Multi-ingredients determination and fingerprint analysis of leaves from *Ilex latifolia* using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry

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**ABSTRACT**

An ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC–QTOF-MS) method integrating multi-ingredients determination and fingerprint analysis has been established for quality assessment and control of leaves from *Ilex latifolia*. The method possesses the advantages of speediness, efficiency, accuracy, and allows the multi-ingredients determination and fingerprint analysis in one chromatographic run within 13 min. Multi-ingredients determination was performed based on the extracted ion chromatograms of the exact pseudo-molecular ions (with a 0.01 Da window), and fingerprint analysis was performed based on the base peak chromatograms, obtained by negative-ion electrospray ionization QTOF-MS. The method validation results demonstrated our developed method possessing desirable specificity, linearity, precision and accuracy. The method was utilized to analyze 22 *I. latifolia* samples from different origins. The quality assessment was achieved by using both similarity analysis (SA) and principal component analysis (PCA), and the results from SA were consistent with those from PCA. Our experimental results demonstrate that the strategy integrated multi-ingredients determination and fingerprint analysis using UPLC–QTOF-MS technique is a useful approach for rapid pharmaceutical analysis, with promising prospects for the differentiation of origin, the determination of authenticity, and the overall quality assessment of herbal medicines.

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1. Introduction

The dried leaves of *Ilex latifolia* has been used as an herbal tea named *ku-ding-cha* in China for adjuvant treatment of headache, cold, diabetes, and hypertension [1]. Previous phytochemical studies revealed the leaves of *I. latifolia* contain bioactive ingredients such as caffeoylquinic acids (CQAs) [2], triterpenes and triterpenoid saponins [3,4]. CQAs possess pharmacological activities such as antiinflammatory, antiviral, and antioxidative [5,6]. Triterpenes and triterpenoid saponins show antidepressant, antibacterial, and antitumor activities [7–9]. High performance liquid chromatography (HPLC) methods have been developed for quality control of the leaves from *I. latifolia* by the determination of several marker compounds such as triterpenoid saponins [10], and CQAs [2]. However, these methods are limited due to only a few components can be accurately determined. It is well accepted that herbal medicines exert their therapeutic effects through multi-components on multiplets [11–14], and one or a few marker compound(s) could not represent their holistic effects. This, together with the chemical complexity and unpredictability, requires a systematical and comprehensive strategy for quality control of the leaves from *I. latifolia* based on the holistic active ingredients.

With regards to quality control of herbal medicines, two methodologies are proposed, one is multi-ingredients determination and the other is fingerprint analysis. Multi-ingredients determination is considered to be a useful approach because most of the active ingredients can be accurately determined during the process of analysis. However, the unpredictable chemical components of herbal medicines together with the arduous work to obtain phytochemical references make the full components determination becoming a very difficult task. Fingerprint analysis views an herbal medicine as an interactive and indivisible whole, and it can

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be utilized to assess and control the quality of herbal medicines systematically and comprehensively even in the absence of reference substances [15–18]. However, the contents of active ingredients cannot be determined by fingerprint analysis, and the quality of herbal medicines cannot be accurately controlled. Thus, a strategy integrating multi-ingredients determination and fingerprint analysis offers a systematical and reliable approach for quality assessment and control of herbal medicines.

Recent years, HPLC coupled with mass spectrometry (MS), with excellent sensitivity, specificity, reasonable qualitative and quantitative capability, has been proven to be a powerful tool for quality control of herbal medicines [19–22]. More recently, ultra-performance liquid chromatography (UPLC) coupled with quadrupole time-of-flight (QTOF) mass spectrometry has been introduced to phytochemical analysis [23–25]. UPLC holds the advantages over conventional HPLC due to its faster analytical speed and greater separation efficiency. In addition, QTOF-MS provides accurate mass measurements for both pseudo-molecular and fragmental ions, allowing fast identification of active ingredients and accurate determination of targeted compounds at low concentration (ppm) level. By using an UPLC–QTOF-MS system, multi-ingredients determination and fingerprint analysis could be achieved in one chromatographic run within a short time, meeting the requirements of speediness, efficiency and accuracy.

The aim of this study is to establish a rapid and reliable UPLC–QTOF-MS method integrating multi-ingredients determination and fingerprint analysis for quality assessment and control of *I. latifolia*.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and methanol of HPLC grade were purchased from Burdick & Jackson (Muskegon, MI, USA). Water for UPLC analysis was purified by a Milli-Q water-purification system (Milford, MA, USA). Formic acid of spectroscopy grade was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Nine reference substances, i.e., 3-O-caffeyloyquinic acid (3-0-CQA), 4-O-CQA, 5-O-CQA, 3,4-di-O-CQA, 3,5-di-O-CQA, 4,5-di-O-CQA, caffeic acid, isoursimarin and rutin, were purchased from Chengdu Push Bio-Technology Co., Ltd. (Sichuan, China). Twelve reference compounds, namely ilekudinoside G, kudinoside A, kudinosides C–G, kudinosides N–O, latifolidosides G–H, and latifoloside Q, were isolated from *I. latifolia* by our group. Their structures (Fig. 1) were elucidated based on their spectral data (UV, IR, MS and NMR), and their purities were found to be more than 98% by HPLC-MS analysis.

The stock solutions of 21 reference compounds for quantitative determination were prepared in methanol and stored in brown volumetric flask at 4 °C. All solutions were diluted to the desired concentration with methanol prior to use.

The samples of dried leaves from *I. latifolia* were collected from Guangdong, Guangxi, Hainan, and Zhejiang provinces of China (details see Table S-1). All samples were authenticated by Prof. Guangxiong Zhou (Jinan University, Guangzhou, China). The voucher samples were deposited in the Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, China. The samples were dried, crushed and sieved through a 0.3-mm stainless-steel sieve before extraction. Sample 1 was used for method development studies.

2.2. Sample extraction

Accelerated solvent extraction (ASE) was used for the extraction of *I. latifolia* samples. An SP-100 QSE system (Shanghai Spectrum Instruments, China) with an 11-ml stainless-steel extraction cell was used for ASE. 1.0 g of sample was placed in an extraction cell, and then the cell was placed into the heating block of the instrument. The sample was extracted under the following conditions: solvent of methanol, temperature of 100 °C, pressure of 10 MPa, static extraction time of 10 min and three extraction cycles. The extract was transferred to a 100-mL volumetric flask and brought up to volume with methanol, and then filtered through a 0.22-μm nylon membrane filter for UPLC–QTOF-MS analysis.

2.3. Instruments

UPLC was performed on a Waters Acquity UPLC system (Waters, Milford, MA, USA) equipped with a binary solvent delivery system, autosampler, column compartment and photodiode-array detection (PDA) system. The samples were separated on a Waters Acquity BEH C18 column (100 mm × 2.1 mm I.D., 1.7 μm) at a temperature of 40 °C, and using water-formic acid (100:0.5, v/v) (solvent A) and acetonitrile (solvent B) as mobile phases at a flow rate of 0.5 mL min⁻¹. A linear solvent gradient was used as follows: 0–1 min, 5% B; 1–4 min, 5–25% B; 4–12 min, 25–40% B; 12–13 min, 40–95% B; and 3 min post-run, 5% B. The injection volume was 2 μL. PDA was set to scan from 200 nm to 400 nm, and 254 nm was used as a detection wavelength.

The above UPLC system was coupled to a Waters Xevo G2 QTOF (Micromass MS Technologies, Manchester, UK) equipped with an electrospray ionization (ESI) source. The capillary voltage and cone voltage were set at 3500 V and 5 V, respectively. The nebulization gas was set to 800 L h⁻¹ at 350 °C. The cone gas was 50 L h⁻¹, and the source temperature was 100 °C. LockSpray™ was used to ensure the mass accuracy and reproducibility. The deprotonated Leucine-enkephalin at m/z 554.2615 was used as the lock mass in negative-ion detection mode, and the MS data were acquired from a mass-to-charge ratio (m/z) range of 100–1500. The MS/MS experiments were performed to the interested precursor ions by ramping collision energies from 25 V to 50 V. A dwell time of 0.2 s was employed with an inter-acquisition delay of 0.01 s. Quantification of UPLC–QTOF-MS was performed under full scan condition by using XICs (with a 0.01 Da window) of the deprotonated molecules.

3. Results and discussion

3.1. Optimization of ASE conditions

ASE is a novel developed extraction technique which uses organic solvents to extract solid/semisolid samples under elevated temperature (50–200 °C) and pressure (500–3000 psi) conditions for short time periods (5–10 min) [26]. The parameters affecting extraction efficiency of ASE include solvent, temperature, static extraction time, and number of extraction cycles [26,27]. These parameters were optimized by employing an univariate design, and using the relative extraction ratios of 5-O-CQA, 3,5-di-O-CQA, rutin, latifoloside H and kudinoside A as performance indicators. The extraction pressure was set at 10 MPa to keep the solvent in the liquid state during the extraction procedure. The optimization of the ASE procedure was performed using *I. latifolia* sample 1.

Different solvents including methanol, ethanol, 90%, 70%, 50% and 30% aqueous ethanol and methanol (v/v) were investigated, and it was found that methanol gives the best extraction efficiency for the five targeted compounds. To find an optimal extraction temperature, a series of experiments were performed at different temperatures (i.e., 60, 80, 100, 120 and 150 °C), the optimal extraction temperature was selected as 100 °C (Fig. S-1A). To evaluate
the influence of static extraction time with extraction efficiency, different times (i.e., 5, 10, 15 and 20 min) were investigated using one extraction cycle mode, the result showed that the optimal static extraction time was 10 min for one cycle (Fig. S-1B). The effect of the number of extraction cycles was determined by running five consecutive extractions on the same sample. The results showed that three extraction cycles were sufficient to extract the five targeted compounds completely (Fig. S-1C).

The extraction efficiencies of 5-O-CQA, 3,5-di-O-CQA, rutin, latifoloside H and kudinoside A by ASE were compared with those by reflux extraction and ultrasonic extraction. The extraction efficiencies of the five compounds by ASE were higher than those by reflux and ultrasonic extraction. Moreover, ASE gave the advantages of shorter extraction time and less extraction solvent than either reflux extraction or ultrasonic extraction.

### 3.2. Optimization of UPLC–QTOF-MS conditions

In order to achieve a good separation for multi-ingredients determination and fingerprint analysis, different elution programs using different elution solvent systems, including water–methanol, water–acetonitrile, water (containing 0.5% formic acid)–methanol and water (containing 0.5% formic acid)–acetonitrile, were investigated. The result showed that a linear gradient elution with water (containing 0.5% formic acid)–acetonitrile gave the best resolution, and most of constituents could be efficiently separated within 13 min (Fig. 2). For most of constituents, especially for the isomers of the triterpenoid saponins, acetonitrile provides better separation efficiency than methanol. Additionally, the addition of formic acid had a substantial effect of increasing the retention time and reducing peak tailing of the organic acids. Different columns
including Waters Acquity BEH C18 (100 mm × 2.1 mm I.D., 1.7 µm), Waters Acquity HSS T3 (50 mm × 2.1 mm I.D., 1.8 µm), Waters Acquity BEH Phenyl (100 mm × 2.1 mm I.D., 1.7 µm), Waters BEH HILIC (100 mm × 2.1 mm I.D., 1.7 µm), Shiseido Capcell pak C18 IF2 (100 mm × 2.1 mm I.D., 2.0 µm), and Agilent Extend C18 RRHD (100 mm × 2.1 mm I.D., 1.8 µm) and different temperatures (i.e., 20, 30, 40, and 50 °C) were also tested. It was found that Waters Acquity BEH C18 column (100 mm × 2.1 mm I.D., 1.7 µm) at a temperature of 40 °C is suitable to separate most of the constituents present in *I. latifolia*.

Different MS parameters (i.e., detection mode, capillary, cone voltage, nebulization gas flow rate, nebulization gas temperature, cone gas flow rate, and source temperature) were investigated. The results showed that negative-ion ESI-MS at the following conditions gave the best sensitivity and specificity: capillary voltage of 3500 V, cone voltage of 5 V, nebulization gas flow rate of 800 L h⁻¹ at the temperature of 350 °C, cone gas of 50 L h⁻¹, and source temperature of 100 °C. More information and better signal-to-noise (S/N) ratios of active ingredients were obtained in negative-ion ESI spectrum than in positive-ion ESI spectrum. By comparison of the chromatograms obtained by PDA at 254 nm (Fig. 2A) and negative-ion ESI-MS (Fig. 2B), most of triterpenoid saponins without conjugated double bond showed very weak signals in the UPLC–PDA chromatogram, while they expressed abundant signals in the negative-ion UPLC–ESI-MS chromatogram. Therefore, the negative-ion ESI-QTOF-MS was suitable for multi-ingredients determination and fingerprint analysis of *I. latifolia*. Collision-induced dissociation (CID) experiments were performed with the collision voltage in the range of 5–50 V, and the collision voltage ramping from 25 V to 50 V was selected.

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**Fig. 2.** Representative chromatograms of *Ilex latifolia* extract obtained by (A) DAD at 254 nm and (B) negative-ion ESI-QTOF-MS; (C) chromatograms of the 21 reference substances obtained by negative-ion ESI-QTOF-MS.
<table>
<thead>
<tr>
<th>Peak</th>
<th>( t_k ) (min)</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>( \lambda_{max} ) (nm)</th>
<th>Experimental weight of negative QTOF-MS (m/z)</th>
<th>MS/MS production (m/z)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.20</td>
<td>( C_{13}H_{23}NO_3 )</td>
<td>361.1373</td>
<td>213</td>
<td>360.1289 [M–H]⁻/–1.7</td>
<td>314.1247, 296.1146, 161.0418, 137.0112, 134.0595</td>
<td>Unidentified</td>
</tr>
<tr>
<td>2</td>
<td>2.30</td>
<td>( C_{12}H_{23}NO_3 )</td>
<td>359.1216</td>
<td>213, 257</td>
<td>358.1145 [M–H]⁻/–2.0</td>
<td>132.0447</td>
<td>Unidentified</td>
</tr>
<tr>
<td>3</td>
<td>2.52</td>
<td>( C_{14}H_{27}O_9 )</td>
<td>354.0951</td>
<td>326</td>
<td>353.0874 [M–H]⁻/–0.3</td>
<td>191.0567, 179.0353, 135.0451</td>
<td>5-O-CQA a)</td>
</tr>
<tr>
<td>4</td>
<td>3.03</td>
<td>( C_{13}H_{25}O_9 )</td>
<td>354.0951</td>
<td>326</td>
<td>353.0872 [M–H]⁻/–0.3</td>
<td>191.0563, 179.0355</td>
<td>3-O-CQA a)</td>
</tr>
<tr>
<td>5</td>
<td>3.16</td>
<td>( C_{14}H_{28}O_9 )</td>
<td>354.0951</td>
<td>326</td>
<td>353.0877 [M–H]⁻/–1.1</td>
<td>191.0562, 179.0356, 173.0458, 135.0452</td>
<td>4-O-CQA a)</td>
</tr>
<tr>
<td>6</td>
<td>3.30</td>
<td>( C_{4}H_{8}O_4 )</td>
<td>180.0423</td>
<td>225</td>
<td>179.0345 [M–H]⁻/–0.6</td>
<td>135.0454</td>
<td>Caffeic acid a)</td>
</tr>
<tr>
<td>7</td>
<td>4.10</td>
<td>( C_{22}H_{30}O_7 )</td>
<td>610.1534</td>
<td>255, 350</td>
<td>609.1450 [M–H]⁻/–1.0</td>
<td>301.0358</td>
<td>Rutin a)</td>
</tr>
<tr>
<td>8</td>
<td>4.27</td>
<td>( C_{22}H_{30}O_7 )</td>
<td>464.0955</td>
<td>258, 350</td>
<td>463.0883 [M–H]⁻/–1.3</td>
<td>301.0349</td>
<td>Isoquercitrin a)</td>
</tr>
<tr>
<td>9</td>
<td>4.61</td>
<td>( C_{22}H_{24}O_12 )</td>
<td>516.1268</td>
<td>326</td>
<td>515.1189 [M–H]⁻/–0.2</td>
<td>353.0873, 191.0558, 179.0352, 173.0456, 135.0452</td>
<td>4,3-Di-O-CQA a)</td>
</tr>
<tr>
<td>10</td>
<td>4.67</td>
<td>( C_{22}H_{22}O_12 )</td>
<td>516.1268</td>
<td>326</td>
<td>515.1186 [M–H]⁻/–0.8</td>
<td>353.0881, 191.0553, 179.0359</td>
<td>3,5-Di-O-CQA a)</td>
</tr>
<tr>
<td>11</td>
<td>4.80</td>
<td>( C_{22}H_{22}O_12 )</td>
<td>516.1268</td>
<td>326</td>
<td>515.1181 [M–H]⁻/–1.7</td>
<td>353.0883, 191.0560, 179.0357, 173.0452</td>
<td>Isomeric di-O-CQA</td>
</tr>
<tr>
<td>12</td>
<td>4.87</td>
<td>( C_{22}H_{22}O_12 )</td>
<td>516.1268</td>
<td>326</td>
<td>515.1188 [M–H]⁻/–0.4</td>
<td>353.0874, 203.0563, 173.0453</td>
<td>3,4-Di-O-CQA a)</td>
</tr>
<tr>
<td>13</td>
<td>5.46</td>
<td>( C_{22}H_{22}O_12 )</td>
<td>516.1268</td>
<td>326</td>
<td>515.1191 [M–H]⁻/0.2</td>
<td>353.0877, 173.0459, 191.0561, 179.0347</td>
<td>Isomeric di-O-CQA</td>
</tr>
<tr>
<td>14</td>
<td>6.43</td>
<td>( C_{20}H_{28}O_6 )</td>
<td>1218.6033</td>
<td>–</td>
<td>1263.5990 [M+HCOO]⁺/–1.6</td>
<td>1217.5951, 909.4859, 747.4331, 601.3740, 469.3329</td>
<td>Unidentified</td>
</tr>
<tr>
<td>15</td>
<td>6.78</td>
<td>( C_{20}H_{106}O_{31} )</td>
<td>1382.6718</td>
<td>–</td>
<td>1381.6600 [M–H]⁻/–2.9</td>
<td>1073.5542, 749.4484, 471.3471</td>
<td>Kudinoside N a)</td>
</tr>
<tr>
<td>16</td>
<td>6.88</td>
<td>( C_{20}H_{106}O_{31} )</td>
<td>1382.6718</td>
<td>–</td>
<td>1381.6631 [M–H]⁻/–0.7</td>
<td>1073.5531, 911.5016, 749.4489, 705.4589</td>
<td>Kudinoside O a)</td>
</tr>
<tr>
<td>17</td>
<td>7.01</td>
<td>( C_{20}H_{106}O_{26} )</td>
<td>1220.6190</td>
<td>–</td>
<td>1265.6152 [M+HCOO]⁺/–1.1</td>
<td>911.5013, 749.4485, 471.3487</td>
<td>Latifoloside G a)</td>
</tr>
<tr>
<td>18</td>
<td>7.11</td>
<td>( C_{20}H_{106}O_{26} )</td>
<td>1220.6190</td>
<td>–</td>
<td>1265.6161 [M+HCOO]⁺/–0.4</td>
<td>911.5006, 749.4484, 471.3485</td>
<td>Latifoloside H a)</td>
</tr>
<tr>
<td>19</td>
<td>7.67</td>
<td>( C_{20}H_{106}O_{22} )</td>
<td>1074.5611</td>
<td>–</td>
<td>1119.5586 [M+HCOO]⁺/–0.1</td>
<td>1073.5545, 911.5017, 749.4475, 603.3912, 471.3489</td>
<td>Kudinoside G a)</td>
</tr>
<tr>
<td>20</td>
<td>7.78</td>
<td>( C_{20}H_{106}O_{22} )</td>
<td>1074.5611</td>
<td>–</td>
<td>1119.5592 [M+HCOO]⁺/0.4</td>
<td>1073.5547, 911.5008, 749.4478, 603.3907, 471.3479</td>
<td>Isomeric latifoloside L</td>
</tr>
<tr>
<td>21</td>
<td>7.93</td>
<td>( C_{20}H_{106}O_{23} )</td>
<td>1088.5403</td>
<td>–</td>
<td>1133.5371 [M+HCOO]⁺/–0.8</td>
<td>925.4807, 701.4278, 555.3685</td>
<td>Kudinoside C a)</td>
</tr>
<tr>
<td>22</td>
<td>8.37</td>
<td>( C_{20}H_{106}O_{15} )</td>
<td>926.4875</td>
<td>–</td>
<td>971.4848 [M+HCOO]⁺/–0.4</td>
<td>925.4812, 701.4263, 555.3694, 493.3681</td>
<td>Kudinoside A a)</td>
</tr>
<tr>
<td>23</td>
<td>9.76</td>
<td>( C_{20}H_{106}O_{22} )</td>
<td>1070.5298</td>
<td>230</td>
<td>1115.5283 [M+HCOO]⁺/0.8</td>
<td>907.4703, 745.4176, 555.3697</td>
<td>Kudinoside E a)</td>
</tr>
</tbody>
</table>
The peak characteristic was obtained by CID fragmentation of each constituent. CID experiments were then performed to the pseudo-molecular ion by adjusting the collision energy to attain abundant fragmentation information, and the accurate mass of each fragment ion was obtained. Thus, structure elucidation of each constituent could be performed based on the characteristic fragmentation patterns. Table 1 listed the retention times ($t_R$), molecular formulas, theoretical molecular weights, $\lambda_{\text{max}}$, pseudo-molecular ions, and characteristic fragment ions of the 28 major peaks in the chromatograms. By comparison of the $t_R$, UV spectra, and exact MS data with those of their references, 21 constituents were identified and confirmed. Among them, there were seven organic acids, i.e., 5-O-CQA (3), 3-O-CQA (4), 4-O-CQA (5), caffeic acid (6), 4,5-di-O-CQA (9), 3,5-di-O-CQA (10) and 3,4-di-O-CQA (12), two flavonoids, i.e., rutin (7) and isoquercitrin (8), and twelve triterpenoid saponins, i.e., kudinoside N (15), kudinoside O (16), latifoloside G (17), latifoloside H (18), kudinoside G (19), kudinoside C (21), kudinoside A (22), kudinoside E (23), kudinoside F (24), latifoloside Q (25), kudinoside D (26), and ilekudinoside G (28). Other four peaks in the chromatogram, i.e., peaks 11, 13, 20 and 27, were tentatively characterized as isomeric di-O-CQA, isomeric di-O-CQA, latifoloside L and ilekudinoside B, respectively, by comparing their exact masses, MS/MS spectra, UV absorptions and retention behaviors with those of reported compounds [17, 28]. While the peaks of 1, 2 and 14 could not be identified due to the absence of references and literatures.

### 3.3. Identification of the active ingredients present in *I. latifolia* by UPLC–QTOF-MS

Identification of the active ingredients present in *I. latifolia* was performed based on the mass spectral information obtained by QTOF-MS and QTOF-MS/MS. According to the accurate mass measurements (error < 5 ppm), the molecular formula (elemental composition) of each constituent was easily deduced. CID experiments were then performed to the pseudo-molecular ion by adjusting the collision energy to attain abundant fragmentation information, and the accurate mass of each fragment ion was obtained. Thus, structure elucidation of each constituent could be performed based on the characteristic fragmentation patterns. Table 1 listed the retention times ($t_R$), molecular formulas, theoretical molecular weights, $\lambda_{\text{max}}$, pseudo-molecular ions, and characteristic fragment ions of the 28 major peaks in the chromatograms. By comparison of the $t_R$, UV spectra, and exact MS data with those of their references, 21 constituents were identified and confirmed. Among them, there were seven organic acids, i.e., 5-O-CQA (3), 3-O-CQA (4), 4-O-CQA (5), caffeic acid (6), 4,5-di-O-CQA (9), 3,5-di-O-CQA (10) and 3,4-di-O-CQA (12), two flavonoids, i.e., rutin (7) and isoquercitrin (8), and twelve triterpenoid saponins, i.e., kudinoside N (15), kudinoside O (16), latifoloside G (17), latifoloside H (18), kudinoside G (19), kudinoside C (21), kudinoside A (22), kudinoside E (23), kudinoside F (24), latifoloside Q (25), kudinoside D (26), and ilekudinoside G (28). Other four peaks in the chromatogram, i.e., peaks 11, 13, 20 and 27, were tentatively characterized as isomeric di-O-CQA, isomeric di-O-CQA, latifoloside L and ilekudinoside B, respectively, by comparing their exact masses, MS/MS spectra, UV absorptions and retention behaviors with those of reported compounds [17, 28]. While the peaks of 1, 2 and 14 could not be identified due to the absence of references and literatures.

### 3.4. Establishment and validation of quantitative method

Multi-ingredients determination was performed based on the peak areas of extracted ion chromatograms of the exact pseudo-molecular ions (with a 0.01 Da window), and the method was validated according to the guidelines of the International Conference on Harmonisation.

#### 3.4.1. Specificity

As shown in Fig. 2B, all of the 28 active ingredients present in *I. latifolia* were well separated in the optimized conditions, and there was no interference for their quantitative determination. Thus, the method had good specificity.

#### 3.4.2. Linearity and ranges

The prepared mixture stock solution containing the 21 references was diluted to form a series of appropriate concentrations for construction of calibration curves. High-correlation coefficient values ($r^2 > 0.9962$) obtained indicated that there were good linear correlations between the concentrations of the investigated compounds and their peak areas within test concentrations (Table 2).

#### 3.4.3. Limits of detection (LOD) and quantification (LOQ)

The LODs and LOQs of each analyte were defined by the concentrations that generated peaks with signal-to-noise values (S/Ns) of 3 and 10, respectively. As shown in Table 2, the LODs and LOQs of the 21 target compounds ranged from 2.4 to 21.6 μg L$^{-1}$ and from 8.1 to 72.0 μg L$^{-1}$, respectively, indicated that the analytical method was acceptable with excellent sensitivity.

#### 3.4.4. Precision, repeatability and stability

Intra- and inter-day variations were evaluated to determine the precision. For the intra-day variability, the mixed working solution with a concentration of 500 μg L$^{-1}$ for each compound was analyzed for six times in a day, whereas the inter-day variability was examined in duplicate using this sample on three consecutive days. The developed method was found to be precise, with the intra- and inter-day precisions for each compound less than 3.8% and 4.9%, respectively.

To evaluate the repeatability, six different solutions made from the same sample (sample 1) were analyzed, and the RSDs were less than 5.7%. For measurements of stability, the sample solution (sample 1) was stored at 25 °C, and repeatedly analyzed in 0, 2, 4, 8, 12, 24 h. The results (RSDs < 4.8%) indicated the sample was stable in 24 h.

#### 3.4.5. Recovery

The recovery was used to evaluate the accuracy of the method, and each analyte was determined using the standard addition method. Certain amounts of individual standards were added into sample 1 for six parallels, and the spiked samples were extracted by ASE and analyzed by UPLC–QTOF-MS. The developed method showed good accuracy, with mean recoveries ranging from 94.7% to 104.9% (Table 2).

The method validation results indicated that the developed method was efficient, accurate and sensitive for multi-ingredients determination of *I. latifolia*.
Table 2
Linear-regression data, LODs, LOQs, precision, repeatability, stability and recovery of the 21 major constituents as determined by UPLC–QTOF-MS.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Regression equation</th>
<th>( r^2 )</th>
<th>Linear range (µg L(^{-1}))</th>
<th>LOD (µg L(^{-1}))</th>
<th>LOQ (µg L(^{-1}))</th>
<th>Precision RSD(^a) (%)</th>
<th>Repeatability (RSD, %, n = 6)</th>
<th>Stability (RSD, %, n = 6)</th>
<th>Recovery(^b) (%)</th>
<th>Mean ± SD (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-O-CQA</td>
<td>( y = 252.0x + 22.08 )</td>
<td>0.9962</td>
<td>100–10,000</td>
<td>13.2</td>
<td>44.1</td>
<td>3.7</td>
<td>4.6</td>
<td>4.4</td>
<td>3.0</td>
<td>96.5 ± 4.2</td>
</tr>
<tr>
<td>3-O-CQA</td>
<td>( y = 229.0x + 23.04 )</td>
<td>0.9986</td>
<td>100–20,000</td>
<td>11.0</td>
<td>36.6</td>
<td>2.9</td>
<td>4.2</td>
<td>3.9</td>
<td>2.8</td>
<td>97.8 ± 2.5</td>
</tr>
<tr>
<td>4-O-CQA</td>
<td>( y = 285.1x – 12.62 )</td>
<td>0.9992</td>
<td>100–10,000</td>
<td>16.8</td>
<td>55.9</td>
<td>2.7</td>
<td>3.6</td>
<td>3.7</td>
<td>3.3</td>
<td>103.1 ± 3.1</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>( y = 320.2x – 14.78 )</td>
<td>0.9995</td>
<td>100–10,000</td>
<td>10.0</td>
<td>33.2</td>
<td>3.0</td>
<td>4.1</td>
<td>4.2</td>
<td>3.5</td>
<td>95.3 ± 2.7</td>
</tr>
<tr>
<td>Rutin</td>
<td>( y = 245.1x + 10.12 )</td>
<td>0.9985</td>
<td>100–10,000</td>
<td>11.7</td>
<td>39.1</td>
<td>3.1</td>
<td>3.9</td>
<td>4.5</td>
<td>3.1</td>
<td>99.6 ± 3.0</td>
</tr>
<tr>
<td>Isoquercitrin</td>
<td>( y = 234.1x + 9.914 )</td>
<td>0.9987</td>
<td>50–10,000</td>
<td>4.0</td>
<td>13.3</td>
<td>2.6</td>
<td>4.2</td>
<td>3.9</td>
<td>2.7</td>
<td>98.8 ± 2.3</td>
</tr>
<tr>
<td>4,5-Di-O-CQA</td>
<td>( y = 318.8x – 22.90 )</td>
<td>0.9987</td>
<td>100–10,000</td>
<td>21.6</td>
<td>72.0</td>
<td>2.9</td>
<td>3.8</td>
<td>4.6</td>
<td>3.0</td>
<td>101.7 ± 3.3</td>
</tr>
<tr>
<td>3,5-Di-O-CQA</td>
<td>( y = 296.8x – 61.98 )</td>
<td>0.9987</td>
<td>100–50,000</td>
<td>13.1</td>
<td>43.7</td>
<td>2.3</td>
<td>4.0</td>
<td>4.5</td>
<td>3.2</td>
<td>104.1 ± 3.7</td>
</tr>
<tr>
<td>3,4-Di-O-CQA</td>
<td>( y = 439.7x + 72.49 )</td>
<td>0.9987</td>
<td>100–50,000</td>
<td>17.7</td>
<td>59.1</td>
<td>2.0</td>
<td>3.7</td>
<td>4.2</td>
<td>2.6</td>
<td>96.8 ± 2.9</td>
</tr>
<tr>
<td>Kudinoside N</td>
<td>( y = 408.8x + 26.80 )</td>
<td>0.9985</td>
<td>50–10,000</td>
<td>4.7</td>
<td>15.8</td>
<td>2.6</td>
<td>3.4</td>
<td>5.6</td>
<td>3.8</td>
<td>102.1 ± 4.7</td>
</tr>
<tr>
<td>Kudinoside O</td>
<td>( y = 396.9x + 21.15 )</td>
<td>0.9986</td>
<td>50–10,000</td>
<td>5.3</td>
<td>17.6</td>
<td>3.4</td>
<td>4.8</td>
<td>4.7</td>
<td>4.2</td>
<td>99.4 ± 2.2</td>
</tr>
<tr>
<td>Latifoloside G</td>
<td>( y = 1234x + 51.26 )</td>
<td>0.9991</td>
<td>50–20,000</td>
<td>2.4</td>
<td>8.1</td>
<td>3.5</td>
<td>4.1</td>
<td>4.6</td>
<td>2.5</td>
<td>101.3 ± 3.3</td>
</tr>
<tr>
<td>Latifoloside H</td>
<td>( y = 1164x + 164.3 )</td>
<td>0.9965</td>
<td>50–50,000</td>
<td>2.7</td>
<td>9.1</td>
<td>2.3</td>
<td>4.5</td>
<td>4.1</td>
<td>3.4</td>
<td>97.9 ± 2.1</td>
</tr>
<tr>
<td>Kudinoside G</td>
<td>( y = 237.3x + 12.17 )</td>
<td>0.9985</td>
<td>100–50,000</td>
<td>9.4</td>
<td>31.2</td>
<td>2.6</td>
<td>4.2</td>
<td>4.6</td>
<td>3.7</td>
<td>104.6 ± 4.5</td>
</tr>
<tr>
<td>Kudinoside C</td>
<td>( y = 250.5x + 4.920 )</td>
<td>0.9998</td>
<td>100–20,000</td>
<td>7.9</td>
<td>26.4</td>
<td>2.1</td>
<td>3.3</td>
<td>3.9</td>
<td>4.0</td>
<td>95.4 ± 4.3</td>
</tr>
<tr>
<td>Kudinoside A</td>
<td>( y = 1086x + 44.89 )</td>
<td>0.9989</td>
<td>50–50,000</td>
<td>4.5</td>
<td>14.9</td>
<td>3.7</td>
<td>4.9</td>
<td>5.3</td>
<td>2.9</td>
<td>94.7 ± 3.4</td>
</tr>
<tr>
<td>Kudinoside E</td>
<td>( y = 308.9x + 0.5562 )</td>
<td>0.9999</td>
<td>50–10,000</td>
<td>9.9</td>
<td>33.0</td>
<td>2.9</td>
<td>3.8</td>
<td>4.7</td>
<td>3.5</td>
<td>103.5 ± 3.8</td>
</tr>
<tr>
<td>Kudinoside F</td>
<td>( y = 503.7x + 22.24 )</td>
<td>0.9990</td>
<td>50–10,000</td>
<td>3.7</td>
<td>12.2</td>
<td>3.6</td>
<td>4.2</td>
<td>4.0</td>
<td>2.9</td>
<td>104.9 ± 4.6</td>
</tr>
<tr>
<td>Latifoloside Q</td>
<td>( y = 887.6x + 32.24 )</td>
<td>0.9991</td>
<td>50–10,000</td>
<td>3.2</td>
<td>10.6</td>
<td>2.5</td>
<td>3.8</td>
<td>4.6</td>
<td>3.2</td>
<td>96.8 ± 3.9</td>
</tr>
<tr>
<td>Kudinoside D</td>
<td>( y = 552.0x + 9.793 )</td>
<td>0.9998</td>
<td>50–20,000</td>
<td>4.9</td>
<td>16.4</td>
<td>2.9</td>
<td>4.0</td>
<td>4.2</td>
<td>3.1</td>
<td>97.8 ± 3.4</td>
</tr>
<tr>
<td>Ilekudinoside G</td>
<td>( y = 284.7x – 0.2857 )</td>
<td>0.9999</td>
<td>100–10,000</td>
<td>9.0</td>
<td>30.1</td>
<td>3.8</td>
<td>3.2</td>
<td>5.7</td>
<td>4.8</td>
<td>102.5 ± 2.9</td>
</tr>
</tbody>
</table>

\(^a\) RSD: relative standard deviation.

\(^b\) The data are presented as the average of six determinations, where standard addition recovery [%] = 100 × (amount found – original amount)/amount spiked.
3.5. Multi-ingredients determination of *L. latifolia* samples

The developed quantitative method was subsequently applied to simultaneous determination of the 21 active ingredients present in 22 *L. latifolia* samples obtained from different location in China. The contents were calculated by the external standard method from three parallel determinations of each sample, and the results are summarized in Table 3. The contents of 3-O-CQA, 3,4-di-O-CQA, 3,4-di-O-CQA, latifoloside G, latifoloside H and kudinoside A were highest in most of *L. latifolia* samples.

3.6. Establishment of UPLC–QTOF-MS fingerprinting method

Fingerprint analysis was performed based on the base peak chromatograms (BPCs) obtained by negative-ion ESI-QTOF-MS. Usually, BPCs were more suitable for fingerprint analysis than total ion chromatograms (TICs) in a LC–MS analysis because the former provides better chromatographic peak shape and higher signal-to-noise ratio of constituents than the latter. The BPC of each sample was exported as its fingerprint with the format of .csv files. Then, the .csv files were transformed into .txt files, and imported into the professional software recommended by State Food and Drug Administration (SFDA), i.e., Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine Version 2004A, for fingerprint analysis. The process includes selection of common peaks in chromatograms, normalization of retention times of all common peaks, and generation of a standardized characteristic fingerprint. The total area of the common peaks must be more than 90% of the whole area in one chromatogram, and there were 28 common peaks shown in all samples. Peaks 4, 10, 17, 22 and 26, with different retention time points covered the whole chromatogram, were selected to normalize the retention times of all common peaks. Using the proposed method, the normalized chromatograms of 22 *L. latifolia* samples were acquired (Fig. S-2).

In order to more accurately describe a full chemical profiling of *L. latifolia*, a standardized characteristic fingerprint was calculated and generated by mean of algorithm using the normalized chromatograms of 22 *L. latifolia* samples. There were 28 common peaks, i.e., chemical markers of *L. latifolia*, in the standardized characteristic fingerprint.

3.7. Similarity analysis (SA)

SFDA suggests that fingerprint analysis should be evaluated in terms of similarity by calculation of the correlation coefficient and/or angle cosine value between each sample’s fingerprint and the standardized characteristic fingerprint. The similarities are in the range of 0–1. The higher the similarity values of a sample, the more the similarity between its fingerprint and the standardized characteristic fingerprint, and the more the desirable quality consistency and stability of the sample. The similarity analysis results were shown in Table 4, samples from Lingnan area (including Guangdong, Guangxi and Hainan provinces, i.e., samples 1–20) possessed the similarities of higher than 0.900, which suggested their quality was in good consistency and stability. While the similarities of the other 2 samples from Zhejiang province (samples 21–22) were 0.502 and 0.466, respectively, showing great variances comparing with Lingnan area samples. Variation of the similarity might be due to a number of factors, such as different origins, production processes, storage conditions, and collection time, etc. To obtain a more comprehensive evaluation of *L. latifolia*, fingerprint analysis should be combined with multi-ingredients determination.
3.8. Principal component analysis (PCA)

PCA is a sophisticated technique widely used for summarizing multivariate variation into a few principle components remaining maximum possible variability. To evaluate the quality variation and differentiate the sources of *I. latifolia* samples, PCA was performed based on the chromatographic fingerprints. In this study, peak areas of the 28 active ingredients were used as variables to observe the variation of samples from different origins.

PCA score plot (Fig. 3A) was utilized to depict the general concentration variation of the characteristic ingredients of *I. latifolia* samples. Seven of the preferential distribution ingredients, which had the highest scores from the two PCs’ absolute values, were latifoloside H (score of 10.40), 3,5-di-O-CQA (score of 9.04), kudinoside G (score of 8.44), kudinoside A (score of 8.42), 3,4-di-O-CQA

![Fig. 3. PCA score plot (A) and loading plot (B) of investigated *Ilex latifolia* samples.](image)

<table>
<thead>
<tr>
<th>Samples no.</th>
<th>Similarity</th>
<th>Samples no.</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.995</td>
<td>12</td>
<td>0.989</td>
</tr>
<tr>
<td>2</td>
<td>0.999</td>
<td>13</td>
<td>0.985</td>
</tr>
<tr>
<td>3</td>
<td>0.996</td>
<td>14</td>
<td>0.990</td>
</tr>
<tr>
<td>4</td>
<td>0.995</td>
<td>15</td>
<td>0.993</td>
</tr>
<tr>
<td>5</td>
<td>0.994</td>
<td>16</td>
<td>0.988</td>
</tr>
<tr>
<td>6</td>
<td>0.995</td>
<td>17</td>
<td>0.991</td>
</tr>
<tr>
<td>7</td>
<td>0.939</td>
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<td>0.979</td>
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<tr>
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<td>0.995</td>
<td>19</td>
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</tr>
<tr>
<td>9</td>
<td>0.995</td>
<td>20</td>
<td>0.993</td>
</tr>
<tr>
<td>10</td>
<td>0.966</td>
<td>21</td>
<td>0.902</td>
</tr>
<tr>
<td>11</td>
<td>0.988</td>
<td>22</td>
<td>0.466</td>
</tr>
</tbody>
</table>

*Table 4: Similarity of the tested samples.*

**Fig. 3.** PCA score plot (A) and loading plot (B) of investigated *Ilex latifolia* samples.
(score of 4.23), 3-O-CQA (score of 3.38) and latifolioside G (score of 3.21). These compounds were considered as the chemical markers of sample variation in the dataset. Kudinoside G exhibited more obviously positive correlation to samples 21 and 22, while other six ingredients expressed greater correlations to samples 1–20.

The origins differentiation of *I. latifolia* samples was shown in the corresponding PCA loading plot (Fig. 3B). Two categories of *I. latifolia* samples from Lingnan area (samples 1–20) and Zhejiang province (samples 21 and 22) were readily divided according to their different locations in accord with similarity analysis. The quality stability was also expressed in the PCA loading plot. As shown in Fig. 3B, samples 7 and 19 were relatively discrete in PCA loading plot, which indicated the samples were less stable when comparing with other Lingnan area samples. The PCA results suggested that the fingerprint analysis is a useful tool to quality assessment and control of *I. latifolia*.

4. Conclusions

In this study, a strategy integrating multi-ingredients determination and fingerprint analysis for quality assessment and control of the leaves from *I. latifolia* based on UPLC–QTOF-MS was proposed. As demonstrated, the method was simple and fast, with a full separation of the 28 major constituents within 13 min. The BPCs were applied to construct fingerprints, which provided valuable information for overall quality assessment and origins tracing of the herbal medicine. Moreover, the common peaks, i.e., the major active ingredients of the herbal medicine, were quickly identified or tentatively characterized according to the accurate mass measurements and fragmentation patterns obtained by QTOF-MS, and the contents of them were quantitatively determined. All the experimental results indicated that the method combining UPLC–QTOF-MS fingerprint and simultaneous determination of multi-ingredients was sensitive, efficient and reliable for quality assessment and control of the leaves from *I. latifolia*.

Acknowledgments

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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.jpba.2013.05.039.

References