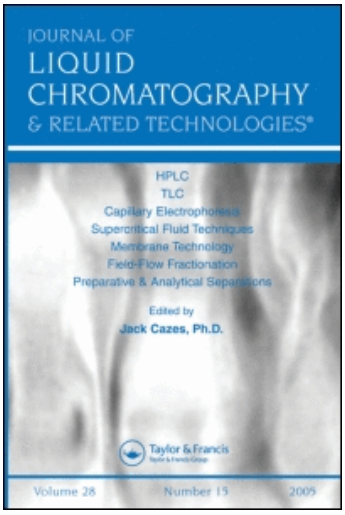


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# SIMULTANEOUS ANALYSIS OF EIGHT COMPONENTS IN "PING-WEI-SAN" BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

A HPLC method for simultaneous determination of eight marker substances was established for the quality control of Chinese medicinal preparation "Ping-Wei-San." These substances were glycyrrhizin in Glycyrrhizae Radix; hesperidin, nobiletin, 3,'4',3,5,6,7,8-heptamethoxyflavone, tangeretin, and 5-hydroxy-3',4',6,7,8-pentane-thoxyflavone in Citri Leiocarpae Exocarpium; honokiol and magnolol in Magnoliae Cortex.

Extracted samples were separated on an Inertsil ODS-80A column at 30°C with a mixture of 20% acetonitrile (pH 2.5) and 70% acetonitrile (pH 2.5) aqueous solution employing a linear gradient elution method at a flow-rate of 1.0 mL/min. The detection wavelength varied with time. It was 275 nm during 0~19 min and 250 nm during 19~80 min.

Relative standard deviations of intra- and inter-day analysis were less than 5%. This separation method could be successfully

applied for the simultaneous determination of eight marker substances in "Ping-Wei-San."

## INTRODUCTION

Recently, due to greater convenience, fewer side effects, and better performance as perceived by patient, concentrated Chinese medicinal preparations have become much more popular. However, crude drugs with different origins, sources, cultural manner, harvest time, pretreatment processes, and manufacturing processes will be of different quality, which will result in significant differences in the same formula when supplied by different factories or even by the same factory. Therefore, quality control of the manufacturing process of the concentrated Chinese medicine preparations will be very critical for their future development.



At present, quality control of the production of concentrated Chinese medicinal preparations is done according to the Draft issued by the Department of Health of the Republic of China, which requires "quantification of at least two marker substances from different Chinese crude drugs in a formula.[1]" In Japan, since 1985, the Ministry of Health and Welfare has required that all concentrated Chinese medicinal preparations submitted for inspection and registration should include a content analysis with at least two chemical components as markers,[2] and since 1998 there is guidance for the marker substances which should be assayed.[3] Concerning these regulations, the goal of analytical method development is to discover more marker substances and simultaneously quantify them in one HPLC method. Such improvement will promote quality control techniques for Chinese medicines.

In this study, the multi-component simultaneous analysis of the preparation Ping-Wei-San was discussed. Ping-Wei-San contains Atractylodis Lanceae Rhizoma, Magnoliae Cortex, Citri Leiocarpae Exocarpium, Zingiberis Rhizoma, Zizyphi Fructus, and Glycyrrhizae Radix. Although a number of analytical methods for these Chinese crude drugs and these marker substances have been reported, there were few analytical methods for Ping-Wei-San. A literature search revealed that only glycyrrhizin, hesperidin, 6-gingerol, honokiol, and magnolol in Ping-Wei-San had been analyzed.[4, 5]

In this study, eight marker substances of Ping-Wei-San were simultaneously analyzed by a reverse phase HPLC method. There were: glycyrrhizin in Glycyrrhizae Radix; hesperidin, nobiletin, 3',4',3,5,6,7,8-heptamethoxyflavone, tangeretin, and 5-hydroxy-3',4',6,7,8-pentamethoxyflavone in Citri Leiocarpae Exocarpium; honokiol and magnolol in Magnoliae Cortex. Also, this analytical method was applied to commercial samples of concentrated Ping-Wei-San.

## EXPERIMENTAL

### Material

The materials for Ping-Wei-San preparation are Atractylodis Lanceae Rhizoma, Magnoliae Cortex, Citri Leiocarpae Exocarpium, Zingiberis Rhizoma, Zizyphi Fructus, and Glycyrrhizae Radix. Each material was obtained from an herbal market, and all herbs were pulverized through a #8 mesh sieve (2.36mm). The samples of commercial concentrated Ping-Wei-San were obtained from three manufactures.

### Chemicals and Reagents

Glycyrrhizin (**2**) was purchased from Yoneyama Co. (Japan). Hesperidin (**1**), nobiletin (**3**), 3',4',3,5,6,7,8-heptamethoxyflavone (**4**), tangeretin (**5**) and 5-hydroxy-3',4',6,7,8-pentamethoxyflavone (**6**) were isolated from Citri Leiocarpae Exocarpium, and honokiol (**7**) and magnolol (**8**) were isolated from Magnoliae Cortex in our laboratory (Figure 1). Naphthalene was purchased from Nacalai Tesque (Japan).

All solvents used were of HPLC grade.



### Standard Decoction Preparation for HPLC

According to Reference. 6, the standard decoction of Ping-Wei-San contains Tractylodis Lanceae Rhizoma 4 g, Magnoliae Cortex 3 g, Citri Leiocarpae Exocarpium 3 g, Zingiberis Rhizoma 1 g, Zizyphi Fructus 2 g, and Glycyrrhizae Radix 1 g. To prepare a standard decoction, 14 g of the pulverized Chinese crude drugs listed above and 280 mL (i.e. 20 times) of water were put into a 1000 mL rounded flask and heated until only about 140 mL (i.e. 10 times) remained. Then, the hot solution was filtered by four layers of gauze, and allowed to cool and adjusted to 140 mL by adding water. A 1.0 mL sample of the solution was removed and adjusted to 5 mL by adding 80% methanol solution, with an appropriate amount of the internal standard solution being added at the same time. After filtering (0.45 μm), this was the standard decoction used for subsequent HPLC analysis.

### Commercial Concentrated Preparations for HPLC

First, the three kinds of commercial product were pulverized and mixed individually. Then, 1/3 of the daily dosage of each commercial product was

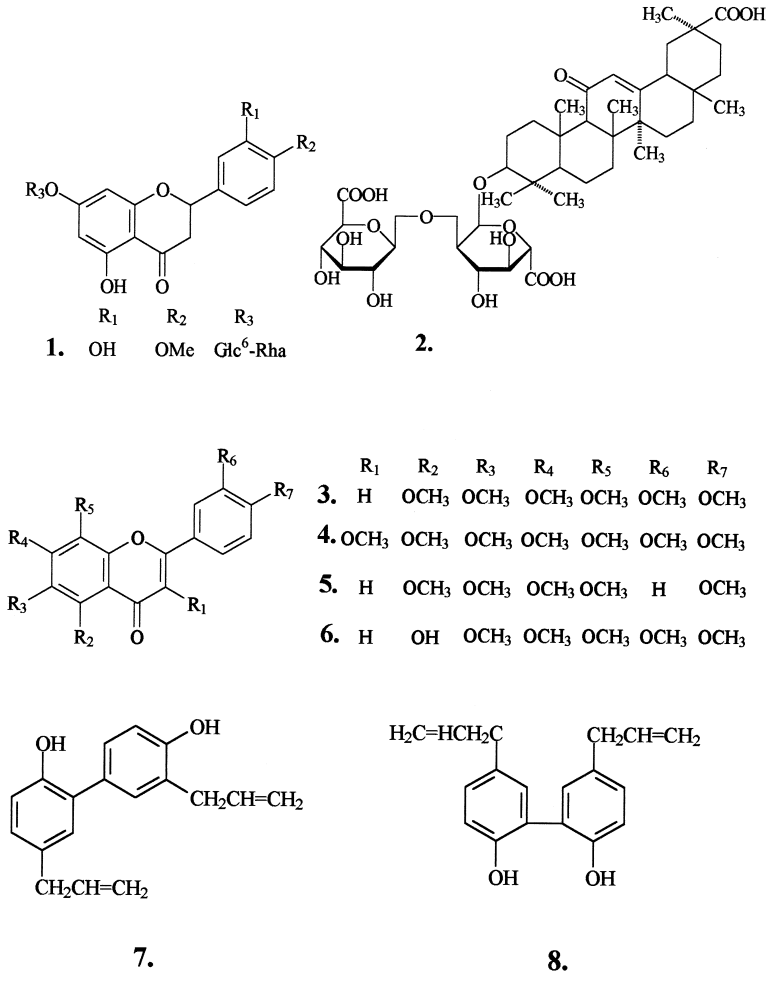


**Figure 1**. Structures of the studied compounds.

weighed accurately into a 100 mL flask, to which was added 40 mL of 80% methanol. After ultrasonic extraction for 30 minutes at 30°C, each was filtered and adjusted to 50 mL by adding 80% methanol. Then, 2 mL samples of each of the solution were adjusted to 5 mL by adding 80% methanol, and as before, the appropriate amount of the internal standard solution was added at the same time. After filtering (0.45 μm), these were the sample solutions used for quantification.

### Liquid Chromatography

The HPLC was conducted with a Waters LC Module 1 system equipped with degasser, column oven, pump, UV detector, and autosampler. Peak areas were calculated with a Waters 746 Data Module. The analysis conditions were listed as follows:

Column: Inertsil ODS-80A (5μm, 4.6 mm × 250 mm)

Column Temperature: 30°C

Mobile Phase: A: 20% acetonitrile (adjusted to pH 2.5 with phosphoric acid). B: 70% acetonitrile (adjusted to pH 2.5 with phosphoric acid). The mixtures of A and B aqueous solutions used in the linear gradient elution are shown in Table 1.

Flow Rate: 1.0 mL/min.

Injection Volume: 20 mL

Detection Wavelength: 275 nm during 0~19 min, 250 nm during 19~80 min.



## RESULTS AND DISCUSSION

### Analytical Conditions for HPLC

Using Inertsil ODS-80A as stationary phase, various kinds of mobile phase were tested to achieve optimal separation. We chose optimal separation

**Table 1**

**Gradient Elution Program Using Mobile Phase A and B**

| Time (min) | Flow Rate (mL/min) | Mobile Phase A[a] (%) | Mobile Phase B[b] (%) |
|---|---|---|---|
| 0 | 1.0 | 100 | 0 |
| 15 | 1.0 | 100 | 0 |
| 20 | 1.0 | 65 | 35 |
| 55 | 1.0 | 55 | 45 |
| 80 | 1.0 | 0 | 100 |

[a] 20% acetonitrile (adjusted to pH 2.5 with phosphoric acid). [b] 70% acetonitrile (adjusted to pH 2.5 with phosphoric acid).

conditions involving two mobile phase systems, which were run following a linear gradient-eluting program (Table 1). One mobile phase system was 20% acetonitrile aqueous solution adjusted to pH 2.5 with phosphoric acid. The other mobile phase system was 70% acetonitrile aqueous solution adjusted to pH 2.5 with phosphoric acid. This mobile phase system was selected for simultaneous quantification of the eight marker substances.

Maximally efficient detection can be obtained by selecting the wavelength where the component has the maximum absorption. Therefore, two stages of detection wavelength were set according to the retention time and the maximum absorption wavelength of each component (275nm for hesperidin, 250nm for other eight compounds).

Chromatograms of the standard decoction of Ping-Wei-San are shown in Figure 2a. Good performance is indicated by the well-separated peaks of each marker substance. The respective retention times were as follows: 14.25 min for hesperidin, 32.08 min for glycyrrhizin, 43.56 min for nobiletin, 48.96 min for 3',4',3,5,6,7,8-heptamethoxyflavone, 54.51 min for tangeretin, 65.00 min for 5-hydroxy-3',4',6,7,8-pentamethoxyflavone, 71.69 min for honokiol, 77.91 min for maganolol, and 72.84 min for the internal standard, naphthalene.



The peaks were detected by a photodiode array detector (Waters), which was used to determine the three-dimensional chromatography, isocline and the identity of UV absorption photography at the top and bottom of each component; the standard decoction was compared to the three kinds of blank solution, which were prepared, respectively, without Citri Leiocarpae Exocarpium, Glycyrrhizae Radix, and Magnoliae Cortex. As shown in Figure 2, there were no peaks present at retention time corresponding to the respective marker substances. Evidently, there was no interaction between components of Ping-Wei-San; therefore, the above conditions can be used for quantification of the marker substances.

**Calibration Line**

The standard solutions of each marker substance were diluted by 80% methanol to sequential concentrations. Each diluted solution contained the internal standard, naphthalene, at 7.45 μg/mL. After filtering through a 0.45 μm medium, 20 μL of each concentration was injected into the HPLC column for analysis. The calibration graphs were plotted after linear regression of the peak area ratio with concentration. The regression equations and correlation coefficients of calibration lines for those marker substances were as follows, where $y$ is the peak area ratio of the marker to the internal standard and $x$ is the concentration of the marker.

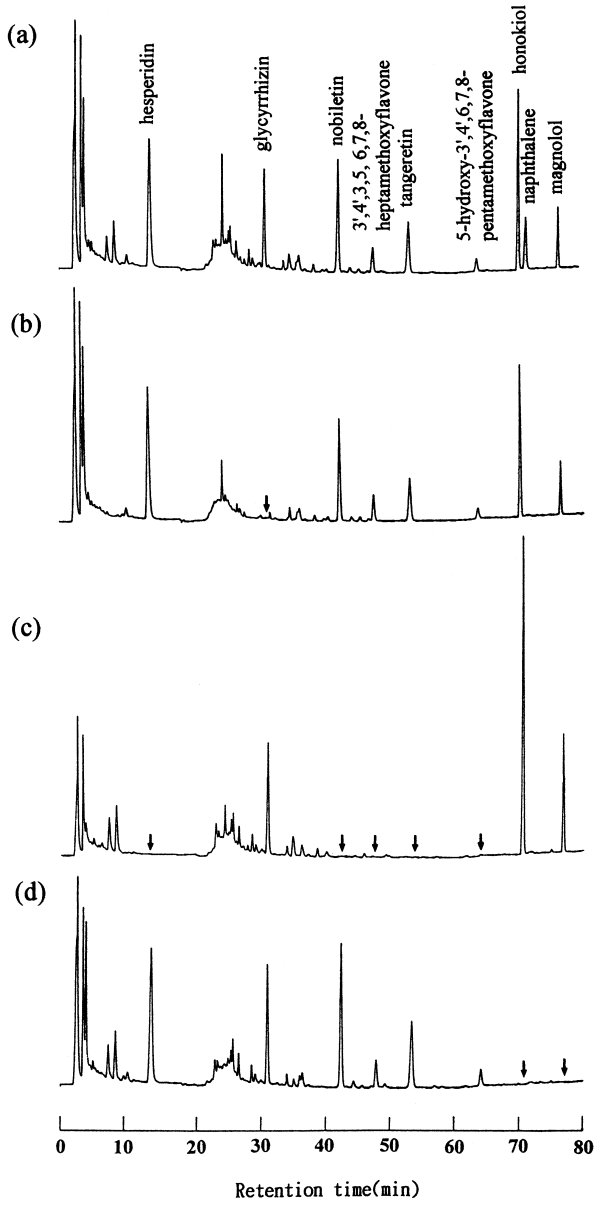


**Figure 2**. Chromatograms of hesperidin, glycyrrhizin, nobiletin, 3', 4', 3,5, 6,7,8-heptamethoxyflavone, tangeretin, 5-hydroxy-3',4',6,7,8- pentamethoxyflavone, honokiol and magnonol in standard decoction of Ping-Wei-San. (a) Ping-Wei-San standard decoction containing internal standard, naphthalene. (b) Ping-Wei-San standard decoction without Glycyrrhizae Radix. (c) Ping-Wei-San standard decoction without Citri Leiocarpae Exocarpium. (d) Ping-Wei-San standard decoction without Magnoliae Cortex.

**Table 2**

**Reproducibilities of Intra-Day and Inter-Day Analysis of Ping-Wei-San**

| Compound | Concentration (μg/mL) | CV(%) Intra-Day (n = 3) | CV(%) Inter-Day (n = 4) |
|---|---|---|---|
| Hesperidin | 35.15 | 1.60 | 1.81 |
| | 53.33 | 0.69 | 0.48 |
| | 62.42 | 2.17 | 0.57 |
| | 71.51 | 0.92 | 0.34 |
| | 109.18 | 1.26 | 1.05 |
| Glycyrrhizin | 29.56 | 0.93 | 1.76 |
| | 41.38 | 0.61 | 0.59 |
| | 47.30 | 2.14 | 0.32 |
| | 53.21 | 0.98 | 0.70 |
| | 67.99 | 1.23 | 0.76 |
| Nobiletin | 0.58 | 0.44 | 0.37 |
| | 3.40 | 0.53 | 1.09 |
| | 6.11 | 2.32 | 0.69 |
| | 8.81 | 0.71 | 0.76 |
| | 14.46 | 1.69 | 0.67 |
| 3',4',3,5,6,7,8 Heptamethoxyflavone | 0.79 | 5.69 | 2.43 |
| | 1.58 | 1.94 | 0.79 |
| | 2.30 | 2.37 | 1.32 |
| | 3.02 | 1.62 | 0.90 |
| | 4.74 | 1.45 | 1.11 |
| Tangeretin | 3.38 | 2.33 | 2.71 |
| | 5.41 | 0.61 | 0.60 |
| | 6.76 | 2.22 | 2.08 |
| | 7.77 | 0.98 | 0.70 |
| | 11.49 | 0.73 | 0.86 |
| 5-Hydroxy-3',4',7,8-pentamethoxyflavone | 0.16 | 1.26 | 2.41 |
| | 1.92 | 0.51 | 0.56 |
| | 3.68 | 2.29 | 2.09 |
| | 5.45 | 0.92 | 0.03 |
| | 7.21 | 2.04 | 2.06 |

**Table 2 (continued)**

**Reproducibilities of Intra-Day and Inter-Day Analysis of Ping-Wei-San**

| Compound | Concentration (μg/mL) | CV(%) Intra-Day (n = 3) | CV(%) Inter-Day (n = 4) |
|---|---|---|---|
| Honokiol | 0.75 | 2.24 | 1.56 |
| | 4.49 | 0.24 | 0.56 |
| | 8.08 | 2.11 | 0.25 |
| | 11.67 | 0.99 | 0.61 |
| | 18.85 | 1.64 | 0.75 |
| Magnolol | 3.56 | 2.51 | 2.77 |
| | 13.88 | 0.64 | 1.31 |
| | 23.50 | 1.81 | 0.87 |
| | 33.11 | 0.92 | 1.20 |
| | 53.14 | 1.82 | 0.46 |



Hesperidin in the concentration range of 35.15, 53.33. 62.42, 71.51, 109.18 μg/mL; $y = 0.16055x - 0.00081$, $r = 0.99982$ ($n = 5$).

Glycyrrhizin in the concentration range of 29.56, 41.38, 47.30, 53.21, 67.99 μg/mL; $y = 0.09491x - 0.12964$, $r = 0.99931$ ($n = 5$).

Nobiletin in the concentration range of 0.58, 3.40, 6.11, 8.81, 14.46 μg/mL; $y = 0.35317x - 0.06978$, $r = 0.99912$ ($n = 5$).

3',4',3,5,6,7,8-Heptamethoxyflavone in the concentration range of 0.79, 1.58, 2.30, 3.02, 4.74 μg/mL; $y = 0.32019x - 0.00605$, $r = 0.99964$ ($n = 5$).

Tangeretin in the concentration range of 3.38, 5.41, 6.76, 7.77, 11.49 μg/mL; $y = 0.22047x - 0.01325$, $r = 0.99946$ ($n = 5$).

5-hydroxy-3',4',6,7,8-pentamethoxyflavone in the concentration range of 0.16, 1.92, 3.68, 5.45, 7.21 μg/mL; $y = 0.30008x + 0.01539$, $r = 0.99960$ ($n = 5$).

Honokiol in the concentration range of 0.75, 4.49, 8.08, 11.67, 18.85 μg/mL; $y = 0.29868x - 0.05797$, $r$?= 0.99980 ($n = 5$).

Magnolol in the concentration range of 3.56, 13.88, 23.50, 33.11, 53.14 μg/mL; $y = 0.16513x - 0.04108$, $r = 0.99996$ ($n = 5$).

**Table 3**

**Recovery of Each Marker Substance in Ping-Wei-San**

| Compound | Added[a] (μg/mL) | Found[a] (μg/mL) | Recovery[b] (%) |
|---|---|---|---|
| Hesperidin | 33.15 | 35.572 | 101.20 ± 5.56 |
| | 62.42 | 61.921 | 99.20 ± 6.15 |
| | 109.18 | 108.306 | 99.20 ± 7.75 |
| Glycyrrhizin | 29.56 | 28.484 | 96.36 ± 6.11 |
| | 47.30 | 46.964 | 99.29 ± 7.78 |
| | 67.99 | 67.929 | 99.91 ± 409 |
| Nobiletin | 0.58 | 0.582 | 100.38 ± 6.37 |
| | 6.11 | 6.022 | 98.56 ± 4.85 |
| | 14.46 | 14.563 | 100.71 ± 6.47 |
| 3',4',3,5,6,7,8-Heptamethoxyflavone | 0.79 | 0.793 | 100.35 ± 7.65 |
| | 2.30 | 2.314 | 100.62 ± 3.17 |
| | 4.74 | 4.738 | 99.90 ± 5.37 |
| Tangeretin | 3.38 | 3.358 | 99.26 ± 7.05 |
| | 6.76 | 6.779 | 100.58 ± 6.97 |
| | 11.49 | 11.618 | 101.11 ± 4.71 |
| 5-Hydroxy-3',4',6,7,8-pentamethoxyflavone | 0.16 | 0.154 | 96.40 ± 2.69 |
| | 3.68 | 3.801 | 103.29 ± 2.82 |
| | 7.21 | 7.151 | 99.18 ± 2.39 |
| Honokiol | 0.75 | 0.721 | 96.15 ± 5.67 |
| | 8.08 | 8.338 | 103.19 ± 6.43 |
| | 18.85 | 19.252 | 102.13 ± 4.72 |
| Magnolol | 3.56 | 3.567 | 100.20 ± 5.88 |
| | 23.50 | 24.370 | 103.70 ± 3.35 |
| | 53.14 | 56.249 | 105.85 ± 2.39 |

[a] Mean, n = 7. [b] Mean ± S.D., n = 7.

**Table 4**

**Contents of Each Marker Substance in Three Commercial Preparations of Ping Wei-San***

| Compound | Commercial Preparation A | Commercial Preparation B | Commercial Preparation C |
|---|---|---|---|
| Hesperidin | 21.810 ± 1.235 | 37.558 ± 1.113 | 21.519 ± 0.574 |
| Glycyrrhizin | 14.660 ± 0.509 | 13.583 ± 0.416 | 19.288 ± 0.467 |
| Nobiletin | 0.233 ± 0.012 | 3.894 ± 0.097 | 4.062 ± 0.094 |
| 3',4',3,5,6,7,8-Heptamethoxyflavone | 0.448 ± 0.024 | 1.316 ± 0.078 | 0.133 ± 0.005 |
| Tangeretin | ------ | 2.196 ± 0.124 | 1.513 ± 0.048 |
| 5-Hydroxy-3',4',6,7,8-pentamethoxyflavone | 0.222± 0.019 | 0.812 ± 0.027 | 0.442 ± 0.019 |
| Honokiol | 1.826 ± 0.054 | 0.644 ± 0.007 | 1.460 ± 0.062 |
| Magnolol | 14.780 ± 1.035 | 4.800 ± 0.084 | 9.706 ± 0.202 |

* Data represented as mean ± S.D. (mg/one daily dose), n = 7.



**Reproducibility Test**

Using the standard solutions with various concentrations, an intra-day test (injecting each concentration three times during 24 hours), and inter-day test (injecting each concentration four times during 7 days with each injection separated by at least 24 hours) was used to check reproducibility. The coefficients of variation (C.V.) were calculated from the experimental results as shown in Table 2. The C.V. values of different concentrations of the eight marker substances in the inter-day or intra-day test were less than 5%, except the compound **4** at 0.79 μg/mL in the intra-day test, and thus showed very good reproducibility.

**Recovery Test**

Good recovery was shown irrespective of concentration (Table 3).

**Analysis of the Commercial Concentrated Preparations**

The commercial concentrated preparations showed the same results as the standard decoction. Therefore, it could be reliably used for simultaneous quantification of the eight marker substances. The measured values, however, as shown in Table 4, were quite different from each other. This might be due to

inconsistencies of temperature, the humidity of additives and/or storage or during the processes of extraction, concentration, drying, and granulation.

## CONCLUSIONS

A multi-component HPLC method was developed for the simultaneous quantification of eight marker substances in Ping-Wei-San. A matrix of 20% acetonitrile and 70% acetonitrile, which were both adjusted to pH 2.5 with phosphoric acid, was used as the mobile phase in a gradient elution program, with an ODS column for the stationary phase. Two detection wavelengths were varied depending on the retention times of the respective components. The internal standard, naphthalene, used to determine the calibration line resulted in a precise and reliable quantification method. The method can be applied for analyzing commercial preparations, is a simple method of sample preparation, and can be adapted for continuous analysis with an auto-sampler. It should be a good application in the quality control of the manufacturing process of Ping-Wei-San in the future.



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