

Simultaneous Determination of Four Anthraquinones in *Polygoni Multiflori Radix* with Single Reference Standard by High Performance Liquid Chromatography

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ABSTRACT

Objective: To establish a rapid, accurate and reliable analytical method for the simultaneous determination of four major anthraquinones in *Polygoni Multiflori Radix* (PMR) using single reference standard.

Methods: The four components including emodin-8-*O*- β -D- (EMG), physcion-8-*O*- β -D-glucoside, emodin and physcion were separated on an ODS C18 column within 13 min and detected at 280 nm. Emodin was selected as the reference standard, and the response factor for each analyte with respect to emodin were calculated. Robustness were also tested including different columns, equipments, temperatures, detection wavelengths, and other chromatographic conditions which might influence stability of response factors.

Results: The method was validated in terms of linearity ($r^2 > 0.9995$), LOQs (0.820–3.05 ng/mL), LODs (0.180–0.920 ng/mL), precision, accuracy (95.8–103.6%, RSD < 2.80%) and stability. A total of 40 batches of PMR were analyzed and the results were found to have no statistically significant differences compared with those obtained using the external standard method.

Conclusion: This work provided a single standard to determine multi-components method for quantitation of four anthraquinones in PMR, which could be applied in the quality control of this herbal drug.

Key Words: *Polygoni Multiflori Radix*, Single standard to determine multi-components method, Anthraquinone, Emodin

INTRODUCTION

Polygoni Multiflori Radix (PMR), with the common name He-Shou-Wu in Chinese, is the dried tuberous root of *Polygonum multiflorum* Thunb^[1]. It is one of the most popular herbal medicines in oriental countries for centuries due to its beneficial effects, such as tonifying the blood, strengthening the bones, blackening the hair and the treatment of hyperlipidemia^[2–4]. In clinic, it is generally used as an anti-aging agent in many herbal preparations. Recently, pharmacological effects of PMR have been well investigated. Lin *et al.* reported that PMR could reduce the contents of very low density lipoprotein and increased the expression of hepatic lipase^[5]. Guan *et al.* demonstrated that the bioactive compounds in PMR would activate tyrosinase and promote melanin biosynthesis^[6].

With the growing interests in the use of natural products, quality control for PMR becomes increasingly important. Modern phytochemical investigations revealed that PMR mainly contains stilbene glycosides, anthraquinones, phosphatides, flavones and tannins^[7]. Free anthraquinones and their glycosides were reported as the important active constituents and often used as chemical markers in the quality control of PMR^[8–9]. Free anthraquinones were determined by the direct assay in the national pharmacopoeia, while the content of their glycosides were often

calculated by subtracting the amount of free anthraquinones from total anthraquinones^[1]. Therefore, a simple, rapid and direct analysis of anthraquinone glycosides is necessary for both dietary and pharmaceutical purposes.

Single standard to determine multi-components (SSDMC) method has proven to be an economic tool for simultaneous determination of multi-components, which only required the limited number of reference standards with low cost^[10–12]. In this work, using emodin as the reference standard, a SSDMC method was established after full validation, four anthraquinones including two free anthraquinones and their glycosides were simultaneously determined. The quantification results from 40 batches of PMR samples determined with SSDMC method and external standard method were comparable, demonstrating its potent application in the quality control of PMR.

MATERIAL AND METHOD

1. Chemical and reagents

Emodin-8-*O*- β -D-glucoside (EMG), physcion-8-*O*- β -D-glucoside (PHG), emodin (EM), and physcion (PH) were purchased from Must Biological Technology Co. Ltd (Chengdu, China). The purity of each compound was determined to be higher than 98% by HPLC. Their structures were shown in Figure 1. HPLC grade of acetonitrile and formic acid with a purity of 98% were purchased from ROE Scientific Inc (Newark, New Castle, USA).

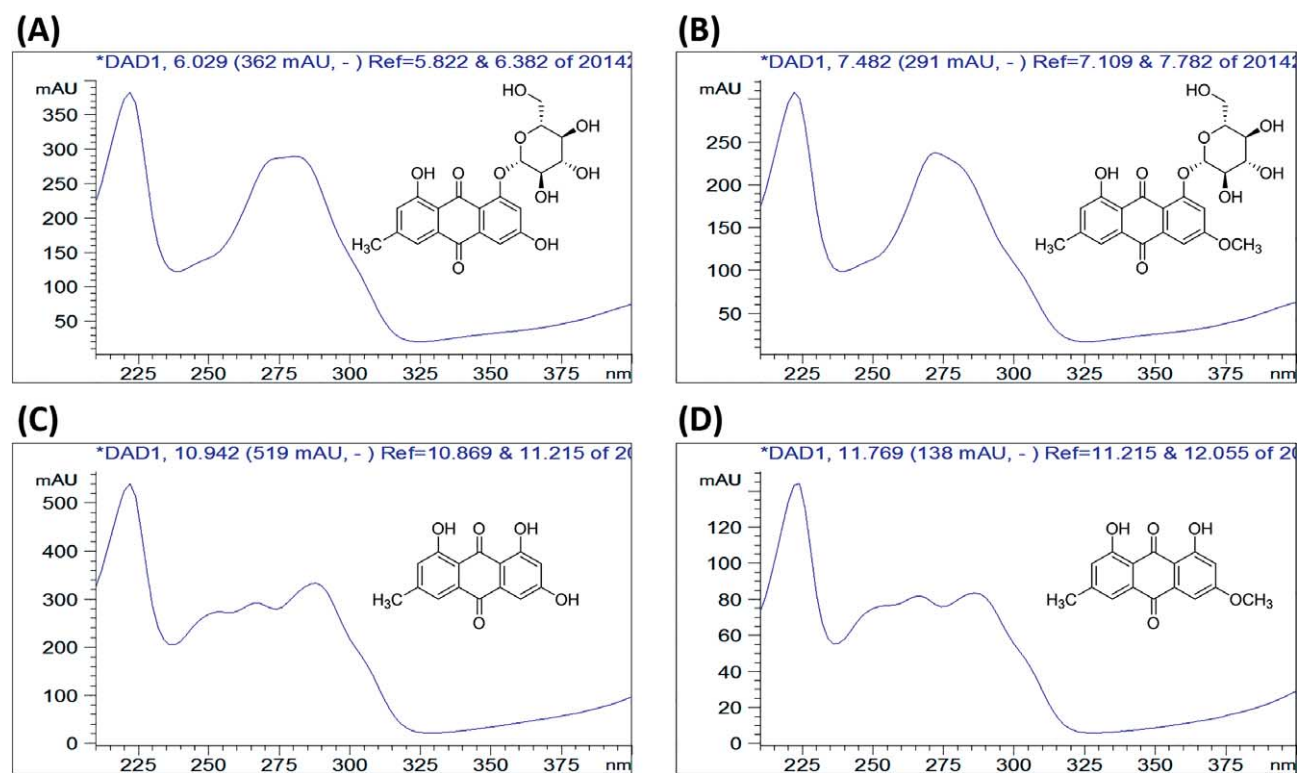


Figure 1. The structures and UV spectra of four anthraquinones. (A) EMG; (B) PHG; (C) EM; (D) PH.

Fourty batches of PMR samples were collected from different provinces in China, as shown in Table S1. All the samples were authenticated by Professor Huijun Li (China Pharmaceutical University). The voucher specimens were deposited in State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, China.

2. Apparatus and chromatographic conditions

Chromatographic analysis was performed on Agilent 1290 HPLC System equipped with a quaternary pump, an online degasser, an auto-plate sampler, and a thermostatically controlled column compartment (Agilent Technologies, Santa Clara, CA, USA). Data analysis was performed using Agilent ChemStation (with LC 3D systems, version B 02.01) (Agilent Technologies, Santa Clara, CA, USA). Two other HPLC system were tested including Shimadzu LC-20A (with a quaternary pump and VWD) and Waters HPLC-PDA 2695-298 systems.

Chromatographic separation was carried out at 30 °C on a Shimadzu ODS C18 column (4.6 mm×150 mm, 5 μm). The mobile phase was a mixture of water containing 0.1% formic acid (A) and acetonitrile (B). The gradient profile was optimized as follows: 0–3 min, 30% B; 3–8 min, 30%–50% B; 8–10 min, 50%–100% B; 10–13 min, 100% B; 13–14 min, 100%–30% B; 14–19 min, 30% B. The flow rate of mobile phase was set at 1.0 mL/min. The sample injection volume was 10 μL. The DAD detector was operated at 280 nm.

3. Standard solutions and sample preparation

Accurately weight EM, PH, EMG, PHG in methanol to prepare the stock solution of mixture reference substances,

for the concentration is 0.048 mg/mL, 0.041 mg/mL, 0.079 mg/mL, 0.053 mg/mL respectively and stored at 4 °C until use. A standard working solution of the mixtures was obtained by diluting stock solutions to desired concentrations (dilution factors = 2, 4, 5, 10, 20, 50).

Transfer about 0.2 g of PMR, finely powdered and accurately weighed. Add accurately 15 mL of methanol, and weigh the filled flask with a precision of ±0.1 mg and then extracted by heating reflux for 60 min. Allow cooling, the resultant mixture was made up to the original weight with methanol. The supernatant was filtered through a 0.22 μm syringe filter and the successive filtrate was collected as the sample solution.

4. Calculation of response factors and relative retention time

The response factor of analyte X (RF_x) was obtained at 6 levels of standard solutions under the same detection conditions and calculated by equation as follows:

$$RF_x = F_x / F_s$$

In which, F_x and F_s were calculated by equation as follows:

$$F_x = r_x / C_x$$

$$F_s = r_s / C_s$$

The r_s and r_x are the peak areas of the reference standard EM and target compound X obtained by chromatographic analyses, respectively. The C_s and C_x is the concentration of

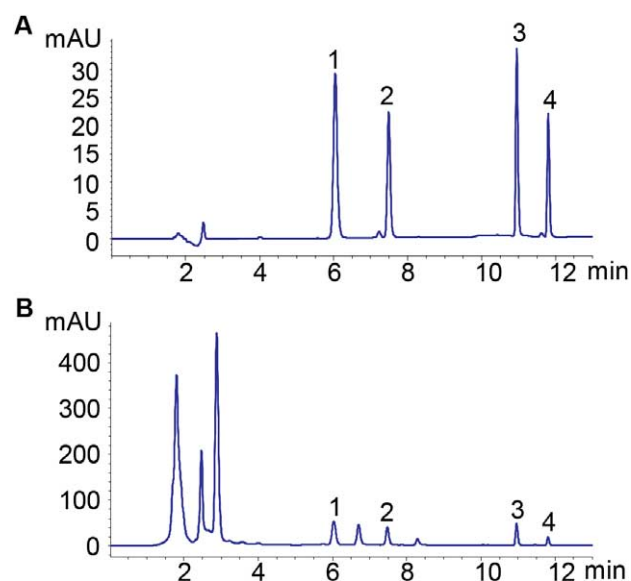


Figure 2. HPLC chromatograms of (A) four mixed reference standards and (B) PMR sample solution. 1-4: EMG, PHG, EM and PH.

EM and X, in $\mu\text{g/mL}$, respectively. Triplicate experiments were performed. The relative retention time of X was calculated as follows:

$$RRT_x = RT_x / RT_s$$

Where RT_x is the retention time of X, RT_s is the retention time of EM.

5. Validation of the SSDMC method

The SSDMC method used for the quantitation of anthraquinones in *Polygoni Multiflori Radix* was validated for linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, stability and robustness. Meanwhile, the SSDMC method was also compared with the conventional external standard method.

Table 1. Ruggedness test of response factor and relative retention time on different columns.

	Response factor			Relative retention time		
	EMG	PHG	PH	EMG	PHG	PH
Column 1	1.15	1.18	1.03	0.55	0.68	1.08
Column 2	1.16	1.17	1.04	0.56	0.67	1.09
Column 3	1.17	1.18	1.04	0.49	0.62	1.08
Column 4	1.15	1.17	1.03	0.61	0.71	1.09
Column 5	1.16	1.19	1.05	0.65	0.74	1.12
Mean	1.16	1.18	1.04	0.57	0.68	1.25
RSD (%)	0.72	0.71	0.81	10.60	6.56	1.38

EMG: emodin-8-O- β -D-glucoside, PHG: physcion-8-O- β -D-glucoside, PH: physcion. Column 1: Shimadzu Inertsil ODS-SP C18 (4.6 \times 150 mm, 5 μm), Column 2: Shimadzu Inersustain C18 (4.6 \times 150 mm, 5 μm), Column 3: Agilent Zorbax Stable Bond C18 (4.6 \times 150 mm, 5 μm), Column 4: Agilent Zorbax Stable Bond C18 (4.6 \times 250 mm, 5 μm); Column 5: Thermo Syncronis C18 (4.6 \times 250 mm, 5 μm).

Table 2. Ruggedness test of response factor and relative retention time on different equipments.

	Response factor			Relative retention time		
	EMG	PHG	PH	EMG	PHG	PH
Agilent	1.17	1.18	1.05	0.57	0.69	1.09
Shimadzu	1.15	1.17	1.03	0.48	0.69	1.09
Waters	1.15	1.16	1.04	0.55	0.68	1.09
Mean	1.15	1.16	1.04	0.53	0.69	1.09
RSD (%)	0.95	0.71	0.80	8.55	0.26	0.13

EMG: emodin-8-O- β -D-glucoside, PHG: physcion-8-O- β -D-glucoside, PH: physcion.

RESULTS AND DISCUSSION

1. Calculation of response factors and relative retention time

In this study, EM was selected as reference standard to determine three other analytes. The RFs of EMG, PHG and PH were 0.72, 0.87 and 0.94, respectively (Table S2). The RSD was less than 3%, showing that the RFs were stable at different levels of standard solutions. The RRTs of PHG, EMG, PH were 1.08, 0.55 and 0.68, respectively.

Table 3. Recovery test of emodin (EM), physcion (PH), emodin-8-O- β -D-glucoside (EMG) and physcion-8-O- β -D-glucoside (PHG).

Analyte	Level	Original (mg)	Spiked (mg)	Found (mg)	Recovery (%)	Average (%)	RSD (%)
EMG	Low	0.037	0.019	0.056	96.4	98.4	1.82
		0.037	0.019	0.056	99.0		
		0.037	0.019	0.056	99.9		
	Medium	0.039	0.039	0.077	100.0	100.8	2.22
		0.039	0.039	0.077	99.6		
		0.039	0.039	0.076	95.8		
	High	0.039	0.058	0.099	101.9	101.9	2.80
		0.040	0.058	0.097	98.2		
		0.039	0.058	0.099	101.9		
PHG	Low	0.022	0.006	0.016	102.8	102.8	2.08
		0.022	0.006	0.016	100.7		
		0.023	0.006	0.016	104.9		
	Medium	0.014	0.016	0.030	96.8	95.8	0.99
		0.014	0.016	0.030	95.0		
		0.014	0.016	0.030	95.4		
	High	0.014	0.033	0.047	100.0	99.6	0.82
		0.014	0.033	0.047	100.1		
		0.014	0.033	0.047	98.7		
EM	Low	0.071	0.032	0.104	105.0	103.6	2.24
		0.071	0.032	0.103	100.9		
		0.071	0.032	0.103	100.9		
	Medium	0.075	0.063	0.139	102.1	100.8	1.53
		0.075	0.063	0.139	101.0		
		0.075	0.063	0.138	99.1		
	High	0.076	0.095	0.175	104.1	102.4	2.08
		0.077	0.095	0.171	100.0		
		0.076	0.095	0.174	103.2		
PH	Low	0.042	0.021	0.063	100.8	100.6	0.39
		0.042	0.021	0.063	100.9		
		0.042	0.021	0.063	100.2		
	Medium	0.044	0.043	0.089	105.2	102.6	2.22
		0.044	0.043	0.088	101.8		
		0.044	0.043	0.087	100.8		
	High	0.045	0.064	0.111	103.7	101.9	2.80
		0.045	0.064	0.108	98.6		
		0.044	0.064	0.111	103.4		

2. Method validation

2.1 Specificity

The specificity was tested by comparing the consistency of the retention time between every analytes in PMR sample and the corresponding reference standards. Figure 2 showed that the four peaks in the chromatogram of PMR could all be located by the position of reference standards. Obviously, no apparent peak overlap was observed and a good resolution was achieved.

2.2 Linearity, LOQs, and LODs

Six level of mixed standard solution samples were analysed separately. The calibration curve was constructed by plotting the peak area of each compound against the concentration of each compound. All the four calibration curves showed good

linearity with correlation coefficients (r^2) no less than 0.9995, as shown in Table S3. The limits of detections (LODs) and quantifications (LOQs) under the chromatographic conditions fall in the range of 0.180–0.920 ng/mL, and 0.820–3.05 ng/mL, respectively.

2.3 Precision

The mixed standard solutions containing three different concentrations (low, medium and high) were analyzed for calculating RSD. To test the precision, the mixed solution was examined in duplicates over three consecutive days. Then the test solutions were prepared independently by 3 operators, in triplicate with the same PMR for the intermediate precision. RSD of all the analytes were within 3.0% which indicated the excellent precision (Table S4 and S5).

Table 4. Quantitative analysis of emodin (EM), physcion (PH), emodin-8-O- β -D-glucoside (EMG), physcion-8-O- β -D-glucoside (PHG) in 40 batches of *Polygoni Multiflori Radix* (n = 2, %).

No.	Origin	EM			PH		EMG		PHG	
		ESM	ESM	SSDCM	ESM	SSDCM	ESM	SSDCM	ESM	SSDCM
1	Yunnan	0.029	0.013	0.013	0.052	0.054	0.038	0.036		
2	Yunnan	0.071	0.023	0.024	0.125	0.129	0.048	0.048		
3	Yunnan	ND	ND	ND	ND	ND	ND	ND		
4	Hubei	0.056	0.023	0.024	0.202	0.205	0.069	0.068		
5	Hubei	0.045	0.023	0.023	0.088	0.090	0.024	0.025		
6	Sichuan	0.001	ND	ND	0.004	0.001	0.003	0.001		
7	Sichuan	0.104	0.034	0.035	0.279	0.287	0.095	0.094		
8	Sichuan	0.053	0.017	0.018	0.179	0.181	0.071	0.069		
9	Sichuan	0.053	0.017	0.018	0.177	0.179	0.070	0.068		
10	Sichuan	0.042	0.014	0.015	0.302	0.301	0.118	0.113		
11	Sichuan	0.061	0.015	0.016	0.272	0.275	0.080	0.078		
12	Sichuan	0.225	0.066	0.068	0.266	0.277	0.065	0.066		
13	Sichuan	0.073	0.022	0.023	0.160	0.165	0.069	0.068		
14	Guizhou	0.043	0.016	0.017	0.084	0.086	0.048	0.047		
15	Guizhou	0.000	0.001	ND	0.004	ND	0.002	ND		
16	Guizhou	0.098	0.039	0.040	0.196	0.203	0.059	0.059		
17	Guizhou	0.005	0.004	0.004	0.002	0.004	0.002	0.003		
18	Guizhou	0.103	0.049	0.050	0.088	0.093	0.040	0.040		
19	Guizhou	0.073	0.039	0.040	0.030	0.034	0.015	0.016		
20	Guizhou	0.010	0.010	0.009	0.034	0.031	0.008	0.008		
21	Guangxi	0.098	0.038	0.038	0.213	0.219	0.087	0.086		
22	Guangxi	ND	ND	ND	ND	ND	ND	ND		
23	Henan	0.238	0.079	0.080	0.079	0.084	0.012	0.013		
24	Henan	0.060	0.033	0.034	0.021	0.024	0.013	0.014		
25	Henan	0.036	0.016	0.016	0.060	0.061	0.019	0.019		
26	Henan	0.070	0.024	0.024	0.023	0.027	0.009	0.011		
27	Zhejiang	0.061	0.028	0.029	0.037	0.041	0.007	0.008		
28	Hebei	0.074	0.025	0.026	0.249	0.254	0.099	0.097		
29	Guangdong	0.013	0.008	0.008	0.051	0.048	0.028	0.025		
30	Guangdong	0.016	0.007	0.007	0.062	0.059	0.031	0.028		
31	Guangdong	0.002	0.001	0.001	0.066	0.034	0.035	0.017		
32	Guangdong	0.003	0.002	0.002	0.077	0.048	0.041	0.025		
33	Guangdong	0.063	0.028	0.029	0.096	0.099	0.053	0.052		
34	Guangdong	0.065	0.029	0.030	0.134	0.138	0.069	0.068		
35	Guangdong	0.046	0.023	0.023	0.144	0.146	0.073	0.071		
36	Hunan	0.005	0.001	0.002	0.058	0.043	0.017	0.013		
37	Hunan	0.042	0.014	0.015	0.062	0.065	0.025	0.025		
38	Hunan	0.023	0.016	0.016	0.073	0.073	0.029	0.028		
39	Shanxi	0.034	0.014	0.014	0.069	0.071	0.050	0.048		
40	Shandong	0.149	0.053	0.054	ND	ND	ND	ND		
	Average	0.059	0.023	0.025	0.111	0.114	0.044	0.043		

ESM: external standard method, SSDCM: single standard to determine multi-components method.

2.4 Robustness

2.4.1 Equipment and columns Through the standard solutions, the ruggedness of RFs and RRTs of EM, PHG, EMG and PH, were compared when using different equipment and columns. As shown in Table 1 and 2, it can be observed that RFs were little influenced by different columns and equipment (RSDs were less than 2.0%). Although analytical columns have a considerable effect on the RRT of EMG (RSD=10.60%), the order of peak location of analytes were unchanged.

2.4.2 Other chromatographic conditions The influences of other chromatographic conditions on the RFs and RRTs of EM, PHG, EMG, and PH were also evaluated. The typical factors included column temperature (25 °C, 30 °C, 35 °C), different detection wavelength (278 nm, 280 nm, 282 nm), pH value of the mobile phase (mobile phase A was set at 0.08%, 0.10%, 0.12% formic acid-water), and flow rate (0.8, 1.0, 1.2 mL/min). The results showed that RFs of EMG, PH were sensitive to the small changes of detection wavelength, however the RSD were still lower than 5%, which warned that the significance of detection wavelength calibration. Meanwhile it showed that RRT was greatly influenced because the chromatographic behaviors of EM, PHG, EMG, PH were different at different flow rate (RSD > 5.0%), and little influenced when the column temperatures (RSD < 3.0%) varied within the defined range.

2.5 Accuracy

Accuracy was calculated as the recovery by the assay of the known added amount of EM, PHG, EMG, PH, in the sample solution. Prepare sample solutions in three different amount levels (low, 50%; medium, 100%; high, 150%) and triplicate experiments at each level. The recovery for each analyte was calculated as follows: recovery (%) = 100 × (amount found – original amount)/amount spiked. The results showed that the

recoveries were in the range 95%-105% with the RSD within 3.0% (Table 3).

2.6 Repeatability and stability test

Repeatability was assessed using 9 test solutions of the same PMR sample covering three different concentration levels (high, 0.3 g; medium, 0.2 g; low, 0.1 g) and triplicate experiments at each level. Separately inject equal volumes of test solution into the chromatograph, record the chromatograms, measure the peak areas for EM, PHG, EMG, PH, then calculate the content.

The stability of sample solution was investigated. It was carried out by comparing the peak areas of EM, PHG, EMG, PH, in the chromatograph of the same sample solution, after storing at room temperature for different time (0, 2, 4, 6, 8, 12, 24 hours) and the RSD was lower than 2.0%. The results were summarized in Table S6 indicating the good stability and repeatability of the sample preparation.

3. Application to real samples

Through the above validation, the SSDMC method showed high repeatability, high stability, good sensitivity, as well as good precision. Then the proposed SSDMC method was applied to assay four anthraquinones in 40 batches of PMR. To evaluate the consistence of result obtained by SSDMC method, all the PMR samples were further determined by external standard method. As listed in Table 4, the SSDMC method could achieve accordant results compared with the external standard method for the analysis of the different PMR samples. It was observed that emodin-8-O-β-D-glucoside was the predominant compound among four analytes, followed by emodin, physcion-8-O-β-D-glucoside and physcion. In addition, various PMR samples from different geographical locations significantly differed in the content of four anthraquinones. As shown in Figure 3, it was found that the PMR from Sichuan had a higher content of anthraquinones than other origin places. Meanwhile, natural

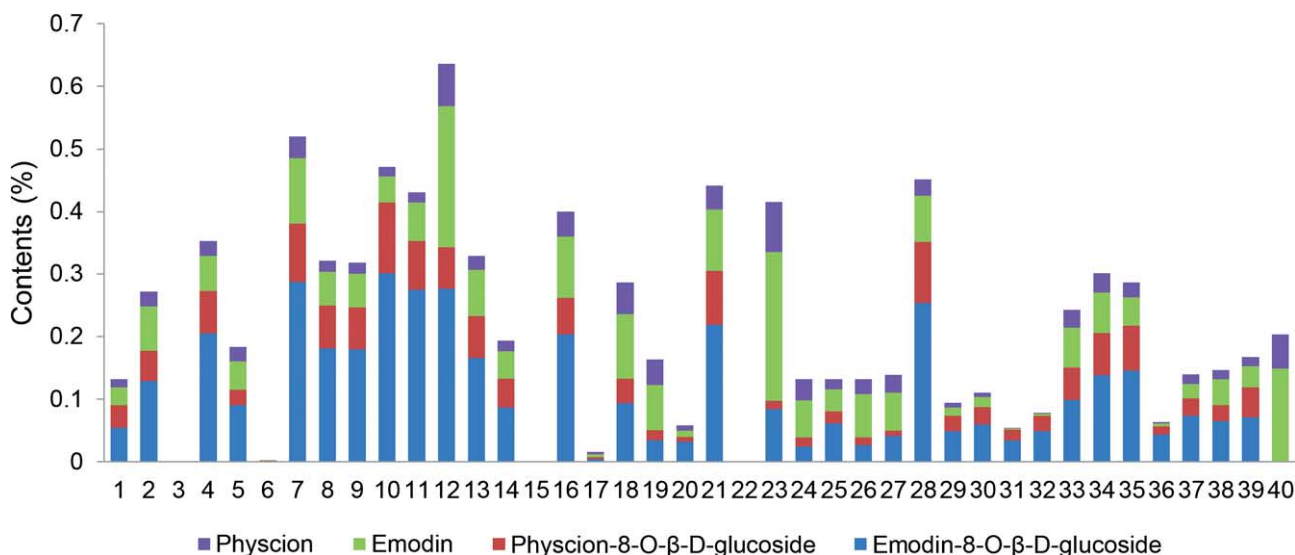


Figure 3. The contents of EM, PH, EMG and PHG in 40 batches of PMR samples determined by two approaches. 1-3, Yunnan; 4-5, Hubei; 6-13, Sichuan; 14-20, Guizhou; 21-22, Guangxi; 23-26, Henan; 27, Zhejiang; 28, Hebei; 29-35, Guangdong; 36-38, Hunan; 39, Shanxi; 40, Shandong.

variations in harvest time, climate and growth environment also related directly to content uniformity in herbal medicines. The results indicated that the direct analysis of anthraquinones is of importance for quality control of PMR.

CONCLUSIONS

In this work, SSDMC method was used for the quantitation of four anthraquinones in PMR with single standard. The results between SSDMC method and conventional external standard method had no notable differences, which proved that SSDMC method is a reliable and stable technique for quantitation of EM, PH, EMG and PHG. Forty batches of PMR samples were successfully determined and compared. A higher amount of anthraquinones was found in the samples from Sichuan province, the variability in the individual anthraquinone content in RMR from the different origin places might be ascribed to environmental factors such as climate, soil, or post-harvest processing. Because of the characteristic of convenience, succinctness, and rapidness, the established methodology- SSDMC method was suitable for routine quality assurance and standardization of the compound from the raw material and commercially available pharmaceutical preparations.

ACKNOWLEDGMENTS

This study was supported by the traditional Chinese Medicine Industry Special Scientific Research (No. 201307002), the National New Drug Innovation Major Project of China (No. 2012ZX09304005002) and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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