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Original article

Quantitative Analysis of Multi-components and Volatile Contituents in Watermelon Frost Powder by HPLC and GC

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ABSTRACT

Objective Watermelon frost powder (WFP, *Mirabilitum Praeparatum*) is a Chinese patent drug commonly used for external application, consisting of dozens of Chinese materia medica. To determine the contents of the multi-components in WFP. **Methods** Quantitative analysis of 10 principal constituents and three principal volatile constituents had been taken in this experiment to investigate the quality of *Glycyrrhizae Radix*, *Coptidis Rhizoma*, *Phellodendri Cortex*, *Rhei Radix* et *Rhizoma*, *Scutellariae Radix*, *Borneolum*, and menthol in WFP by HPLC and GC. **Results** Comparing with raw materials, the contents of liquirtin and glycyrrhetinic acid were distinct, and the contents of rhein in WFP and raw *Rhei Radix* et *Rhizoma* were different. The possible reasons might lie in the input status of raw materials in the processes of production or others. In GC test, isoborneol, the constituent in *Borneolum syntheticum*, had been detected out, and should not exist in nature *Borneolum*. **Conclusion** The inspection standard of WFP should be perfected and some weakness involved in this experiment requires further explanation and research.

Key words

GC; glycyrrhetinic acid; HPLC; liquirtin; quality control; volatile materials; watermelon frost powder

1. Introduction

Chinese materia medica (CMM, including herbs, animals, mineral, and patent drugs) is the important part of traditional Chinese medicines (TCM), which are widely applied all over the world. As a common form, powder (Feng and Zhang, 1997) is consisted of one or more CMM. Ideal degree of grinding and larger specific surface area is benefit to absorb or disperse to tissues, which is the main advantage of powder. According to *Chinese Pharmacopeia 2010* (ChP2010) and *Drug Standards* issued by the Ministry of Health, the number of powder products of CMM is over 200.

Watermelon frost powder (WFP, *Mirabilitum Praeparatum*) is a Chinese patent drug consisted of 14 kinds of CMM. In clinic, WFP can reduce swelling, alleviate pain from inflammation, and treat mouth ulcer and gingival bleeding (Huang and Hua, 1997).

Watermelon frost (derived from processed product of watermelon and glauber salts), *Phellodendri Cortex*, *Coptidis Rhizoma*, *Glycyrrhizae Radix*, *Rhei Radix* et *Rhizoma*, *Scutellariae Radix*, *Borneolum*, *Belamcandae Rhizoma*, and *Sophorae Tonkinensis Radix* are main parts of WFP. There are also volatile compounds as *Borneolum* and menthol to enhance the pharmacodynamic effects.

Berberine is an alkaloid currently used to control the quality of *Coptidis Rhizoma* and *Phellodendri Cortex*, while baicalin is commonly used to control the quality of *Scutellariae Radix* or its related products according to ChP2010.

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Contents of free anthraquinones, such as emodin, physcione, rhein, chrysophanol, and aloe-emodin (Fu et al, 2011), are the main indexes to evaluate the quality of *Rhei Radix* et *Rhizoma*. And flavonoids are the important ingredients in *Glycyrrhizae Radix* for required biological activity (Gao et al, 2009). Contents of liquirtin and ammonium glycyrrhetate are used for quality control of *Glycyrrhizae Radix*. Both *Borneolum* and menthol belong to volatile substances. *Borneolum* is classified into natural *Borneolum* and *Borneolum Syntheticum* (Zeng, 2011). And borneol is the major constituent in *Borneolum* which is always divided into two types of epimerides such as endo-configuration and exo-configuration (isoborneol) (Zhang and Chen, 1984).

According to ChP2010 and *Drug Standards* issued by the Ministry of Health, the detection methods contain TLC and HPLC for two qualitative and quantitative analyses of WFP. The contents of berberine and borneol are the only two indexes for the quantitative analyses by HPLC and GC. It is not comprehensive to evaluate the quality of WFP composed with more than 10 kinds of CMM. In this study, we selected 13 principal constituents from seven kinds of CMM in quantitative analysis by HPLC and GC, in order to investigate the quality of WFP and to improve the quality standards.

2. Materials and methods

2.1 Chemicals and materials

All the reference materials involving in this experiment were obtained from National Institute for Food and Drug Control (Beijing, China). The purities of liquiritin, ammonium glycyrrhizinate, and glycyrrhetinic acid were more than 99.8%. For emodin, physcion, chrysophanol, aloe-emodin, and rhein, the purities of chrysophanol and rhein were determined to 98.0% and 99.6%, respectively, and those of other three compounds were more than 98%. The purities of baicalin, berberine, menthol, and isoborneol were more than 99.8%, and that of borneol was determined to 99.3%.

Acetonitrile and phosphoric acid for HPLC were obtained from Fisher Scientific (USA). Methanol, cyclohexanone, and absolute ethyl alcohol of analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Water with high purity was obtained from Milipore, Milli-Q Purification System (USA). The nitrogen, hydrogen, and air used in GC experiment were all of chromatographic grade.

The products of WFP were obtained from Guilin Sanjin Pharmaceutical Co., Ltd. (Guangxi, China). *Glycyrrhizae Radix* and *Rhei Radix* et *Rhizoma* were collected and indentified by National Institute for Food and Drug Control.

2.2 Apparatus

Shimadzu LC−20A HPLC was used in this experiment with the UV Detector (Shimadzu, Japan). The HPLC system contains a quaternary solvent delivery, an on-line degasser, an auto-sampler, and an analytical workstation (LC solution) (Japan). The chromatographic column was Agilent Zorbax SB-C_{18} column (250 mm × 4.6 mm, 5 μm), obtained from Agilent Technologies (USA).

Agilent 7890A GC was used in this experiment with the Flame-ionization Detector (FID). The GC system contains an auto-sampler and an analytical workstation (Agilent Chemstation). Samples were separated on an Agilent DB-FFAP capillary column (30 m × 0.53 mm, 1.00 μm) (Agilent Technologies, USA).

A Branson SB3500 Ultrasonic Generator (150 W, 42 kHz) (China) and a Mettle-Toledo XS105 Electronic Balance (USA) were used for sample preparation.

2.3 Chromatographic conditions

2.3.1 Chromatographic conditions for HPLC

The mobile phase was mixture of acetonitrile (A) and water containing 0.1% phosphoric acid (B). The elute gradient was as following: The initial composition was 15% A, then 18% A in 10 min, 35% A in 30 min, 50% A in 40 min, finally 75% A in 70 min. The mobile flow rate was 1.0 mL/min. The UV detector was operated at 254 nm, and the column temperature was 30 ^{o}C.

2.3.2 Chromatographic conditions for GC

Gas chromatography was equipped with a FID detector and a split/splitless injector at 180 ^{o}C and operated at 250 ^{o}C; The oven temperature was as following: The initial temperature was 60 ^{o}C, programmed from 60 ^{o}C to 80 ^{o}C with a heating rate of 3 ^{o}C/min, then increased to 160 ^{o}C with a heating rate of 30 ^{o}C/min, and held for 5 min. Nitrogen was used as carrier gas, the flow rate was 4 mL/min, and the split ratio was 30:1.

2.4 Preparation of solutions

2.4.1 Preparation of standard solutions

In HPLC experiment, stock solutions of liquiritin, glycyrrhetinic acid, emodin, physcion, chrysophanol, aloe-emodin, rhein, baicalin, and berberine were separately prepared and the appropriate amounts in methanol were accurately weighed, and ammonium glycyrrhizinate was prepared and the appropriate amounts in 90% methanol were accurately weighed. Calibration reference solutions were prepared by serially diluting the stock solution. All the solutions were stored under refrigeration at 4 ^{o}C and they were filtered through 0.22 μm membrane before injected into HPLC system.

In GC experiment, cyclohexanone was accurately weighed as 1.3323 g and was dissolved into the internal standard (IS) solution with 444.10 μg/mL. The stock solutions of menthol, isoborneol, and borneol were separately prepared and dissolved with IS solution. Calibration standard solutions were prepared by serially diluting the stock solution. All the solutions were stored under the same conditions as HPLC experiment and they were filtered through 0.22 μm membrane before injection into the GC system.

2.4.2 Preparation of sample solutions

In HPLC experiment, sample solutions were prepared by accurately weighing 0.5 g products of CMM powders with *Mirabilitum Praeparatum* into 50 mL glass stopper concial flask, 40 mL methanol was added and then sonicated for 30 min. After that, the solution was filtered into 50 mL amber volumetric flask, and then diluted to volume with methanol.

Glycyrrhize Radix and *Rhei Radix* et *Rhizoma* were smashed with grinder and screened with sieve before pretreatment. The powder was accurately weighed 0.2 g into 50 mL glass stopper concial flask, 20 mL methanol was added and then sonicated for 30 min. The solution was filtered and filtrate was transferred into 25 mL amber volumetric flask, diluted to volume with methanol.

In GC experiment, sample was accurately weighed 0.2 g into 25 ml glass stopper conical flask, 20 mL IS solution was added in, and then sonicated for 20 min. The solution was filtered into 25 mL amber volumetric flask, and then diluted to volume with IS solution. All solutions were filtered through 0.22 μm membrane filters before injection into the HPLC and GC systems.

3. Results and discussion

3.1 Calibration curves

For HPLC, stock solution containing 10 reference materials accurately weighed was prepared and diluted to series appropriate concentration. Calibration curves of each compound were performed with different concentration. Diluted solutions were injected into the HPLC, spectra of peak area versus concentration of reference solutions were plotted, and then calibration curves were calculated by linear regression. Results of linearity were demonstrated in Table 1. The determination coefficient of *r*-square was calculated by means of the least-square analysis and the calibration lines were obtained. For GC, cyclohexanone was accurately weighed as IS solution. Series diluted solutions were injected into the GC, and peak area ratios of cyclohexanone and three target compounds versus concentration of reference solutions were plotted to calculate calibration curves. Results were demonstrated in Table 1. Calibration curves demonstrated the ideal linearity with excellently correlated coefficients and gave a wide calibration range for quantitative analysis by HPLC and GC.

3.2 Limits of detection and quantification

The limits of detection (LOD) and limits of quantification (LOQ) values were estimated from the decreasing concentration of reference solutions, in which the response of the LOD and LOQ was equivalent to three and ten times of background noise, respectively. Results of LOD and LOQ for HPLC under the present chromatographic conditions are shown in Table 1. The LOD values of each compound were in the range of 0.12−1.36 μg/mL, and LOQ values were in the range of 0.30−4.08 μg/mL, respectively. For GC, the LOD values of each compound were in the range of 1.17−3.06 μg/mL, and LOQ values were in the range of 5.14−9.19 μg/mL, respectively. These indicated the high sensitivity of the HPLC and GC analysis conditions.

3.3 Inter- and intra-day precisions

For HPLC and GC, the inter-day precision of the analysis was determined for six times by continuously analyzing concentration-known samples in one day. And the intra-day precision was determined once by analyzing the samples for 6 d. The results are shown in Table 2, which indicated that the RSD values of intra- and inter-day precisions were less than 0.91% and 1.02% for 10 compounds by HPLC, respectively. And then the RSD values were less than 0.55% and 0.61% by GC, respectively. Results indicated that this method was sufficiently precise for the quantitative evaluation.

3.4 Repeatability

For HPLC and GC, analytic repeatability was examined by the injection of six samples prepared with the same

Table 1 Calibration curves, LOD, and LOQ for WFP by HPLC and GC

Components	Linear ranges / (μg·mL^{-1})	Equations[a]	r^2	LOD[b] / (μg·mL^{-1})	LOQ[b] / (μg·mL^{-1})
liquirtin	27.28− 682.00	y = 6595.9x−207 83	0.999 4	1.36	4.08
ammonium glycyrrhizinate	4.08− 101.90	y = 7516.2x−5294.9	0.999 7	0.61	1.83
glycyrrhetinic acid	21.30− 532.50	y = 138 69x−415 65	0.999 1	0.62	1.86
baicalin	10.34− 516.80	y = 7151.9x−168 64	0.999 9	0.12	0.36
berberine	9.26− 462.80	y = 177 13x + 130 85	0.999 8	0.10	0.30
aloe-emodin	0.85− 21.25	y = 127 37x−207 83	0.999 6	0.28	0.85
rhein	0.77− 19.33	y = 330 93x−3482.3	0.999 7	0.13	0.40
chrysophanol	1.33− 33.28	y = 195 73x + 4854.1	0.999 6	0.30	1.00
physcion	26.62− 665.50	y = 130 53x − 207 83	0.999 8	0.25	0.75
emodin	1.84− 46.00	y = 325 59x − 2078.3	0.999 9	0.30	0.92
menthol	10.28−2056.00	y = 0.003x + 0.0109	0.999 8	1.71	5.14
borneol	10.38−2075.00	y = 0.003x + 0.0188	0.999 8	1.73	5.19
isoborneol	18.38−3676.00	y = 0.003x + 0.0099	0.999 9	3.06	9.19

[a] y and x refer to the peak areas and concentration of analytes, respectively.

[b] LOQ and LOD were defined as the concentration for which the signal-to-noise ratio was 10 and 3, respectively.

preparation procedures. And the repeatability of the solution was in the range of 1.14%–1.48% by HPLC (Table 2). Besides, the repeatability on GC was in the range of 0.74%–1.31%.

3.5 Stability

The stability of 10 compounds from WFP sample solution was analyzed by HPLC, which were made at different time as follows: 0, 2, 4, 8, 12, and 24 h. Peak areas were recorded and RSD values were less than 2% (Table 2). For GC, RSD values of stability were also less than 2%. The results indicated that the samples were stable in 24 h.

3.6 Recovery

Recovery showed the proximity between the experimental and real values. It ensured that no loss or uptake occurred during the processes. Same treatment processes were operated by HPLC and GC. Accurate amounts of references were added into WFP samples. Then the samples were processed for six times and the recoveries were calculated and analyzed. The average recoveries of 10 investigated compounds by HPLC spiked into WFP were in the range of 96.4%−99.9%, and RSD values were all less than 2% (Table 2). For GC, the average recoveries of three compounds were in the range of 92.1%−100.4%, and RSD values were also less than 2%. This method was sufficiently accurate and reliable for the quantitative analysis of target analytes.

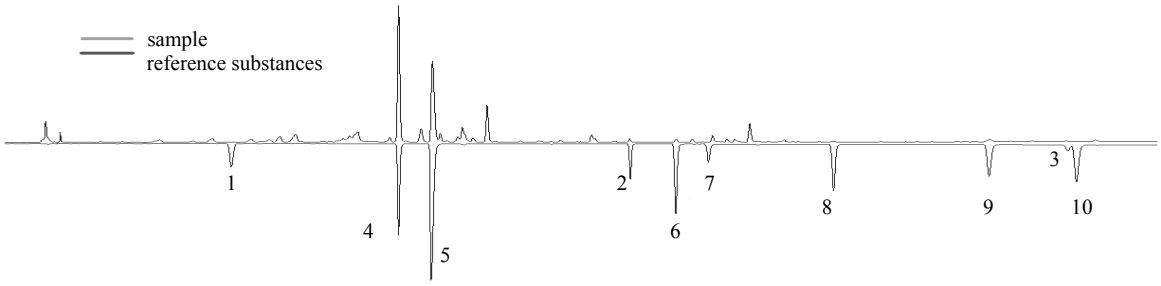
Table 2 Precision, accuracy, recovery, repeatability, and stability results for WFP by HPLC and GC

Components	RSD of inter-day precision / %	RSD of intra-day precision / %	Recoveries		Repeatabilities / %	Stabilities / %
			Ranges of recovery / %	RSD / %		
liquirtin	1.02	0.91	96.4− 99.9	1.01	1.43	1.65
ammonium glycyrrhizinate	0.91	0.49	97.9− 98.0	1.45	1.14	1.13
glycyrrhetinic acid	0.31	0.42	97.5− 99.6	0.85	1.23	1.75
baicalin	0.87	0.62	98.3− 99.2	0.12	1.25	1.98
berberine	1.01	0.66	97.9− 98.1	0.13	1.22	1.91
aloe-emodin	0.92	0.81	96.4− 99.8	0.54	1.41	1.45
rhein	0.85	0.79	97.3− 98.5	0.21	1.21	0.32
chrysophanol	0.51	0.88	98.1− 99.6	0.12	1.45	0.76
physcion	1.01	0.72	95.3− 98.6	1.65	1.48	0.91
emodin	0.77	0.21	96.8− 99.8	1.42	1.19	1.42
menthol	0.53	0.41	93.9−100.4	1.78	1.31	0.52
borneol	0.54	0.61	92.6−100.2	1.89	1.29	0.67
isoborneol	0.55	0.59	92.1− 99.6	1.66	0.74	0.87

3.7 Content determination for WFP

There were multi-components in each herb or material of WFP. Ten primary components were selected for quantitative analysis by HPLC in this study. Spectra of sample and reference solution are displayed with a sharp contrast in Figure 1 and the results of quantitative analysis are shown in Table 3. In the spectra of sample, peaks had better baseline separation, so that the quantitative analysis by HPLC was stable and reliable.

Glycyrrhizae Radix is an important herb for clinical application in TCM theories. In this study, three compounds such as glycyrrhetinic acid, ammonium glycyrrhetate, and liquiritin were chosen for the quantitative analysis. Results indicated that liquiritin and glycyrrhetinic acid had not been detected out. By contrast, the concentration of ammonium glycyrrhetate attained to (1.22 ± 0.32) mg/g in WFP. Liquiritin existed in *Glycyrrhizae Radix* naturally, and it was incomprehensible and suspectable not to be detected out. The



1: liquiritin 2: ammonium glycyrrhizinate 3: glycyrrhtinic acid 4: baicalin 5: berberine 6: aloe-emodin 7: rhein 8: emodin 9: chrysophanol 10: physcion

Figure 1 HPLC Chromatogram of sample and 10 reference substances

crude materials of *Glycyrrhizae Radix* were investigated by the same method and the results are shown in Figure 2 and Table 3. The contents of liquiritin and ammonium glycyrrhizinate were significantly higher than that of glycyrrhetinic acid in crude *Glycyrrhizae Radix*. The concentration of liquiritin and ammonium glycyrrhizinate was (12.80 ± 1.72) and (16.86 ± 2.22) mg/g, and 22.78 and 29.99 times higher than that of glycyrrhetinic acid, respectively. The concentration of glycyrrhetinic acid was (0.56 ± 0.19) mg/g. The contents of three compounds were distinct in WFP and *Glycyrrhizae Radix*, and it indicated that the quality of materials in WFP also should be inspected and worthy for attention.

Rhei Radix et *Rhizoma* is a commonly used traditional Chinese herb in clinic. Results of quantitative analysis demonstrated that the highest concentration of chrysophanol was detected as (0.51 ± 0.18) mg/g, which was 8.84 and 10.47 times higher than that of emodin and aloe-emodin. The concentration of emodin and aloe-emodin was (0.06 ± 0.01) and (0.05 ± 0.02) mg/g, respectively (Figure 3 and Table 3).

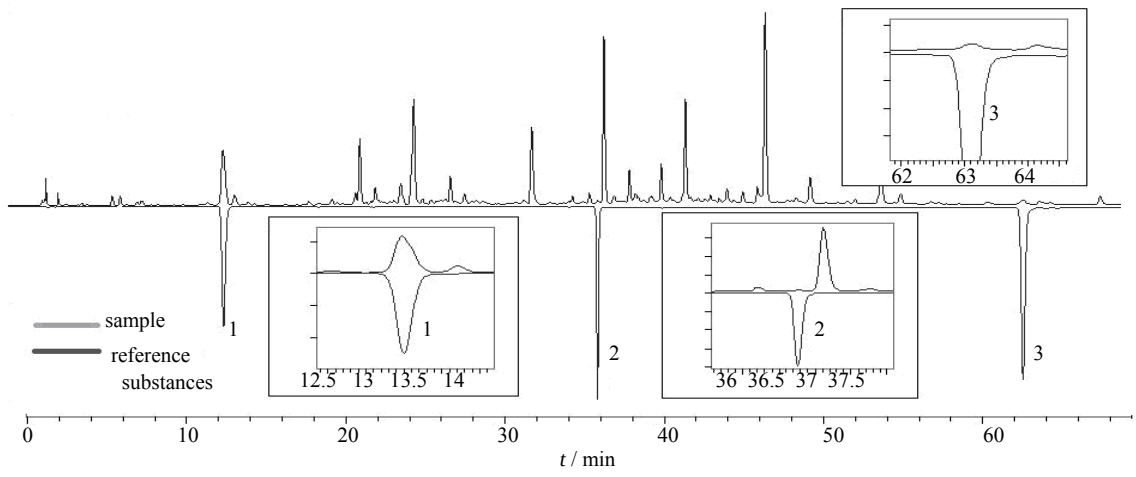
The concentration of physcione (0.20 ± 0.03) mg/g was ranked the second and only next to that of chrysophanol. Furthermore, rhein had not been detected out in WFP by HPLC. Same as *Glycyrrhizae Radix*, we investigated the status of free anthraquinones in crude *Rhei Radix* et *Rhizoma*. Chrysophanol had the highest concentration of (0.57 ± 0.10) mg/g, which was 3.04 and 5.95 times higher than those of emodin and aloe-emodin, respectively. The concentration was (0.19 ± 0.02) and (0.10 ± 0.02) mg/g for emodin and aloe-emodin. Similarly, the concentration of physcione was (0.20 ± 0.04) mg/g, also ranked the second and only next to that of chrysophanol. It was worthwhile to note that the higher concentration of rhein as (1.76 ± 0.43) μg /mL was observed in *Rhei Radix* et *Rhizoma.* So the absence of rhein in WFP needed to be researched in future due to its importance of pharmacodynamic effects.

Scutellariae Radix, *Coptidis Radix*, and *Phellodendrii Cortex* are all the important Chinese medicinal herbs applied in clinic. Results of quantitative analysis for berberine and baicalin are shown in Table 3. The concentration was (30.02 ± 2.99) and (14.44 ± 1.22) mg/g for berberine and baicalin in WFP, obviously higher than that of other components inspected in this study. The concentration of baicalin was 2.09 times higher than that of berberine. It suggested that the quality of *Scutellariae Radix* in production processes was also worthy of noting.

According to the TCM theories, mint and borneolum are both widely used in practice, with the clinical function of inducing resuscitation. The spectra and results by GC are shown in Figure 4 and Table 3. The concentration of menthol, borneol, and isoborneol was (2.41 ± 0.49), (6.06 ± 0.07), and

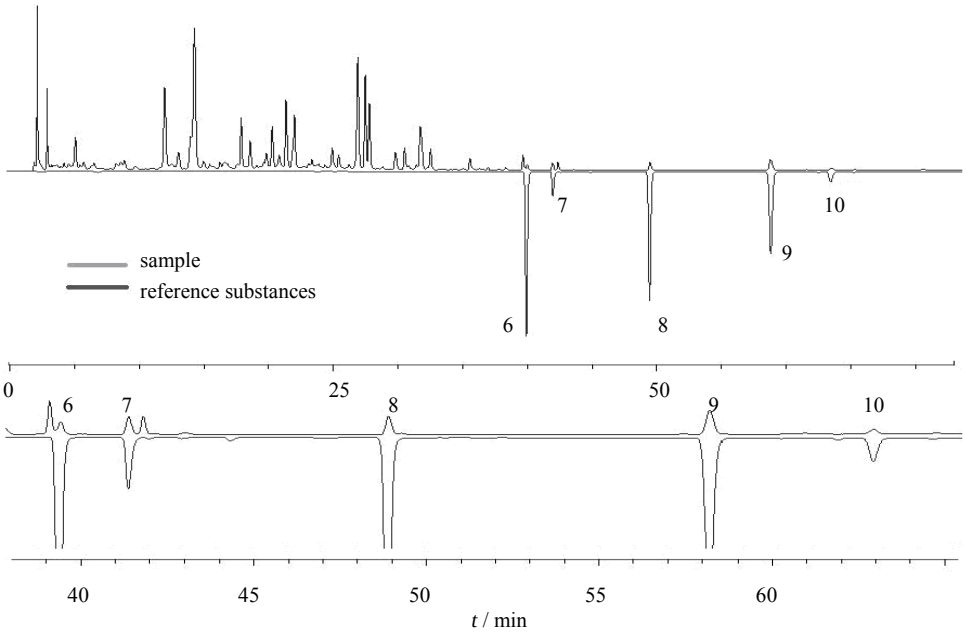
Table 3 Concentration of components in WFP and crude materials ($\bar{x} \pm s$, $n = 6$)

Drugs	Components	Contents / (mg·g^{-1})	Drugs	Components	Contents / (mg·g^{-1})
Glycyrrhizae Radix	lliquiritin	12.80 ± 1.72	WFP	aloe-emodin	0.05 ± 0.02
	ammonium glycyrrhetate	16.86 ± 2.22		emodin	0.06 ± 0.01
	glycyrrhetinic acid	0.56 ± 0.19		physcione	0.19 ± 0.03
Rhei Radix et *Rhizoma*	aloe-emodin	0.10 ± 0.02		chrysophanol	0.51 ± 0.18
	emodin	0.19 ± 0.02		rhein	–
	physcione	0.20 ± 0.04		baicalin	30.02 ± 2.99
	chrysophanol	0.57 ± 0.10		berberine	14.44 ± 1.22
	rhein	0.18 ± 0.04		menthol	2.41 ± 0.49
WFP	lliquiritin	–		borneol	6.06 ± 0.07
	ammonium glycyrrhetate	1.22 ± 0.32		isoborneol	3.55 ± 0.23
	glycyrrhetinic acid	–			



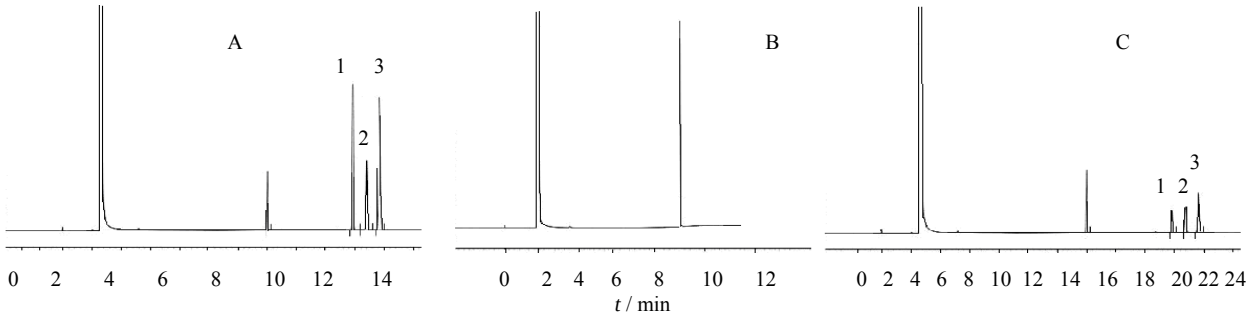
1: liquirtin 2: ammonium glycyrrhizinate 3: glycyrrhtinic acid

Figure 2 HPLC Chromatogram of three components in *Glycyrrhizae Radix*



6: aloe-emodin 7: rhein 8: emodin 9: chrysophanol 10: physcion

Figure 3 HPLC Chromatogram of five free anthraquinones in crude *Rhei Radix* et *Rhizoma*



1: menthol 2: isoborneol 3: borneol

Figure 4 GC of menthol, borneol, and isoborneol as standard reference substances in WFP (A), blank control (B), and sample (C)

(3.55 ± 0.23) mg/g, respectively. Results showed that the content of isoborneol was 58.6% of that of borneol. In ChP2010, the standard of quality control of WFP only referred to quantitative analysis of borneol. The prescription did not define natural and synthetic borneolum, so that the potential risks of application for synthetic borneolum might be ignored. So the application of natural and synthetic borneolum should be paid more attention to in the future.

4. Conclusion

As this study of WFP, according to ChP2010, berberine and borneol are the only two indexes for quality control, and not enough for this production consisting of dozens of herbs and minerals. The components in *Glycyrrhizae Radix*, *Rhei Radix* et *Rhizoma*, *Scutellariae Radix*, and some volatile constituents are investigated in recent study and the methods by both HPLC and GC achieved the requirements. But some problems also need to be further researched, which makes the inspection standards more reasonable in the future.

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