tangeretin and 5-hydroxy-6,7,8,3’0,4’0–pentamethoxyflavone were found to be much higher in the peel of Citrus reticulata ‘Chachi’ than in other cultivars. On the contrary, the content of 3,5,6,7,8,3’0,4’0–heptamethoxyflavone was found to be higher in the CRP cultivars with content ranging from 1.896 to 4.390 mg/g, compared with Citrus reticulata ‘Chachi’. The results showed that there was a great deal of regional variability in content of major bioactive flavonoids in CRP across China.

3.4.2. Quality assessment of CRP by HCA

To evaluate the CRP variations, HCA, a multivariate analysis technique, was performed based on the characteristics of the contents of six bioactive flavonoids. The content of six analytes in 12 CRP samples formed a 12 × 6 matrix. Distances between the 12 samples were calculated using the SPSS software. The HCA results demonstrated significant variations in the content of these flavonoids in the samples from different regions in China. As shown in Fig. 3, sample No. 6–12 (Citrus reticulata ‘Chachi’) from Xinhui District, Guangdong Province could be included in one cluster, and sample No. 1–5 (Citrus reticulata ‘Erythrosa’, Citrus reticulata ‘Unshiu’ and Citrus reticulata ‘Subcompressa’) from other provinces in China. The chemical variation may be caused by a lot of factors, such as their genetic origin, growing environment, time of collection, and storage conditions, especially the genetic origin (Li et al., 2011; Zheng et al., 2009).

4. Conclusions

In this paper, six bioactive flavonoids in CRP were simultaneously characterised and quantified by the developed RRLC–ESI-MSn method for the first time. Among the tested flavonoids, 5-hydroxy-6,7,8,3’0,4’0–pentamethoxyflavone showed the highest anti-proliferative activity against both A549 and HepG2 cell lines. The established RRLC–ESI-MSn method demonstrated superiority in terms of time savings and sensitivity for quantitative analysis. HCA indicated that chemical profile of Citrus reticulata ‘Chachi’ could be easily distinguished from other cultivars. The proposed method could be readily utilised as a quality control method for CRP and other traditional Chinese medicines. The comparative activity of Citrus reticulata ‘Chachi’ and other cultivars is still under investigation in our laboratory, and will be reported in the near future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.06.077.

References


