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Method Development and Validation for Simultaneous Analysis of Eleven Components for Quality Control of Geumgwesingihwan Using HPLC–DAD and UPLC–MS/MS

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Abstract: Geumgwesingihwan (GGSGH) is an oriental herbal formula made by adding *Achyranthes bidentate* Blume and *Plantago asiatica* L. to Yukmijiwhanghwan. It has been used for the treatment of edema since ancient times. The purpose of this study is to develop and validate a method for simultaneous quantification of 11 components: gallic acid (**1**), 5-(hydroxymethyl)furfural (**2**), geniposidic acid (**3**), morroniside (**4**), loganin (**5**), paeoniflorin (**6**), acteoside (**7**), cornuside (**8**), benzoic acid (**9**), benzoylpaeoniflorin (**10**), and paeonol (**11**), using high-performance liquid chromatography with a diode array detector (HPLC–DAD) and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). Compounds **1–11** were separated on a Capcell Pak UG 80 C_{18} column (250 mm × 4.6 mm, 5 μm) using a mobile phase of a distilled water–acetonitrile system, both containing 0.1% formic acid. In UPLC–MS/MS, compounds **1–11** were separated on an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm) using a mobile phase of a distilled water–acetonitrile system containing 1.0% acetic acid. Using these methods, samples of GGSGH were determined to contain 0.13–2.87 mg/g (HPLC–DAD) and not detected–4.60 mg/g (UPLC–MS/MS) of compounds **1–11**. The developed HPLC–DAD assays for simultaneous determination of all analytes were validated with respect to linearity, limits of detection and quantification, recovery, and precision. The established HPLC assay will be used to obtain basic data for quality evaluation of GGSGH and related oriental herbal formulas.

Keywords: Geumgwesingihwan; simultaneous analysis; high-performance liquid chromatography; validation; ultra-performance liquid chromatography–tandem mass spectrometry

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1. Introduction

Oriental herbal formulas have been prepared and taken in the form of tang (decoctions), pills, granules, and powders for a long time. In Korean medicine, a decoction, which is a method of boiling in water, is the most widely used method among the manufacturing methods [1,2]. In Asian countries including Korea, China, and Japan, oriental herbal formulas have been widely used for the treatment and prevention of diseases [3]. In addition, they have been reported to have fewer side effects than synthetic drugs [4,5]. Nevertheless, modern scientific research is required to confirm their biological efficacy and maintain consistent quality control. For this purpose, various analytical methods using high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry should be reported.

Geumgwesingihwan (GGSGH) is an ancient oriental herbal formula recorded in a classic of traditional Korean medicine, "*Bangyakhappyeon*", written by Hwang in the Joseon Dynasty period. It has long been used for the treatment of edema in Korea [6]. GGSGH is a formula made in Korea by adding *Achyranthes bidentate* Blume and *Plantago asiatica* L. to "Yukmijiwhanghwan" [6]. The formula is composed of eight botanical medicines: *Rehmannia glutinosa* (Gaertn.) DC., *Dioscorea japonica* Thunb., *Cornus officinalis* Siebold &

Zucc., *Poria cocos* Wolf, *Paeonia suffruticosa* Andrews, *Alisma plantago-aquatica* subsp. *orientale* (Sam.) Sam., *A. bidentate*, and *P. asiatica* L. [6].

GGSGH contains a variety of ingredients, the main ones being a furan derivative (e.g., 5-(hydroxymethyl)furfural) from *R. glutinosa* [7], an alkaloid (e.g., allatoin) and steroid (dioscin) from *D. japonica* [8,9], iridoids (e.g., morroniside and loganin) from *C. officinalis* [10], triterpenoids (e.g., polyporenic acid C and pachymic acid) from *P. cocos* [11], a monoterpenoid (e.g., paeoniflorin) and phenol (e.g., paeonol) from *P. suffruticosa* [12], triterpenoids (e.g., alisol B and alisol B acetate) from *A. plantago-aquatica* [13], a steroid (e.g., ecdysterone) from *A. bidentate* [14], and an iridoid (e.g., geniposidic acid) and phenylpropanoid (e.g., acteoside) from *P. asiatica* [15].

Several analytical methods have been developed and reported for quality evaluation of each of the constituent raw herbs of GGSGH, using analytical instruments such as HPLC [7–12,14], ultra-performance liquid chromatography [13], and liquid chromatography with mass spectrometry [11,16]. However, no analysis method has been reported for quality control of GGSGH based on these analytical approaches. Today, quality control of oriental herbal formulas composed of two or more herbal medicines are often performed with these analytical instruments.

Herein, we report the development and validation of a simultaneous assay with the widely used and convenient HPLC coupled with a diode array detector (DAD), for efficient quality control of GGSGH using 11 analytes: gallic acid (**1**), 5-(hydroxymethyl)furfural (**2**), geniposidic acid (**3**), morroniside (**4**), loganin (**5**), paeoniflorin (**6**), acteoside (**7**), cornuside (**8**), benzoic acid (**9**), benzoylpaeoniflorin (**10**), and paeonol (**11**). In addition, simultaneous analysis of these analytes was performed using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS).

2. Materials and Methods

2.1. Plant Materials

As presented in Table S1, 8 botanical herbal medicines, which are the constituents of GGSGH, were purchased from Kwangmyungdang Pharmaceuticals (Ulsan, Korea), a specialized herbal medicine manufacturing company, in November 2017. Each raw plant material was morphologically identified according to the guideline "The Dispensatory on the Visual and Organoleptic Examination of Herbal Medicine" by Dr. Goya Choi, Korea Institute of Oriental Medicine (KIOM, Daejeon, Korea) [17]. A specimen (2018CA04–1 to 2018CA04–8) of each raw material was deposited at the Korean Medicine Science Research Division, KIOM.

2.2. Chemicals and Reagents

The 11 analytes used for the quality control of GGSGH were purchased from various standard compound manufacturing companies: compounds **1** (CAS No. 149-91-7, 100.0%, Catalog No. G7384), 5-(hydroxymethyl)furfural, **2** (CAS No. 67-47-0, ≥99.0%, Catalog No. W501808), **9** (CAS No. 65-85-0, 99.9%, Catalog No. 242381), and **11** (CAS No. 552-41-0, 99.9%, Catalog No. H35803) from Merck KGaA (Darmstadt, Germany); compound **3** (CAS No. 27741-01-1, ≥98.0%, Catalog No. 078-05841) from Fujifilm Wako Pure Chemical Co., Ltd. (Osaka, Japan); compounds **6** (CAS No. 23180-57-6, 99.4%, Catalog No.DR10579), **8** (CAS No. 131189-57-6, 98.7%, Catalog No.DR10598), and **10** (CAS No. 38642-49-8, ≥98.0%, Catalog No. DR10582) from Shanghai Sunny Biotech Co., Ltd. (Shanghai, China); and compounds **4** (CAS No. 25406-64-8, 99.9%, Catalog No. BP0960), **5** (CAS No. 18524-94-2, ≥98.0%, Catalog No. BP0884), and **7** (CAS No. 61276-17-3, 99.7%, Catalog No. BP0124) from Biopurify Phytochemicals (Chengdu, China) (Figure S1). The solvents methanol (MeOH), acetonitrile (ACN), and distilled water (DW) were HPLC- or MS-grade and were purchased from JT Baker (Phillipsburg, NJ, USA). The acids trifluoroacetic acid (TFA, ≥99.0%), formic acid (FA, 98.0–100.0%, analytical-grade), and glacial acetic acid (AA, ≥100.0%, ACS reagent-grade) were purchased from Merck KGaA (Darmstadt, Germany).

2.3. Preparation of GGSGH Sample

A GGSGH sample (GGSGH–1) was extracted at KIOM using a previously reported preparation protocol [18,19]. Each raw herb presented in Table S1 was mixed with water (50 L) and boiled at 100 °C for 2 h, using an electric extractor (COSMOS-660, Kyungseo E&P, Incheon, Korea). The extract was then filtered using a sieve (53 μm mesh) and freeze-dried with an LP100R freeze dryer (IlShinBioBase, Yangju, Korea). After extraction and freeze-drying, the powdered sample weighed 1355.6 g (yield 27.1%) and was used in the analysis while refrigerated (ca. 4 °C). A second sample, GGSGH–2, was provided by Wonkwang University and was prepared using the same protocol.

2.4. Preparation of Sample Solutions and Standard Solutions for HPLC–DAD Analysis

Sample solutions for HPLC simultaneous analysis of compounds **1**–**11** in lyophilized GGSGH samples were prepared at a concentration of 10 mg/mL using 70% MeOH. For the quantification of compounds **1**, **2**, **4**, **6**, and **11**, the prepared sample solution was subsequently diluted twofold with 70% MeOH prior to analysis.

A standard stock solution of each reference standard compound was prepared, stored under refrigeration at a concentration of 1000 μg/mL in MeOH and diluted prior to use.

All solutions were filtered with a 0.2 μm syringe filter (Pall Life Sciences, Ann Arbor, MI, USA) before HPLC analysis.

2.5. HPLC Equipment and Simultaneous Analysis Conditions

The method was developed with a Shimadzu Prominence LC-20A series instrument equipped with DAD (Tokyo, Japan), and data were collected and processed using LCSolution software (version 1.24, SP1, Shimadzu Corporation, Kyoto, Japan). Under the analysis conditions shown in Table S2, compounds **1**–**11** were separated on a reversed-phase Capcell Pak UG80 C_{18} column (250 mm × 4.6 mm, 5 μm, Shiseido, Tokyo, Japan).

2.6. Validation Method for the Developed HPLC Simultaneous Analysis Method

The developed HPLC method for efficient quality assessment of GGSGH was validated with respect to linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, and precision according to the guidelines [20]. Briefly, the linearity was evaluated as the coefficient of determination (r^2) value, using the regression equation of the calibration curves of analytes prepared in the concentration ranges of 0.31–20.00 μg/mL (compounds **1**, **3**, **5**, and **7**–**10**) and 0.63–40.00 μg/mL (compounds **2**, **4**, **6**, and **11**). LOD and LOQ were calculated from the regression equation: LOD = 3.3 × σ/S and LOQ = 10 × σ/S, where σ is the standard deviation of the y-intercept and S is the slope of the regression equation. The extraction recovery (%) was evaluated by the standard addition method in which three different concentrations were added to a known sample. The recovery was calculated as: recovery (%) = found amount/spiked amount × 100. Finally, intra-day and inter-day precisions were evaluated by calculating the relative standard deviation (RSD, %) values after measuring the standard solution five times per day on three consecutive days. The repeatability was measured six times and evaluated by calculating the RSD for the area and retention time of each peak.

2.7. System Suitability Test

The suitability of the analysis system was assessed by evaluating the capacity factor (k'), selectivity factor (α), resolution (Rs), number of theoretical plates (N), and tailing factor (Tf) parameters according to the guidelines [21].

2.8. Simultaneous Analysis of GGSGH Samples Using UPLC–MS/MS

Simultaneous analysis of the eleven analytes was conducted in GGSGH samples using a UPLC–MS/MS multiple reaction monitoring (MRM) method. Analysis of these analytes was performed using a Waters Acquity UPLC H-Class system coupled with a Xevo TQ-S micro-MS (Milford, MA, USA), operated via MassLynx (version 4.2, Waters Corporation,

Milford, MA, USA). The UPLC and MS/MS analysis conditions are shown in Table S3, and the MRM parameters of each analyte for simultaneous analysis are shown in Table 1.

Table 1. MRM parameters of each marker analyte for UPLC–MS/MS analysis.

Analyte [a]	Ion Mode	Exact Mass (Da)	Precursor Ion (Q1, *m*/*z*)	Product Ion (Q3, *m*/*z*)	Cone Voltage (V)	Collision Energy (eV)
1	−	170.02	169.0	125.0	40	13
2	+	126.03	126.9	109.0	25	8
3	−	374.12	373.0	122.9	50	15
4		406.15	450.8	160.9	30	15
5	+	390.15	391.1	228.9	12	8
6	−	480.16	478.9	448.9	60	5
7	+	624.21	625.1	163.0	16	28
8	+	542.16	543.1	211.0	16	14
9		122.04	122.5	79	15	8
10	−	584.19	583.0	553.0	70	5
11	+	166.06	166.9	42.9	16	16

[a] Gallic acid (**1**), 5-(hydroxymethyl)furfural (**2**), geniposidic acid (**3**), morroniside (**4**), loganin (**5**), paeoniflorin (**6**), acteoside (**7**), cornuside (**8**), benzoic acid (**9**), benzoylpaeoniflorin (**10**), and paeonol (**11**).

3. Results and Discussion

3.1. HPLC Phytochemical Profiling of Each Raw Herb Composing GGSGH

As shown in Figure S2, HPLC profile analysis of the constituent herbs of GGSGH was conducted using a Capcell Pak UG80 C_{18} column (250 mm × 4.6 mm, 5 μm, Shiseido, Tokyo, Japan) and a DW–ACN mobile system (both containing 0.1% FA) for 5-(hydroxymethyl)furfural from *R. glutinosa*, dioscin from *D. japonica*, gallic acid, morroniside, loganin, cornin, cornuside, and sweroside from *C. officinalis*, polyporenic acid C and pachymic acid from *P. cocos*, gallic acid, paeoniflorin, benzoic acid, benzoylpaeoniflorin, and paeonol from *P. suffruticosa*, alisol B and alisol B acetate from *A. plantago-aquatica*, ecdysterone from *A. bidentate*, and geniposidic acid and acteoside from *P. asiatica*. Furthermore, these components were identified as target components for the development of an analytical method for quality evaluation of GGSGH by HPLC (Figure S2).

3.2. Selection of Components for Continuous Quality Evaluation of GGSGH

Based on the HPLC profiling performed in Section 3.1, the presence or absence of a total of 19 components (presented in Section 3.1) in GGSGH was investigated. As shown in Figure S3, the investigated components were detected in each raw herb, but only 11 components were detected in GGSGH, a mixture of eight botanical medicines. As a result, 11 components (compounds **1–11**) were selected as components for continuous quality evaluation of GGSGH (Figure 1).

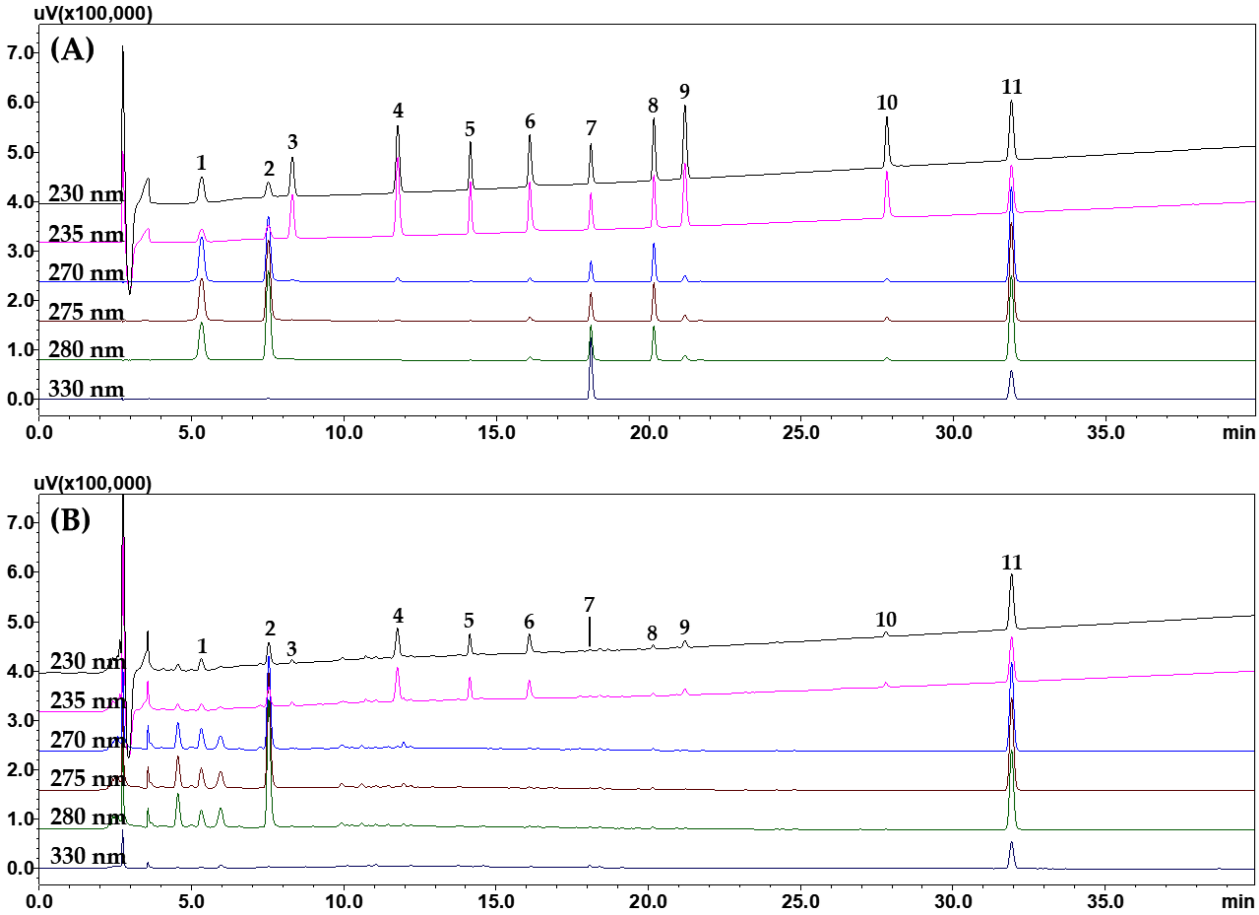

Figure 1. HPLC chromatograms of the standard solution (**A**) and GGSGH sample (**B**) monitored at different UV wavelengths. Gallic acid (**1**), 5-(hydroxymethyl)furfural (**2**), geniposidic acid (**3**), morroniside (**4**), loganin (**5**), paeoniflorin (**6**), acteoside (**7**), cornuside (**8**), benzoic acid (**9**), benzoylpaeoniflorin (**10**), and paeonol (**11**). The concentration of each analyte in the mixed standard solution was: 10.00 μg/mL (compound **5**), 20.00 μg/mL (compounds **1**, **2**, **9**, and **11**), 40.00 μg/mL (compounds **4** and **10**), and 50.00 μg/mL (compounds **3** and **6**–**8**).

3.3. Optimization of HPLC Simultaneous Analysis Conditions

Optimization of the simultaneous analysis of components of GGSGH was achieved according to the following steps. First, a series of reversed-phase C_{18} columns (4.6 mm × 250 mm, 5 μm) such as Gemini (Phenomenex, Torrance, CA, USA), Hypersil GOLD (Thermo Fisher Scientific Inc., San Jose, CA, USA), XBridge (Waters, Milford, MA, USA), Quasar SPP (PerkinElmer, Seoul, Korea), and Capcell Pak UG80 (Shiseido, Tokyo, Japan) were compared with respect to their efficiency for the chromatographic separation of the components selected in Section 2.2 (Table S4). Secondly, following the column comparison, the system was optimized further by adding FA, trifluoroacetic acid (TFA), or glacial acetic acid (AA) to the mobile-phase system to improve separation performance (Table S5). Finally, after establishing the optimal column type and mobile-phase acid additive, the effects of different column oven temperatures (30, 35, 40, and 45 °C) were compared (Table S6). By comparing the above conditions, the optimal conditions for simultaneous analysis of compounds **1**–**11** in GGSGH were established to be a Capcell Pak UG80 C_{18} column, with a column temperature of 40 °C, and gradient elution of the DW–ACN mobile-phase system with both phases containing 0.1% FA. All components were separated within 35 min without interference from neighboring peaks (Figure 1).

3.4. Validation Method for the Developed HPLC Simultaneous Analysis

For the consistent quality control of GGSGH, a simultaneous analysis method using HPLC–DAD was developed for the first time. The assay, which was based on the analysis of 11 components, was validated by testing the linearity, LOD, LOQ, and precision. The results are summarized in Table 2. For all analytes, the r^2 values and residuals show good linearity over the tested ranges ≥0.9998 and <3.0%, respectively (Table 1 and Figure S4). In this assay, the LOD and LOQ values of all analytes based on the regression equation of each analyte were calculated as 0.01–0.17 μg/mL and 0.03–0.22 μg/mL. The extract recovery values (%) of compounds **1–11** were in the range 97.78–104.20%, showing good results (Table 3). Table 4 shows the validation results for intra-day and inter-day precision, which were all evaluated with respect to the RSD. Analysis of all compounds confirmed the good precision of the method, with an RSD value of less than 2.0%. The RSD values of repeatability for the retention times and peak areas of compounds **1–11** were all found to be less than 1.0% (Table S7). The validation results confirm that the HPLC method developed in this study is appropriate for use as an assay for quality control of compounds **1–11** as components of GGSGH.

Table 2. Detection wavelength, linear range, regression equation, r^2, LOD, LOQ, and retention time of each analyte for a simultaneous determination (n = 3).

Analyte [a]	Detection Wavelength (nm)	Linear Range (μg/mL)	Regression Equation [b] y=ax+b	r^2	LOD (μg/mL)	LOQ (μg/mL)	Retention Time (min)
1	270	0.31–20.00	y = 55,170.65x + 3666.55	0.9998	0.03	0.10	5.32
2	280	0.63–40.00	y = 94,130.28x + 14147.39	0.9998	0.04	0.13	7.50
3	235	0.31–20.00	y = 13,975.16x + 1218.48	0.9999	0.02	0.05	8.29
4	235	0.63–40.00	y = 33,876.65x + 5744.90	0.9998	0.05	0.14	11.75
5	235	0.31–20.00	y = 57,411.73x + 5068.48	0.9998	0.01	0.03	14.13
6	230	0.63–40.00	y = 15,482.56x − 2650.38	0.9999	0.07	0.22	16.08
7	330	0.31–20.00	y = 15,904.03x + 1722.64	0.9999	0.03	0.08	18.09
8	270	0.31–20.00	y = 9735.69x + 978.96	0.9998	0.04	0.12	20.32
9	230	0.31–20.00	y = 60,978.26x + 6475.75	0.9998	0.02	0.06	21.17
10	230	0.31–20.00	y = 20,261.17x + 476.60	0.9999	0.06	0.19	27.79
11	275	0.63–40.00	y = 192,362.00x + 22,923.35	0.9999	0.03	0.08	31.89

[a] Details of the substances are presented in Table 1; [b] y and x represent the respective peak areas at different concentrations (μg/mL) of each reference standard compound.

Table 3. Extract recovery (%) for a simultaneous determination of 11 analytes in the developed HPLC method.

Analyte [a]	Spiked Amount (μg/mL)	Found Amount (μg/mL)	Recovery (%) [b]	SD	RSD (%)
1	1.00	1.01	100.55	1.43	1.42
	2.00	2.03	101.32	0.74	0.73
	4.00	4.05	101.34	1.64	1.61
2	2.00	2.02	101.17	0.80	0.79
	5.00	5.06	101.17	1.53	1.51
	10.00	10.11	101.09	0.52	0.52
3	1.00	1.01	101.44	1.29	1.28
	2.00	2.02	100.90	1.25	1.24
	4.00	4.10	102.47	0.59	0.57
4	2.00	1.98	99.06	1.36	1.37
	4.00	4.12	102.88	1.50	1.46
	8.00	8.15	101.87	0.29	0.28
5	1.00	1.01	100.66	1.15	1.14
	2.00	2.00	99.84	1.01	1.01
	4.00	4.17	104.20	0.51	0.49
6	2.00	1.99	99.15	1.52	1.53
	5.00	5.12	102.42	0.91	0.89
	10.00	10.04	100.56	0.38	0.38
7	1.00	1.02	101.99	1.18	1.16
	2.00	2.02	101.13	0.73	0.72
	4.00	4.14	103.53	0.41	0.40
8	1.00	1.01	100.92	1.00	1.00
	2.00	2.06	103.10	1.05	1.01
	4.00	4.03	100.78	0.95	0.94
9	1.00	0.99	99.25	0.90	0.90
	2.00	1.96	98.03	0.58	0.59
	4.00	3.96	99.11	0.37	0.37
10	1.00	1.01	100.77	1.38	1.37
	2.00	1.96	98.12	1.52	1.55
	4.00	4.07	101.75	0.81	0.80
11	2.00	1.96	97.78	1.97	2.01
	4.00	4.12	102.96	0.67	0.65
	8.00	8.06	100.78	0.39	0.38

[a] Details of the substances are presented in Table 1; [b] recovery (%) = found amount/spiked amount × 100.

Table 4. Validation of the precision of the developed HPLC method using 11 analytes.

Analyte [a]	Conc. (μg/mL)	Intra-Day (n = 5)			Inter-Day (n = 5)		
		Observed Conc. (μg/mL)	Precision (RSD, %)	Accuracy (%)	Observed Conc. (μg/mL)	Precision (RSD, %)	Accuracy (%)
1	5.00	5.09	0.17	101.77	5.15	1.11	102.98
	10.00	9.86	1.39	98.64	9.93	0.98	99.27
	20.00	19.67	0.88	98.33	19.83	1.06	99.13
2	10.00	10.29	0.46	102.86	10.42	1.15	104.21
	20.00	19.87	1.64	99.37	20.02	1.18	100.09
	40.00	39.44	0.98	98.59	39.89	1.33	99.72
3	5.00	5.07	0.24	101.50	5.13	1.20	102.59
	10.00	9.90	0.85	98.95	9.93	0.63	99.26
	20.00	19.66	0.37	98.29	19.78	0.95	98.88
4	10.00	10.14	0.16	101.38	10.27	1.19	102.73
	20.00	19.71	1.62	98.54	19.84	1.05	99.19
	40.00	39.25	0.84	98.13	39.53	1.05	98.83
5	5.00	5.07	0.30	101.36	5.13	1.15	102.64
	10.00	9.84	1.08	98.36	9.92	0.92	99.18
	20.00	19.61	0.90	98.05	19.76	1.04	98.80
6	10.00	9.88	0.38	98.84	9.98	1.23	99.79
	20.00	19.33	1.56	96.64	19.43	1.06	97.14
	40.00	39.41	0.96	98.51	39.76	1.22	99.39
7	5.00	5.03	0.22	100.56	5.09	1.18	101.86
	10.00	9.82	1.46	98.20	9.88	1.01	98.82
	20.00	19.62	0.69	98.10	19.72	0.87	98.61
8	5.00	5.07	0.21	101.38	5.13	1.11	102.65
	10.00	9.85	1.67	98.46	9.90	1.08	99.04
	20.00	19.66	0.68	98.32	19.77	1.01	98.85
9	5.00	5.14	0.17	102.81	5.21	1.18	104.17
	10.00	9.95	1.27	99.52	10.07	1.31	100.65
	20.00	19.84	1.19	99.20	20.09	1.40	100.44
10	5.00	5.01	0.33	100.23	5.07	1.16	101.36
	10.00	9.80	1.45	97.96	9.85	1.23	98.54
	20.00	20.27	0.50	101.37	20.03	1.26	100.13
11	10.00	10.30	0.18	103.04	10.43	1.12	104.31
	20.00	20.03	1.23	100.13	20.29	1.35	101.47
	40.00	39.87	1.19	99.68	40.40	1.39	100.99

[a] Details of the substances are presented in Table 1.

3.5. System Suitability Test

The system suitability evaluation results show that the system is appropriate with respect to k' (0.95–10.70), α (1.06–1.85), Rs ($\geq$3.20), N ($\geq$13,320.17), and Tf (1.02–1.38) (Table S8).

*3.6. Simultaneous Determination of Compounds **1**–**11** in GGSGH Samples Using HPLC*

The HPLC method developed for quality control of GGSGH using 11 components was successfully applied to the quantitative analysis of real samples. Appropriate chromatographic separations were achieved using a reversed-phase column and were monitored simultaneously for quantification at 230 nm (compounds **6**, **9**, and **10**), 235 nm (compounds **3**–**5**), 270 nm (compounds **1** and **8**), 275 nm (compound **11**), 280 nm (compound **2**), and 330 nm (compound **7**). All compounds were detected at concentrations of 0.13–2.87 mg/g within 35 min (Table 5, Figure 1). Among these components, compound **2** (main component of *R. glutinosa*) was determined to be present in the highest concentration.

Table 5. Contents of compounds **1**–**11** in GGSGH samples (n = 3).

Analyte [a]	HPLC Analysis				LC–MS/MS Analysis			
	GGSGH–1 [b]		GGSGH–2		GGSGH–1		GGSGH–2	
	Mean (mg/g) ± SD ($\times 10^{-2}$)	RSD (%)	Mean (mg/g) ± SD ($\times 10^{-2}$)	RSD (%)	Mean (mg/g) ± SD ($\times 10^{-1}$)	RSD (%)	Mean (mg/g) ± SD ($\times 10^{-1}$)	RSD (%)
1	0.89 ± 0.07	0.08	0.44 ± 0.14	0.31	3.47 ± 0.71	2.04	1.27 ± 0.08	0.66
2	2.38 ± 0.22	0.09	2.87 ± 3.87	1.35	3.90 ± 0.07	0.18	4.60 ± 1.23	2.67
3	0.35 ± 0.18	0.50	0.55 ± 0.29	0.53	0.35 ± 0.10	2.76	0.58 ± 0.06	1.07
4	1.64 ± 0.17	0.10	1.34 ± 0.22	0.16	ND [c]	–	ND	–
5	0.46 ± 0.20	0.44	0.41 ± 0.22	0.53	1.52 ± 0.66	4.36	1.45 ± 0.79	5.42
6	1.94 ± 0.66	0.34	1.39 ± 0.23	0.17	2.48 ± 0.85	3.44	1.79 ± 0.43	2.40
7	0.22 ± 0.14	0.64	0.19 ± 0.35	1.85	0.15 ± 0.01	0.91	0.12 ± 0.08	6.66
8	0.32 ± 0.12	0.37	0.25 ± 0.05	0.21	0.27 ± 0.03	0.94	0.21 ± 0.02	1.07
9	0.18 ± 0.03	0.19	0.13 ± 0.07	0.53	ND	–	ND	–
10	0.40 ± 0.26	0.64	0.22 ± 0.29	1.30	0.64 ± 0.12	1.95	0.37 ± 0.09	2.35
11	1.78 ± 0.15	0.08	0.43 ± 0.02	0.04	3.67 ± 0.37	1.01	0.99 ± 0.21	2.12

[a] Details of the substances are presented in Table 1; [b] GGSGH–1: sample prepared at KIOM, GGSGH–2: sample provided by Wonkwang University; [c] not detected.

*3.7. Simultaneous Determination of Compounds **1**–**11** in GGSGH Samples Using UPLC–MS/MS*

A total of 11 analytes were detected using positive and negative ion modes of the UPLC–MS/MS MRM mode (Table 1). As a result of analyzing compounds **1**–**11** in GGSGH samples using the established analytical method, compounds **4** and **9** were not detected. Therefore, they were excluded from the quantitative analysis. As shown in Table S9, calibration curves for quantitative analyses of all analytes were prepared at different concentration levels, and the linearity of each analyte was good with $r^2 \geq 0.9985$. Representative total ion chromatograms of the standard solution and the sample solution are shown in Figure 2. Except for compounds **4** and **9**, the analytes were detected at concentrations of 0.12–4.60 mg/g in the lyophilized GGSGH samples (Table 5). Using UPLC–MS/MS, compound **2** was detected at the highest concentration as in the HPLC–DAD analysis.

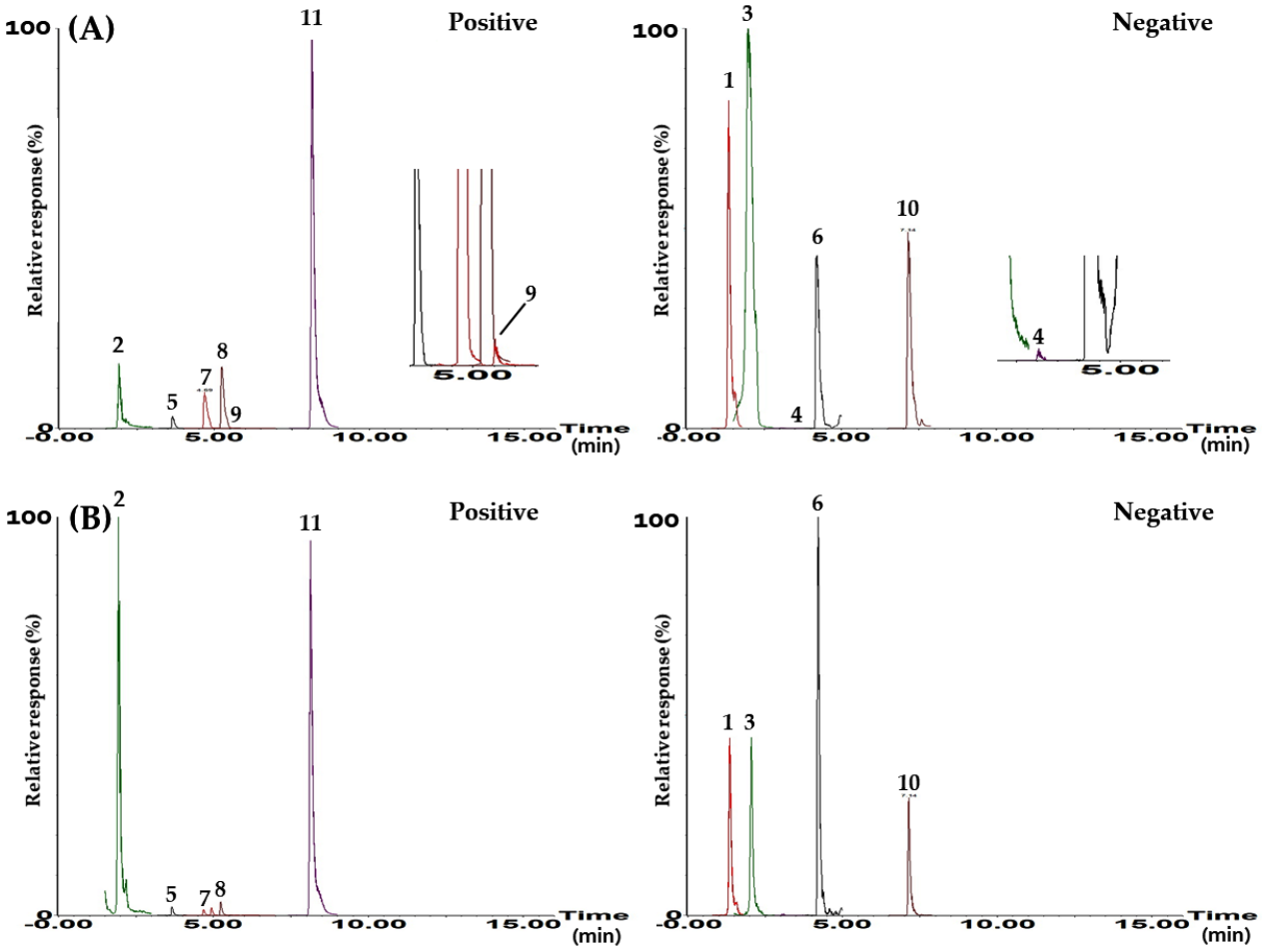

Figure 2. Total ion chromatograms of the mixed reference standard (**A**) and GGSGH sample (**B**) using LC–MS/MS MRM mode. Gallic acid (**1**), 5-(hydroxymethyl)furfural (**2**), geniposidic acid (**3**), morroniside (**4**), loganin (**5**), paeoniflorin (**6**), acteoside (**7**), cornuside (**8**), benzoic acid (**9**), benzoylpaeoniflorin (**10**), and paeonol (**11**).

4. Conclusions

Using the widely used HPLC system and the more sensitive and accurate UPLC–MS/MS system, a simultaneous analysis method for consistent quality control of the oriental herbal formula GGSGH was developed for the first time. The developed HPLC assay was validated with respect to linearity, LOD, LOQ, recovery, and precision, and was satisfactorily applied to the simultaneous analysis of real samples. These analytical methods will provide valuable data for quality standardization of GGSGH as well as other herbal formulas.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9080213/s1. Figure S1: Chemical structures of the 11 selected components for quality control of GGSGH; Figure S2: HPLC profiling of the raw botanical herbs and their main components. A: *R. glutinosa*; B: *D. japonica*; C: *C. officinalis*; D: *P. cocos*; E: *P. suffruticosa*; F: *A. plantago-aquatica*; G: *A. bidentate*; H: *P. asiatica*; Figure S3: HPLC chromatograms of the solution of investigated 19-component standard mixture (A) and 70% methanol solution of GGSGH sample (B). Gallic acid (**1**), 5-(hydroxymethyl)furfural (**2**), geniposidic acid (**3**), morroniside (**4**), loganin (**5**), cornin (6), paeoniflorin (**7**), acteoside (**8**), ecdysterone (**9**), cornuside (**10**), benzoic acid (**11**), sweroside (**12**), benzoylpaeoniflorin (**13**), paeonol (**14**), dioscin (**15**), polyporenic acid C (**16**), alisol B (**17**), alisol B acetate (**18**), and pachymic acid (**19**); Figure S4: Residual plots for evaluating linearity of 11 components and their calibration curves. Gallic acid (A), 5-(hydroxymethyl)furfural (B), geniposidic acid (C), morroniside (D), loganin (E), paeoniflorin (F), acteoside (G), cornuside (H),

benzoic acid (I), benzoylpaeoniflorin (J), and paeonol (K); Table S1: Composition of GGSGH; Table S2: HPLC chromatographic conditions for analyzing the 11 analytes of GGSGH; Table S3: UPLC–MS/MS conditions for simultaneous analysis of the 11 analytes in GGSGH samples; Table S4: Parameter comparison of analytes according to different columns; Table S5: Effect of column oven temperatures on selected column; Table S6: Effect of acids on selected column and column oven temperature; Table S7: Repeatability for retention times and peak areas of compounds **1**–**11**; Table S8: System suitability of compounds **1**–**11**; Table S9: The retention time, linear range, regression equation, r^2, and LOD of the analytes from GGSGH using LC–MS/MS MRM mode.

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