Available online at SciVarse ScienceDirect

**Chinese Herbal Medicines (CHM)**

ISSN 1674-6384

Journal homepage: www.tiprpress.com E-mail: chm@tiprpress.com

## Original article

# Simultaneous Determination of Seven Components in Gamboge and Its Processed Products Using a Single Reference Standard

Min Xu†, Hong-sheng Tan†, Wen-wei Fu, Ling-yun Pan, Yue-xun Tang, Shen-da Huang, Yan-feng Xiu*

*School of Pharmacy, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China*

ARTICLE INFO

*Article history*

Received: July 12, 2016

Revised: September 10, 2016

Accepted: October 21, 2016

Available online:

December 29, 2016

DOI:

10.1016/S1674-6384(17)60074-1

ABSTRACT

**Objective** To establish a quality control method for simultaneous determination of multiple components in gamboge. **Methods** A single reference standard for the determination of multiple components (SSDMC) with HPLC was proposed. Seven major components of gamboge including gambogenic acid (S), β-morellic acid (C1), 2*R*-30-hydroxygambogic acid (C2), isogambogenic acid (C3), gambogellic acid (C4), 2*R*-gambogic acid (C5), and 2*S*-gambogic acid (C6) were simultaneously analyzed using gambogenic acid as reference standard. The credibility and feasibility of SSDMC method were validated with respect to linearity, limits of detection and quantification, precision, stability, repeatability, accuracy, ruggedness, and robustness. The relative conversion factors (RCFs) of S and C1-6 were calculated. Twelve batches of gamboge including crude and processed products were successfully analyzed by applying the SSDMC and traditional external standard (ES) methods. **Results** The SSDMC method was credible and feasible. The RCFs of S and C1-6 were 1.000, 0.913, 0.864, 1.064, 0.777, 0.921, and 0.919, respectively. No significant difference was observed in the contents of the seven components between SSDMC and ES methods. The heat-processing technique caused a reduction in the seven components. **Conclusion** SSDMC is a simple, reliable, and effective method for the analysis of the complex multiple components in gamboge, and it is also a practical and economical approach.

*Key words*

gamboge; HPLC; quality control; relative conversion factor; single standard for determination of multiple components



## 1. Introduction

Gamboge, the resin exuded from the plant *Garcinia hanburyi* Hook. F., is a folk medicine used in India, Thailand, Cambodia, and other Southeast Asian countries. Gamboge has also been used in traditional Chinese medicine (TCM) for hundreds of years under the Chinese name *Tenghuang* (Nanjing University of Traditional Chinese Medicine, 2006).

* **Corresponding author: Xiu YF** E-mail: xiu_yf@163.com Fax: +86-21-5132 2197

† These two authors contributed equally to this work.

Fund: Science and Technology Commission of Shanghai Municipality (13ZR1442000); Shanghai Municipal Education Commission (2014YSN20) Support Program

Because of gamboge's toxicity, which is obvious due to stimulation of the gastrointestinal tract which affects the duodenum and jejunum edema (Dou et al, 2013), gamboge is usually used in external treatments for chronic dermatitis, scabies, tinea, hemorrhoids, bedsores, and malignant boils (Nanjing University of Traditional Chinese Medicine, 2006; Ou et al, 2011). When gamboge is administered orally, it must be specially processed. The TCM processing technique can reduce the toxicity of TCMs and retain their medicinal activities as much as possible (Pharmacopoeia Committee of P. R. China, 2015). For gamboge, a heat-processing method has been proven to reduce its toxicity (Zhao et al, 2016).

The bioactive effects of gamboge are highly related to its components. Modern chemical and pharmacological studies have revealed that the major components of gamboge are caged xanthones, of which gambogic acid is the most representative. These major components have significant antitumor and anticancer (Anantachoke et al, 2012; Zhang et al, 2013; Qi et al, 2015), anti-inflammatory and antipsoriatic (Wen et al, 2014), anti-HIV (Reutrakul et al, 2007), and neurotrophic activities (Jang et al, 2007). Recently, over 40 different xanthones have been isolated from *G. hanburyi* (Anantachoke et al, 2012; Han et al, 2009). In the past, HPLC equipped with ELSD (Yang et al, 1999) or UV (Zhang et al, 2003; Han et al, 2006; Song et al, 2007; Li et al, 2008a) detector and UPLC-MS (Zhou et al, 2008) were established as the methods of choice for the analysis of caged xanthones in gamboge samples. However, reference standards are needed to utilize these methods, but only gambogic and gambogenic acids are available for purchase. Due to the lack of reference standards, the above methods have not been widely used in the quality control of gamboge. Simultaneous determination of multiple components is an effective method of controlling the quality of herbal medicines (Li et al, 2008b). Therefore, it is meaningful to establish a method for simultaneous determination of the major components in gamboge and its processed products, without the need for authentic reference standards for each individual compound.

We isolated and purified a large quantity of caged xanthones from gamboge in the laboratory (Xu et al, 2016). In this work, we optimized and established a single reference standard for determination of multiple components in TCMs abbreviated as the SSDMC method (Hou et al, 2011; Chen et al, 2016; Shi et al, 2015) for simultaneous determination of seven components in gamboge. Gambogenic acid was used as the reference standard, while the other six components included β-morellic acid, 2*R*-30-hydroxygambogic acid, isogambogenic acid, gambogellic acid, 2*R*-gambogic acid, and 2*S*-gambogic acid. The method was validated with respect to linearity, and the limits of detection and quantification, precision, stability, repeatability, accuracy, ruggedness, and robustness. Twelve batches of gamboge including crude and processed products were analyzed using the SSDMC method. The contents of seven components were compared with those obtained by the traditional external standard method. The contents of seven components in processed gamboge were also compared with that of the crude gamboge.

## 2. Materials and methods

### *2.1 Materials and reagents*

Six batches of gamboge were purchased from different herbal markets in China and India, including gamboge (SG) from India (SG-1 and SG-2) and Anhui (SG3 and SG4), Shanghai (SG5) and Yunnan (SG6) provinces, China. All samples were identified by Associate Prof. Yan-feng Xiu (School of Pharmacy, Shanghai University of Traditional Chinese Medicine). The specimen samples were retained at School of Pharmacy, Shanghai University of Traditional Chinese Medicine.

To investigate the effects of processing techniques on the major components in gamboge, water-boiled gamboge was prepared. All six batches of gamboge were used to prepare the heat-processed gamboge. The water-boiled gamboge (WBG) was prepared as follows (Nanjing University of Traditional Chinese Medicine, 2006): 50 g of crude gamboge powder was boiled in a beaker and then filtered. The filtrate was boiled for 3 h while stirring constantly and more hot water was added midway. Finally, the decoction was concentrated and dried below 50 ºC under vacuum. Six batches of crude gamboge were processed and marked as WBG1－6.

Seven standard compounds, gambogenic acid, β-morellic acid, 2*R*-30-hydroxygambogic acid, isogambogenic acid, gambogellic acid, 2*R*-gambogic acid, and 2*S*-gambogic acid were obtained from the Engineering Research Center of Shanghai Colleges for TCM New Drug Discovery. The structures of compounds (Figure 1) were fully characterized by NMR and MS. The purity of the compounds was analyzed using the HPLC peak area normalization method and was above 97%. Acetonitrile of HPLC grade was purchased from Fisher Scientific (USA). The water used for HPLC was redistilled. Other chemicals and solvents were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

### *2.2 Instrumentation*

The Agilent 1200 HPLC System, comprised of a quaternary solvent delivery system, an online degasser, an auto-sampler, a column temperature controller, and a variable wavelength detector (VWD), coupled with Agilent ChemStation software (Agilent Technologies, USA) was used for analysis. Chromatography was performed on a Diamonsil C8 column (250 mm × 4.6 mm, 5 μm) and SB−3200D ultrasonic bath (Scientz, Ningbo, China) was used for sample preparation.

### *2.3 Reparation of solutions*

#### *2.3.1 Reparation of standard solutions*

Stock solutions of standard compounds were prepared and stored at −20 ºC. Accurately weighed gambogenic acid (S), β-morellic acid (C1), 2*R*-30-hydroxygambogic acid (C2), isogambogenic acid (C3), gambogellic acid (C4), 2*R*-gambogic acid (C5), and 2*S*-gambogic acid (C6) were dissolved

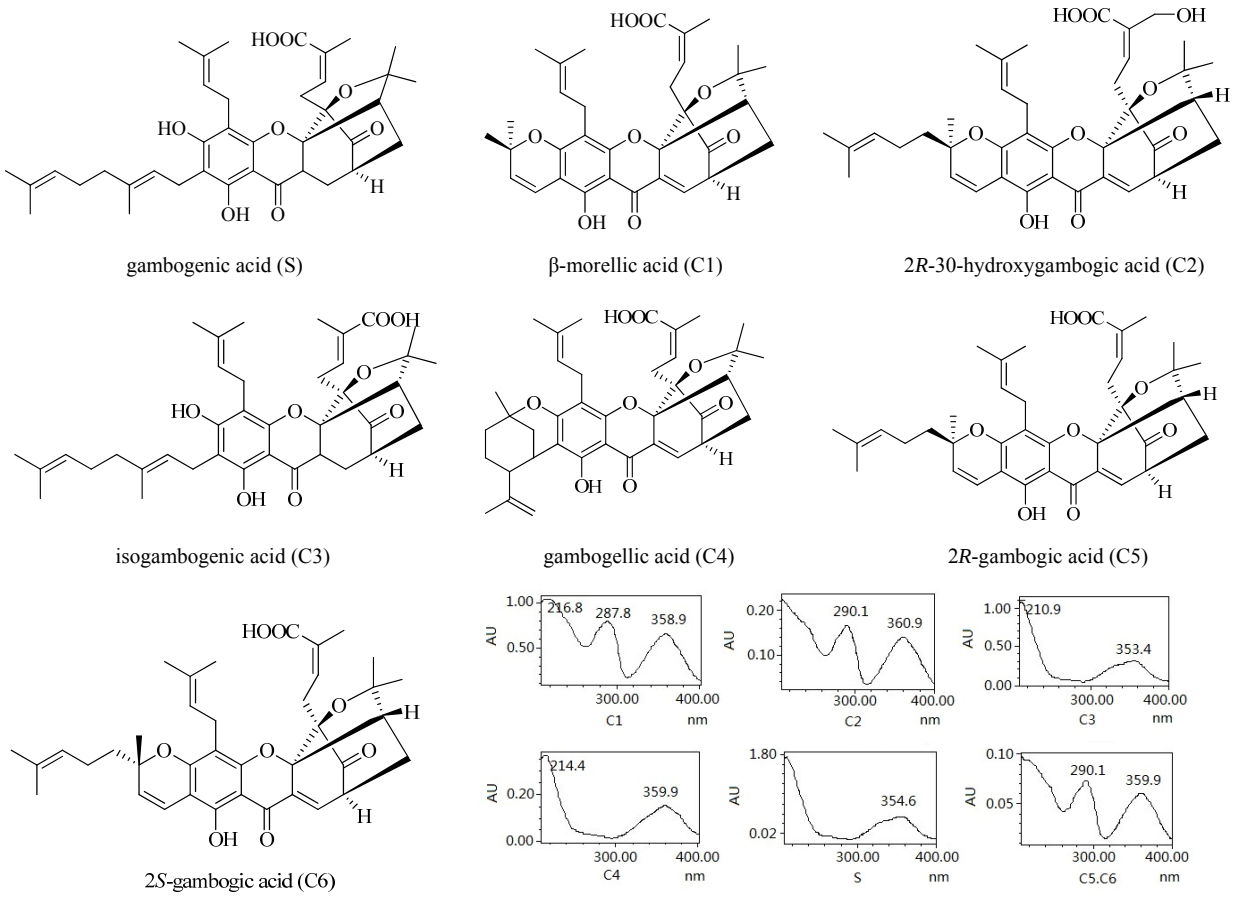


**Figure 1 Chemical structures of seven reference standards and their UV spectra**

in a 10 mL amber volumetric flask and diluted to the desired volume with methanol. The concentrations of S and C1-6 were 2.904, 1.887, 2.334, 1.998, 1.423, 7.096, and 6.868 mg/mL, respectively. Gambogic acid was stored in methanol at room temperature for a week, and underwent derivation (Han et al, 2005), thus, the stock solutions were stored at −20 ºC.

#### *2.3.2 Sample solutions*

An accurately weighed sample (approximately 0.1 g) of gamboge powder filtered through an 80 micron mesh was transferred to a conical flask fitted with a plug. The powder was extracted ultrasonically with methanol (20 mL) for 20 min, and then filtered. The residue was washed again with methanol and then all the filtrates were combined. The solution was diluted with methanol to 25 mL, filtered through a 0.45 μm nylon membrane filter and stored at 4 $^{o}$C for further use.

### *2.4 Hromatographic conditions*

The mobile phase was a mixture of acetonitrile (mobile phase A) and water containing 0.1% glacial acetic acid (mobile phase B). The elution gradient was as follows: 0–20 min, 70% A; 20–50 min, 70%–80% A; 50–60 min, 80% A. The flow rate was 1.0 mL/min and the column temperature was 30 ºC. The injection volume was 10 μL, and the detection wavelength was 360 nm (see Figure 1 for UV spectra of the seven reference standards**)**.

### *2.5 Calculation of relative conversion factor and relative retention time*

Methanol stock solutions containing seven standard compounds: S and C1−C6 were prepared and diluted to appropriate concentrations ranges for the construction of calibration curves. Each calibration curve was performed in triplicate with seven concentration levels. The calibration curves of all compounds were constructed by plotting the peak area versus the concentrations of each standard compound. The relative conversion factor (RCF) was calculated based on the linearity data and the following equations:

$$RCF_{xi} = (A_{si} / C_{si}) / (A_{xi} / C_{xi}) \quad (i = 1 - N) \qquad (1)$$

$$RCF_x = (\Sigma\, RCF_{xi}) / N \qquad (2)$$

Where $A_{si}$ and $A_{xi}$ represent the peak areas of gambogenic acid (S) and other standard compounds (X) at the concentration level $i$, respectively. $C_{si}$ and $C_{xi}$ are the concentrations of the standard gambogenic acid (S) and other standard compounds (X) at the concentration level $i$, respectively. N is the number of linearity data points, which was 7 in this work.

To identify the peaks with a single reference standard in the chromatogram, the relative retention time (RRT) was calculated. The RRT of the reference standard X ($RRT_x$) was calculated as the ratio of the retention time of the other standard compounds X ($RT_x$) and gambogenic acid (S) ($RT_s$):

$$RRT_x = RT_x / RT_s$$

### *2.6 Ruggedness and robustness of RCF and RRT*

Different equipment and columns were tested to validate the ruggedness of RCF and RRT. Three columns (250 mm × 4.6 mm, 5 μm) and two chromatographic instruments were chosen for this experiment. Columns included Diamonsil C8, Silversil C8 (Dikma Technologies, China), and Ultimate XB-C8 (Welch Materials, Inc., China), and the two HPLC-systems were Agilent 1200 and Waters e2695. The RCF and RRT were calculated using those columns and instruments. The robustness test was performed to examine the effects of operational factors, such as different analysts, concentration of glacial acetic acid (0.1% ± 0.01%), UV detection wavelength (360 ± 2 nm), time program of the mobile phase (20 ± 5/50 ± 5/60 ± 5 min), flow rate (1.0 ± 0.1 mL/min), and column temperature (30 ± 5 ºC).

### *2.7 Validation of analytical method*

Gambogenic acid, used as the external reference standard, exhibited good separation from the other components in the chromatogram. The SSDMC method was validated with respect to linearity, limits of detection and quantification, precision (intra- and inter-day variability), stability, repeatability, accuracy, ruggedness, and robustness. The results of the precision and accuracy analyses calculated for the new method were statistically compared with the results calculated using the traditional external standard method using the paired *t*-test.

## 3. Results and discussion

### *3.1 Calculation of RCF and RRT*

Selection of the reference standard is a notable factor for SSDMC method (Yang et al, 2015). According to the four requirements defined earlier (Hou et al, 2011), the single reference standard used in SSDMC should be abundant in the sample, stable, easily accessible and have a maximum UV absorption at the detection wavelength. Thus, gambogenic acid was selected from the components in gamboge. This acid was tested as the reference standard in the calculation of RCF of the other six components. The RCFs were calculated as described in section 2.5 and the results were shown in Table 1. The RCFs of C1−6 were 0.913, 0.864, 1.064, 0.777, 0.921, and 0.919, respectively.

The calculation of RRT was shown in Table 1. The RSD values of $RRT_s$ (< 0.20%) suggested that the RRTs obtained on the same instrument were highly reproducible. The peaks without the standards could be identified according to the $RRT_x$ of the components.

### *3.2 Validation of SSDMC method*

#### *3.2.1 Linearity, limits of detection, and quantification*

Figure 2 showed that the seven peaks in the chromatogram of gamboge could all be identified with the corresponding standards. Seven different concentration levels of mixed standard solutions were injected for HPLC analysis. The calibration curves of seven reference standards were plotted as the peak area versus concentration. As shown in Table 1, the results exhibited good linearity ($r^2 \geq 0.9993$) within the test range. The linearity requirement was met when analyzing the samples in the test ranges for S and C1−6. The limits of detection (LODs) and limits of quantification (LOQs) were calculated as the signal-to-noise ratios (S/N) of 3 and 10 for the individual compound (Table 1). The LODs and LOQs of seven standard compounds at the detection wavelength (360 nm) were in the ranges of 0.61−1.78 μg/mL and 1.73−4.02 μg/mL, respectively.

#### *3.2.2 Precision and stability*

Method precision was determined by comparing the intra- and inter-day variability, as shown in Table 2. Variations in the peak area (A) and retention time (RT) were examined. To calculate the RSD%, mixed standard solutions with a 10 μL injection volume were analyzed in six replicates in one day or in triplicate for three consecutive days. The results indicated that the relative standard deviations (RSDs) of A and RT were less than 0.70% and 0.31% for intra-day and 1.60% and 0.33% for inter-day variability, respectively.

The stability of the crude gamboge sample solution, SG1, was analyzed by measuring the peak area (A) and retention time (RT) after storage for 0, 2, 4, 6, 8, 12, 16, and 24 h. A and RT of seven components were analyzed and the RSD percentage was calculated, as shown in Table 2. The results of stability evaluation indicated that the sample solution was stable within a 24 h period after preparation and that the RSDs of A and RT were 0.61%−3.24% and 0.53%−1.13%, respectively.

**Table 1 Results of linearity, LOD, LOQ, RCF, and RRT studies**

| Standards | Linearity equations[a] | $r^2$ | Ranges / (μg·mL$^{-1}$) | LOD / (μg·mL$^{-1}$) | LOQ / (μg·mL$^{-1}$) | RCF | | RRT | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | RCF | RSD / % | RRT | RSD / % |
| S | $y = 13.59x + 14.04$ | 1.0000 | 25.05 − 501.00 | 0.64 | 1.94 | 1.000 | 0.00 | 1.000 | 0.00 |
| C1 | $y = 15.07x - 8.71$ | 0.9993 | 12.06 − 241.20 | 0.61 | 1.73 | 0.913 | 1.86 | 0.601 | 0.19 |
| C2 | $y = 15.73x - 4.10$ | 1.0000 | 2.60 − 52.00 | 0.78 | 2.58 | 0.864 | 2.19 | 0.719 | 0.11 |
| C3 | $y = 13.02x - 2.78$ | 1.0000 | 5.25 − 105.00 | 0.86 | 2.68 | 1.064 | 2.11 | 0.851 | 0.08 |
| C4 | $y = 18.10x - 18.77$ | 0.9994 | 3.45 − 69.00 | 1.78 | 2.56 | 0.777 | 3.95 | 1.149 | 0.08 |
| C5 | $y = 14.86x + 7.20$ | 1.0000 | 69.50 − 1390.00 | 1.61 | 4.02 | 0.921 | 0.68 | 1.361 | 0.11 |
| C6 | $y = 14.94x - 8.50$ | 1.0000 | 67.50 − 1350.00 | 1.61 | 4.02 | 0.919 | 1.04 | 1.421 | 0.11 |

[a]*y* was peak area of standard compound; *x* was concentration of standard compound.

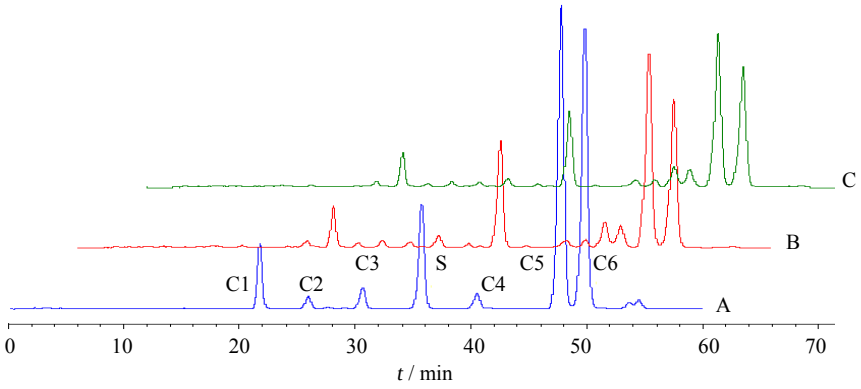


**Figure 2 HPLC of seven mixed reference standards and samples**

A: mixed reference standards; B: crude gamboge (SG); C: water-boiled gamboge (WBG); S: gambogenic acid; C1: β-morellic acid; C2: 2*R*-30-hydroxygambogic acid; C3: isogambogenic acid; C4: gambogellic acid; C5: 2*R*-gambogic acid; C6: 2*S*-gambogic acid

**Table 2 Results of precision and stability tests**

| Standards | Precision RSD / % | | | | Stability RSD / % | |
|---|---|---|---|---|---|---|
| | Intra -day | | Inter -day | | | |
| | A | RT | A | RT | A | RT |
| S | 0.68 | 0.22 | 1.24 | 0.29 | 1.67 | 0.53 |
| C1 | 0.47 | 0.31 | 0.99 | 0.26 | 0.61 | 0.87 |
| C2 | 0.51 | 0.27 | 0.99 | 0.29 | 1.57 | 0.96 |
| C3 | 0.70 | 0.27 | 1.21 | 0.33 | 3.24 | 0.94 |
| C4 | 0.68 | 0.24 | 1.60 | 0.18 | 3.10 | 1.13 |
| C5 | 0.57 | 0.21 | 1.01 | 0.16 | 0.98 | 1.01 |
| C6 | 0.46 | 0.21 | 0.95 | 0.16 | 0.99 | 1.04 |

### *3.2.3 Repeatability and accuracy*

Six independent analytical sample solutions from gamboge SG1 were prepared as described in section 2.3.2. They were analyzed under chromatographic conditions defined in section 2.4. The contents and RSDs of seven components were obtained using two methods: SSDMC and external standard (ES) methods. The *P* values were calculated using paired *t*-test between the results of two methods. Reproducible results were shown in Table 3. The RSDs of the results calculated using SSDMC method, in comparison with ES method, were in the ranges of 0.71% to 2.66% and 0.72% to 2.76%, respectively, which demonstrated that the methods were reproducible. The results were consentaneous and without statistically significant differences between the two methods. The results obtained from the two methods showed no remarkable differences using *t*-test analysis ($P > 0.05$).

The recovery test was performed in order to evaluate the method accuracy. The mixed standards solution was added to a certain amount of SG1 samples and extracted as described in section 2.3.2. The recovery of each compound was calculated using the equation: recovery (%) = (observed amount − original amount) / spiked amount. The results calculated using the SSDMC and ES methods are listed in Table 3. The recoveries of target components all fell in the range of 95% to 105% with the RSDs less than 3%, except for the RSD of C1 (3.26%). The results obtained from the two methods showed no significant differences using *t*-test analysis ($P > 0.05$).

**Table 3 Results of repeatability and recovery tests**

| Standards | Repeatability | | | | | Accuracy | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SSDMC method | | ES method | | *P* | SSDMC method | | ES method | | *P* |
| | g / 100 g | RSD% | g / 100 g | RSD% | | Recovery / % | RSD / % | Recovery / % | RSD / % | |
| S | 8.77 | 1.69 | 8.77 | 1.69 | 1.00 | 102.79 | 1.47 | 102.79 | 1.47 | 1.00 |
| C1 | 3.49 | 1.97 | 3.52 | 2.01 | 0.49 | 101.54 | 3.26 | 99.80 | 3.26 | 0.38 |
| C2 | 0.49 | 2.22 | 0.49 | 2.30 | 0.81 | 100.80 | 2.00 | 100.58 | 1.89 | 0.85 |
| C3 | 1.14 | 0.71 | 1.15 | 0.72 | 0.50 | 105.13 | 1.71 | 103.97 | 1.65 | 0.28 |
| C4 | 1.04 | 2.66 | 1.05 | 2.76 | 0.71 | 102.17 | 2.90 | 101.48 | 2.69 | 0.69 |
| C5 | 18.05 | 1.62 | 17.99 | 1.64 | 0.76 | 102.75 | 1.18 | 102.68 | 1.17 | 0.93 |
| C6 | 14.31 | 1.64 | 14.28 | 1.68 | 0.82 | 102.19 | 0.62 | 102.25 | 0.60 | 0.87 |

#### *3.2.4 Ruggedness and robustness of RCF and RRT*

In order to introduce the SSDMC method to different laboratories, the ruggedness and robustness of RCF was further examined. Two different types of chromatographic instruments, three columns, and other operational factors were varied in order to compare the variations in RCF and RRT, as described in section 2.6. The results of these analyses are listed in Table 4. These experiments demonstrated that the values of RCF and RRT obtained using different equipment or columns were remarkably similar. The RSD values of RCFs and RRTs were all less than 2.72% and 2.94%, respectively, and demonstrated that modern HPLC instruments and columns could generally meet the analytical requirements. As far as the other operational factors were concerned, different analysts, concentration of glacial acetic acid (0.1% ± 0.01%), UV detection wavelength (360 ± 2 nm), time program of the mobile phase (20 ± 5/50 ± 5/60 ± 5 min), flow rate (1.0 ± 0.1 mL/min) and column temperature (30 ± 5 ºC) did not exert a significant influence on the RCFs and RRTs of the seven compounds.

**Table 4 Ruggedness and robustness of RCF and RRT**

| Factors | | RCFs | | | | | | RRTs | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C1 | C2 | C3 | C4 | C5 | C6 | C1 | C2 | C3 | C4 | C5 | C6 |
| | Diamonsil C8 | 0.91 | 0.86 | 1.06 | 0.78 | 0.92 | 0.92 | 0.60 | 0.72 | 0.85 | 1.15 | 1.36 | 1.42 |
| Agilent 1200 | Ultimate XB-C8 | 0.91 | 0.85 | 1.04 | 0.78 | 0.93 | 0.94 | 0.62 | 0.74 | 0.85 | 1.19 | 1.43 | 1.50 |
| | Silversil C8 | 0.92 | 0.86 | 1.04 | 0.80 | 0.94 | 0.95 | 0.60 | 0.71 | 0.85 | 1.16 | 1.38 | 1.45 |
| | Diamonsil C8 | 0.92 | 0.88 | 1.07 | 0.84 | 0.91 | 0.91 | 0.60 | 0.72 | 0.85 | 1.13 | 1.35 | 1.41 |
| Waters e2695 | Ultimate XB-C8 | 0.90 | 0.86 | 1.04 | 0.79 | 0.91 | 0.92 | 0.61 | 0.74 | 0.85 | 1.20 | 1.42 | 1.51 |
| | Silversil C8 | 0.91 | 0.87 | 1.06 | 0.81 | 0.92 | 0.93 | 0.60 | 0.72 | 0.85 | 1.16 | 1.40 | 1.47 |
| RSD / % | | 0.94 | 1.44 | 1.42 | 2.72 | 1.31 | 1.60 | 1.46 | 1.72 | 0.40 | 2.28 | 2.53 | 2.94 |
| | analyst 1 | 0.89 | 0.81 | 1.02 | 0.80 | 0.89 | 0.90 | 0.61 | 0.71 | 0.84 | 1.12 | 1.33 | 1.40 |
| Different analysts | analyst 2 | 0.91 | 0.86 | 1.06 | 0.78 | 0.92 | 0.92 | 0.60 | 0.72 | 0.85 | 1.15 | 1.36 | 1.42 |
| | analyst 3 | 0.90 | 0.84 | 1.05 | 0.82 | 0.91 | 0.91 | 0.61 | 0.73 | 0.86 | 1.14 | 1.34 | 1.40 |
| Concentration of glacial | 0.09% | 0.90 | 0.84 | 1.05 | 0.82 | 0.90 | 0.90 | 0.61 | 0.73 | 0.86 | 1.14 | 1.34 | 1.40 |
| acetic acid (± 0.01%) | 0.10% | 0.91 | 0.86 | 1.06 | 0.78 | 0.92 | 0.92 | 0.60 | 0.72 | 0.85 | 1.15 | 1.36 | 1.42 |
| | 0.11% | 0.90 | 0.84 | 1.05 | 0.82 | 0.91 | 0.91 | 0.61 | 0.73 | 0.86 | 1.14 | 1.34 | 1.40 |
| | 358 nm | 0.91 | 0.85 | 1.05 | 0.80 | 0.92 | 0.93 | 0.59 | 0.71 | 0.84 | 1.14 | 1.35 | 1.41 |
| Detection wavelength(± 2 nm) | 360 nm | 0.91 | 0.86 | 1.06 | 0.78 | 0.92 | 0.92 | 0.60 | 0.72 | 0.85 | 1.15 | 1.36 | 1.42 |
| | 362 nm | 0.90 | 0.84 | 1.04 | 0.81 | 0.92 | 0.91 | 0.60 | 0.72 | 0.85 | 1.14 | 1.36 | 1.41 |
| Time program of mobile | 15/45/55 min | 0.91 | 0.85 | 1.05 | 0.75 | 0.92 | 0.93 | 0.64 | 0.75 | 0.87 | 1.14 | 1.32 | 1.38 |
| phase (± 5 min) | 20/50/60 min | 0.91 | 0.86 | 1.06 | 0.78 | 0.92 | 0.92 | 0.60 | 0.72 | 0.85 | 1.15 | 1.36 | 1.42 |
| | 25/55/65 min | 0.91 | 0.85 | 1.05 | 0.82 | 0.92 | 0.93 | 0.58 | 0.70 | 0.84 | 1.15 | 1.37 | 1.43 |
| | 0.9 mL/min | 0.91 | 0.84 | 1.05 | 0.75 | 0.91 | 0.92 | 0.62 | 0.74 | 0.86 | 1.14 | 1.33 | 1.39 |
| Flow rate (± 0.1 mL/min) | 1.0 mL/min | 0.91 | 0.86 | 1.06 | 0.78 | 0.92 | 0.92 | 0.60 | 0.72 | 0.85 | 1.15 | 1.36 | 1.42 |
| | 1.1 mL/min | 0.91 | 0.84 | 1.05 | 0.81 | 0.92 | 0.92 | 0.59 | 0.71 | 0.85 | 1.15 | 1.37 | 1.43 |
| Column temperature | 25 ºC | 0.90 | 0.83 | 1.05 | 0.76 | 0.90 | 0.91 | 0.61 | 0.72 | 0.86 | 1.15 | 1.35 | 1.41 |
| (± 5 ºC) | 30 ºC | 0.91 | 0.86 | 1.06 | 0.78 | 0.92 | 0.92 | 0.60 | 0.72 | 0.85 | 1.15 | 1.36 | 1.42 |
| | 35 ºC | 0.90 | 0.83 | 1.05 | 0.82 | 0.91 | 0.91 | 0.61 | 0.73 | 0.86 | 1.13 | 1.34 | 1.39 |
| RSD / % | | 0.77 | 1.84 | 1.07 | 2.98 | 0.94 | 0.79 | 2.16 | 1.64 | 0.88 | 0.64 | 0.98 | 0.99 |

### *3.3 Application to gamboge samples*

Seven components were analyzed in six batches of gamboge from different habitats, as well as their processed products, using the validated SSDMC and traditional ES method. The typical chromatograms of the two groups of samples are shown in Figure 2. The number of observed peaks was not significantly different between the crude gamboge and its processed product. The quantitative results were summarized in Table 5. It was evident from Table 5 that the contents of S and C1−C6 were in the ranges of 8.56−13.19, 2.08−3.88, 0.49−0.87, 1.07−1.71, 0.47−1.24, 17.83−24.14, and 12.75−18.86 g/100 g, respectively, and that C5 and C6 (gambogic acid) were the main components in gamboge. The contents of seven components in different gamboge samples obtained by SSDMC method were consistent with that determined by the traditional ES method. Due to the difficulties and expenses associated with the preparation of all standard compounds, the application of the traditional external reference method was limited. Because the SSDMC method only required a minimum number of standard compounds and the content of components could be obtained directly using multiple conversion factors, it was an economically and environmentally friendly method for the simultaneous determination of multiple components. Therefore, it was worthwhile to establish SSDMC method and obtain the relative conversion factor values.

The contents of analyzed components showed a decreasing tendency after processing, as shown in Table 5. To compare the statistical significance between the processed and crude gamboge, the relative content was calculated and shown in Figure 3. The relative content of each component in processed

**Table 5 Contents of seven components in samples analyzed using SSDMC and ES methods** (g/100 g)

| Samples | S | C1 | | C2 | | C3 | | C4 | | C5 | | C6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ES | ES | SSDMC | ES | SSDMC | ES | SSDMC | ES | SSDMC | ES | SSDMC | ES | SSDMC |
| SG1 | 8.83 | 3.52 | 3.55 | 0.49 | 0.49 | 1.17 | 1.17 | 1.07 | 1.08 | 18.05 | 18.05 | 14.32 | 14.28 |
| SG2 | 11.04 | 2.84 | 2.84 | 0.59 | 0.59 | 1.38 | 1.38 | 0.47 | 0.45 | 19.26 | 19.24 | 15.20 | 15.14 |
| SG3 | 8.56 | 3.72 | 3.76 | 0.54 | 0.54 | 1.11 | 1.11 | 1.20 | 1.22 | 18.31 | 18.33 | 14.67 | 14.64 |
| SG4 | 8.66 | 3.76 | 3.80 | 0.52 | 0.52 | 1.07 | 1.06 | 1.24 | 1.25 | 18.47 | 18.48 | 14.82 | 14.79 |
| SG5 | 13.19 | 3.88 | 3.90 | 0.78 | 0.79 | 1.71 | 1.72 | 1.07 | 1.07 | 24.14 | 24.14 | 18.87 | 18.86 |
| SG6 | 9.22 | 2.08 | 2.06 | 0.87 | 0.88 | 1.46 | 1.47 | 0.73 | 0.73 | 17.83 | 17.83 | 12.75 | 12.68 |
| WBG1 | 7.47 | 3.03 | 3.04 | 0.36 | 0.36 | 1.01 | 1.01 | 0.92 | 0.92 | 15.33 | 15.32 | 12.18 | 12.12 |
| WBG2 | 8.07 | 2.54 | 2.54 | 0.46 | 0.46 | 1.07 | 1.07 | 0.41 | 0.39 | 14.89 | 14.87 | 12.23 | 12.16 |
| WBG3 | 7.08 | 2.82 | 2.83 | 0.39 | 0.38 | 0.93 | 0.93 | 0.95 | 0.95 | 14.93 | 14.93 | 12.35 | 12.31 |
| WBG4 | 6.09 | 2.51 | 2.52 | 0.43 | 0.43 | 0.93 | 0.93 | 1.02 | 1.03 | 15.77 | 15.82 | 12.66 | 12.64 |
| WBG5 | 9.16 | 3.16 | 3.18 | 0.57 | 0.57 | 1.25 | 1.25 | 0.95 | 0.96 | 19.09 | 19.10 | 15.14 | 15.11 |
| WBG6 | 7.10 | 1.64 | 1.61 | 0.71 | 0.71 | 1.06 | 1.06 | 0.52 | 0.51 | 15.00 | 15.00 | 11.21 | 11.14 |

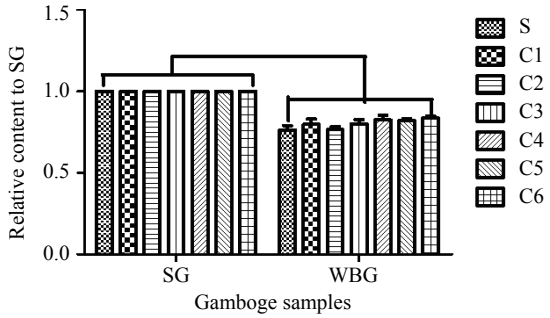


**Figure 3 Relative contents of seven components in processed gamboge (WBG) and crude gamboge (SG)**

gamboge (WBG) was equal to the ratio of each component and the other corresponding component in crude gamboge (SG). In Figure 3, compared with SG, the relative content of all seven components in WBG was markedly reduced, and this reduction was a statistically significant difference ($P < 0.01$). The result was compared for the two samples using a *t*-test based on the relative content of C1−C6 in gamboge. It was concluded that the heat-processing method could cause a reduction in the major components of gamboge.

## 4. Conclusion

In this work, the single reference standard for multiple components (SSDMC) determination method was used to determine the components of gamboge and the RCFs of gambogenic acid, β-morellic acid, 2*R*-30-hydroxygambogic acid, isogambogenic acid, gambogellic acid, 2*R*-gambogic acid, and 2*S*-gambogic acid were obtained as 1.000, 0.913, 0.864, 1.064, 0.777, 0.921, and 0.919, respectively. The RCFs can be used to determine the contents of seven components in gamboge without all standard reference compounds except gambogenic acid. The method was validated and presented a significant advance in the quality control of gamboge, which made multiple component analysis method more practical and easier to use.

The processed products were successfully analyzed and the results were compared with crude gamboge. The contents of seven components in gamboge were reduced after processing. This study focused on the major components in gamboge and its products, since it has been proven that the toxicity decreased after processing. Whether the studied components are related to the toxicity of gamboge remains to be investigated further.

### Conflict of interest statement

The authors declare no conflict of interest.

### References

Anantachoke N, Tuchinda P, Kuhakarn C, Pohmakotr M, Reutrakul V, 2012. Prenylated caged xanthones: Chemistry and biology. *Pharm Biol* 50(1): 78-91.

Chen JJ, Zhou Y, Huang XL, Yu HY, 2016. Determination of content of Shuxuening Injection based on quantitative analysis of multi-components by single marker. *Chin Tradit Herb Drugs* 47(11): 1890-1896.

Dou J, Wen HM, Yu HL, Chai C, Bian HM, 2013. Pathological study on toxicity of gamboges *in vivo* and *in vitro*. *Chin Exp Trad Med Formu J* 19(5): 279-282.

Han QB, Cheung S, Tai J, Qiao CF, Song JZ, Xu HX, 2005. Stability and cytotoxicity of gambogic acid and its derivative, gambogoic acid. *Biol Pharm Bull* 28(12): 2335-2337.

Han QB, Song JZ, Qiao CF, Xu HX, 2006. Characterization and quantitative analysis of xanthones in gamboges. *Chin J Nat Med* 4(3): 210-214.

Han QB, Xu HX, 2009. Caged Garcinia xanthones: development since 1937. *Curr Med Chem* 16(28): 3775-3796.

Hou J J, Wu WY, Da J, Yao S, Long H L, Yang Z, Cai LY, Yang M, Liu X, Jiang BH, Guo DA, 2011. Ruggedness and robustness of conversion factors in method of simultaneous determination of multi-components with single reference standard. *J Chromatogr A* 1218(33): 5618-5627.

Jang SW, Okada M, Sayeed L, Xiao G, Stein D, Jin P, Ye KQ, 2007. Gambogic amide, a selective agonist for TrkA receptor that possesses robust neurotrophic activity, prevents neuronal cell death. *Proc Natl Acad Sci USA* 104(41): 16329-16334.

Li P, Qi LW, Liu EH, Zhou JL, Wen XD, 2008a. Analysis of Chinese herbal medicines with holistic approaches and integrated

evaluation models. *Trend Anal Chem* 27(1): 66-77.

Li SL, Song JZ, Han QB, Qiao CF, Xu HX, 2008b. Improved high performance liquid chromatographic method for simultaneous determination of 12 cytotoxic caged xanthones in gamboges, a potential anticancer resin from Garcinia hanburyi. *Biomed Chromatogr* 22(6): 637-644.

Nanjing University of Traditional Chinese Medicine, 2006. *Dictionary of Chinese Traditional Medicines*. Shanghai Scientific and Technical Publishers: Shanghai.

Ou SP, Wang S, Yang QY, Chen ZH, Wu HM, Yang M, 2011. Advances in studies on dried resin of *Garcinia hanburyi* processing in toxicity reducing and efficacy enhancing effects. Chin Tradit Herb Drugs 42(12): 2560-2563.

Pharmacopoeia Committee of P. R. China, 2015. *Parmacopoeia of eople's Republic of China*. China Medical Science and Technology Press: Beijing.

Qi Q, Lu N, Li CL, Zhao J, Liu W, You QD, Guo QL, 2015. Involvement of RECK in gambogic acid induced anti-invasive effect in A549 human lung carcinoma cells. *Mol Carcinogen* 54(S1): E13-E25.

Reutrakul V, Anantachoke N, Pohmakotr M, Jaipetch T, Sophasan C, Yoosook C, 2007. Cytotoxic and anti-HIV-1 caged xanthones from the resin and fruit of arcinia hanburyi. *Planta Med* 73(1): 33-40.

Shi W, Wang ZZ, Ni FY, Li JC, Sun XL, Li Y, Xiao W, 2015. Application of QAMS method in quantitative evaluation of Liuwei Dihuang Soft Capsule. *Chin Tradit Herb Drugs* 46(19): 2880-2886.

Song JZ, YipYK, Han QB, Qiao CF, Xu HX, 2007. Rapid determination of polyprenylated xanthones in gamboges resin of Garcinia hanburyi by HPLC. *J Sep Sci* 30(3): 304-309.

Wen JL, Pei HY, Wang XH, Xie CF, Li SC, Huang L, Qiu N, Wang W, Cheng X, Chen LJ, 2014. Gambogic acid exhibits anti-psoriatic efficacy through inhibition of angiogenesis and inflammation. *J Dermatol Sci* 74(3): 242-250.

Xu M, Fu WW, Zhang BJ, Tan HS, Xiu YF, Xu HX, 2016. Combinative application of pH-zone-refining and conventional high-speed counter-current chromatography for preparative separation of caged polyprenylated xanthones from gamboge. *J Sep Sci* 39(3): 559-565.

Yang H, Cong XD, Jiang WL, Liu LX, 1999. Determination of the content of gambogic acid and its derivatives in gamboges by HPLC-ELSD. *J Chin Pharm Uni* 30(3): 202-205.

Yang TW, Zhao C, Fan Y, Qi LW, Li P, 2015. Design of ultraviolet wavelength and standard solution concentrations in relative response factors for simultaneous determination of multi components with single reference standard in herbal medicines. *J Pharm Biomed Anal* 114: 280-287.

Zhao Q, Fang Y, Pan LY, Xu M, Wang HY, Xiu YF, 2016. Attenuation mechanism of gamboges during processing based on inflammatory toxicity and AQP3, AQP4 protein and mRNA expressions in rat gastric and duodenal tissues. *China J Chin Mater Med* 41(9): 1627-1634.

Zhang X, Liu WY, Feng F, You QD, Zhang ZX, 2003. HPLC fingerprinting of gamboges. *Chin J Pharm* 34(12): 605-606.

Zhang XJ, Li X, Sun HP, Wang XJ, Zhao L, Gao Y, Liu XR, Zhang SL, Wang YY, Yang YR, Zeng S, Guo QL, You QD, 2013. *Garcinia xanthones* as orally active antitumor agents. *J Med Chem* 56(1): 276-292.

Zhou Y, Liu X, Yang J, Han QB, Song JZ, Li SL, Qiao CF, Ding LS, Xu HX, 2008. Analysis of caged xanthones from the resin of *Garcinia hanburyi* using ultra-performance liquid chromatography/ electrospray ionization quadrupole time-of-flight tandem mass spectrometry. *Anal Chim Acta* 629(1-2): 104-118.