Simultaneous Determination of 10 Flavonoids in Crude and Wine-Processed *Radix scutellariae* by UHPLC

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Abstract

*Radix scutellariae* is a well-known traditional Chinese medicine used for the treatments of inflammation, pyrexia, hepatitis, etc. Flavonoids are its main active compounds. The aim of this study is to develop and validate the ultra high-performance liquid chromatography (UHPLC) method for simultaneous determination of 10 flavonoids (baicalin, wogonoside, baicalein, wogonin, oroxylin A, chrysin, scutellarin, oroxylin A-7-O-glucuronide, apigenin and apigenin-7-glucuronide) in crude and wine-processed *R. scutellariae*. The quantitative determination was conducted by UHPLC. Optimal separation was achieved by gradient elution with mobile phase consisting of 0.01% aqueous formic acid and methanol on a Waters ACQUITY UHPLC BEH C₁₈ column. Detection wavelength was set at 275 nm. Method validation was accomplished with linearity, precision and recovery tests. All calibration curves showed good linearity (R² > 0.9993). The limit of detection and limit of quantification of these compounds were from 0.08 to 0.24 μg/mL and from 0.23 to 0.76 μg/mL, respectively. The average recoveries of these compounds were from 96.95 to 109.51% with relative standard deviation (RSD) values from 2.14 to 3.26% for crude *R. scutellariae*, while from 94.73 to 108.38% with RSD values from 1.83 to 3.47% for wine-processed *R. scutellariae*. The developed method can be applied to the intrinsic quality control of crude and wine-processed *R. scutellariae*.

Introduction

*Radix scutellariae*, the dried roots of *Scutellaria baicalensis Georgi*, is a well-known traditional Chinese medicine (TCM) used for the treatments of inflammation, pyrexia, jaundice, diarrhea, hepatitis, etc. (1–3). Both crude *R. scutellariae* and its wine-processed product were frequently applied in clinics. Flavonoids are considered to be the main active compounds of *R. scutellariae*. Baicalin, wogonoside, baicalein, wogonin, oroxylin A, chrysin, scutellarin, oroxylin A-7-O-glucuronide, apigenin and apigenin-7-glucuronide (structures in Figure 1), as the major flavonoid glycosides and aglycons with high contents (4, 5), were investigated intensively and found to exert anti-inflammatory, antioxidative anti-hepatitis B virus, antitumor, antiallergic and anxiolytic properties (6–10).

Up to now, several analytical methods, including high-performance liquid chromatography-ultraviolet spectrophotometry detector (HPLC-UV) (11, 12), high-performance liquid chromatography-electrochemical detector (HPLC-ECD) (13), ultraviolet spectrophotometry (UV) (14, 15), high performance capillary electrophoresis (HPCE) (16, 17), infrared spectrophotometry (IR) (18, 19) and liquid chromatography–mass spectrometry (LC–MS) (20, 21), have been reported for the determination of flavonoids in *R. scutellariae*. Simultaneous determination of three to six components in *R. scutellariae* by
HPLC has been reported (22–25), but determination of more than six components at one time by ultra high-performance liquid chromatography (UHPLC) has never been investigated. The quality of *R. scutellariae* can be comprehensively evaluated with more active constituents since other flavonoids including chrysin, scutellarin, scutellarein, apigenin and apigenin-7-glucuronide also possess anti-inflammatory activities (26–29). Besides, these constituents with good pharmacological effects on hepatic injury, cardio cerebral vascular disease, acute lung injury, cancer, etc. are related to the efficacy of *R. scutellariae*; therefore, it is necessary to develop a method for the determination of these constituents from *R. scutellariae* (30–32). Moreover, comparing with HPLC, UHPLC could both improve the resolution of peaks and save analytic time (33). Therefore, a rapid, simple and reliable UHPLC method for simultaneous determination of 10 flavonoids in *R. scutellariae* might be developed for controlling the intrinsic quality of crude and wine-processed *R. scutellariae*.

**Experimental**

**Materials and reagents**

Reference substances (baicalin, wogonoside, baicalein, wogonin, oroxylin A and scutellarin; purity >99%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Reference substances (oroxynin A-7-O-glucuronide, chrysin, apigenin and apigenin-7-glucuronide; purity >98%) were purchased from Shanghai Yuanye Biotech Co. Ltd (Shanghai, China).

The samples of crude *R. scutellariae* were collected from three different suppliers (Shandong, Anhui and Zhejiang in China) and authenticated by Professor Xunhong Liu (Nanjing University of Chinese Medicine, Nanjing, PR China). The samples of wine-processed *R. scutellariae* were prepared using their corresponding samples of crude *R. scutellariae* according to Pharmacopoeia of the People’s Republic of China (2010) (1): the crude *R. scutellariae* slices were mixed with the rice wine (100 : 12.5, *R. scutellariae*/wine, w/w) and allowed...
to completely absorb the rice wine. Then the moistened slices were stir-fried in a metallic pan over a low flame at 120–150°C until they were completely dried. These voucher specimens are deposited in Jiangsu Key Laboratory of Chinese Medicine Processing (Nanjing University of Chinese Medicine).

HPLC-grade methanol was purchased from Merck (Darmstadt, Germany), and HPLC-grade formic acid was purchased from Tedia Co. Ltd (Fairfield, OH, USA). The purified water used in the study was prepared using the Milli-Q system (Millipore, Bedford, MA, USA). Other chemicals used were all of analytical grade.

Figure 2. Typical chromatograms of mixed standard solution (A), crude *Radix scutellariae* sample solution (B) and wine-processed *R. scutellariae* sample solution (C). Suppliers: Shandong (a), Anhui (b) and Zhejiang (c). Reference compounds: scutellarin (1), apigenin-7-glucuronide (2), baicalin (3), oroxylin A-7-O-glucuronide (4), wogonoside (5), apigenin (6), baicalein (7), wogonin (8), chrysin (9) and oroxylin A (10). This figure is available in black and white in print and in color at *JCS* online.

Table I. Linearity, Correlation Coefficient ($R^2$), LOD and LOQ of Compounds Determined

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linear range (µg/mL)</th>
<th>Regression equation*</th>
<th>$R^2$</th>
<th>LOD (µg/mL)</th>
<th>LOQ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scutellarin</td>
<td>14.6–234</td>
<td>$y = 5591.2x - 10,865$</td>
<td>0.9998</td>
<td>0.21</td>
<td>0.68</td>
</tr>
<tr>
<td>Apigenin-7-glucuronide</td>
<td>12.8–206</td>
<td>$y = 4984.5x + 3398.6$</td>
<td>0.9995</td>
<td>0.24</td>
<td>0.76</td>
</tr>
<tr>
<td>Baicalin</td>
<td>837.5–13,400</td>
<td>$y = 8504.3x - 318,603$</td>
<td>0.9998</td>
<td>0.16</td>
<td>0.48</td>
</tr>
<tr>
<td>Oxyrulin A-7-O-glucuronide</td>
<td>65.6–1,050</td>
<td>$y = 7772.6x - 7531.2$</td>
<td>0.9999</td>
<td>0.17</td>
<td>0.53</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>131.3–2,100</td>
<td>$y = 9627.9x - 8584.9$</td>
<td>0.9998</td>
<td>0.14</td>
<td>0.41</td>
</tr>
<tr>
<td>Apigenin</td>
<td>7.1–114</td>
<td>$y = 7055.6x + 2598.6$</td>
<td>0.9996</td>
<td>0.18</td>
<td>0.55</td>
</tr>
<tr>
<td>Baicalein</td>
<td>136.3–2,180</td>
<td>$y = 13145.6x + 25,125$</td>
<td>0.9993</td>
<td>0.09</td>
<td>0.25</td>
</tr>
<tr>
<td>Wogonin</td>
<td>73.1–1,170</td>
<td>$y = 13,335x + 66,676$</td>
<td>0.9995</td>
<td>0.08</td>
<td>0.23</td>
</tr>
<tr>
<td>Chrysin</td>
<td>7.6–122</td>
<td>$y = 13,253x + 4318.7$</td>
<td>0.9996</td>
<td>0.08</td>
<td>0.23</td>
</tr>
<tr>
<td>Oxyrulin A</td>
<td>13.1–209</td>
<td>$y = 13054.9x - 1,990$</td>
<td>0.9998</td>
<td>0.09</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* $y$: peak area, $x$: concentration (µg/mL).
Chromatographic system
Analysis was conducted on a Waters Acquity UHPLC system (Waters, Milford, MA, USA). Separation was performed on a Waters ACQUITY UHPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm, Waters, Ireland) maintained at 35°C. The mobile phase consisted of aqueous formic acid (0.01%, v/v) (A) and methanol (B) using a linear gradient elution program as follows: 15–35% B at 0–3 min, 35% B at 3–12 min, 35–50% B at 12–14 min, 50% B at 14–28 min, 50–15% B at 28–28.5 min and 15% B at 28.5–30 min. The flow rate was 0.2 mL/min, and the injection volume was 5 µL. Detection wavelength was set at 275 nm.

Preparation of mixed standard and sample solutions
A mixed standard stock solution of baicalin (1,340 µg/mL), wogonoside (210 µg/mL), baicalein (218 µg/mL), wogonin (117 µg/mL), oroxylin A (20.9 µg/mL), oroxylin A-7-O-glucuronide (105 µg/mL), chrysin (12.2 µg/mL), scutellarin (23.4 µg/mL), apigenin (11.4 µg/mL) and apigenin-7-glucuronide (20.6 µg/mL) was prepared in methanol and stored at 4°C. The mixed calibration standard working solutions were freshly prepared by appropriate dilution of the mixed standard stock solution.

For the preparation of sample solution, 0.1 g powder of crude or wine-processed R. scutellariae was accurately weighted and transferred into a dark brown calibrated flask, then extracted with 5 mL of 70% methanol in an ultrasonic bath for 30 min and subsequently cooled to room temperature; 70% methanol was added to compensate the lost weight. The solution was filtered through a 0.22-µm membrane before injection.

Quantitative analysis
The prepared sample solutions were analyzed using the validated method. The target compounds were identified based on comparison of retention time, and quantitative analyses were performed by means of external standard methods.

Results
Optimization of HPLC chromatographic conditions
In order to develop a better method to determine 10 compounds in a single run in crude and wine-processed R. scutellariae, chromatographic conditions were optimized. Different mobile phase compositions were tested: (i) water–methanol, (ii) water–acetonitrile, (iii) aqueous formic acid (0.1%, v/v)–methanol and (iv) aqueous formic acid (0.01%, v/v)–methanol. As a result, the mobile phase consisted of aqueous formic acid (0.01%, v/v) and methanol using a linear gradient elution was the best for separation. Column temperatures were also tested from 25 to 40°C and finally set at 35°C. The UHPLC chromatograms of the 10 compounds under the optimized conditions were shown in Figure 2. The peaks of compounds were identified by comparing retention time and spectra with those of reference substances.

Table II. Contents of Ten Flavonoids in Crude and Wine-Processed Radix scutellariae from Different Suppliers (mean ± SD, n=3)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Supplier</th>
<th>Scutellarin</th>
<th>Apigenin</th>
<th>Apigenin-7-O-glucuronide</th>
<th>Oroxylin</th>
<th>Chrysin</th>
<th>Oroxylin A-7-O-glucuronide</th>
<th>Wogonoside</th>
<th>Wogonin</th>
<th>Wogonin</th>
<th>Chlorogenic Acid</th>
<th>Baicalein</th>
<th>Wogonol</th>
<th>Baicalin</th>
<th>Wogonol</th>
<th>Wogonol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Radix scutellariae</td>
<td>Shandong</td>
<td>0.162 ± 0.0018</td>
<td>0.211 ± 0.0021</td>
<td>11.32 ± 0.21</td>
<td>1.13 ± 0.07</td>
<td>11.1 ± 0.07</td>
<td>1.1 ± 0.07</td>
<td>1.02 ± 0.07</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
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<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
</tr>
<tr>
<td></td>
<td>Anhui</td>
<td>0.157 ± 0.0019</td>
<td>0.194 ± 0.0023</td>
<td>10.73 ± 0.19</td>
<td>1.11 ± 0.07</td>
<td>11.3 ± 0.07</td>
<td>1.1 ± 0.07</td>
<td>1.02 ± 0.07</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
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<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
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<tr>
<td></td>
<td>Zhejiang</td>
<td>0.153 ± 0.0010</td>
<td>0.183 ± 0.0021</td>
<td>10.57 ± 0.18</td>
<td>1.10 ± 0.07</td>
<td>11.2 ± 0.07</td>
<td>1.1 ± 0.07</td>
<td>1.02 ± 0.07</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
</tr>
<tr>
<td>Wine-processed Radix scutellariae</td>
<td>Shandong</td>
<td>0.171 ± 0.0014</td>
<td>0.218 ± 0.0018</td>
<td>15.28 ± 0.25</td>
<td>1.21 ± 0.07</td>
<td>2.58 ± 0.07</td>
<td>0.041 ± 0.0010</td>
<td>1.43 ± 0.007</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
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<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
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<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
</tr>
<tr>
<td></td>
<td>Anhui</td>
<td>0.162 ± 0.0017</td>
<td>0.189 ± 0.0017</td>
<td>13.21 ± 0.24</td>
<td>1.15 ± 0.07</td>
<td>2.56 ± 0.07</td>
<td>0.049 ± 0.0010</td>
<td>1.34 ± 0.007</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
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<td>0.049 ± 0.0010</td>
<td>0.049 ± 0.0010</td>
</tr>
<tr>
<td></td>
<td>Zhejiang</td>
<td>0.154 ± 0.0016</td>
<td>0.186 ± 0.0019</td>
<td>13.17 ± 0.22</td>
<td>1.18 ± 0.07</td>
<td>2.49 ± 0.07</td>
<td>0.038 ± 0.0010</td>
<td>1.22 ± 0.007</td>
<td>0.038 ± 0.0010</td>
<td>0.038 ± 0.0010</td>
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</table>

Calibration curves, limit of detection and limit of quantification
Method validation was executed by linearity, precision and accuracy tests on the basis of International Conference on Harmonization (ICH) guidelines. Linearity was confirmed by the correlation coefficient ($R^2$). To calculate the regression equations, five different concentrations of the standard solution were used to draw up the calibration curves. The regression equation formed was $y = ax + b$ ($a$ was the slope of the calibration curve, $b$ was the intercept of the calibration curve, $y$ axis
was the value of the peak area and x axis was the concentration of the component. All correlation coefficients of the calibration curves of 10 components showed good linearity \((R^2 > 0.9993)\). The limit of detection (LOD) and the limit of quantification (LOQ) in chromatographic conditions were determined at signal-to-noise ratio of 3 and 10, respectively. The regression equations and correlation coefficients \((R^2)\) of 10 compounds were given in Table I.

**Precision, repeatability and stability**

The precision of the developed method was evaluated by performing intra- and interday assays with replicate \((n = 5)\) injection of a mixed standard solution. Intraday precisions were measured for injections at 4-h intervals during the same day, whereas interday precisions were measured on three consecutive days. The relative standard deviations \((RSDs)\) of the peak areas of 10 components were 0.36–1.15% for intraday precisions and 0.52–2.21% for interday precisions.

In order to confirm the repeatability, one crude *R. scutellariae* sample and one wine-processed *R. scutellariae* sample were treated six times as the same procedure of sample solution preparation and analyzed, respectively. The RSD values of the contents of 10 components were 0.16–1.82% for crude *R. scutellariae* sample and 0.18–2.26% for wine-processed *R. scutellariae* sample.

The stability was tested and analyzed with one crude *R. scutellariae* sample and one wine-processed *R. scutellariae* sample at room temperature at 0, 2, 4, 8, 12, 24 and 48 h within 2 days, respectively. The RSD values of the peak areas of 10 components were 0.22–2.14% for crude *R. scutellariae* sample and 0.19–2.74% for wine-processed *R. scutellariae* sample, indicating that the sample solutions were stable within 48 h.

**Accuracy**

Accuracy was determined by the recovery test. The appropriate amounts of crude and wine-processed *R. scutellariae* powders were weighed and added with known amounts of the mixed standard stock solutions, respectively. They were then treated and analyzed with the above described method for the preparation of sample solutions. Each sample was analyzed in six replicates. The total amount of each analyte was calculated from the corresponding calibration curve.

\[
\text{Recovery (\%)} = \frac{\text{Amount}_{\text{determined}} - \text{Amount}_{\text{original}}}{\text{Amount}_{\text{spiked}}} \times 100
\]

Where Amount\(_{\text{determined}}\) is the determined total of each analyte, Amount\(_{\text{original}}\) is the original amount of each analyte in crude or wine-processed *R. scutellariae* samples measured and Amount\(_{\text{spiked}}\) is the spiked amount of each analyte. For comparison, an unspiked sample was prepared and analyzed simultaneously. The average recoveries of the compounds were 96.95–109.51% with RSD values ranging from 2.14 to 3.26% for crude *R. scutellariae* sample, while 94.73–108.38% with RSD values ranging from 1.83 to 3.47% for wine-processed *R. scutellariae* sample \((n = 6)\).

**Sample analysis**

The contents of 10 flavonoids in crude and wine-processed *R. scutellariae* samples were listed in Table II. The results showed that comparing with the contents of 10 compounds in crude *R. scutellariae* samples, the contents of 10 compounds in processed *R. scutellariae* products varied significantly. After being wine-processed, the contents of scutellarin, apigenin-7-glucuronide and chrysin varied slightly, while the contents of baicalin, oroxylin A-7-O-glucuronide and wogonoside markedly increased, and the contents of apigenin, baicalein, wogonin and oroxylin A slightly decreased, which might be due to the processing of *R. scutellariae*. Thus, it is necessary to establish a valid analytical method for intrinsic quality control of crude and wine-processed *R. scutellariae*.

**Discussion**

An UHPLC-PDA method has been developed to simultaneously determine baicalin, wogonoside, baicalein, wogonin, oroxylin A, oroxylin A-7-O-glucuronide, chrysin, scutellarin, apigenin and apigenin-7-glucuronide in crude and wine-processed *R. scutellariae*. This new established method is validated to be rapid, precise and accurate, which can be used as a valid analytical method for intrinsic quality control of crude and wine-processed *R. scutellariae*.

TCM processing is an important part of Chinese folk medicine. After processing with heat, water or wine, the different degrees of changes of a component in quality and quantity caused by changed physical and chemical properties of TCM may result in significant alteration of clinical efficacy. In general, many TCMs contain ingredients such as glycosides and corresponding specific enzymes for decomposing these glycosides. Under certain temperature and humidity, enzymes could easily decompose these glycosides into aglucones, some of which might be further oxidized into ineffective components. Therefore, this wine-processing technology developed by ancient pharmaceutical workers could be used to kill the enzymes in *R. scutellariae*, and avoid hydrolysis of glycosides at higher temperature during wine processing, which resulted in higher contents of flavonoid glycosides and total flavonoids in wine-processed *R. scutellariae* (34). Our study suggested that wine-processing approach may be an effective way in maintaining the concentration of active components in *R. scutellariae*.

**Supplementary Material**

Supplementary materials are available at [Journal of Chromatographic Science](http://chromsci.oxfordjournals.org).

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**Conflict of interest statement.** The authors declare no conflict of interest.

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