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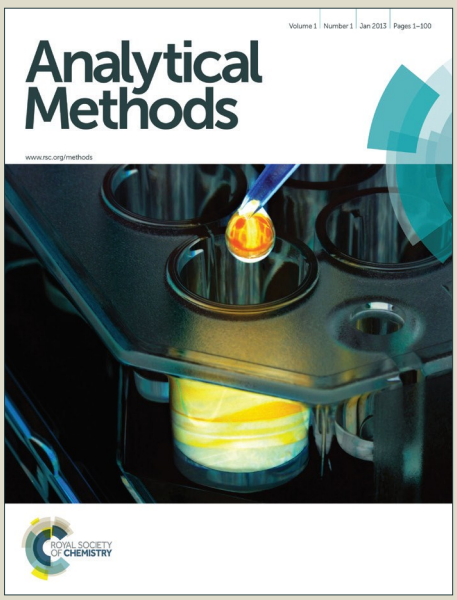

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A practical method for the simultaneous quantitative determination of twelve anthraquinone derivatives in rhubarb by a single-marker based on ultra-performance liquid chromatography and chemometrics analysis

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Abstract: Anthraquinone derivatives are the major bioactive components in rhubarb. Unfortunately, due to the lack of standard substances for quantitative analysis, the full quality control of rhubarb has been limited. This study aimed to solve the difficulty of lack of anthraquinone glycosides for quantitative analysis. A simple but very applicable analytical method for simultaneous quantification analysis of 12 anthraquinone derivatives in rhubarb by a single-marker based on ultra-performance liquid chromatography was developed. The UPLC method was performed on a Waters Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 μm) with a binary gradient mobile phase consisting of a mixture of methanol and 0.1% (v/v) aqueous phosphoric acid. Relative calibration factors of other components were calculated with emodin as internal reference substance. The new QAMS method was successfully applied to the quantitative determination of 12 anthraquinone derivatives in rhubarb with good chromatographic separation. Test result shown that the difference of anthraquinone glycosides content in these samples was obvious, quantitative analysis of anthraquinone derivatives will contribute significantly to improving the quality control of rhubarb. In conclusion, the devised technique is suitable for the quality control of rhubarb.

1. Introduction

In recent years, quality control of phytomedicines is attracting more and more attention and research. With the rapid development of high-performance liquid chromatography (HPLC), the quantitative analysis of chemical components using external standard method (ESM) has been proven to be a quite quickly way of implementing quality control of herbal medicines. However, there do existing some defects of existing ESM method, such as needs a large number of high-purity (>98.00%) chemical standard substances which are generally not available, and what's worse, these high-purity standard substances are usually very expensive. In an aim to solve the problem, a unique quantitative analysis of multi-component by a single-marker (QAMS) method has been listed officially in Chinese Pharmacopoeia towards the quality control of *Coptis Rhizoma* [1]. The subsequent results, several literatures have been reported about the application of the QAMS analysis method for the quality control of phytomedicines, such as *Angelica Sinensis* (Oliv.) Diels, *Panax notoginseng* (Burk.) F.H. Chen [2, 3], etc. Additionally, Chemometric analysis, especially HPLC fingerprint similarity analysis (SA), principal component analysis (PCA), and clustering analysis have been used as a useful approach for quality control of phytomedicines.

Rhubarb is an herb of worldwide fame. It has historically been used in clinical therapy under traditional Chinese medical theories owing to its multiple

pharmacological effects including laxative, antibacterial, and hemostatic effects [4-6]. Now, rhubarb is officially listed in the European, Chinese and Japanese Pharmacopoeia [7-9] from the following three important plant sources: the root of *Rheum tanguticum* Maxim.ex Balf, *Rheum. officinale* Baill and *Rheum. palmatum* L. Rhubarb is nowadays used in the clinical treatment of chronic renal failure, acute pancreatitis, icteric hepatitis, and cancer, under the guidance of modern medical theories [10-15]. Anthraquinone glucosides and free-anthraquinones are a series of major bioactive components in rhubarb (**Fig. 1**), several important pharmacological effects of anthraquinone derivatives include relaxing the bowels, anti-inflammatory [16], Anti-cancer cells [17], and antiplatelet [18]. Unfortunately, despite the extensive phytochemical research and some pharmacological studies on rhubarb, the full quality control of rhubarb has been limited attribute the cause to the following three main reasons. Firstly, high-purity anthraquinone derivative monomers are extremely expensive or difficult to obtain. Secondly, these pure anthraquinone derivative monomers usually are very expensive, such as the price of rhein-8-O-β-D-glucoside now sold for selling for more than $200 a milligram, resulting in a high-cost quality control. Last but not least, the existing LC-MS/MS analytical methods is really limited in practical use because of high cost and complexity of using. In fact, there are few quality control method listed in Chinese Pharmacopoeia using mass spectrometer, meanwhile lots of drug regulatory agencies are not equipped with mass spectrometer. Last but not least, good separation of anthraquinone derivatives as a complex mixture in rhubarb is very difficult to obtain by conventional HPLC method. Therefore, it is

regrettable that Chinese, European and Japanese Pharmacopoeias didn't provide a quantitative analysis method for anthraquinone glucosides. So far, although there are several reports on the analysis of anthraquinone glucosides in rhubarb by HPLC or LC-MS/MS, but these analytical methods are really limited in practical use [19-22].

In this study, we aim to solve the problems of the lack of high-purity standard substances and low resolution of existing HPLC analytical methods. To this end, a simple but own wide applicable QAMS analytical method based on ultra-performance liquid chromatography (UPLC) was developed and successfully applied to simultaneous quantification of 12 anthraquinone derivatives in *Rheum tanguticum* Maxim.ex Balf., *Rheum. palmatum* L. and *Rheum officinale* Baill.

2. Experimental

2.1. Materials and reagents

During the study, 24 batches of rhubarb raw materials (named samples Rh01–Rh24) were collected from the Qinghai, Sichuan and Gansu provinces in china. All the samples were identified as *Rheum tanguticum* Maxim.ex Balf. (Samples Rh01–Rh08), *Rheum palmatum* L. (samples Rh09–Rh16) or *Rheum. officinale* Baill.. (Samples Rh17–Rh24).

A total of 12 anthraquinone derivatives standards, including anthraquinone glycosides aloe-emodin-8-O-β-D-glucoside (**1**), rhein-8-O-β-D-glucoside (**3**), chrysophanol-8-O-β-D-glucoside (**5**), emodin-8-O-β-D-glucoside (**6**), and physcion-8-O-β-D-glucoside (**7**) as well as free anthraquinones aloe-emodin (**8**), rhein (**9**), emodin (**10**), chrysophanol (**11**), and physcion (**12**), isomers sennoside B (**2**) and

sennoside A (**4**) were supplied by Chengdu Chroma Biotechnology Company were used as chemical standard reference substances for quantitative analysis. Each of the 12 anthraquinones has a purity of more than 99.00%, as validated by HPLC-DAD. Methanol and phosphoric acid [85% (w/w) in H_2O] for quantitative analysis were of HPLC grade (Thermo Fisher Scientific, USA). High-purity water was obtained from a Millipore Milli-Q water purification system (Millipore, Synergy, USA). All other chemical reagents were purchased from Sigma-Aldrich, China.

2.2. UPLC instrumentation and conditions

Quantitative analysis was performed on a Waters Acquity ultra-performance liquid chromatograph system equipped with a binary solvent delivery pump (Waters, USA), an auto sample manager, and a photoelectric diode array detector. Data collection and integration were performed by the Empower 2 software. The chromatographic separation was performed using a Waters Acquity BEH C18 column (2.1 mm × 100 mm, 1.7μm). The mobile phase, consisting of a mixture of methanol (A) and 0.1% (v/v) aqueous phosphoric acid (B), had a flow rate of 0.20 mL/min. The binary gradient elution protocol was as follows: 0.00–5.00 min with 39–42% A, 5.01–7.00 min with 42–51% A , 7.01–12.00 min with 51–56% A, 12.01–15.00 min with 56–70% A, 15.01–17.00 min with 70–77% A, 17.01–21.00 min with 77–78% A, and 21.01–25.00 min with 78–85% A. The detector wavelength was set at 410 nm. The injection volume was 2.0 μL, and the column temperature was maintained at 30 °C.

2.3. Standard solution preparation

Each of the 12 standard substances was accurately weighed into a 50-mL brown

volumetric glass flask, dissolved in 2.00 ml of DMSO and diluted with 48.00 mL of methanol to make a stock solution of 15.00 – 60.00 μg/mL. The standard solution were stable for four weeks at 2-4 °C. Different concentrations of working solutions for UPLC analysis were diluted from this stock solution.

2.4. Sample solution preparation

Each of the 24 rhubarb samples (0.2000–0.2100 gram) was accurately weighed into a 50-mL brown glass flask volumetric with 25 ml 80% (v/v) methanol aqueous solution and was then extracted by ultrasonic treatment for 60 minutes (500 W, 40 KHz). After ultrasonic processing, the lost weight was made up using 80% methanol aqueous solution. Each sample solution was filtered through a 0.22-μm microfiltration membrane (Agilent Technologies, USA) before UPLC injection.

2.5. Validation of UPLC method

The UPLC method was rigorously validated in terms of its sensitivity, accuracy stability, and linearity according to the Chinese Pharmacopoeia of "Traditional Chinese Medicine Quality Analysis Methods Validation Guidelines" and the International Conference on Harmonization Guidelines (ICH Q2B, validation of analytical procedures, methodology) [23, 24].

2.5.1. Sensitivity of the analysis

The limit of detection (LOD) and the limit of quantification (LOQ) for 12 anthraquinone derivatives were analyzed aim to evaluate the sensitivity of the developed UPLC analytic method. The LOD and the LOQ for the 12 anthraquinone derivates were estimated by injecting a series of dilute solutions with known

concentrations, which were detected at signal/noise (S/N) ratios of approximately 3: 1 and 10: 1, respectively.

2.5.2. Linear regression equation and calibration curve of the analysis

The calibration curve was employed to assess the correlation between the peak area (y) and the injection concentration (x, μg/ml) of each anthraquinones, and the results were described by the linear regression equation (y = ax + b). Six injections were performed with concentrations from 0.15–60.00 μg/mL to obtain the absorption plots. Test solutions to evaluate the linearity were prepared by diluting the mixed standard stock solution. The linearity study was also carried out at the LOQ level.

2.5.3. Precision and stability of the analysis

Intra- and inter-day variations were used to evaluate the precision of the developed UPLC method. The relative standard deviation (RSD) was used to evaluate the variation range of the results. Intra- and inter-day repeatability was determined by six replicate analyses of sample Rh01 within one and two consecutive days, respectively. The stability of the analyzed components in sample solution was tested by analyzing sample Rh01, and the peak areas of the analyzed components at 0, 2, 4, 8, 24, and 48 h were recorded. Variations in the content were expressed as RSD values.

2.5.4. Accuracy of the analysis

The accuracy of the quantitative analysis method was evaluated based on the recovery of each standard substance when spiked into the matrix. A certain amount of

each of the 12 standard reference substances was added to sample Rh01 (0.1000–0.1100g); then, the mixtures were extracted as described in Section 2.4, and analyzed using the developed UPLC method. The recovery of each added standard substance was calculated by the following formula: recovery% = [(C_{mea} – C_{sam})/(C_{add})]×100, where C_{mea} is the measured amount of the mixture of sample Rh01; C_{sam} and C_{add} represent the mean value of the detected anthraquinone derivatives in sample Rh01 and added reference substances, respectively.

2.5.5. Robustness of the analysis

To verify the robustness of the method, the final experimental conditions were altered and the resolutions were examined. The flow rate, percentage of organic modifier, and column temperature were varied by (±) 5.0%, (±) 5.0%, and (±) 3 °C, respectively.

2.6. Sample concentration calculation

The QAMS method based on the theory as follows [25]: Within a certain linear range, it is found that there exists a directly proportional relationship between the concentration of chemical component and the response of a detector. To develop a QAMS method, an easily available and inexpensive chemical component in the herb is chosen as the internal referring substance, the relative calibration factors (RCF, *fsi*) of the other every analyzed components are calculated according to the equation (1):

$$f_{si} = \frac{f_s}{f_i} = \frac{A_s / C_s}{A_i / C_i} \qquad (1)$$

A*s* is represent the peak area of IRS, *Cs* is present the UPLC inject concentration of the IRS, A*i* is represent the peak area of the component *i*, and *Ci* is represent the

UPLC inject concentration of the component *i* in sample solution. From equation (1), we can export equation (2):

$$C_i = f_{si} \times C_s \times \frac{A_i}{A_s} \qquad (2)$$

We can calculate the concentration of each analyte component in samples using equation (2).

2.7. Data analysis

Chromatographic fingerprint analysis and similarity analysis (SA) were performed on a professional analysis software supplied by the State Food and Drug Administration (SFDA, 2004). The software was used to evaluate the similarities of chromatographic spectra. Heat map analysis and clustering analysis were performed on a professional analysis software named Heml-1.0. Principal component analysis (PCA) was performed on a software SIMCA-P 13.0 (Umetrics Corporation)

3. Results and disscusion

3.1. Optimization of sample prepation conditions

In order to obtain completely extraction, the influence factors involved in the sample extraction procedure including extraction solvent, extraction method and extraction time were optimized. A series of different concentration of methanol solutions as extraction solvents, including 60%, 70%, 80%, 90%and 100% were compared. By comparing peak areas of the 12 anthraquinone derivatives, it was found that, the peak areas of the 12 investigated components got the maximum values when using 80% methanol. Therefore, 80% methanol was preferred as the extraction solvent. After comparing two extraction methods such as ultrasonication, heating

reflux extraction, ultrasonication was found to be the better suitable extraction method. Rhubarb sample powder (0.2000-0.2100 gram) was ultrasonic extracted with 25 mL 80% methanol for 0.5 h, 1.0 h, 1.5 and 2.0 h. After comparing the peak areas of the 12 investigated components obtained using different extraction times, it was found that the anthraquinone derivatives were almost completely extracted within 1.0 h. Hence, 1.0 h was preferred as the optimal extraction time.

3.2. Validation of the UPLC analytical method

Linearity, sensitivity, precision, stability, and accuracy analyses were performed to validate the developed UPLC method. Linear regression equations of the 12 anthraquinone derivatives were obtained at six concentration levels in triplicate, as summarized in **Table 1**. The linearity was expressed in terms of the correlation coefficient (R^2). The results showed the majority of correlation between the peak areas and the concentration of UPLC injection within the acceptable range, with R^2 values ranging from 0.9990-1.0000. The calibration curves and the 95% confidence ellipses of all components are shown in supporting information (Fig. 1). The LODs and LOQs of the 12 anthraquinones are summarized in **Table 1**. The RSDs for inter- and intraday variation repeatability are summarized in supporting information (Table 1). The overall inter- and intraday variations were not more than 4.07% and 4.77%, respectively. The variations in the concentrations of the analyzed components in sample Rh01 were 0.45%-4.77%, indicating that the analyzed components in the sample solutions were stable for at least 48 h. The calculated recoveries of the 12 anthraquinone derivatives are summarized in supporting information (Table 2). The

recoveries of the investigated components ranged from 93.88% to 101.55%, and the RSDs were less than ±5.0%. Although the recoveries of sennoside A and sennoside B were not very ideal, but still within the acceptable range. Thus result indicating the good reliability and accuracy of the developed method. The test results showed satisfactory chromatographic resolutions under the conditions where the analytical parameters were varied.

3.3. Selection of internal reference standard substance

Emodin is easily available and inexpensive; moreover, it is one of the most pharmacologically active component, showing anti-microbico [26], Anti-inflammation [27], anti-tumor [28, 29]. Owing to its extensive pharmacological effects and cheap standard substance, it is the most commonly used reference standard substance for quality control of rhubarb (Chinese Pharmacopoeia 2015). In our previous investigation, it was also found that the retention time error of emodin was minimal in diverse instruments and chromatographic columns. Therefore, emodin was chosen as the internal reference substance for our study.

3.4. Calculation of the relative calibration factors

Relative calibration factors (RCFs) were calculated according to the formula (1) outlined in section of “Experimental section”. The emodin was used as internal reference substance (IRS). Relative calibration factors of 11 anthraquinone derivatives are shown in **Table 2**. The concentration of 11 anthraquinone derivatives in rhubarb were calculated according to the formula (2), respectively.

3.5. Evaluation the robustness of relative calibration factors

In order to assess the robustness of these relative calibration factors, the influence of different column temperatures and flow rates on RCFs were investigated, as shown in **Table 3**. The accuracy of RCFs were also relatively high. Therefore, QAMS is suitable for quantifying the multi-component in rhubarb, when authenticated standard substances are unavailable.

3.6. The comparison of QAMS and ESM

The developed UPLC-based QAMS analytical method was applied to the simultaneous determination of 12 anthraquinone derivatives in three plant species of rhubarbs. The amount of individual anthraquinone derivative in 24 batches of samples was calculated, the results of 10 samples are shown in **Table 4**, and the rest of 14 samples are shown in supporting information (Table 3). The typical UPLC-UV chromatograms of standard reference solution, sample solution (Rh01: *Rheum tanguticum* Maxim.ex Balf.) are shown in **Fig. 1**. In order to verify the accuracy of QAMS method, the content of individual anthraquinone derivative in samples were quantitative determined with conventional external standard method (ESM). Comparative analysis results indicated there was no significant difference between the two analytical methods, and RSDs were below 5%, as shown in **Table 4**.

3.7. Chemometric analysis

The chromatograms of 24 samples were imported into a similarity evaluation system for chromatographic fingerprint of TCM software. Analyses confirmed the presence of 16 common peaks and identified the 12 chemical compounds through the reference substances. The chromatographic fingerprint profiles showed abundant

diversity of chemical components in three different kinds of rhubarb, as shown in **Fig 2.** Similarities of each chromatogram from the 24 samples were calculated with the reference fingerprint, which was the median of all chromatograms. Similarities of *Rheum tanguticum* Maxim.ex Balf. samples (Rh01-Rh08) ranged from 0.831 to 0.876, *Rheum. palmatum L.* samples (Rh09-Rh16) ranged from 0.806 to 0.857, while chromatograms of *Rheum. officinale* Baill samples (Rh17-Rh24) ranged from 0.903 to 0.915. Test results implied that the chemical components in three species of rhubarbs differ greatly. With content difference of 5 free anthraquinone derivatives as measures, adopting the hierarchical clustering method attempt to classify these different rhubarb samples, the result shown that it's almost impossible to distinguish these rhubarb samples. Further, with content difference of 12 anthraquinone derivatives as measures, the hierarchical clustering method could distinguish between these different rhubarb samples, but this classification is not very obvious. Considering the contribution rate of bioactive components, with content difference of 5 free anthraquinone derivatives as measures, adopting the principal component analysis (PCA) method try to classify those different rhubarb samples, and the score plot result shown that it was hard to distinguish between these different rhubarb samples. On the contrary, with content difference of 12 anthraquinone derivatives as measures, the PCA score plot could easily distinguished the boundary among these three categories of rhubarbs. It is implied that quantitative analysis of 12 anthraquinone derivatives is important for improving the quality control of rhubarb. The detailed results of the chemometric analysis are shown in **Fig. 3**.

4. Conclusions

Rhubarb from three plant species including *Rheum tanguticum* Maxim.ex Balf., *Rheum. palmatum* L., and *Rheum officinale* Baill. As is known to all, there do exist a really significantly quality attributes differences among three plant species. Therefore, quality control is crucial to ensure the efficacy and safety of widely used rhubarb. Unfortunately, the existing analysis methods are complicated and high cost. Aim at solving the problem of lack of anthraquinone glycoside standard substances and low chromatographic resolution for quantitative analysis, this study has provided a simple but possess wide applicability QAMS analytical method for simultaneous quantitative analysis of 12 anthraquinone derivatives in rhubarbs. In conclusion, this study will be beneficial for a broader shift toward using approach to quality control of rhubarb, and the developed UPLC-based QAMS analytical method will be a promising tool for improving the quality control of rhubarb.

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Conflict of interests

The authors have declared no conflict of interests.

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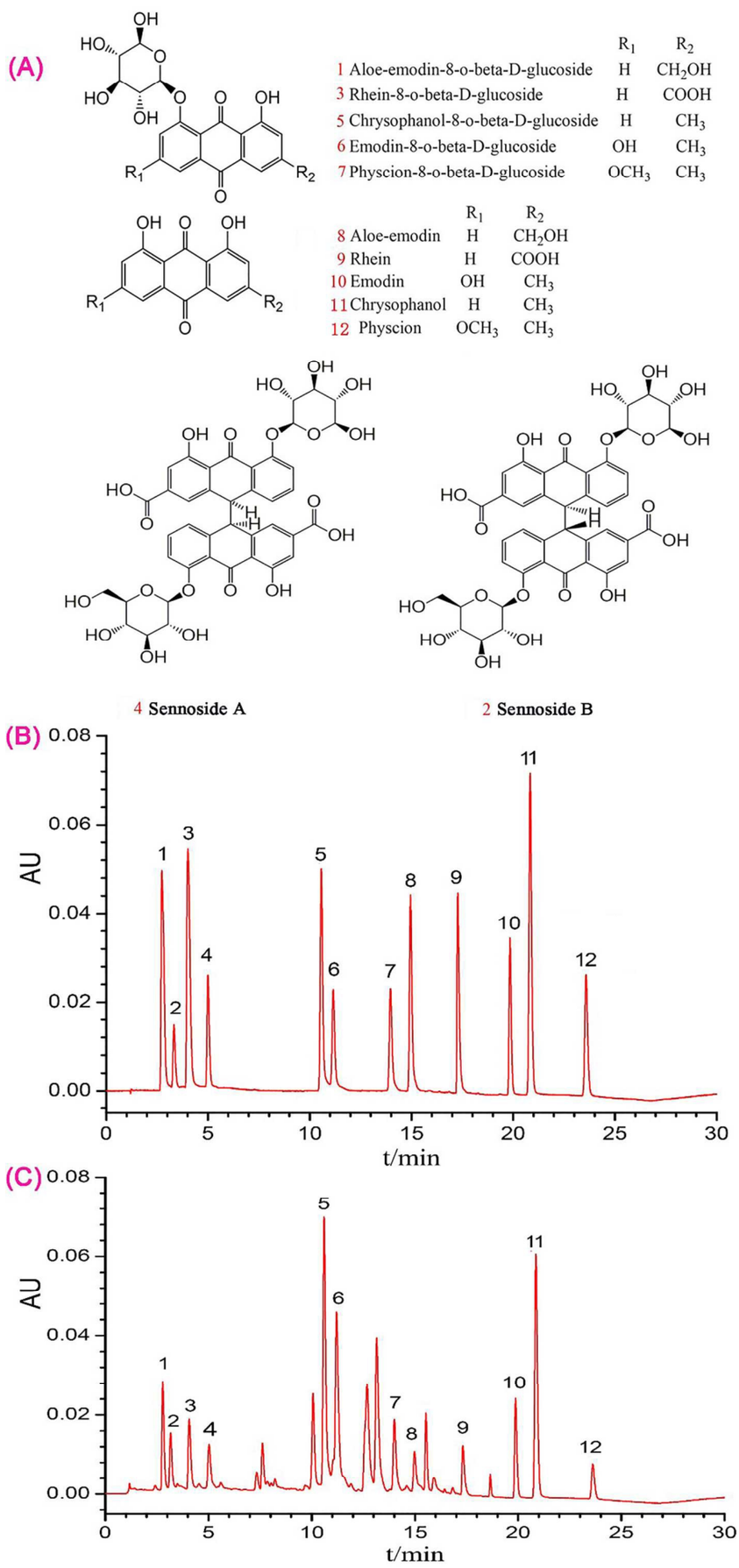

Figure 1. (A) The chemical structures of 12 anthraquinone derivatives **:** aloe-emodin-8-O-β-D-glucoside (**1**), sennoside B (**2**), rhein-8-O-β-D-glucoside (**3**), sennoside A (**4**), chrysophanol-8-O-β-D-glucoside (**5**), emodin-8-O-β-D-glucoside (**6**), and physcion-8-O-β-D-glucoside (**7**) aloe-emodin (**8**), rhein (**9**), emodin (**10**), chrysophanol (**11**), and physcion (**12**); the typical UPLC-UV chromatograms of (B) standard substance solution,
(C) sample Rh01: *Rheum tanguticum* Maxim.ex Balf.

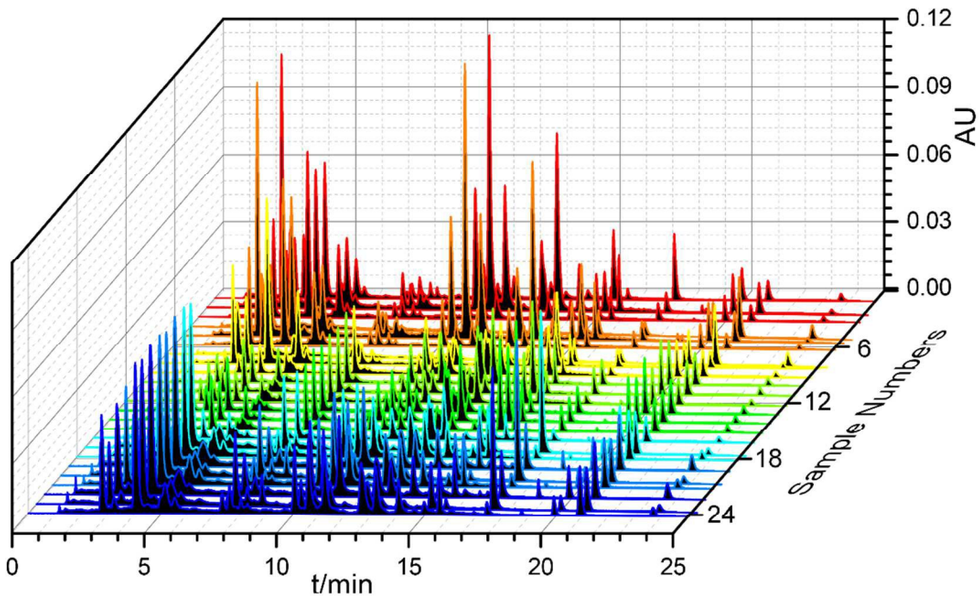

Figure 2. The chromatographic fingerprint of 24 batches of rhubarb samples

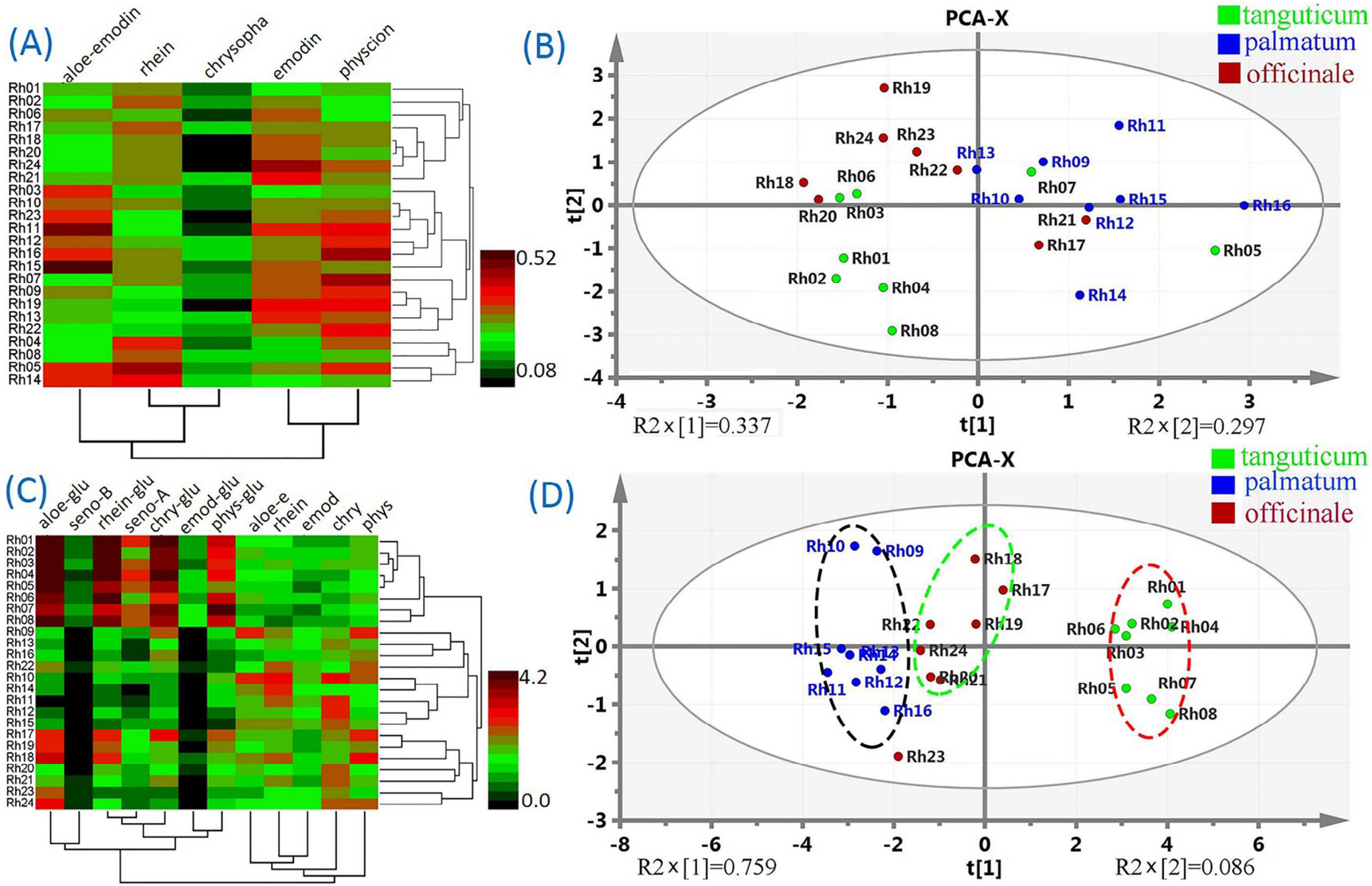

Figure 3. Chemometric figures of rhubarb samples: (A) Heat map and hierarchical clustering analysis of total content of five anthraquinones in accordance with Chinese Pharmacopoeia requirements, (B) Score plot obtained by principal components analysis in accordance with Chinese Pharmacopoeia requirements, (C) Heat map and hierarchical clustering analysis of content of 12 anthraquinones derivatives in accordance with QAMS, (D) Score plot obtained by principal components analysis in accordance with QAMS.

Table 1 Linearity and sensitivity of the UPLC analysis

Components	RT(min)	Linear regression equation	R^2	Linear range (μg/mL)	LOD [b](μg/mL)	LOQ [c](μg/mL)
Aloe-emodin-8-O-β-D-glucoside	2.73	y=5311805x-27489[a]	0.9994	2.00-50.00	0.50	2.00
Sennoside B	3.27	y=302281x+2769	0.9991	0.60-18.00	0.20	0.60
Rhein-8-O-β-D-glucoside	4.13	y=5362761x-43121	0.9992	2.40-60.00	0.60	2.40
Sennoside A	5.29	y=290745x+3017	0.9990	1.50-15.00	0.50	1.50
Chrysophanol-8-O-β-D-glucoside	10.59	y=4887728x-3665	0.9994	3.36-42.00	0.84	3.36
Emodin-8-O-β-D-glucoside	11.17	y=4934766x-6736	0.9995	3.00-20.00	1.00	3.00
Physcion-8-O-β-D-glucoside	13.99	y=5469086x-4318	0.9993	2.00-20.00	0.80	2.00
Aloe-emodin	14.96	y=9928489x-10565	0.9997	2.00-20.00	0.80	2.00
Rhein	17.30	y=8915511x-10171	0.9994	2.00-20.00	0.80	2.00
Emodin	19.85	y=7532254x-5767	0.9995	3.00-36.00	1.00	3.00
Chrysophanol	20.84	y=10073945x+517	1.0000	3.00-32.00	1.03	3.00
Physcion	23.58	y=9260815x-1532	1.0000	2.70-15.00	0.90	2.70

[a] y is peak area, x is injection concentration (μg/mL).

[b] LOD: Limit of detection (S/N=3.0).

[c] LOQ: Limit of quantification (S/N=10.0).

Table 2 RCFs of each analyte in rhubarb (mean, n=6)

RCFs[a]	Concentration Numbers						Mean	RSD (%)
	1	2	3	4	5	6		
f Aloe-emodin-8-O-β-D-glucoside /emodin	1.48	1.47	1.48	1.47	1.48	1.48	1.48	0.62
f Sennoside B /emodin	0.41	0.42	0.44	0.43	0.42	0.41	0.42	2.77
f Rhein-8-O-β-D-glucoside)/emodin	1.47	1.46	1.47	1.46	1.48	1.47	1.47	0.55
f Sennoside A /emodin	0.62	0.63	0.64	0.63	0.64	0.64	0.63	1.29
f Chrysophanol-8-O-β-D-glucoside /emodin	1.47	1.48	1.46	1.47	1.48	1.49	1.49	0.71
f Emodin-8-O-β-D-glucoside)/emodin	1.50	1.52	1.53	1.52	1.50	1.52	1.51	0.84
f Physcion-8-O-β-D-glucoside /emodin	1.28	1.29	1.28	1.27	1.28	1.29	1.28	0.59
f Aloe-emodin /emodin	0.74	0.73	0.74	0.73	0.74	0.72	0.73	1.16
f Rhein /emodin	0.84	0.83	0.84	0.82	0.83	0.85	0.83	0.94
f Chrysophanol /emodin	0.74	0.74	0.73	0.72	0.75	0.74	0.74	1.06
f Physcion /emodin	0.83	0.82	0.84	0.83	0.83	0.83	0.83	0.77

[a] RCF: Relative Calibration Factors

Table 3 Influence of different flow rates and column temperatures on RCFs (mean, n=3)

RCFs	Flow Rate (mL/min)			Column Temperature (℃)		
	0.16	0.20	0.24	27	30	33
f Aloe-emodin-8-O-β-D-glucoside /emodin	1.48	1.48	1.50	1.48	1.46	1.44
f Sennoside B /emodin	0.41	0.42	0.42	0.41	0.42	0.42
f Rhein-8-O-β-D-glucoside)/emodin	1.47	1.47	1.49	1.48	1.47	1.47
f Sennoside A /emodin	0.63	0.62	0.63	0.62	0.62	0.63
f Chrysophanol-8-O-β-D-glucoside /emodin	1.47	1.47	1.44	1.47	1.45	1.47
f Emodin-8-O-β-D-glucoside)/emodin	1.50	1.50	1.53	1.53	1.53	1.51
f Physcion-8-O-β-D-glucoside /emodin	1.28	1.28	1.31	1.31	1.30	1.33
f Aloe-emodin /emodin	0.74	0.74	0.75	0.74	0.73	0.76
f Rhein /emodin	0.84	0.84	0.84	0.84	0.83	0.85
f Chrysophanol /emodin	0.74	0.74	0.73	0.75	0.74	0.74
f Physcion /emodin	0.83	0.83	0.81	0.84	0.83	0.81

Table 4 Result comparing between external standard method (ESM) and QAMS method (mean, mg/g, n=3)

Sample	Aloe-emodin-8-O-β-D-glu			Rhein-8-O-β-D-glucoside			Chrysophanol-8-O-β-D-glu			Emodin-8-O-β-D-glucoside			Physcion-8-O-β-D-glucoside			Sennoside B		
	ESM[a]	QAMS	RSD (%)	ESM	QAMS	RSD (%)	ESM	QAMS	RSD (%)	ESM	QAMS	RSD (%)	ESM	QAMS	RSD (%)	ESM	QAMS	RSD (%)
Rh01	4.422	4.418	0.681	5.136	5.142	0.952	4.686	4.677	3.894	3.515	3.508	1.022	2.789	2.767	0.563	0.669	0.603	2.645
Rh02	4.976	4.921	1.754	4.672	4.592	4.627	3.941	3.992	2.793	3.229	3.201	2.187	1.983	1.929	1.676	0.815	0.769	4.553
Rh03	5.037	5.019	1.913	4.323	4.367	3.362	4.015	4.033	3.127	2.993	2.912	1.894	2.432	2.416	0.993	0.982	0.937	3.814
Rh04	5.372	5.366	2.684	4.597	4.602	3.721	4.632	4.617	2.021	3.267	3.229	0.776	2.885	2.817	1.127	0.591	0.533	1.649
Rh09	1.202	1.238	2.947	1.328	1.337	4.088	2.675	2.661	3.784	1.343	1.325	3.264	1.027	1.012	3.089	---	---	---
Rh10	1.087	1.104	4.183	1.203	1.215	2.446	1.128	1.133	4.902	1.875	1.867	4.808	0.884	0.901	3.215	0.241	0.203	3.711
Rh11	----[b]	----	----	0.662	0.673	3.935	1.013	1.008	4.067	1.224	1.258	1.237	0.309	0.317	4.279	0.296	0.284	2.548
Rh17	2.717	2.695	1.724	2.993	2.954	1.461	3.021	3.017	2.883	2.489	2.443	1.882	1.775	1.712	2.663	0.131	0.112	3.701
Rh18	3.448	3.417	1.694	2.671	2.695	2.874	1.752	1.803	4.895	1.967	2.023	0.781	1.329	1.318	2.767	0.254	0.208	4.882
Rh19	2.723	2.709	1.272	2.566	2.527	1.993	2.013	2.066	2.904	2.174	2.168	3.094	1.625	1.619	3.012	---	---	---
Sample	Aloe-emodin			Rhein			Chrysophanol			Emodin			Physcion			Sennoside A		
	ESMa	QAMS	RSD (%)	ESM	QAMS	RSD (%)	ESM	QAMS	RSD (%)	ESM		RSD (%)	ESM	QAMS	RSD (%)	ESM	QAMS	RSD (%)
Rh01	1.634	1.631	3.291	1.758	1.746	1.904	1.184	1.118	0.906	2.037		1.553	0.892	0.899	3.289	1.144	1.117	3.459
Rh02	1.932	1.927	3.232	1.421	1.433	2.632	1.365	1.351	3.775	1.874		2.632	1.259	1.247	2.016	1.021	0.931	4.633
Rh03	1.361	1.344	2.801	1.887	1.821	1.077	1.822	1.837	2.309	1.945		1.904	1.307	1.349	3.992	0.817	0.808	2.119
Rh04	1.537	1.568	3.089	1.729	1.698	0.898	1.043	1.012	2.447	1.768		3.077	1.095	1.068	4.089	1.268	1.215	3.064
Rh09	2.994	2.987	0.863	2.342	2.353	1.023	1.988	1.903	1.012	2.723		2.892	1.766	1.722	2.906	0.277	0.241	1.794
Rh10	2.873	2.889	2.119	2.991	3.014	4.065	3.002	3.019	2.879	2.606		0.793	1.841	1.837	3.114	0.138	0.129	4.021
Rh11	1.973	1.926	2.033	2.446	2.483	0.673	2.901	2.883	4.732	1.681		4.324	1.972	1.949	2.871	0.124	0.108	4.796
Rh17	1.884	1.845	1.754	1.965	1.917	1.347	2.317	2.364	1.319	2.824		3.905	0.996	1.014	4.893	0.433	0.415	3.274
Rh18	2.103	2.176	3.038	2.457	2.433	2.081	2.015	2.037	4.792	3.019		4.071	1.443	1.472	3.124	0.765	0.727	2.382
Rh19	1.783	1.703	4.065	2.056	2.082	1.442	1.902	1.883	3.084	2.346		2.908	1.067	1.077	4.073	0.093	0.088	1.908

ESM[a]: represent external standard method. ---[b] ＜LOQ: Limit of quantification was not reached.

This study aimed to solve the difficulty of lack of anthraquinone derivatives standards for quantitative analysis. A simple but very applicable analytical method for simultaneous quantification analysis of 12 anthraquinone derivatives in rhubarb by a single-marker based on ultra-performance liquid chromatography was developed.