Research Article

High-performance liquid chromatography with photodiode array detection and chemometrics method for the analysis of multiple components in the traditional Chinese medicine Shuanghuanglian oral liquid

Shuanghuanglian oral liquid, a traditional Chinese medicine preparation, is a mixture of three herbs (Flos Lonicerae, Radix Scutellariae and Fructus Forsythiae). In this study, the quantitative analysis of three main active compounds, chlorogenic acid, forsythin and baicalin in samples from different manufacturers was performed rapidly by high-performance liquid chromatography coupled with photodiode array detection followed by Contour Projection coupled to stepwise regression treatment of the obtained three-dimensional spectra in which the partial overlap between adjacent target components existed. The method was validated for linearity (R > 0.9940), precision (RSD < 1.25%), recovery (92.20–102.50%), limit of detection (0.01–0.02 μg/mL) and limit of quantification (0.03–0.07 μg/mL). The results indicated that the combination of the three-dimensional spectra of traditional Chinese medicine and Contour Projection-stepwise regression offered an accurate, simple, low-cost and eco-friendly way for the rapid quantitative analysis of Shuanghuanglian oral liquid samples.

Keywords: Chemometrics / Contour projection / High-performance liquid chromatography / Quantitative analysis / Three-dimensional spectroscopy

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1 Introduction

Herbal medicine (HM) (or traditional Chinese preparation) has been widely used for centuries in oriental countries and plays a more and more important role owing to the effective therapeutic performance, low toxicity and rare side effects. Different from western medicine, the HM preparations are usually a combination of several herbs in composite formula [1], and a single herb also contains multiple active components. These components may vary significantly due to various factors such as growth location, harvest time, drying and storage conditions, which make the difficulty for the QC of HM preparations. Therefore, a reliable and effective method for the quality assessment of herbal formulas is therefore imperative.

Shuanghuanglian (SHL) preparation is a traditional Chinese formula, which comprises three herbs: Flos Lonicerae, Radix Scutellariae and Fructus Forsythiae [2], commonly used to treat acute upper respiratory tract infection, acute bronchitis and pneumonia [3, 4]. Particularly, SHL oral liquid has been widely used as effective clinical therapeutics [5].

By now, several strategies have been developed for the quantification of the main active components in SHL oral liquid samples, such as HPLC [6–9], CE [10] and rapid-resolution liquid chromatography (RRLC) [11]. However, these methods suffer from a few disadvantages such as a higher ratio of organic solvent and a longer time for the optimization of the experimental conditions, which are difficult to meet the requirements of environment-friendly and rapid analysis. Therefore, the fast, simple and environmentally friendly analytical methods still need to be proposed.

Currently, for the purpose of the rapid quantitative determination of the multiple target compounds in complex samples, a large number of methods [12–16] have been developed. In addition, in our previous study, the accumulated projection (AP) method was proposed and utilized for the rapid simultaneous quantification of multiple active components [17]. Furthermore, the image processing techniques had been reported for the quantitative analysis of
multiple target compounds such as digital image recognition technique [18], Zernike moments method (ZM) [19], wavelet moment method (WM) [20, 21] and Tchebichef moment method (TM) [22]. As all mentioned above, chemometric techniques can make it possible to quantify a complicated system and the main virtue of these methods is the reduction of experimental time and organic reagent.

In the present study, for the first time, we have established a quantitative model using the contour projection-stepwise (CP-stepwise) regression chemometric technique based on 3D HPLC with photodiode array detection (PAD) for quantifying the main active compounds in SHL oral liquid samples. This combined method was compared with partial least squares regression (PLSR) and conventional HPLC separation methods. This method can be severed as a fast quantitative tool, and a simpler, more cost-effective and environmentally friendly tool instead of the separation techniques. This method was successfully used to the quantitative analysis of seven batches of SHL oral liquid samples.

2 Materials and methods

2.1 Reagents and materials

Acetonitrile and methanol were of HPLC grade (DIKMA, USA), and phosphoric acid was of analytical grade (FUCHEN, China); chlorogenic acid, forsythin and baicalin were all of HPLC grade (ABCRI, Germany) and were used without further purification. The chemical structures of target compounds are illustrated in Fig. 1.

A total of seven batches of SHL oral liquid samples were purchased from local drug markets (Lanzhou, China). According to the Chinese Pharmacopoeia (Version 2010), chlorogenic acid, forsythin and baicalin are the marker compounds for the QC of SHL oral liquid [23].

2.2 Preparation of standards solutions

The standard samples of chlorogenic acid, forsythin and baicalin were accurately weighted and then dissolved with methanol to give the concentration of 1650, 1580 and 750 μg/mL, respectively. A mixed standard solution was prepared by mixing standard solution with methanol to obtain a series of concentrations for the calibration curves in the range of 11.55–107.25 μg/mL for chlorogenic acid, 15.80–118.50 μg/mL for forsythin and 24.00–302.50 μg/mL for baicalin.

2.3 Instrumentation and chromatographic conditions

A Waters (USA) HPLC system coupled with a 2998 PAD, 1525 binary HPLC pump, manual injector and a reversed-phased C18 column (4.6 × 250 mm, 5 μm) was employed.

A gradient elution program including 0.2% v/v phosphoric acid aqueous solution (A) and acetonitrile (B) was used to analyze the standards and samples. The gradient elution

![Figure 1. Chemical structures of chlorogenic acid, baicalin and forsythin.](image-url)
Figure 2. HPLC spectrum of a mixed standard solution; the 3D spectrum (A), the CP plot (B) and the maximum absorption wavelengths plot for chlorogenic acid, baicalin and forsythin (C).

program was as follows: 0–3.5 min, 27% v/v B; 3.5–6.0 min, 27–60% B; 6.0–7.0 min, 60–40% B; 7.0–10.0 min, 40–27% and then return to the initial conditions within the next 20 min. The temperature ranged from 30 to 34 °C; scan wavelength ranged from 220 to 400 nm; flow rate was set at 1.0 mL/min; injection volume was 5 μL. The real samples and standard solutions were filtered through a 0.22 μm filter (Shanghai, China) before HPLC analysis.

As the comparison, traditional baseline separation was also carried out for the same target components in the same samples. Gradient elution conditions were as follows: 0–6.0 min, 14% v/v B (1.0 mL/min); 6.0–15.0 min, 14–35% B (1.0–0.6 mL/min); 15–25 min, 35–5% B (0.6 mL/min); 25–30 min, 5–14% (0.6–1.0 mL/min); 30–50 min 14% B (1.0 mL/min). The wavelength used to measure chlorogenic acid, baicalin and forsythin were 326, 278 and 226 nm, respectively. The analytical results were used as a standard to evaluate the proposed method.

Peak identification of the three target components was finished by injection of single corresponding standard. The HPLC chromatograms of the mixed standards are illustrated in Fig. 2A.

3 Chemometric approach

The overall scheme of the combined method for the quantitative analysis of multiple compounds is shown in Fig. 3. As can be seen in Fig. 3, the scheme consisted of three main steps. They were contour projection, retention time alignment and stepwise regression. In this section, the combined method was described in detail.

3.1 Contour Projection (CP)

CP method was proposed to transfer each 3D HPLC spectrum to an interesting chromatographic profile (Fig. 2B), in which each value was the maximum absorption intensity in all scanning wavelengths at each retention time point. In this study, as for the maximum absorption wavelengths of the
investigated target components differ from one to another (Fig. 2C), the CP method could obtain the intensity of each component at corresponding maximum absorption wavelength accurately for improving the sensitivity and selectivity without choosing appropriate absorption one at different wavelengths [24, 25].

3.2 Retention time alignment

In real LC measurement, chromatographic peak shifts from run to run are inevitable due to subtle changes of the experimental conditions, such as minor changes in mobile phase composition, pressure and temperature. Chromatogram alignment methods [26, 27] are proposed to solve this problem. In this study, before model building, the interval correlation optimized shifting algorithm (icoshift) [28] was employed for the alignment of the shifted peaks.

3.3 Stepwise regression

After the alignment of chromatographic profiles, the linear models were established by means of stepwise regression (adding and removing independent variants from a multilinear model based on their statistical significances in a regression), respectively [19]. The regression equations for chlorogenic acid, forsythin and baicalin were all given in the form of $Y = A \times X + B$, where $Y$ (dependent variable) and $X$ (independent variable) were the concentrations of mixed standard solutions ($\mu$g/mL) and the aligned CP values that selected by stepwise regression, respectively.

The performance of the established models was presented with their statistical parameters such as the correlation coefficients ($R$), adjusted correlation coefficients ($R_{adj}$), correlation coefficient of leave-one-out ($R_{LOO-cv}$), root mean square errors (RMSE) and $F$-test, $p$-value.

All the calculation programs used were written in M-file and carried out on MATLAB 7.0, with PC (CPU 3.50 GHz, 32.0 GB RAM).

4 Results and discussion

4.1 Linear modeling

The aligned CP chromatographic profiles ($n = 21$) were divided into retention time dimensions for the purpose of re-
Table 1. The linear equations of the three investigated components

<table>
<thead>
<tr>
<th>Methods</th>
<th>Analytes</th>
<th>Regressive equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-stepwise</td>
<td>Chlorogenic acid</td>
<td>$y^a = 0.21 + 240.85 \times X_{43}$</td>
</tr>
<tr>
<td></td>
<td>Forsythin</td>
<td>$y^a = 11.88 + 561.26 \times X_{53} + 306.99 \times X_{69}$</td>
</tr>
<tr>
<td></td>
<td>Baicalin</td>
<td>$y^a = -86.73 + 305.48 \times X_{21} + 55201 \times X_{45}$</td>
</tr>
</tbody>
</table>
| Traditional calibration | Chlorogenic acid | $y^b = 15079 \times X_{\text{con}.
|                      | Forsythin        | $y^b = 19461 \times X_{\text{con}.
|                      | Baicalin         | $y^b = 15800 \times X_{\text{con}.

*For the CP-stepwise method:
$X_m$: selected aligned CP value at $m^{th}$ retention time point in each region
$Y^a$: experimental concentration for each target compound ($\mu g/mL$)

*For the traditional calibration method
$X_{\text{con}}$: experimental concentration ($\mu g/mL$)
$Y^b$: peak area.

Producing the impact of irrelevant information, and the regions were chosen by visual inspection. The first region that ranged from 2.40 to 3.24 min (101 retention time points) included only chlorogenic acid; the second region ranged from 6.99 to 7.83 min (101 retention time points) involved the co-eluted peaks of forsythin and baicalin. By means of stepwise regression, the linear models that describe the relationship between the selected CP data (associated with the concentrations of the three components) and the concentrations of the three components in standard samples were established (Table 1). The relationship of the experimental concentrations (Exp. con) and the calculated concentrations (Cal. con) of the three target compounds in standard samples are presented in Fig. 4.

4.2 Validation of the established models

The performances of the linear models were calculated and are summarized in Table 2. The minimum correlation coefficient of 0.9940 indicated good linearity for the three target compounds between the concentrations and the selected CP data within the test ranges. The $R_{adj}$ and $R_{LOO-cv}$ were higher than 0.9790, indicating a good prediction ability of these established models. F-test values were higher than 326 and their
Table 2. Summary of the performances of the established linear models

<table>
<thead>
<tr>
<th>Item</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-stepwise</td>
<td>Correlation coefficient ($R$)</td>
<td>0.9970</td>
<td>0.9965</td>
</tr>
<tr>
<td></td>
<td>Adjusted correlation coefficient ($R_{adj}$)</td>
<td>0.9965</td>
<td>0.9960</td>
</tr>
<tr>
<td></td>
<td>Root mean square error (RMSE)</td>
<td>2.90</td>
<td>3.21</td>
</tr>
<tr>
<td></td>
<td>$F$-test value</td>
<td>1380</td>
<td>780</td>
</tr>
<tr>
<td></td>
<td>$p$-value</td>
<td>$2.40 \times 10^{-20}$</td>
<td>$2.13 \times 10^{-18}$</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient of LOO ($R_{LOO}$)</td>
<td>0.9898</td>
<td>0.9815</td>
</tr>
<tr>
<td></td>
<td>Precisions (RSD, %, $n = 6$) intra-day</td>
<td>0.67</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Reversibility (%)</td>
<td>102.50</td>
<td>93.32</td>
</tr>
<tr>
<td></td>
<td>LOD (ug/mL)</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>LOQ (ug/mL)</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient ($R$)</td>
<td>0.9955</td>
<td>0.9919</td>
</tr>
<tr>
<td></td>
<td>Adjusted correlation coefficient ($R_{adj}$)</td>
<td>0.9905</td>
<td>0.9769</td>
</tr>
<tr>
<td></td>
<td>Root mean square error (RMSE)</td>
<td>3.17</td>
<td>4.33</td>
</tr>
<tr>
<td></td>
<td>$F$-test value</td>
<td>2410</td>
<td>767</td>
</tr>
<tr>
<td>PLSR</td>
<td>Correlation coefficient of LOO ($R_{LOO}$)</td>
<td>0.9943</td>
<td>0.9842</td>
</tr>
<tr>
<td></td>
<td>Precisions (RSD, %, $n = 6$) inter-day</td>
<td>0.75</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>105.0</td>
<td>87.5</td>
</tr>
<tr>
<td></td>
<td>LOD (ug/mL)</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>LOQ (ug/mL)</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>Traditional calibration</td>
<td>Correlation coefficient ($R$)</td>
<td>0.9980</td>
<td>0.9989</td>
</tr>
<tr>
<td></td>
<td>Adjusted correlation coefficient ($R_{adj}$)</td>
<td>0.9975</td>
<td>0.9985</td>
</tr>
<tr>
<td></td>
<td>Root mean square error (RMSE)</td>
<td>1.63</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td>$F$-test value</td>
<td>996</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>$p$-value</td>
<td>$6.01 \times 10^{-6}$</td>
<td>$1.84 \times 10^{-6}$</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient of LOO ($R_{LOO}$)</td>
<td>0.9933</td>
<td>0.9975</td>
</tr>
<tr>
<td></td>
<td>Precisions (RSD, %, $n = 6$) inter-day</td>
<td>0.35</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>92.36</td>
<td>93.30</td>
</tr>
<tr>
<td></td>
<td>LOD (ug/mL)</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>LOQ (ug/mL)</td>
<td>0.03</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Model 1 for chlorogenic acid; Model 2 for forsythin; Model 3 for baicalin.

$p$-values were smaller than $4.19 \times 10^{-15}$, which proved that the linear relationships between the concentrations and the selected CP data were significant. These statistical parameters illustrated that the outstanding models could be applied to real sample analyses.

4.3 Validation of the quantitative analysis

4.3.1 LOD and LOQ

The LOD and LOQ under the present chromatographic conditions was determined by injecting a series of standard solutions until the $S/N$ of at least threefold ($S/N > 3$) and 10-fold ($S/N > 10$) for LOD and LOQ, respectively. The high sensitivity at the chromatographic condition was confirmed by LOD (0.01–0.02 µg/mL), and LOQ (0.03–0.07 µg/mL) (Table 2).

4.3.2 Precision

The precision of the proposed method was determined in terms of intra- and inter-day test. For intra-day precision test, the mixed standard solution was tested for six replicates within the same day, and for inter-day precision test, the same solution was analyzed twice per day for three consecutive days. Variations were carried out by RSD and was calculated by the formula $RSD(\%) = (SD/mean) \times 100\%$. The overall intra- and inter-day precisions (94.00 µg/mL for chlorogenic acid, 88.48 µg/mL for forsythin and 167.50 µg/mL for baicalin) of the three analytes were less than 1.25%, which demonstrated good precise for this method.

4.3.3 Recoveries

The accuracy of the method was estimated by means of a recovery test, which was determined by the standard addition method. The quantity of each investigated components was obtained from the established calibration curve and the recovery was estimated by the following formula:

$$\text{Recovery(%) } = \left( \frac{\text{detected concentration} - \text{initial concentration}}{\text{spiked concentration}} \right) \times 100$$  (1)
The recovery of 92.20–102.50% for the target components at one concentration level (15.80 μg/mL for chlorogenic acid, 16.60 μg/mL for forsythin and 30.00 μg/mL for baicalin), which showed that the quantitative method has satisfactory accuracy.

4.4 Sample analysis

The described method was subsequently used to the simultaneous determination of the three target components in seven batches of SHL oral liquid samples. Triplicate analysis was performed for each real sample. The aligned CP chromatographic profiles of the real samples were also divided into two regions as the same as those illustrated in Section 4.1. The obtained concentrations of the three components in the real samples were summarized in Table 3, in which the contents of chlorogenic acid, forsythin and baicalin were higher than 0.73, 0.55 and 18.30 mg/mL, respectively. The QC of the SHL preparations is officially listed in the Chinese Pharmacopoeia (Vision 2010) and chlorogenic acid, forsythin and baicalin are used as assessment markers, which should be higher than 0.6, 0.3 and 10 mg/mL, respectively [23]. These values demonstrated the availability of the proposed method and qualities of the seven batches of SHL oral liquid samples reach the requirements of the Chinese Pharmacopoeia standard. In addition, the differences in the content of the three components among different manufacturers may attribute to many factors, including raw materials (plant age, harvest time, weather conditions, soil conditions, etc.) and/or the preparation technologies of different manufactures [29].

4.5 Comparison

4.5.1 Comparison with partial least squares regression (PLSR) method

PLSR is an algorithm for building linear regression models that has been applied in several fields [30, 31]. In this study, the PLSR method was implemented on the same samples to further validate the proposed method.

According to the Chinese Pharmacopoeia, the maximum absorption wavelength of chlorogenic acid, forsythin and baicalin was 326, 278 and 226 nm, respectively. Therefore, the chromatograms of the three target compounds at the maximum absorption wavelengths have been chosen first, respectively, followed by the retention time alignment using icoshift method, and then, the same regions (Section 4.1) have been chosen to establish the PLSR models. The optimum number of latent variables employed in each model was determined based on the minimum value of root mean square error of cross validation (RMSECV), and one, six and seven latent variables were chosen for chlorogenic acid, forsythin and baicalin, respectively.

The related statistical parameters were listed in Table 2. As for the parameters of model 1 that obtained from the PLSR method, \( R_{LOO-CV} \) was higher than that of the CP-stepwise method, indicating that the predictive power of this model was higher, and \( F \)-test value and \( p \)-value were consistently higher and consistently lower than that of CP-stepwise method, respectively, demonstrating that PLSR method is more significant. However, as for the parameters of model 2 and model 3, the \( R \), \( R_{adj} \), \( R_{LOO-CV} \) and \( F \)-test values were consistently lower than that of the CP-stepwise method, and \( p \)-values were consistently higher than that of CP-stepwise method, indicating that predictive power of these two models is higher and CP-stepwise method is more significant. Moreover, compared with the precisions and recoveries obtained from PLSR method, the results from the CP-stepwise method were satisfactory.

From the results obtained above, we are confident that the CP-stepwise method is more appropriate for simultaneous determination of the three target components, especially for the co-eluted analytes (forsythin and baicalin). Moreover, compared with PLSR method, the CP-stepwise method can give the linear regression equation, which is simpler and more intuitive.

4.5.2 Comparison with traditional method

The separation by the traditional method (Fig. 5A–C) takes a longer time (21 min) than that of the proposed method (7.5 min) (Fig. 5D). Moreover, it was tedious to choose these wavelengths while using traditional calibration method.

The equations obtained using traditional calibration method are listed in Table 1. The performances (Table 2) obtained from the traditional method were similar with that of CP-stepwise method. These results proved that both methods allow for accurate quantitative determination of the three target compounds in SHL oral liquid samples. However, with the application of CP-stepwise method, the same quantitative analysis results (Table 3) were obtained with a shorter analysis time. Therefore, the CP-stepwise method was especially recommended for the quantitative analysis of the chromatograms suffering from the partial overlapping of target compounds’ peaks.

In our experiment, the CP-stepwise method serve as a replacement of time-consuming chemical analysis, which allows one to rapid quantify the concentrations of the three target components in SHL oral liquid samples. Moreover, the CP-stepwise method could obtain the intensity of each component at corresponding maximum absorption wavelength accurately for improving the sensitivity and selectivity without artificial selection of appropriate absorption wavelength. Therefore, the proposed CP-stepwise method have the advantages over traditional calibration method in terms of analytical speed, time saving and solvent saving. In a word, the proposed method might be a powerful tool for the fast, low-cost and simultaneous determination of components of interesting in real complex samples and yield satisfactory results.
**Table 3.** The concentrations (mg/mL) of the three detected components in SHL oral liquid samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Batch number</th>
<th>CP-stepwise</th>
<th>PLSR</th>
<th>Traditional calibration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chlorogenic acid</td>
<td>Forsythin</td>
<td>Baicalin</td>
</tr>
<tr>
<td>S1</td>
<td>112040924</td>
<td>0.80</td>
<td>0.70</td>
<td>18.30</td>
</tr>
<tr>
<td>S2</td>
<td>130505092</td>
<td>0.73</td>
<td>0.78</td>
<td>23.21</td>
</tr>
<tr>
<td>S3</td>
<td>12110625</td>
<td>1.07</td>
<td>0.60</td>
<td>21.73</td>
</tr>
<tr>
<td>S4</td>
<td>12111243</td>
<td>1.05</td>
<td>0.55</td>
<td>22.10</td>
</tr>
<tr>
<td>S5</td>
<td>13011961</td>
<td>0.83</td>
<td>0.65</td>
<td>20.46</td>
</tr>
<tr>
<td>S6</td>
<td>120919</td>
<td>0.74</td>
<td>0.55</td>
<td>22.91</td>
</tr>
<tr>
<td>S7</td>
<td>120610</td>
<td>0.85</td>
<td>0.55</td>
<td>24.03</td>
</tr>
</tbody>
</table>

**Figure 5.** Typical chromatograms of three compounds; (A, B, C) baseline separated, (D) partially overlapped. Peak identification: (1) chlorogenic acid; (2) baicalin and (3) forsythin.

**5 Concluding remarks**

In this work, a rapid, low-cost, simple, environmentally friendly and effective combined HPLC–PAD and CP-stepwise method was established to successfully determine the three active components in SHL oral liquid samples based on 3D HPLC–PAD spectra, although there existed the partial overlapping of target compounds’ peaks. The feasibility of the proposed method was proved by quantifying seven batches of real samples. Compared with the quantitative results by traditional method, the same results can be obtained from the proposed method with a shorter time. Therefore, the combined method could provide an efficient way to the accurate determination of multiple target components in herbal preparations. More research work will be carried out to extend the application of the proposed method.
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The authors have declared no conflict of interest.

6 References
