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Determination of liquiritin, glycyrrhizin, hesperidin, cinnamic acid, cinnamaldehyde, honokiol and magnolol in the traditional Chinese medicinal preparation Wuu-Ji-San by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method for the determination of liquiritin, glycyrrhizin, hesperidin, cinnamic acid, cinnamaldehyde, honokiol and magnolol in a traditional Chinese medicinal preparation, Wuu-Ji-San, which contains Glycyrrhizae Radix, Citri Leiocarpae Exocarpium, Aurantii Fructus, Cinniamomi Ramulus and Magnoliae Cortex, was established. The samples were separated with a Cosmosil $5C_{18}$-AR column by linear gradient elution using 0.03% (v/v) phosphoric acid–acetonitrile (0 min, 95:5; 52 min, 30:70) as the mobile phase at a flow-rate of 1.0 ml/min and the eluate was detected at 254 nm. *n*-Propyl benzoate was used as the internal standard and seven regression equations showing linear relationships between the peak-area ratios of each marker to *n*-propyl benzoate and concentration were obtained. The recoveries of the markers listed above were 84.77, 87.07, 79.81, 72.71, 72.72, 77.15 and 73.74%, respectively. The relative standard deviations were less than 5% ($n = 5$). Very satisfactory and reproducible results were obtained within 52 min for the simultaneous determination of the seven markers. Different processes such as concentration by reduced-pressure evaporation, freeze-drying and spray-drying were studied with regard to their effects on the marker contents. There were only minor effects on most of the markers except cinnamic acid and cinnamaldehyde. Three commercial concentrated products of Wuu-Ji-San were also analysed. The contents of the marker substances in the commercial preparations were different from those in a standard decoction.

1. Introduction

Quantitative studies on the constituents of Chinese medicinal preparations generally started from the examination of a single herb component and then proceeded with the analysis of traditional prescriptions containing that specific constituent. As a result, most analytical work on Chinese medicine is confined to a single herb, e.g., the determination of glycyrrhizin and liquiritin in Glycyrrhizae Radix [1–3], hesperidin in Citrus Pericarpium [4,5] and magnolol and honokiol in Magnoliae Cortex [6].

In Taiwan, the Department of Health will request that all concentrated herbal preparations submitted for registration should include the determination of at least two chemical constituents as markers after 1995. Further, in order to promote the Good Manufacture Practice (GMP) of Chinese medicinal preparations, our

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aim was to develop simple and expedient analytical methods for routine quality control.

In recent years, we have established many analytical methods for Chinese medicinal preparations, some of which have been published in this journal [7–9]. In this study, we selected a Chinese medicinal preparation, Wuu-Ji-San, and employed HPLC to determine simultaneously the contents of seven marker substances. During the search for the optimum conditions, we found that using an aqueous acid–acetonitrile eluent is a feasible way to perform the analysis. *n*-Propyl benzoate was used as the internal standard and there was no interference at the same retention time.

2. Experimental

2.1. Materials

According to Ref. [10], the materials used to prepare Wuu-Ji-San are Poria, Atractylodis Rhizoma, Pinelliae Tuber, Citri Leiocarpae Exocarpium and Atractylodis Lanceae Rhizoma (2.0 g each) and Paeoniae Radix, Cnidii Rhizoma, Angelicae Radix, Magnoliae Cortex, Angelicae Dahuricae Radix, Aurantii Fructus, Platycodi Radix, Cinniamomi Ramulus, Zingiberis Siccatum Rhizoma, Ephedrae Herba, Zizyphi Fructus, Glycyrrhizae Radix and Zingiberis Rhizoma (1.2 g each). Each material was obtained from the market and pulverized (8 mesh). For concentrated commercial products of Wuu-Ji-San, three different brands were purchased from the market.

2.2. Chemicals and reagents

The structures of the marker substances are shown in Fig. 1. Glycyrrhizin, cinnamic acid, cinnamaldehyde, honokiol and hesperidin were purchased from Nacalai Tesque (Kyoto, Japan), liquiritin, magnolol and the internal standard *n*-propyl benzoate from Wako (Osaka, Japan), acetonitrile and methanol (HPLC grade) from Labscan (Dublin, Ireland) and phosphoric acid (analytical-reagent grade) from Kanto (Tokyo, Japan). Ultrapure distilled water with a resistivity greater than 18 MΩ was used.

2.3. Instrumentation

HPLC was conducted with a Waters Model 625 system equipped with a Waters Model 486 UV detector and a Rheodyne Model 9125-080 injector (Millipore, Boston, MA, USA). Peak areas were calculated with a Shiunn Haw computing integrator. A Cosmosil $5C_{18}$-AR (5 μm) reversed-phase column (150 × 4.6 mm I.D.) (Nacalai Tesque) was used.

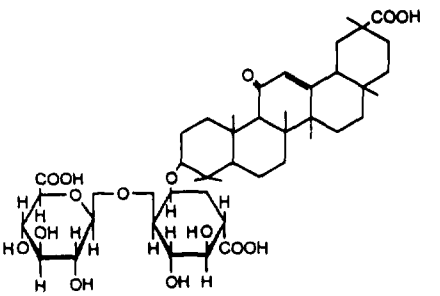
Concentration by reduced-pressure evaporation, freeze-drying and spray-drying of a standard decoction were carried out with a Rotavapor (R110/RE120/EL-13; Büchi, Flawil, Switzerland), freeze drier (Freeze Mobile 3; Virtis, Gardiner, NY, USA) and a mini spray dryer (Büchi Model 190), respectively.

2.4. Liquid chromatography

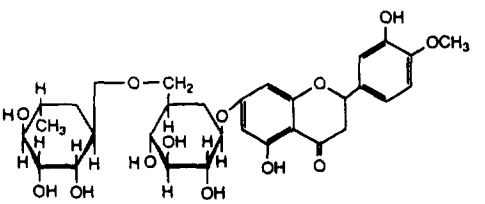
The mobile phase was a gradient of 0.03% (v/v) phosphoric acid–acetonitrile (0 min, 95:5; 52 min, 30:70), filtered through a 0.45-μm Millipore filter and degassed prior to use. The analyses were carried out at a flow-rate of 1.0 ml/min with UV detection at 254 nm.

2.5. Preparation of standard solution

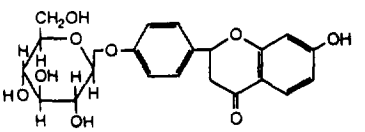
To prepare a standard solution containing liquiritin, hesperidin, cinnamic acid, cinnamaldehyde, glycyrrhizin, honokiol and magnolol, accurately weighed amounts of each compound were dissolved in 70% methanol to give serial concentrations with the ranges 8.1–36.5, 15.36–122.88, 0.476–2.14, 0.011–0.050, 10.6–47.9, 0.667–3.00 and 1.78–8.00 μg/ml, respectively. An appropriate volume of internal standard solution was then added. Calibration graphs were plotted after linear regression of the peak-area ratios with concentrations.



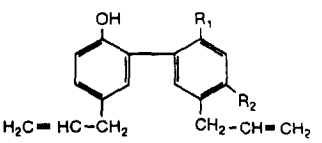
Glycyrrhizin



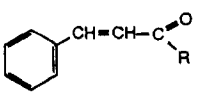
Hesperidin



Liquiritin



	R_1	R_2
Magnolol	OH	H
Honokiol	H	OH



	R
Cinnamic Acid	OH
Cinnamaldehyde	H

Fig. 1. Structures of marker substances.

2.6. *Preparation of sample solutions*

Standard decoction

Amounts of crude drugs equivalent to a daily dose of Wuu-Ji-San were weighed and pulverized, a twentyfold mass of water was added and the mixture was boiled for more than 30 min to halve the original volume. After filtration while hot, the filtrate was diluted with methanol to give a 70% methanol solution and then a suitable amount of internal standard was added to the solution to give a concentration of 0.15 μg/ml of *n*-propyl benzoate.

Interference test

Amounts of crude drugs equivalent to a daily

Table 1
Inter- and intra-day relative standard deviations ($n = 5$) for liquiritin, glycyrrhizin, hesperidin, cinnamic acid, cinnamaldehyde, magnolol and honokiol in Wuu-Ji-San

Marker substance	Concentration (μg/ml)	Peak area of marker substance/peak area of *n*-propyl benzoate		R.S.D. (%)[a]	
		Inter-day	Intra-day	Inter-day	Intra-day
Liquiritin	8.1	0.154	0.154	0.81	0.72
Hesperidin	15.36	0.086	0.086	1.79	1.78
Cinnamic acid	0.47	0.081	0.081	3.53	2.01
Cinnamaldehyde	0.01	0.748	0.747	0.35	0.42
Glycyrrhizin	10.6	0.181	0.181	1.97	2.01
Honokiol	0.67	0.048	0.048	2.15	1.29
Magnolol	1.78	0.035	0.035	4.67	4.78

[a] $n = 5$ with 95% confidence limits.

dose of Wuu-Ji-San without, one at a time, Glycyrrhizae Radix, Citri Leiocarpae Exocarpium, Cinniamomi Ramulus, Magnoliae Cortex, Citri Leiocarpae Exocarpium and Aurantii Fructus were weighed and pulverized, a twentyfold mass of water was added and the mixture was boiled for more than 30 min to halve the original

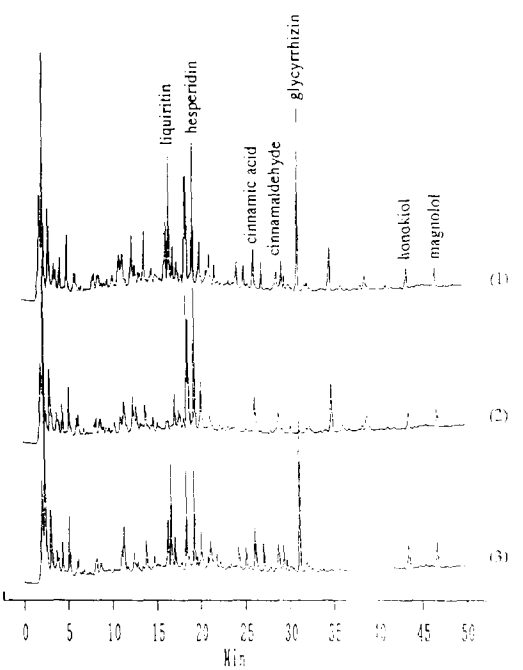


Fig. 2. Chromatograms of liquiritin, hesperidin, cinnamic acid, cinnamaldehyde, glycyrrhizin, honokiol and magnolol in Wuu-Ji-San. (1) Standard decoction; (2) standard decoction without Glycyrrhizae Radix; (3) standard decoction without Citri Leiocarpae Exocarpium.

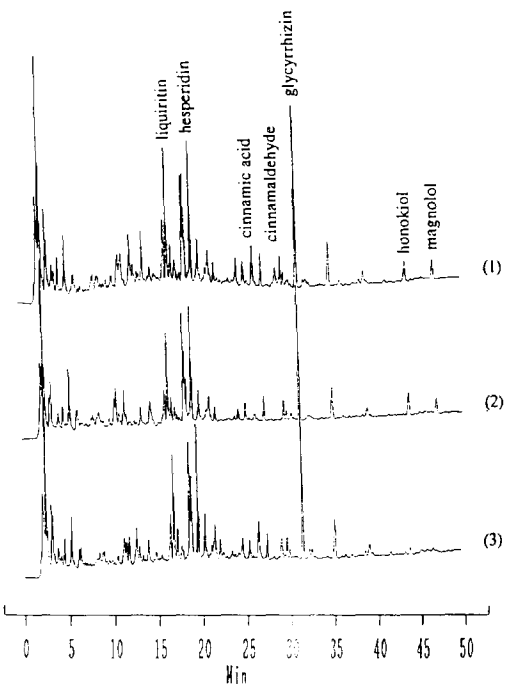


Fig. 3. Chromatograms of liquiritin, hesperidin, cinnamic acid, cinnamaldehyde, glycyrrhizin, honokiol and magnolol in Wuu-Ji-San. (1) Standard decoction; (2) standard decoction without Cinniamomi Ramulus; (3) standard decoction without Magnoliae Cortex.

Table 2
Recovery of liquiritin, glycyrrhizin, hesperidin, cinnamic acid, cinnamaldehyde, magnolol and honokiol in Wuu-Ji-San

Marker substance	Added (μg/ml)	Found (μg/ml)	Recovery (%)	Mean ± S.D.[a] (%)	R.S.D. (%)
Liquiritin	16.20	13.80	85.19	84.77 ± 3.45	3.53
	12.15	10.60	87.24		
	8.10	7.10	87.65		
	4.05	3.20	79.01		
Glycyrrhizin	21.20	18.30	86.32	87.07 ± 1.91	1.09
	15.90	13.90	87.42		
	10.60	9.10	85.85		
	5.30	4.70	88.68		
Hesperidin	15.36	12.30	80.08	79.81 ± 0.41	0.45
	30.72	24.30	79.10		
	61.44	49.20	80.08		
	122.88	98.30	79.99		
Cinnamic acid	0.95	0.70	73.61	72.71 ± 7.55	8.99
	0.71	0.50	70.13		
	0.48	0.30	63.08		
	0.24	0.19	84.03		
Cinnamaldehyde	0.022	0.015	68.18	72.72 ± 3.21	3.83
	0.017	0.012	72.72		
	0.011	0.009	77.27		
	0.006	0.004	72.72		
Honokiol	1.33	1.07	80.21	77.15 ± 2.98	3.35
	1.00	0.80	79.96		
	0.67	0.49	73.46		
	0.33	0.25	74.96		
Magnolol	3.56	2.70	75.84	73.74 ± 4.15	4.88
	2.67	2.10	78.65		
	1.78	1.30	73.03		
	0.89	0.60	67.42		

[a] $n = 5$ with 95% confidence limits.

volume. After filtration while hot, the filtrate was diluted with methanol to give a 70% methanol solution.

Contents of liquiritin, glycyrrhizin, hesperidin, cinnamic acid, cinnamaldehyde, honokiol and magnolol in crude drug

Amounts of individual crude drugs equivalent to a daily dose of Wuu-Ji-San was weighed and pulverized, a twentyfold mass of water was added and the mixture was boiled for more than 30 min to halve the original volume. After filtration while hot, the filtrate was diluted with methanol to give a 70% methanol solution and then a suitable amount of internal standard was added to the solution to give a concentration of 0.15 μg/ml of *n*-propyl benzoate.

Concentrated products of standard decoction

Concentration of a standard decoction by reduced-pressure evaporation, freeze-drying and spray-drying was carried out. The residues obtained were dissolved in a suitable amount of 70% methanol and internal standard was then added to give a concentration of 0.15 μg/ml of *n*-propyl benzoate.

Concentrated herbal preparations from market

An amount of the concentrated herbal preparation equivalent to a daily dose was weighed accurately and extracted with a twentyfold mass of water for 30 min in an ultrasonic bath. After extraction, the samples were filtered and diluted with methanol to give a 70% methanol solution and internal standard was then added to give a concentration of 0.15 μg/ml of *n*-propyl benzoate.

2.7. Solutions for recovery study

An appropriate amount of the concentrated herbal preparation from the market was weighed accurately and extracted with 70% methanol for 20 min in an ultrasonic bath, the filtrate was divided into five portions (one as a control group), each portion (except the control) was spiked with different concentrations of standard solution to add various concentrations of liquiritin (16.20, 12.15, 8.10, 4.05 μg/ml), glycyrrhizin (21.20, 15.90, 10.60, 5.30 μg/ml), hesperidin (15.36, 30.72, 61.44, 122.88 μg/ml), cinnamic acid (0.95, 0.71, 0.48, 0.24 μg/ml), cinnamaldehyde (0.022, 0.017, 0.011, 0.006 μg/ml), honokiol (1.33, 1.00, 0.67, 0.33 μg/ml) or magnolol (3.56, 2.67, 1.78, 0.89 μg/ml), and internal standard was then added to give a concentration of 0.15 μg/ml of *n*-propyl benzoate. All samples were filtered through a 0.45-μm Millipore filter and were injected for HPLC analysis to calculate the recovery.

3. Results and discussion

To check the precision of this method, we injected standard solutions of liquiritin, glycyrrhizin, hesperidin, cinnamic acid, cin-

Table 3
Contents of seven marker substances in a standard decoction and three different commercial preparations of Wuu-Ji-San

Sample	Liquiritin		Glycyrrhizin		Hesperidin	
	Mean ± S.D.[a] (mg/g)	R.S.D. (%)	Mean ± S.D.[a] (mg/g)	R.S.D. (%)	Mean ± S.D.[a] (mg/g)	R.S.D. (%)
Standard decoction	13.71 ± 0.57	3.78	21.14 ± 0.08	0.34	3.75 ± 0.16	4.02
Commercial preparation A	11.72 ± 0.39	2.91	32.86 ± 1.49	3.94	N.D.[b]	–
Commercial preparation B	N.D.[b]	–	11.59 ± 0.43	3.22	11.15 ± 0.46	3.63
Commercial preparation C	2.88 ± 0.10	3.08	7.25 ± 0.11	1.33	9.02 ± 0.34	3.34

Sample	Cinnamaldehyde		Cinnamic acid		Magnolol		Honokiol	
	Mean ± S.D.[a] (mg/g)	R.S.D. (%)	Mean ± S.D.[a] (mg/g)	R.S.D. (%)	Mean ± S.D.[a] (mg/g)	R.S.D. (%)	Mean ± S.D.[a] (mg/g)	R.S.D. (%)
Standard decoction	0.89 ± 0.05	5.24	0.69 ± 0.04	5.56	3.58 ± 0.26	6.33	0.80 ± 0.04	5.30
Commercial preparation A	10.80 ± 0.63	5.00	2.18 ± 0.16	6.39	5.21 ± 0.42	7.05	3.50 ± 0.11	2.75
Commercial preparation B	0.96 ± 0.07	6.94	0.32 ± 0.02	5.97	1.39 ± 0.11	7.31	0.31 ± 0.02	7.70
Commercial preparation C	7.60 ± 0.12	1.36	0.43 ± 0.02	4.56	3.09 ± 0.26	7.52	2.47 ± 0.07	2.66

[a] $n = 5$ with 95% confidence limits.
[b] Not determined.

namaldehyde, honokiol and magnolol at the concentrations of 8.1, 15.36, 0.47, 0.01, 10.6, 0.67 and 1.78 μg/ml, respectively, five times on the same day. The intra-day relative standard deviations (R.S.D.s) were 0.72, 1.78, 2.01, 0.42, 2.01, 1.29 and 4.78%, respectively. The inter-day R.S.D.s obtained for a 5-day period were 0.81, 1.79, 3.53, 0.35, 1.97, 2.15 and 4.67%, respectively (Table 1). The recoveries of liquiritin, glycyrrhizin, hesperidin, cinnamic acid, cinnamaldehyde, honokiol and magnolol were 84.77, 87.07, 79.81, 72.71, 72.72, 77.15 and 73.74%, respectively (Table 2). For herbal analysis, the values mentioned above indicated acceptable precision and accuracy.

Calibration graphs for liquiritin, hesperidin, cinnamic acid, cinnamaldehyde, glycyrrhizin, honokiol and magnolol were obtained over the ranges 8.1–36.5, 15.36–122.88, 0.476–2.14, 0.011–0.050, 10.6–47.9, 0.667–3.00 and 1.78–8.00 μg/ml, respectively. The regression equations were $y = 5.165951 \cdot 10^{-2}x + 3.466 \cdot 10^{-4}$ ($r = 0.9999$) for liquiritin, $y = 5.761677x + 6.605 \cdot 10^{-2}$ ($r = 0.9998$) for glycyrrhizin, $y = 1.816894 \cdot 10^{-3}x - 5.224 \cdot 10^{-4}$ ($r = 0.9991$) for hesperidin, $y = 6.281 \cdot 10^{-2}x + 5.866181$ ($r = 0.9989$) for cinnamic acid, $y = 3.15 \cdot 10^{-7}x + 0.1493414 \cdot 10^{-4}$ ($r = 0.9999$) for cinnamaldehyde, $y = 6.246814x + 2.059 \cdot 10^{-2}$ ($r = 0.9958$) for magnolol and $y = 14.28926x - 4.032 \cdot 10^{-2}$ ($r = 0.9994$) for honokiol in Wuu-Ji-San, where y is the peak-area ratio of the marker to the internal standard and x is the concentration of the marker. These results showed good linear relationships between peak-area ratio and concentration.

To ensure the specificity and selectivity of the method, we prepared five blank decoctions for comparison. They were combinations excluding, one at a time, Glycyrrhizae Radix, Citri Leiocarpae Exocarpium, Cinniamomi Ramulus, Magnoliae Cortex, Citri Leiocarpae Exocarpium and Aurantii Fructus. The chromatograms are shown in Figs. 2–4. The retention times of the marker

Table 4
Contents of the seven marker substances in standard decoction and in products after concentration by various processes

Sample	Liquiritin		Glycyrrhizin		Hesperidin	
	Mean ± S.D.[a] (mg/g)	R.S.D. (%)	Mean ± S.D.[a] (mg/g)	R.S.D. (%)	Mean ± S.D.[a] (mg/g)	R.S.D. (%)
Standard decoction	13.71 ± 0.57	3.78	21.14 ± 0.08	0.34	3.57 ± 0.16	4.02
Concentration under reduced pressure	13.44 ± 0.76	4.92	18.78 ± 0.93	4.30	3.59 ± 0.25	6.15
Freeze-drying	13.41 ± 0.82	5.30	18.32 ± 0.77	3.68	3.59 ± 0.27	6.64
Spray-drying	13.79 ± 0.62	3.92	19.16 ± 0.88	4.00	3.56 ± 0.19	4.54

Sample	Cinnamaldehyde		Cinnamic acid		Magnolol		Honokiol	
	Mean ± S.D.[a] (μg/g)	R.S.D. (%)	Mean ± S.D.[a] (mg/g)	R.S.D. (%)	Mean ± S.D.[a] (μg/g)	R.S.D. (%)	Mean ± S.D.[a] (mg/g)	R.S.D. (%)
Standard decoction	0.89 ± 0.05	5.24	0.69 ± 0.04	5.56	3.58 ± 0.26	6.33	0.80 ± 0.04	5.30
Concentration under reduced pressure	N.D.[b]	–	N.D.[b]	–	3.25 ± 0.32	8.58	0.48 ± 0.03	6.88
Freeze-drying	N.D.[b]	–	N.D.[b]	–	3.01 ± 0.27	5.85	0.62 ± 0.04	5.85
Spray-drying	N.D.[b]	–	N.D.[b]	–	3.47 ± 0.18	4.62	0.51 ± 0.01	1.06

[a] $n = 5$ with 95% confidence limits.
[b] Not determined.

Table 5
Contents of marker substances in crude drug and turnover ratio in standard decoction of Wuu-Ji-San

Crude drug	Daily dose (g)	Marker substance	Content of marker substance in crude drug (mg/g)[a]		Theoretical content of marker substance in standard decoction (daily dose, A)	Content of marker substance in standard decoction (mg/g) (daily dose, B)	Turnover ratio [B/A (%)]
			Mean ± S.D.	R.S.D.			
Glycyrrhizae Radix	1.2	Liquiritin	37.56 ± 1.15	2.65	45.07	16.45	36.50
		Glycyrrhizin	89.21 ± 1.65	1.60	107.05	25.37	23.70
Citri Leiocarpae Exocarpium	2.0	Hesperidin	24.03 ± 1.04	3.77	76.90	11.42	14.85
Aurantii Fructus	1.2						
Cinniamomi Ramulus	1.2	Cinnamic acid	1.92 ± 0.02	1.13	2.30	0.83	36.09
		Cinnamaldehyde[b]	1.45 ± 0.02	1.41	1.74	1.07	61.49
Magnoliae Cortex	1.2	Magnolol	41.51 ± 1.58	3.30	49.81	4.30	8.63
		Honokiol	5.82 ± 0.41	6.07	6.98	0.96	13.75

[a] $n = 5$ with 95% confidence limits.
[b] μg/g.

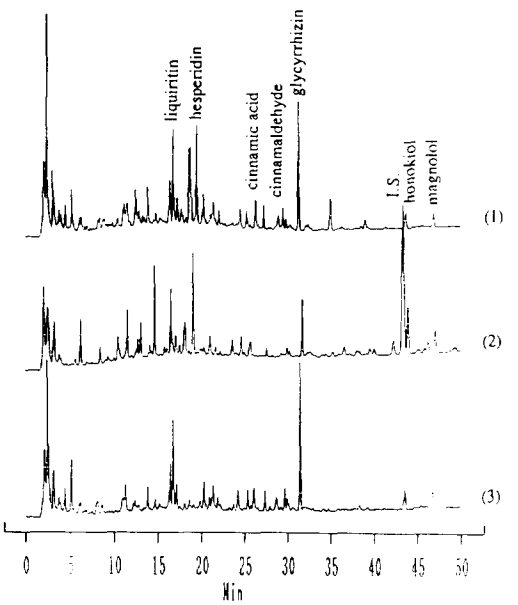


Fig. 4. Chromatograms of liquiritin, hesperidin, cinnamic acid, cinnamaldehyde, glycyrrhizin, honokiol and magnolol in Wuu-Ji-San. (1) Standard decoction; (2) commercial preparation; (3) standard decoction without Citri Leiocarpae Exocarpium and Aurantii Fructus.

substances and internal standard, i.e., liquiritin, hesperidin, cinnamic acid, cinnamaldehyde, glycyrrhizin, *n*-propyl benzoate, honokiol and magnolol, were 16, 19, 26, 29, 31, 43, 44 and 47 min, respectively. On inspection of the three-dimensional chromatograms, these eight constituents all showed good purity. There was no peak found at their retention times in blank decoctions. The three commerical preparations also showed satisfactory separations.

The contents of marker substances in commercial preparations differ greatly among each other and from those in the standard decoction, as shown in Table 3. This is probably due to the different sources of the crude drugs and different manufacturing processes. The effects of different concentration processes were also investigated using reduced-pressure evaporation, spray-drying and freeze-drying, and the results are given in Table 4. Neither cinnamaldehyde nor cinnamic acid was detected after these three kinds of concentration process. This may be due to the volatility of cinnamaldehyde and the low content of cinnamic acid. In addition, it showed only minor effects on the contents of the other five markers.

The turnover ratios of these constituents were defined as the percentage yields of these constituents detected in the Chinese medicinal preparations, calculated on the basis of their contents in the respective crude drugs. The turnover ratios of these markers vary greatly among each other (from 61.49% to 8.63%), as shown in Table 5. Wuu-Ji-San contains eighteen kinds of crude drugs, and each crude drug has been known to contain many chemical constituents. In the process of preparing decoctions, whether the molecular interactions cause poor turnover ratios is a complicated problem and needs further study.

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