STUDIES ON THE COMPONENT ANALYSIS IN TONIC WINE PREPARATION OF SHYR-CHYUAN-DAH-BUU-YAW-JYO

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A facile HPLC method for the resolution and quantitative measurement of six marker substances, the active ingredients in tonic wine preparation of Shyr-Chyuan-Dah-Buu-Yaw-Jyo, was established under the gradient elution in the reversed-phase mode. These marker substances included ferulic acid (Angelicae Sinensis Radix and Cnidii Rhizoma), paeoniflorin (Paeoniae Radix), glycyrrhizin (Glycyrrhizae Radix), cinnamic acid, cinnamaldehyde (Cinnamomi Cortex), and calycosin (Astragali Radix). Different manufacture conditions were performed to evaluate quality of Shyr-Chyuan-Dah-Buu-Yaw-Jyo.

Extracted samples were analyzed with reversed-phase column (Inertsil 5 ODS-2, 4.6 i.d. × 250 mm) at 30 °C and eluted with a mixture of 10% acetonitrile and 60% acetonitrile (each adjusted to pH 2.8 with phosphoric acid) aqueous solution in gradient manner at a flow-rate of 1.0 mL/min, and detected at 240 nm.

Relative coefficients of variations of intra- and inter-day analysis were less than 5%. All the recoveries were 92.30~115.00%. This method could be applied for the simultaneous determination of six marker substances in "Shyr-Chyuan-Dah-Buu- Yaw-Jyo".

Key words: Shyr-Chyuan-Dah-Buu-Yaw-Jyo, HPLC.

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INTRODUCTION

Since 2001, the Department of Health (DOH), Taiwan has been promoting the HPLC method for quantitative analysis of ingredients in Chinese medicinal preparation. Especially when the manufacturer's license expired and need to be renewed[1], these documents should also be included. Therefore, precise and reliable method for marker substances analysis is an important factor in upgrading the qualities of Chinese medicinal preparations.

In recent years, a number of analytical methods for Chinese medicinal preparations have been established in our laboratory[2-12]. In the present study, we selected Shyr-Chyuan-Dah-Buu-Yaw-Jyo, a popular tonic wine preparation of Chinese medicine in Taiwan for HPLC analysis. This wine contains Angelicae Sinensis Radix, Cnidii Rhizoma, Paeoniae Radix, Rehmanniae Radix, Codonopsitis Radix, Poria, Atractylodis Rhizoma, Glycyrrhizae Radix, Cinnamonomi Cortex, and Astragali Radix. Although a number of analytical methods for these Chinese crude drugs and these marker substances have been reported[13-22], but no analytical methods for Shyr-Chyuan-Dah-Buu-Yaw-Jyo has been reported. The tonic wine is known to weakness of chi and blood circulation, lassitude of the extremities and the trunk etc.

In this study, six marker substances including ferulic acid in Angelicae Sinensis Radix and Cnidii Rhizoma, paeoniflorin in Paeoniae Radix, glycyrrhizin in Glycyrrhizae Radix, cinnamic acid, cinnamaldehyde in Cin-namomi Cortex, calycosin in Astragali Radix are resolved and quantitatively measured through a reversed-phase HPLC approach. The method developed demonstrated to be facile in the routine analysis for quality control by quantitatively determining the active ingredients in this tonic wine preparation from different manufactures.

MATERIALS AND METHODS

Materials

Materials for the preparation of Shyr-Chyuan-Dah-Buu-Yaw-Jyo preparation are Angelicae Radix, Cnidii Rhizoma, Paeoniae Radix, Rehmanniae Radix, Codonopsitis Radix, Poria, Atractylodis Rhizoma, Glycyrrhizae Radix, Cinnamonomi Cortex, and Astragali Radix. They were all purchased from herbal market, and were pulverized through a #8 mesh sieve (2.36 mm) for use. The origin of crude drugs were verified by microscopic and TLC examination. Voucher specimens were deposited in the department of Plant Industry, National Pingtung University Science and Technology.

Tonic wine preparations of Shyr-Chyuan-Dah-Buu-Yaw-Jyo were obtained from Department of Food Science, National Chia-Yi University in Taiwan.

Chemicals and Reagents

The structures of marker substances are shown in Fig. 1. Cinnamic acid and cinnamaldehyde were purchased from Fluka Chemie AG (Switzerland), and ferulic acid, glycyrrhizin and internal standard methyl paraben were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Paeoniflorin and calycosin were purified and identified in our laboratory.

95% ethanol was purchased from Taiwan Tabacco and Wine Board (R.O.C.). Acetonitrile and methanol (HPLC grade) were obtained from Mallinckrodt, Inc. (New Jersey, USA), and phosphoric acid (analytical-reagent grade) from Kanto Chemical (Tokyo, Japan). Ultra-pure distilled water with a resistivity greater than 18 MΩ was prepared with a mini-Q system (Millipore, Bedford, MA, USA). Samples for HPLC were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA). All other reagents were analytical grade.

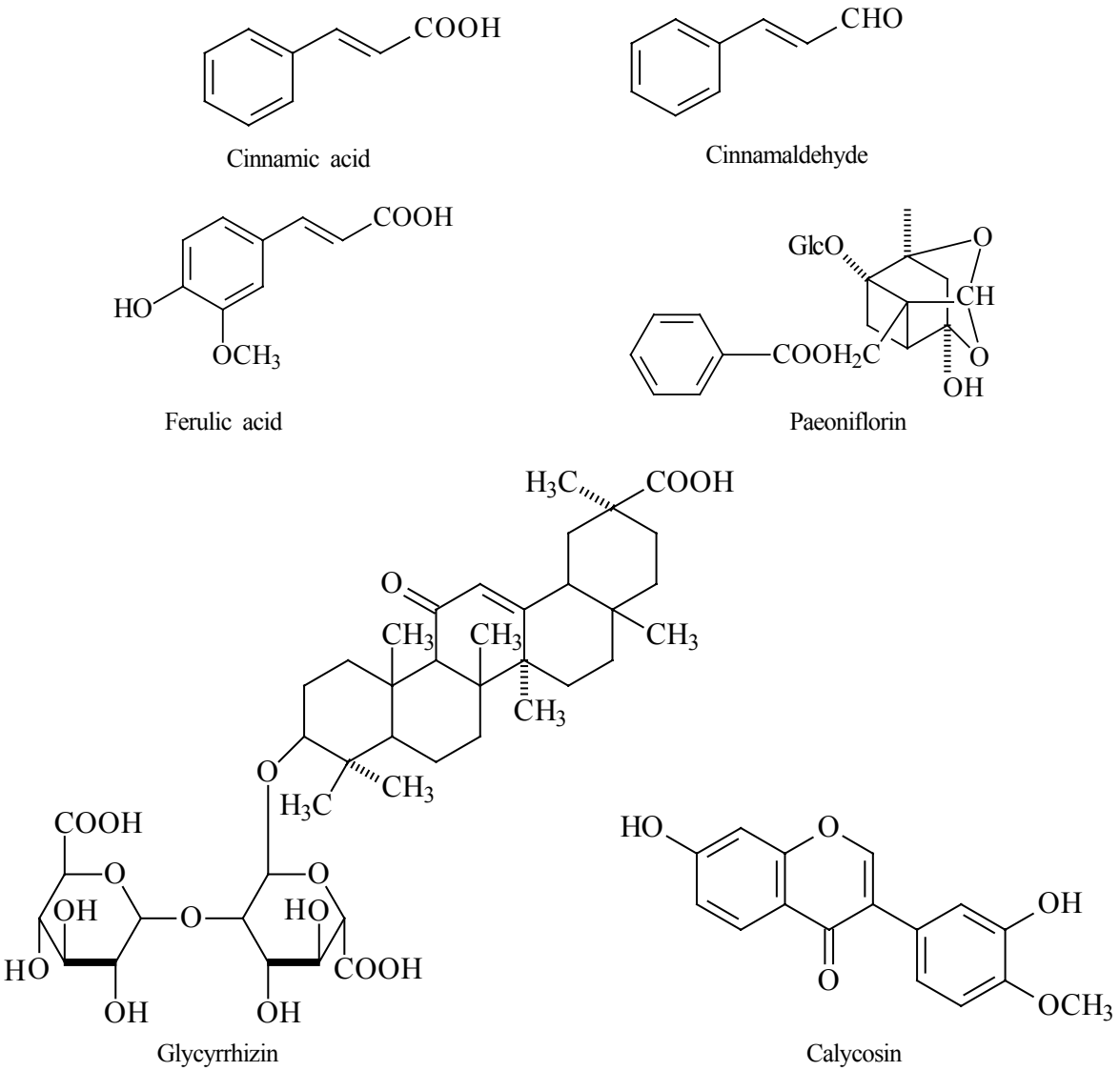


Fig. 1. Structures of the marker substances in Shyr-Chyuan-Dah- Buu-Yaw-Jyo.

Preparation of Standard and Internal Standard Solutions

1. Preparation of standard solution

The standard solutions were prepared by dissolving the amount of each marker substance as indicated in the parenthesis in 70% methanol solution to obtain the desired concentration: paeoniflorin (256.0 μg/mL), ferulic acid (216.0 μg/mL), cinnamic acid (248.0 μg/mL), cinnamaldehyde (2,000.0 μg/mL), glycyrrhizin (4,152.0 μg/mL), and calycosin (24.0 μg/mL).

2. Preparation of internal standard solution

The internal standard solution (400 μg/mL) was prepared by dissolving 100.0 mg of methylparaben in 70% methanol solution to obtain a total volume of 250 mL.

Preparation of Test Solution

According to Reference 23, 37.5 mg of Angelicae Sinensis Radix, 15.0 mg of Cnidii Rhizoma, 22.5 mg of Paeoniae Radix, 30.0 mg of Rehmanniae Radix, 20.0 mg of Codonopsitis Radix, 30.0 mg of Poria, 30.0 mg of Atractylodis Rhizoma, 10.0 mg of Glycyrrhizae Radix, 12.5 mg of Cinnamonomi Cortex, and 22.5 mg of Astragali Radix were used to make Shyr-Chyuan-Dah-Buu-Yaw-Jyo. The above-mentioned Chinese crude drugs were extracted with ethanol at six different concentrations and six different extraction conditions were denoted as A, B, C, D, E and F and were as follow. Their products were obtained from Department of Food Science, National Chia-Yi University in Taiwan.

A: Extracted with 50% ethanol and stored at room temperature (30 °C) for thirty days.

B: Extracted with 70% ethanol and stored at room temperature (30 °C) for thirty days.

C: Extracted with 50% ethanol and stored at room temperature (30 °C) for sixty days.

D: Extracted with 70% ethanol and stored at room temperature (30 °C) for sixty days.

E. Extracted with 50% ethanol and stored at room temperature (30 °C) for ninety days.

F. Extracted with 70% ethanol and stored at room temperature (30 °C) for ninety days.

A 1.0 mL sample of each solution was removed by micropipette, while 1.0 mL internal standard solution was added to each solution to afford a concentration of 200.0 μg/mL. The resulted solution was used as test solution for subsequent HPLC analysis after filtration through a 0.45 μm membrane filter.

HPLC Analysis

HPLC separation was conducted by a Hitachi system equipped with a degasser DG-2410, pump L-7100, UV/Vis detector L-7420, photodiode array detector L-4500 and autosampler L-7200. Peak areas were calculated with a D-7000 HSM software.

A reversed phase column Inertsil 5 ODS-2 (GL Science, 4.6 mm i.d. × 250 mm) was used. The column

oven was set at 30 °C. The mobile phases consisting of 10% and 60% acetonitrile (each adjusted to pH 2.8 with phosphoric acid) aqueous solutions in gradient elution are shown in Table 1. The detection wavelength was set at 240 nm. The flow rate was set at 1.0 mL/min. The volume for each injection was 20 μL.

Calibration Method

Standard solutions of each marker substance were prepared from the stock solutions by adding 70% methanol to give concentrations of paeoniflorin: 8.0, 16.0, 32.0, 64.0, 128.0 μg/mL; ferulic acid: 6.75, 13.5, 27.0, 54.0, 108.0 μg/mL; cinnamic acid: 7.75, 15.5, 31.0, 62.0, 124.0 μg/mL; cinnamaldehyde: 62.5, 125.0, 250.0, 500.0, 1,000.0 μg/mL; glycyrrhizin: 129.75, 259.5, 519.0, 1038.0, 2076.0 μg/mL; calycosin: 0.75, 1.5, 3.0, 6.0, 12.0 μg/mL.

Each standard solution contained the internal standard (methylparaben) at 200.0 μg/mL. All standard solutions were filtered and 20 μL of each was injected into the HPLC column for analysis. The calibration curve was plotted by using the ratio of the peak areas of standard solution and internal standard solution as the y-axis, and concentrations as the x-axis. Linear regression method was used to evaluate the equation of $y = ax + b$ and the correlation coefficient.

Validation

1. Precision

Standard stock solutions were diluted with 80% methanol to three different concentrations. Intra-day test (injecting each concentration three times within 24 hours), and an inter-day test (injecting each concentration four times over 7 days with each injection separated by at least 24 hours) were run to check reproducibility. The

Table 1. Gradient elution program using mobile phase A and B

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	90	10
10	80	20
13	77	23
23	77	23
30	50	50
40	40	60
50	30	70
60	0	100
65	0	100
70	90	10
75	90	10

Flow rate l.0 mL/min.
A: 10% acetonitrile (adjusted to pH 2.8 with phosphoric acid).
B: 60% acetonitrile (adjusted to pH 2.8 with phosphoric acid).

standard deviation (S.D.) and relative standard deviation (R.S.D.) were calculated.

2. Accuracy

Each standard stock solution of a series of various concentrations was spiked into an ethanol solution of Shyr-Chyuan-Dah-Buu-Yaw-Jyo, and then refluxed at 80 °C for 3 hrs. Internal standard solution was added to each solution to afford a concentration of 200.0 μg/mL. Then the solution was filtered and subjected to HPLC analysis in triplicates. The recovery (%) was calculated by the equation of ((C3-C2)/C1) × 100%, in which C1 represents the amount of each standard spiked, C2 represents the amount of each marker in ethanol solution of Shyr-Chyuan-Dah-Buu-Yaw-Jyo, and C3 represents the total amount of each marker in the solution.

3. Limit of detection test

To evaluate the HPLC method's limit of detection (LOD), prepared various concentrations of each standard stock solution were analyzed. LOD were based on a signal to noise (S/N) ratio with 3:1 as the minimum of that observed in the standard.

RESULTS AND DISCUSSION

Separation of Marker Substances by HPLC

The HPLC chromatograms of the marker substances and the ethanol extract of Shyr-Chyuan-Dah-Buu-Yaw-Jyo are shown in Fig. 2. The chromatogram indicated that the peaks of paeoniflorin, ferulic acid, calycosin, cinnamic acid, cinnamaldehyde, glycyrrhizin, and the internal standard have been well separated. The respective retention times were as follow: 12.31 min for paeoniflorin, 17.53 min for ferulic acid, 35.41 min for calycosin, 36.41 min for cinnamic acid, 39.15 min for cinnamaldehyde, 50.27 min for glycyrrhizin, and 25.56 min for the internal standard, methylparaben.

The ethanol extract of Shyr-Chyuan-Dah-Buu-Yaw-Jyo was compared to the five kinds of blank solutions, which were prepared by the deletion of material of Paeoniae Radix, Angelicae Sinensis Radix, Cnidii Rhizoma, Astragali Radix, Cinnamomi Cortex, Glycyrrhizae Radix, respectively. As shown in Fig. 3B to 3F, no peak of the deleted material was observed at retention times corresponding to the respective marker substances. Apparently, there was no interaction among components of Shyr-Chyuan-Dah-Buu-Yaw-Jyo. Therefore, the above conditions can be used for quantification of the marker substances.

Calibration Line

The linear regression equations, correlation coefficients and concentration range of calibration lines for marker substances were listed in Table 2. All calibration curves were in good linearity with correlation coefficients of 0.9997~1.0000.

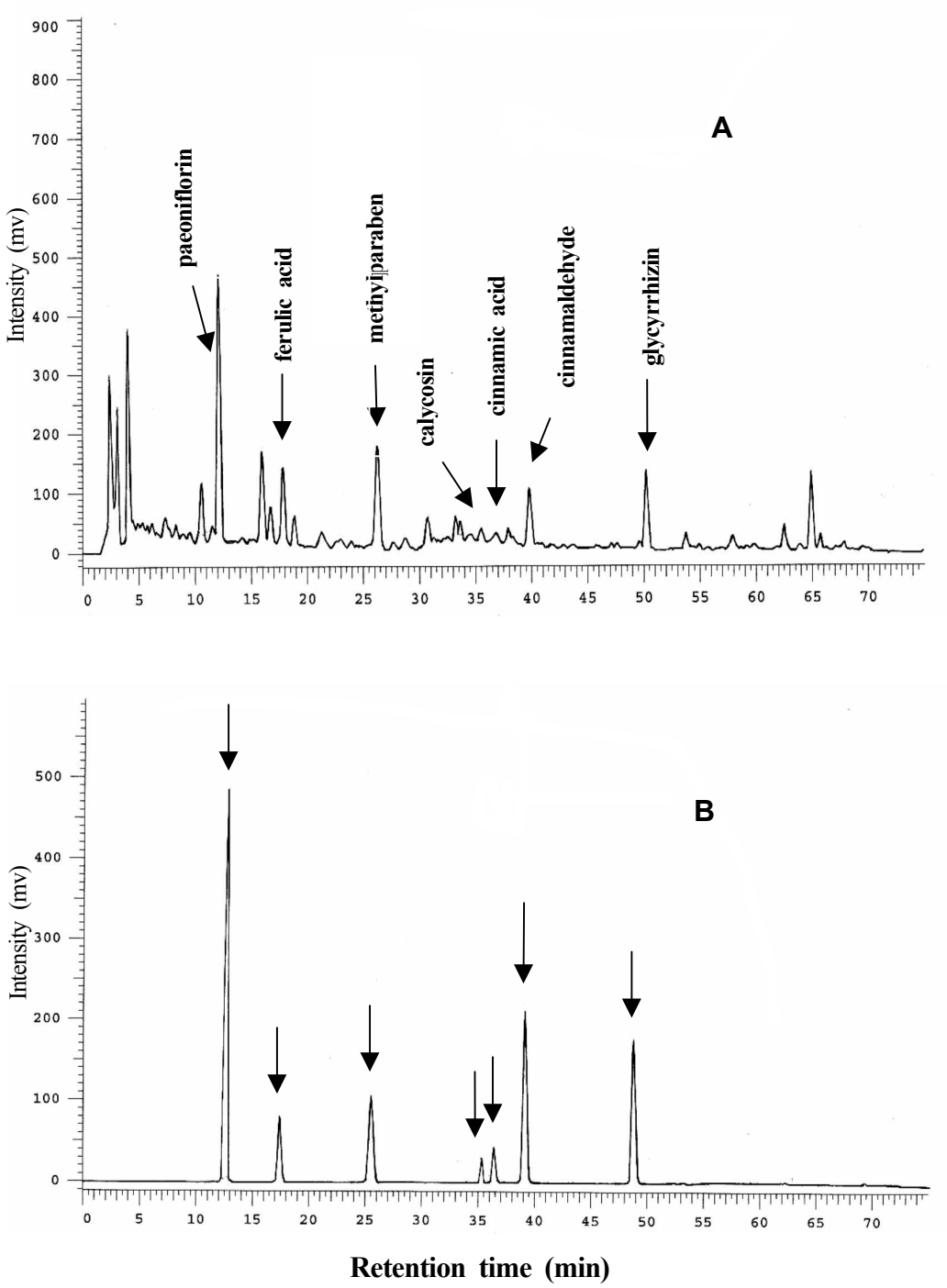
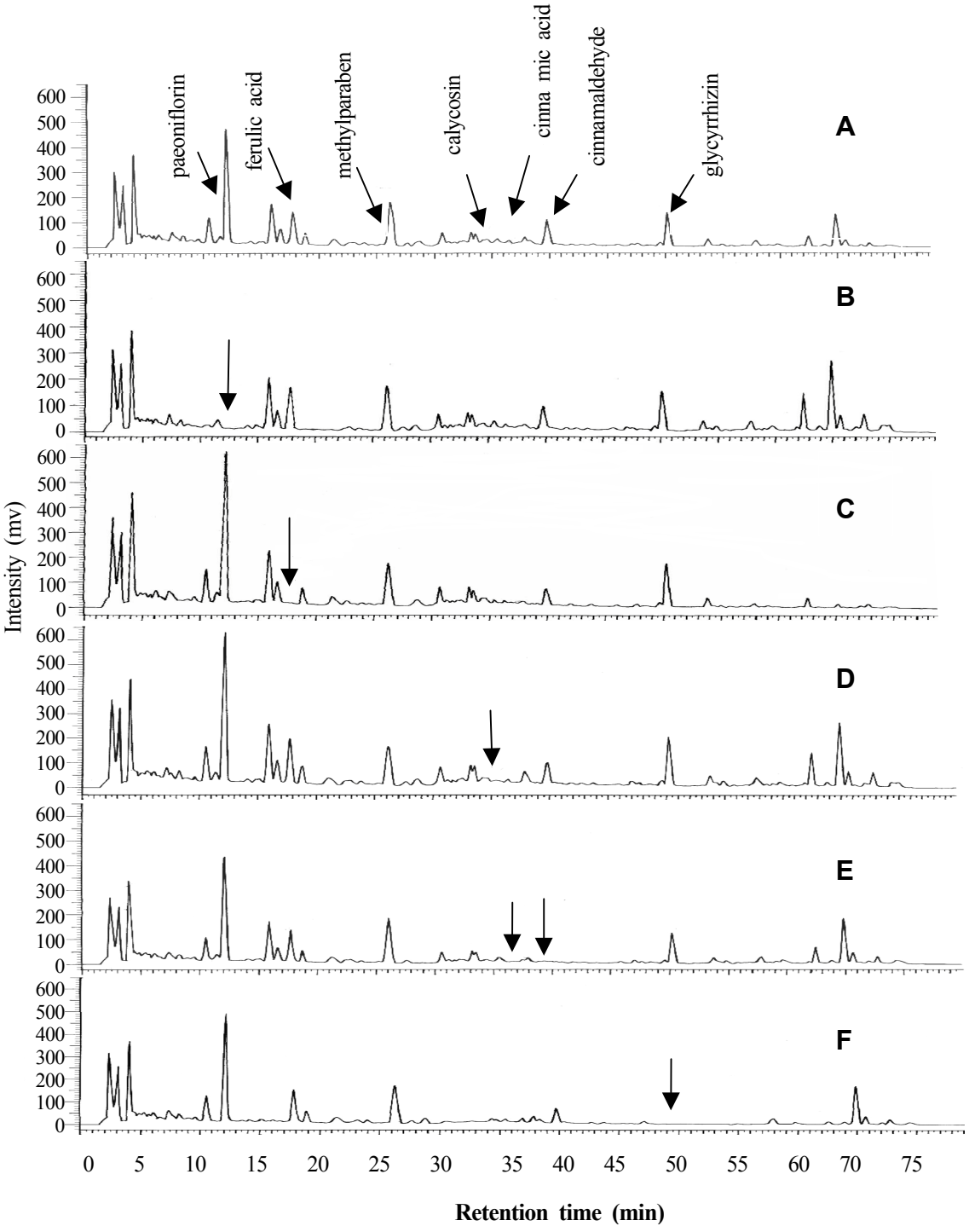


Fig. 2. HPLC Chromatograms of (A) marker substances in 95% ethanol extractions of Shyr-Chyuan-Dah-Buu-Yaw-Jyo, (B) marker substances.

Precision and Accuracy

The relative standard deviations of the intra-day and inter-day analysis were 0.07~3.60% and 0.37~4.67% respectively, suggesting that the method had very good reproducibility (Table 3).



A: Ethanol extract of Shyr-Chyuan-Dah-Buu-Yaw-Jyo containing internal standard, methyl paraben.
B: Ethanol extract of Shyr-Chyuan-Dah-Buu-Yaw-Jyo without Paeoniae Radix.
C: Ethanol extract of Shyr-Chyuan-Dah-Buu-Yaw-Jyo without Angelicae Sinensis Radix and Cnidii Rhizoma.
D: Ethanol extract of Shyr-Chyuan-Dah-Buu-Yaw-Jyo without Astragali Radix.
E: Ethanol extract of Shyr-Chyuan-Dah-Buu-Yaw-Jyo without Cinnamomi Cortex.
F. Ethanol extract of Shyr-Chyuan-Dah-Buu-Yaw-Jyo without Glycyrrhizae Radix.

Fig. 3. Chromatograms of marker substances in 95% ethanol extracts of Shyr-Chyuan-Dah-Buu-Yaw-Jyo made from incomplete materials.

Table 2. Calibration curves of marker substances

Compound	Concentration range μg/mL	Regression equation	r
paeoniflorin	8.00~128.00	y = 0.0024 x - 0.0030	0.9997
ferulic acid	6.75~108.00	y = 0.0055 x + 0.0017	1.0000
cinnamic acid	7.75~124.00	y = 0.0024 x + 0.0018	0.9999
cinnamaldehyde	62.5~1000.00	y = 0.0026 x - 0.0009	1.0000
glycyrrhizin	129.75~2076.00	y = 0.0007 x + 0.0157	0.9999
calycosin	0.75~12.00	y = 0.0198 x - 0.0003	0.9999

Table 3. Reproducibilities of intra-day and inter-day analysis of Shyr-Chyuan-Dah-Buu-Yaw-Jyo

Compound	Concentration	Mean ± S.D. (R.S.D %)	
	(μg/mL)	intra-day (n = 5)	inter-day (n = 4)
Paeoniflorin	128.00	127.48 ± 0.09 (0.07)	127.13 ± 0.70 (0.55)
	32.00	32.89 ± 0.02 (0.07)	33.24 ± 0.14 (0.43)
	8.00	8.14 ± 0.06 (0.77)	7.61 ± 0.04 (0.58)
Ferulic acid	108.00	108.72 ± 1.37 (1.26)	108.93 ± 1.43 (1.31)
	27.00	27.84 ± 0.03 (0.10)	27.37 ± 0.22 (0.81)
	6.75	6.58 ± 0.02 (0.31)	6.24 ± 0.03 (0.53)
Cinnamic acid	124.00	123.39 ± 0.62 (0.50)	125.13 ± 0.93 (0.74)
	31.00	31.23 ± 0.09 (0.30)	31.79 ± 0.10 (0.31)
	7.75	7.86 ± 0.24 (3.05)	7.43 ± 0.07 (0.90)
Cinnamaldehyde	1000.00	1001.57 ± 3.10 (0.31)	998.38 ± 10.28 (1.03)
	250.00	250.59 ± 0.23 (0.09)	250.97 ± 1.71 (0.68)
	62.50	61.91 ± 0.25 (0.41)	63.08 ± 0.79 (1.25)
Glycyrrhizin	2076.00	2078.15 ± 10.81 (0.52)	2077.27 ± 7.69 (0.37)
	519.00	519.49 ± 0.99 (0.19)	520.14 ± 2.13 (0.41)
	129.75	130.24 ± 2.11 (1.62)	130.83 ± 2.46 (1.88)
Calycosin	12.00	12.14 ± 0.06 (0.45)	11.54 ± 0.14 (1.17)
	3.00	3.21 ± 0.08 (2.37)	3.34 ± 0.11 (3.33)
	0.75	0.76 ± 0.03 (3.60)	0.77 ± 0.04 (4.67)

Recoveries of the analysis were shown in Table 4. All the recoveries are greater than 92.30%.

LOD of these marker substances under above conditions was 5 ng/mL.

Analysis of the Sample Solutions

Significant differences in individual and total component contents were observed among six different ethanol concentrations and extraction conditions of Shyr-Chyuan-Dah-Buu-Yaw-Jyo (Fig. 4, Table 5). The quantitative analysis indicated that with the increase of ethanol concentration, the content of marker substances are increased, since there were little difference in content of marker substances between 30, 60 or 90 days. The optimized extraction condition is 70% ethanol and 30 days.

Table 4. Recovery of six marker substances from Shyr-Chyuan-Dah-Buu-Yaw-Jyo

Compound	Concentration (μg/mL)	Recovery (%) Mean ± S.D. (R.S.D %)
Paeoniflorin	128.00	101.56 ± 0.24 (0.24)
	32.00	103.13 ± 0.67 (0.65)
	8.00	115.00 ± 0.29 (0.25)
Ferulic acid	108.00	99.58 ± 0.44 (0.45)
	27.00	107.81 ± 0.41 (0.38)
	6.75	106.18 ± 0.23 (0.22)
Cinnamic acid	124.00	102.71 ± 3.36 (3.27)
	31.00	107.22 ± 3.80 (3.55)
	7.75	93.68 ± 2.23 (2.38)
Cinnamaldehyde	1000.00	98.65 ± 0.49 (0.50)
	250.00	96.54 ± 0.40 (0.41)
	62.50	92.30 ± 0.28 (0.30)
Glycyrrhizin	2076.00	103.85 ± 0.22 (0.21)
	519.00	104.64 ± 0.06 (0.06)
	129.75	111.93 ± 0.88 (0.79)
Calycosin	12.00	99.25 ± 1.22 (1.23)
	3.00	107.00 ± 1.16 (1.08)
	0.75	109.33 ± 1.45 (1.33)

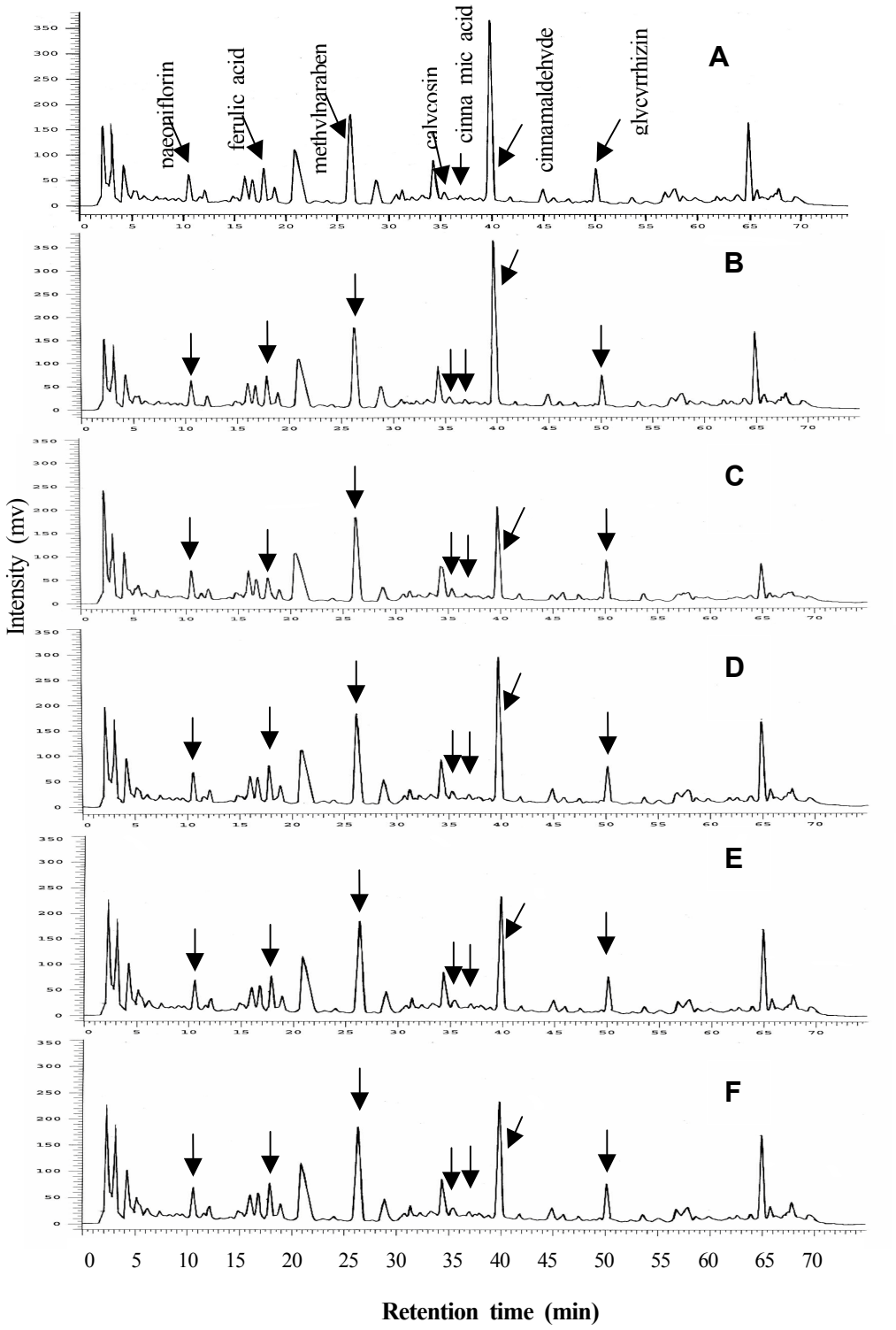
Table 5. Contents of marker substances in various manufacture process of Shyr-Chyuan-Dah-Buu-Yaw-

Compound	1	2	3	4	5	6
Paeoniflorin	1.97 ± 0.82	1.71 ± 0.73	1.78 ± 1.71	2.25 ± 0.24	2.06 ± 3.16	2.37 ± 0.92
Ferulic acid	0.84 ± 5.00	2.91 ± 2.10	2.22 ± 0.91	2.02 ± 4.03	1.57 ± 1.21	2.39 ± 1.15
Cinnamic acid	2.56 ± 2.22	2.09 ± 0.28	2.05 ± 0.23	2.69 ± 1.71	2.24 ± 0.09	2.58 ± 0.34
Cinnamaldehyde	22.79 ± 1.48	57.66 ± 0.73	44.85 ± 0.09	52.91 ± 1.69	43.15 ± 0.14	49.96 ± 0.10
Glycyrrhizin	33.00 ± 3.87	30.29 ± 3.01	37.34 ± 1.92	31.02 ± 2.37	30.72 ± 1.23	35.28 ± 4.16
Calycosin	0.07 ± 0.03	0.17 ± 0.02	0.51 ± 0.03	0.53 ± 0.33	0.41 ± 1.33	0.53 ± 3.25
Total	61.23	92.93	86.70	91.42	80.15	93.11

Data represented as mean (mg/30mL) ± C.V. value (%).
1. Extracted with 50% ethanol and stored at room temperature (30 °C) for thirty days.
2. Extracted with 70% ethanol and stored at room temperature (30 °C) for thirty days.
3. Extracted with 50% ethanol and stored at room temperature (30 °C) for sixty days.
4. Extracted with 70% ethanol and stored at room temperature (30 °C) for sixty days.
5. Extracted with 50% ethanol and stored at room temperature (30 °C) for ninety days.
6. Extracted with 70% ethanol and stored at room temperature (30 °C) for ninety days.

CONCLUSIONS

A multi-component HPLC method was developed for the simultaneous quantification of six marker substances in Shyr-Chyuan-Dah-Buu-Yaw-Jyo. A matrix of 10% acetonitrile and 60% acetonitrile, which were



A: Extracted with 50% ethanol and stored at room temperature (30 °C) for thirty days.
B: Extracted with 70% ethanol and stored at room temperature (30 °C) for thirty days.
C: Extracted with 50% ethanol and stored at room temperature (30 °C) for sixty days.
D: Extracted with 70% ethanol and stored at room temperature (30 °C) for sixty days.
E: Extracted with 50% ethanol and stored at room temperature (30 °C) for ninty days.
F: Extracted with 70% ethanol and stored at room temperature (30 °C) for ninty days.

Fig. 4. HPLC chromatograms of six kinds of 95% extraction conditions in Shyr-Chyuan-Dah-Buu-Yaw-Jyo.

both adjusted to pH 2.8 with phosphoric acid, was used as the mobile phase in a gradient elution program, with an ODS column for the stationary phase. UV 240 nm was used for the detection of the marker substances. The internal standard used to determine the calibration line resulted in a precise and reliable quantification method. The results of the quantitative analysis showed that the optimal extraction condition of Shyr-Chyuan-Dah-Buu-Yaw-Jyo was 70% ethanol and 30 days. This method can be used to establish the standards for quality control to ensure accuracy, efficiency in the manufacturing process of Shyr-Chyuan-Dah-Buu-Yaw-Jyo in future.

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十全大補藥酒成分分析之研究

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使用 HPLC 進行十全大補藥酒之成分分析，開發出包含當歸、川芎中 ferulic acid、白芍中 paeoniflorin、甘草中 glycyrrhizin、肉桂中 cinnamic acid，cinnamaldehyde 及黃耆中 calycosin 等六種指標成分之多成分同時定量分析方法。同時以不同製程條件探討製程對十全大補藥酒品質之影響。

十全大補藥酒之試料通過保持在 30 ℃ 恒溫之 HPLC 層析管（Inertsil ODS-2, 4.6 mm i.d. × 250 mm），移動相採用 10% 及 60% acetonitrile（各用磷酸調整 pH 值為 2.8）之混合溶液，進行梯度沖提法，以 1.0 mL/分之流速沖提。六種指標成分之檢測使用 UV 偵測器，偵測波長設定 240 nm。

本法之回收率，同日間及異日間的變異係數均在 5% 以下，回收率在 92.30~115.00%之間。這個分析法對於十全大補藥酒製劑中六種指標成分是安定且值得信賴之定量法。

關鍵詞：十全大補藥酒、高效液相層析法。

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