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*Research Article*

# Simultaneous Determination of Six Major Constituents of the Herbal Formula Insampaedok-san Using HPLC-PDA

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A simple, rapid, and accurate high-performance liquid chromatographic method was applied to the quantitative analysis of six components of a traditional herbal formulation, Insampaedok-san (ISPDS): liquiritin (**1**), ferulic acid (**2**), naringin (**3**), hesperidin (**4**), neohesperidin (**5**), and glycyrrhizin (**6**). The six components were separated within 35 min using a Gemini $C_{18}$ column maintained at 40°C. The mobile phase was composed of 1.0% (v/v) aqueous acetic acid (A) and 1.0% (v/v) acetic acid in acetonitrile (B) by gradient elution. The flow rate was 1.0 mL/min and the detector was a photodiode array (PDA) set at 254, 280, and 320 nm. The calibration curves showed good linearity ($R^2 = 1.0000$) for different concentration ranges. The recovery of each component was in the range of 92.62%–105.96%, with a relative standard deviation (RSD) of less than 4.0%. The RSDs for intra- and interday precision were 0.04%–1.70% and 0.06%–2.56%, respectively. The concentration of each of the six components of ISPDS was in the range 0.72–9.88 mg $g^{-1}$.

## 1. Introduction

Herbal Medicines, including herbal formulas and prescriptions, have long been used in Korea, China, Japan, and other Asian countries to prevent and treat various diseases. These herbal medicines have few side effects and exhibit multiple activities [1–3]. Insampaedok-san (ISPDS), a traditional Korean herbal prescription, is a renowned remedy used for the treatment of the common cold and influenza presenting with fever, chills, headache, muscular pain, and cough [4]. ISPDS contains 12 species of medicinal herb (Table 1) and exhibits biological activity, such as anti-inflammatory [5], analgesia [6], antipyresis [6], and neuroprotective [7] effects. Recently, reports on its safety have included consideration of acute toxicity [8, 9]. In addition, the simultaneous analysis of four constituents of fermented ISPDS using high-performance liquid chromatography (HPLC) has been reported [7]. However, this method conducted simultaneous analysis of fermented ISPDS using *Lactobacillus casei* KFRI 129, and required a long separation time for the constituents of about 65 min. There have been few reports on the quality control of the components of ISPDS, excluding research by Weon et al. [7]. Therefore, we conducted simultaneous determination of the major components for the quality control of ISPDS using a simple, rapid, and accurate HPLC-PDA method. Currently, HPLC is frequently coupled with a photodiode array (PDA) detector, because it is a convenient and widely applicable method for the rapid separation and identification of multiple components of herbal extracts and medicines [1, 10, 11].

In this study, we performed a quantitative determination and method validation using the HPLC-PDA method for six components of ISPDS: four flavonoids (liquiritin (**1**), naringin (**3**), hesperidin (**4**), and neohesperidin (**5**)), one phenolic acid (ferulic acid (**2**)), and one triterpenoid saponin (glycyrrhizin (**6**)) (Figure 1).

## 2. Experimental

*2.1. Reagents and Materials.* The reference compounds **1**, **4**, and **5** were purchased from NPC BioTechnology Inc.

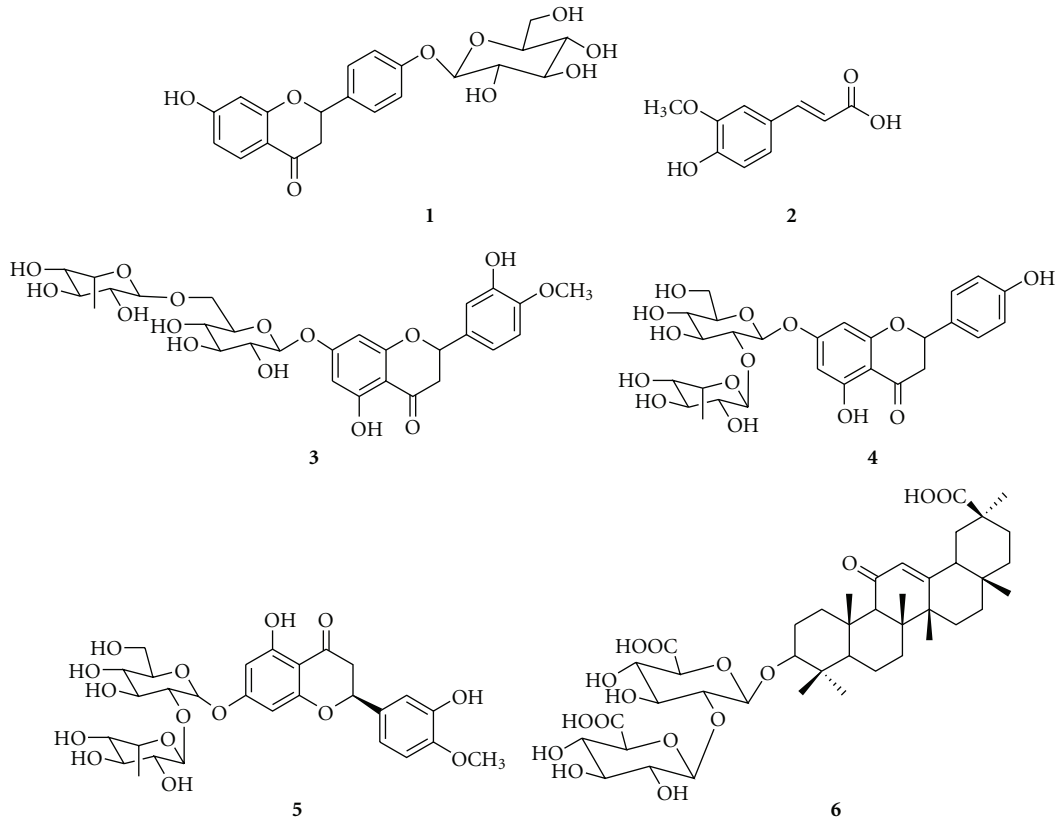


Figure 1: Chemical structure of the six marker compounds **1–6** in Insampaedok-san.

Table 1: Crude components of Insampaedok-san.

| Scientific name | Latin name | Amount (g) | Company of purchase | Source |
|---|---|---|---|---|
| *Panax ginseng* | Ginseng radix alba | 3.75 | Omniherb | Geumsan, Korea |
| *Bupleurum falcatum* | Bupleuri radix | 3.75 | HMAX | China |
| *Angelica decursiva* | Angelicae decursivae radix | 3.75 | HMAX | China |
| *Ostericum koreanum* | Osterici radix | 3.75 | HMAX | China |
| *Aralia continentalis* | Araliae continentalis radix | 3.75 | Omniherb | Yeongcheon, Korea |
| *Citrus aurantium* | Aurantii fructus immaturus | 3.75 | HMAX | China |
| *Platycodon grandiflorum* | Platycodi radix | 3.75 | Omniherb | Yeongcheon, Korea |
| *Cnidium officinale* | Cnidii rhizoma | 3.75 | Omniherb | Yeongcheon, Korea |
| *Poria cocos* | Hoelen | 3.75 | HMAX | China |
| *Glycyrrhiza uralensis* | Glycyrrhizae radix | 3.75 | HMAX | China |
| *Mentha arvensis* | Menthae herba | 3.75 | Omniherb | Yeongcheon, Korea |
| *Zingiber officinale* | Zingiberis rhizoma crudus | 3.75 | Omniherb | Yeongcheon, Korea |
| Total amount | | 45.00 | | |

(Daejeon, Republic of Korea), Biopurify Phytochemicals Ltd (Chengdu, China), and ChromaDex (Irvine, CA, USA), respectively. Compounds **2**, **3**, and **6** were obtained from Wako Chemicals (Osaka, Japan). The purity of the six reference standards was at least 98.0%, according to the HPLC data. The HPLC-grade solvents used—methanol, acetonitrile, and water—were obtained from J.T. Baker (Phillipsburg, NJ, USA). The glacial acetic acid used was of analytical reagent grade, and was procured from Junsei Chemical Co. Ltd. (Tokyo, Japan).

The ISPDS samples used, consisting of 12 herbal medicines, were purchased from Omniherb (Yeongcheon, Republic of Korea) and HMAX (Jecheon, Republic of Korea). The origin of each herbal medicine was confirmed taxonomically by Prof. Je-Hyun Lee and Young-Bae Seo, at Dongguk University, Gyeongju, Republic of Korea, and

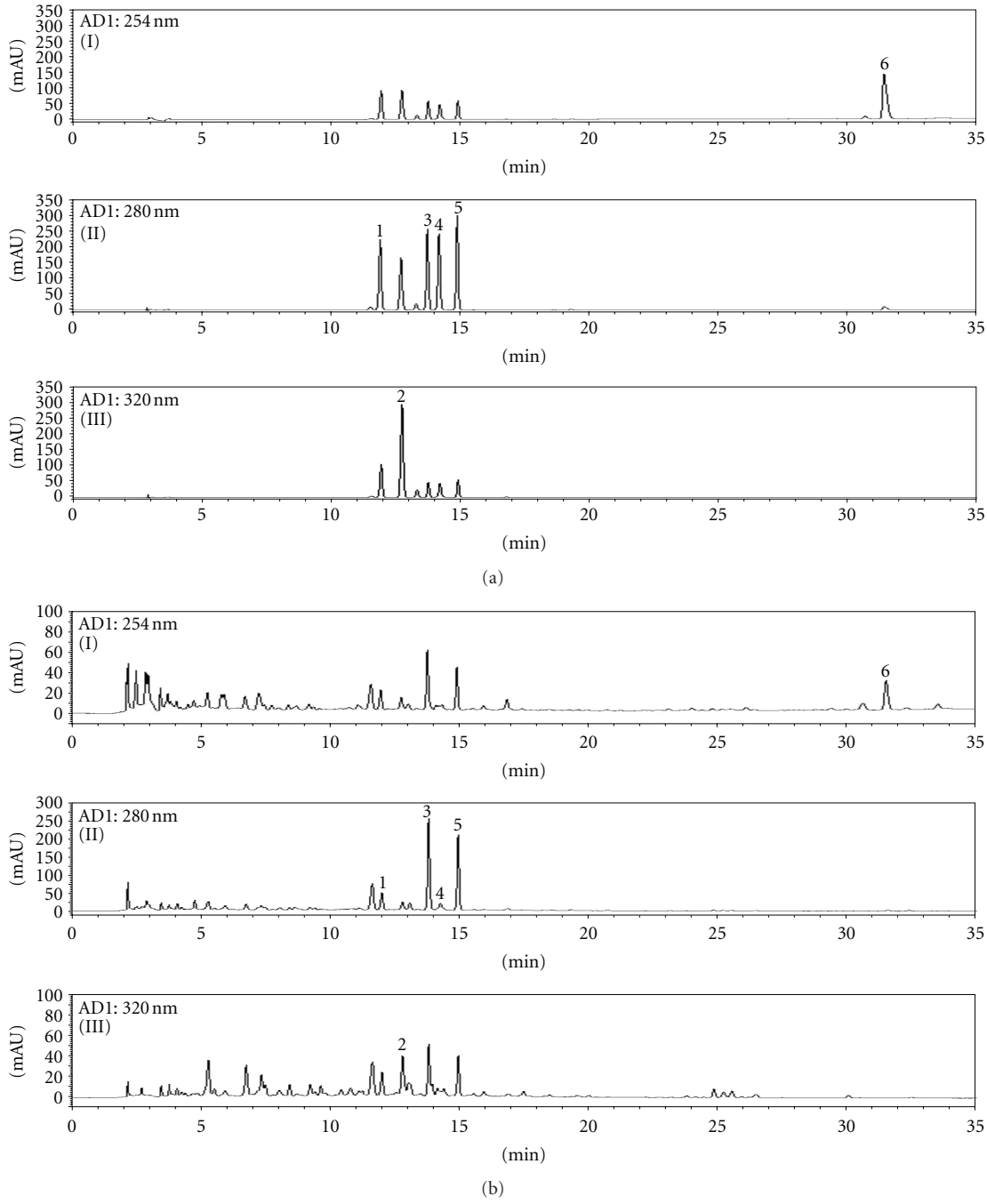


Figure 2: HPLC chromatograms of a standard mixture (a) of six components and Insampaedok-san sample (b), with detection at (I) 254 nm, (II) 280 nm, and (III) 320 nm. The peaks are ascribed to liquiritin (**1**), ferulic acid (**2**), naringin (**3**), hesperidin (**4**), neohesperidin (**5**), and glycyrrhizin (**6**).

Daejeon University, Daejeon, Republic of Korea, respectively. The composition of the ISPDS samples is shown in Table 1. A voucher specimen (2008-KE14-1 through KE14-12) has been deposited at the Basic Herbal Medicine Research Group, Korea Institute of Oriental Medicine.

*2.2. Preparation of Standard and Sample Solutions.* Standard stock solutions of compounds **1**–**6** (all at 1000 $\mu$g/mL) were prepared in methanol and stored below 4°C. Working standard solutions were prepared by serial dilution of the stock solutions with methanol.

A decoction of ISPDS was prepared in our laboratory from a mixture of chopped crude herbs (Table 1, total weight = 3.51 kg), extracted in 35 L of distilled water at 100°C for 2 h in a herb extractor (COSMOS-660, Kyungseo Machine Co., Inchon, Korea). After filtration using a standard sieve

Table 2: Linearity, correlation coefficient, LOD, and LOQ of the marker compounds ($n = 3$).

| Compound | Linear range ($\mu$g mL$^{-1}$) | Regression equation[a] | Correlation coefficient ($R^2$) | LOD[b] ($\mu$g mL$^{-1}$) | LOQ[c] ($\mu$g mL$^{-1}$) |
|---|---|---|---|---|---|
| **1** | 0.78–100.00 | $Y = 16{,}546.59x + 327.71$ | 1.0000 | 0.09 | 0.29 |
| **2** | 0.39–50.00 | $Y = 46{,}824.31x - 4{,}190.75$ | 1.0000 | 0.03 | 0.09 |
| **3** | 3.13–400.00 | $Y = 15{,}866.42x + 3{,}406.43$ | 1.0000 | 0.08 | 0.27 |
| **4** | 0.39–50.00 | $Y = 18{,}561.95x - 244.18$ | 1.0000 | 0.08 | 0.26 |
| **5** | 1.56–200.00 | $Y = 19{,}357.24x + 1{,}447.17$ | 1.0000 | 0.07 | 0.22 |
| **6** | 0.78–100.00 | $Y = 8{,}513.98x + 1{,}124.60$ | 1.0000 | 0.09 | 0.31 |

[a] $Y$ = peak area (mAU) of the components. $x$ = concentration ($\mu$g mL$^{-1}$) of the components.
[b] LOD = 3 × signal-to-noise (S/N) ratio.
[c] LOQ = 10 × signal-to-noise (S/N) ratio.

Table 3: Precision and accuracy of the analytical results ($n = 5$).

| Compound | Fortified conc. ($\mu$g mL$^{-1}$) | Intraday | | | Interday | | |
|---|---|---|---|---|---|---|---|
| | | Observed conc. ($\mu$g mL$^{-1}$) | Precision (%) | Accuracy (%) | Observed conc. ($\mu$g mL$^{-1}$) | Precision (%) | Accuracy (%) |
| | 4.00 | 4.07 | 1.63 | 101.70 | 4.07 | 0.85 | 101.73 |
| **1** | 10.00 | 10.05 | 1.69 | 100.46 | 9.82 | 0.22 | 98.16 |
| | 20.00 | 19.96 | 0.36 | 99.82 | 20.08 | 0.06 | 100.39 |
| | 2.00 | 1.98 | 0.44 | 99.10 | 2.03 | 1.13 | 101.66 |
| **2** | 4.00 | 3.96 | 1.14 | 99.02 | 4.10 | 2.56 | 102.39 |
| | 8.00 | 8.02 | 0.28 | 100.30 | 7.94 | 0.70 | 99.30 |
| | 20.00 | 19.70 | 1.22 | 98.50 | 19.21 | 0.80 | 96.04 |
| **3** | 50.00 | 50.21 | 0.60 | 100.42 | 50.11 | 0.23 | 100.21 |
| | 100.00 | 99.96 | 0.12 | 99.96 | 100.11 | 0.08 | 100.11 |
| | 3.00 | 2.94 | 1.37 | 98.12 | 2.96 | 0.55 | 98.82 |
| **4** | 8.00 | 8.52 | 0.28 | 106.51 | 8.42 | 0.83 | 105.27 |
| | 15.00 | 14.73 | 0.13 | 98.22 | 14.78 | 0.25 | 98.55 |
| | 10.00 | 10.05 | 1.70 | 100.47 | 9.76 | 0.60 | 97.63 |
| **5** | 35.00 | 34.64 | 1.19 | 98.97 | 34.58 | 0.52 | 98.81 |
| | 70.00 | 70.11 | 0.21 | 100.16 | 70.28 | 0.12 | 100.40 |
| | 6.00 | 6.34 | 0.82 | 105.67 | 6.34 | 0.84 | 105.62 |
| **6** | 15.00 | 14.50 | 0.20 | 96.64 | 14.44 | 0.65 | 96.29 |
| | 30.00 | 30.18 | 0.04 | 100.61 | 30.21 | 0.13 | 100.70 |

(no. 270, 53 $\mu$m), the solution was evaporated to dryness and freeze-dried (852.3 g). The yield of the ISPDS extract was 24.28%. For HPLC analysis, lyophilized ISPDS powder (200 mg) was accurately weighed and dissolved in distilled water (20 mL). All the solutions were filtered through a SmartPor GHP syringe filter (pore size = 0.2 $\mu$m, Woongki Science, Seoul, Korea) before HPLC analysis.

*2.3. HPLC Analysis.* HPLC analysis was performed using a Shimadzu HPLC system (Shimadzu Co., Kyoto, Japan) equipped with a solvent delivery unit (LC-20AD), online degasser (DGU-20A3), column oven (CTO-20A), autosample injector with cooler (SIL-20AC), and PDA detector (SPD-M20A). The data processor used the LC solution software package v.1.24 (Shimadzu Co., Kyoto, Japan). Column separation was carried out using a Gemini $C_{18}$ analytical column (250 × 4.6 mm, particle size = 5 $\mu$m, Phenomenex, Torrance, CA, USA) and the column temperature was maintained at 40°C. The mobile phases were 1.0% (v/v) aqueous acetic acid (A) and 1.0% (v/v) acetic acid in acetonitrile (B). The gradient flow was as follows: (A)/(B) = 85/15 (0 min) → (A)/(B) = 35/65 (40 min) → (A)/(B) = 0/100 (45 min, hold for 5 min) → (A)/(B) = 85/15 (55 min, hold for 15 min). Analysis was performed using a flow rate of 1.0 mL/min and monitored at 254 nm for compound **6**, 280 nm for compounds **1** and **3**–**5**, and 320 nm for compound **2**. The injection volume used was 10 $\mu$L.

*2.4. Linearity, Limits of Detection, and Quantification.* Calibration curves of components **1**–**6** were calculated from the peak areas of standard solutions in the following concentration range: compounds **1** and **6**, 0.78–100.00 $\mu$g mL$^{-1}$; compounds **2** and **4**, 0.39–50.00 $\mu$g mL$^{-1}$; compound **3**, 3.13–400.00 $\mu$g mL$^{-1}$; and compound **5**, 1.56–200.00 $\mu$g mL$^{-1}$.

Table 4: Recovery levels for the six marker compounds ($n = 5$).

| Compound | Original mean level ($\mu$g mL$^{-1}$) | Spiked level ($\mu$g mL$^{-1}$) | Detected mean level ($\mu$g mL$^{-1}$) | Recovery mean (%) | RSD (%) |
|---|---|---|---|---|---|
| **1** | 24.91 | 4.00 | 29.03 | 103.19 | 1.62 |
| | | 10.00 | 34.92 | 101.33 | 1.42 |
| | | 20.00 | 45.42 | 102.57 | 0.73 |
| **2** | 7.44 | 2.00 | 9.53 | 104.43 | 0.99 |
| | | 4.00 | 11.58 | 103.34 | 0.82 |
| | | 8.00 | 15.40 | 99.50 | 3.89 |
| **3** | 104.43 | 20.00 | 124.26 | 99.16 | 1.81 |
| | | 50.00 | 157.15 | 105.46 | 0.63 |
| | | 100.00 | 210.39 | 105.96 | 0.31 |
| **4** | 14.55 | 3.00 | 17.56 | 100.46 | 1.04 |
| | | 8.00 | 22.81 | 103.28 | 0.47 |
| | | 15.00 | 28.93 | 95.88 | 1.34 |
| **5** | 71.86 | 10.00 | 81.39 | 95.32 | 1.79 |
| | | 35.00 | 106.78 | 99.78 | 0.92 |
| | | 70.00 | 143.45 | 102.27 | 0.33 |
| **6** | 33.02 | 6.00 | 39.11 | 101.53 | 1.09 |
| | | 15.00 | 46.91 | 92.62 | 0.73 |
| | | 30.00 | 62.09 | 96.90 | 0.21 |

Limit of detection (LOD) and limit of quantification (LOQ) were determined based on signal-to-noise (S/N) ratios of 3 and 10, respectively.

*2.5. Precision and Accuracy.* Reproducibility was assessed by analyzing six independently prepared standard solutions. The relative standard deviation (RSD) of the analyte peak areas and peak retention times for each standard were calculated. Intra- and interday precision values were determined using a standard addition method to prepare spiked samples, employing both standards and controls. The accuracy of the HPLC method was estimated from the recovery tests. The recovery tests were performed adding known concentrations (low, medium, and high) of the reference standards to the ISPDS samples. The average recovery value was calculated using the following formula:

$$\text{Recovery (\%)} = \frac{\left(\text{Amount}_{\text{determined}} - \text{Amount}_{\text{original}}\right)}{\text{Amount}_{\text{spiked}}} \times 100. \quad (1)$$

## 3. Results and Discussion

*3.1. Optimization of HPLC Separation.* We obtained good separation chromatograms using mobile phases consisting of (A) 1.0% (v/v) aqueous acetic acid and (B) 1.0% (v/v) acetic acid in acetonitrile. Quantitation was achieved using PDA detection in the region 190–400 nm, based on retention times and UV spectra compared with those of the standards. The UV wavelength was recorded at 254 nm for compound **6**, 280 nm for compounds **1** and **3–5**, and 320 nm for compound **2**. Using these optimized chromatography conditions, the six components were eluted within a period of 35 min. The retention times of compounds **1–6** were 11.92, 14.80, 15.55, 14.20, 14.90, and 31.35 min, respectively. Representative HPLC chromatograms of standards and the extract are shown in Figure 2.

*3.2. Linearity, Range, LOD, and LOQ.* Calibration curves of compounds **1–6** were obtained using standard solutions. The linearity of the peak area ($y$) versus the concentration ($x$, $\mu$g mL$^{-1}$) curve for each component was used to calculate the amount of each main component of ISPDS. All the coefficients ($R^2$) of determination of the calibration curves for the six constituents were 1.0000. The linear equations and coefficients ($R^2$) of determination of the calibration curves are summarized in Table 2. In addition, the LOD and LOQ values were in the range 0.03–0.09 $\mu$g mL$^{-1}$ and 0.09–0.31 $\mu$g mL$^{-1}$, respectively. These data are shown in Table 2.

*3.3. Precision and Accuracy.* The reproducibility, or intra-assay precision, was assessed by repeatedly measuring the retention times and peak areas for six independently prepared samples of analyte. The reproducibility for all compounds was better than RSD = 1.00% for peak responses and better than RSD = 0.03% for the retention times (data not shown). Thus, the HPLC assay showed good repeatability under optimized conditions and this method was very stable. In addition, to test the precision and accuracy of our analytical method, the intra- and interday variations in the measurements of the six major constituents were determined; these are summarized in Table 3. Briefly, the

Table 5: Content of the six marker compounds of Insampaedok-san ($n = 3$).

| Component | Content ($mg\,g^{-1}$) | | |
|---|---|---|---|
| | Mean | SD | RSD (%) |
| **1** | 2.33 | $0.22 \times 10^{-1}$ | 0.96 |
| **2** | 0.72 | $0.02 \times 10^{-1}$ | 0.29 |
| **3** | 9.88 | $0.33 \times 10^{-1}$ | 0.34 |
| **4** | 1.42 | $0.34 \times 10^{-1}$ | 2.42 |
| **5** | 6.82 | $0.28 \times 10^{-1}$ | 0.41 |
| **6** | 3.28 | $0.08 \times 10^{-1}$ | 0.24 |

intra- and interday precisions were in the range 0.13%–1.70% and 0.12%–2.56%, respectively. The intraday accuracy was in the range 96.64%–106.51% and the interday accuracy was 96.04%–105.62%. The recovery of the six components was in the range 92.62%–105.96% and the RSD was in the range 0.21%–3.89% (Table 4).

*3.4. Sample Analysis.* Our newly established analytical method was applied to the simultaneous determination of the six components of ISPDS. Figure 2 shows chromatograms of the reference components and a water extract of ISPDS, with detection of eluents at 254, 280, and 320 nm. The amounts of compounds **1–6** (liquiritin, ferulic acid, naringin, hesperidin, neohesperidin, and glycyrrhizin, resp.) were 2.33, 0.72, 9.88, 1.42, 6.82, and 3.28 $mg\,g^{-1}$, respectively. The analytical results for each component identified are summarized in Table 5.

## 4. Conclusion

We have developed a simple and rapid HPLC method for the simultaneous separation and determination of six components of ISPDS to evaluate their quality in ISPDS samples. In this work, the simultaneous determination of these six marker compounds in ISPDS was validated with respect to linearity, precision, and accuracy. Our method will be of great usefulness improving quality control and analysis of ISPDS samples.

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