Quantitative Analytical Method for Eighteen Marker Compounds in Ongyeong-tang, a Traditional Herbal Formula, Using High-Performance Liquid Chromatography for Quality Assessment

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ABSTRACT

Objectives : Ongyeong-tang (OGT) is a traditional herbal formula used to cure gynaecological disorders. OGT consists of 12 herbal medicines containing various bioactive components. Therefore, the development of suitable analytical method for the marker compounds is necessary for the quality control of OGT.

Methods : Determination of the 18 marker compounds in OGT preparations was quantitatively performed by high-performance liquid chromatography-photodiode array detection analysis. The marker compounds were separated on a reversed-phase C_{18} column and the analytical method was successfully validated, which was applied to compare OGT extracts from laboratory preparation and commercial OGT granules.

Results : Limit of detection and limit of quantification values were in the ranges of 0.001-0.016 μg/mL and 0.003-0.047 μg/mL, respectively. Precision was 0.03-3.71 % within a day and 0.03-3.81 % over four consecutive days. Recovery of marker compounds ranged from 90.63-108.26 %, with relative standard deviation (RSD) values ⟨4.0 %. Reproducibility was ⟨2.5 % of the RSD value. The 18 marker compounds were stable within 16 h at 10 ℃, with the RSD value ⟨3.5 %. Quantitative analysis results showed that the quantities of the 18 marker compounds varied among OGT samples. Pearson coefficient evaluation and principal component analysis demonstrated that an OGT water extract produced by a laboratory method clearly differed from commercial OGT granules.

Conclusions : The developed analytical method was simple, precise, and reliable. Therefore, it can be used for the quality assessment of OGT preparations.

Key words : Ongyeong-tang, Quantitative analysis, Method development, Pearson coefficient, Principal component analysis

Introduction

Ongyeong-tang (OGT), a traditional herbal formula for treating gynaecological disorders, consists of 12 herbal medicines, i.e., *Liriope platyphylla* F.T.Wang & Tang, *Angelica gigas* Nakai, *Panax ginseng* C.A.Mey., *Pinellia ternata* (Thunb.) Makino, *Paeonia lactiflora* Pall., *Chidium officinale* Makino., *Paeonia suffruticosa* Andrews, *Equus asinus* L., *Glycyrrhiza uralensis* Fisch., *Euodia ruticarpa* (A. Juss.) Benth, *Cinnamomum cassia* Presl, and *Zingiber officinale* Roscoe. OGT has been traditionally used for the treatment of menstrual diseases such as irregular menstruation, advanced or delayed menstruation, and uterine bleeding[1]. Clinical studies reported that OGT can treat anovulatory women with high plasma luteinizing hormone levels, improve gonadotropin pulsatile or estradiol

secretion in the treatment of anovulatory women, or adjunctively treat hormone replacement therapy−resistant menopausal women with depressive ilness[2-5]. OGT has also shown pharmacological effects such as stimulatory effects on ovarian steroidogenesis and the ovulatory process in the ovary, inhibitory effects on the corticotrophin−releasing factor−induced increase in locomotor activity, and the proliferation of uterine endometrial tissue[6-9]. OGT is a combination of these 12 herbal medicines with a specific compositional ratio and is generally prescribed as a form of decoction produced by boiling with water. Multiple chemical constituents can be extracted from the compositional herbs; their chemical interaction is thought to contribute their therapeutic effects. Identification of the chemical compounds in OGT and the determination of their quantities may be a fundamental step towards further pharmacological or clinical experiments. However, the quantification of OGT based on a single compound, or several chemical compounds is challenging, because a few compounds cannot reflect all the chemical characteristics of OGT. Therefore, the simultaneous determination of various chemical compounds with different structure is necessary for understanding the full chemical spectrum of OGT. Thus, the quality assessment of OGT preparations can be performed using quantitative analysis of multiple components as marker compounds.

High−performance liquid chromatography combined with photodiode array detection (HPLC−PDA) has been widely used for the chemical analysis of herbal formulae; it is a simple, precise, and rapid technique for the detection of multiple chemical compounds[10-12]. Thus, HPLC−PDA methods are used for the quality control of herbal preparations[13-15]. Various bioactive marker compounds were reported in the compositional herbs of OGT: gallic acid, oxypaeoniflorin, (+)−catechin, paeoniflorin, 1,2,3,4,6−pentagalloylglucose, benzoylpaeoniflorin, and paeonol from *P. lactiflora* and *P. suffruticosa*; chlorogenic acid, caffeic acid, and ferulic acid from *C. officinale*; liquiritin, ononin, and glycyrrhizin from *G. uralensis*; nodakenin from *A. gigas*; coumarin, cinnamic acid, and cinnamaldehyde from *C. cassia*; and 6−gingerol from *Z. officinale*[16-25]. Some of these bioactive compounds were also used as the marker compounds for HPLC analysis of herbal medicines[26,27]. Therefore, the above 18 chemical compounds can be used as the marker compounds for the quantitative analysis and quality assessment of OGT preparations.

In the present study, we developed analytical methods for the 18 marker compounds in an OGT water extract (OGTWE) by a validated method using HPLC−PDA. The amounts of marker compounds were quantified. An OGTWE and commercial granules were compared for the quality assessment of OGT preparations.

Materials and methods

1. Chemicals and reagents

HPLC−grade methanol, acetonitrile, and water were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Formic acid was purchased from Fluka (Seelze, Germany). Trifluoroacetic acid was purchased from Alfa Aesar (Ward Hill, MA, USA). Gallic acid (1), (+)−catechin (3), ferulic acid (7), coumarin (11), and cinnamic acid (13) were purchased from Sigma−Aldrich (St Louis, MO, USA). Oxypaeoniflorin (2), nodakenin (9), 1,2,3,4,6−pentagalloylglucose (10), ononin (12), and benzoylpaeoniflorin (15) were supplied by Chengdu Biopurify Phytochemicals (Chengdu, China). Chlorogenic acid (4) and caffeic acid (5) were purchased from Acros Organics (Morris Plains, NJ, USA). Paeoniflorin (6), cinnamaldehyde (14), and paeonol (16), 6−gingerol (17), and glycyrrhizin (18) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Liquiritin (8) was supplied by NPC Biotechnology (Geumsan, Korea). The chemical structures of the standard compounds are shown in Fig. 1. The purity of all reagents and marker compounds was ≥ 98 %.
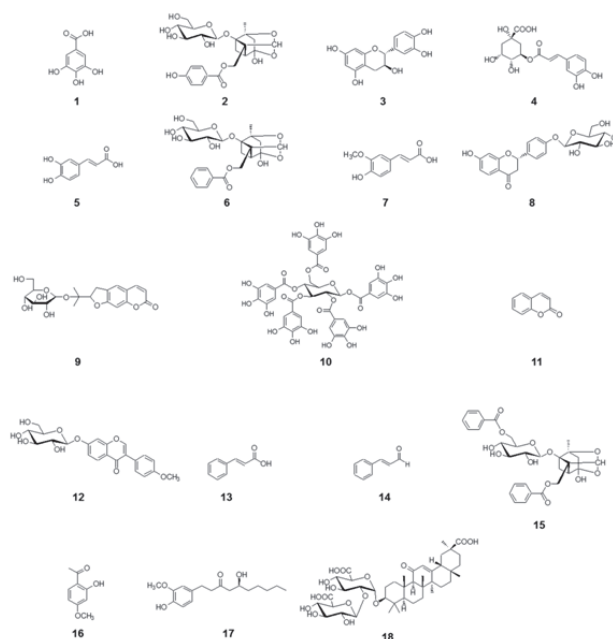
Fig. 1. Chemical structures of the 18 standard compounds in Ongyeong−tang (OGT). (1) Gallic acid, (2) oxypaeoniflorin, (3) (+)−catechin, (4) chlorogenic acid, (5) caffeic acid, (6) paeoniflorin, (7) ferulic acid, (8) liquiritin, (9) nodakenin, (10) 1,2,3,4,6−pentagalloylglucose, (11) coumarin, (12) ononin, (13) cinnamic acid, (14) cinnamaldehyde, (15) benzoylpaeoniflorin, (16) paeonol, (17) 6−gingerol, and (18) glycyrrhizin.

Compositional herbal medicines purchased from the herbal medicine company, Kwangmyungdang Medicinal Herbs (Ulsan, Korea), and from the commercial samples are listed in Table 1. A voucher specimen (2014−KE41−1

-12) was deposited in the Herbal Medicine Formulation Research Group of the Korea Institute of Oriental Medicine.

Table 1. Composition of Ongyeong-tang water extract (OGTWE), OGT01, and OGT02

Herbal medicine	OGTWE		OGT01 (g)	OGT02 (g)
	Original region	Amount (g)		
Liriope platyphylla F.T.Wang & Tang	Milyaong, Gyeongnam, Korea	7.50	3.33	3.33
Angelica gigas Nakai	Bonghwa, Gyeongbuk, Korea	5.63	1.00	1.00
Panax ginseng C.A.Mey.	Yeongju, Gyeongbuk, Korea	3.75	0.67	0.67
Pinellia ternata (Thunb.) Makino	China	3.75	1.67	1.67
Paeonia lactiflora Pall.	Euiseong, Gyeongbuk, Korea	3.75	0.67	0.67
Cnidium officinale Makino.	Yeongyang, Gyeongbuk, Korea	3.75	0.67	0.67
Paeonia suffruticosa Andrews	Jecheon, Chungbuk, Korea	3.75	0.67	0.67
Equus asinus L.	China	2.81	0.67	0.67
Glycyrrhiza uralensis Fisch.	China	2.81	0.67	0.67
Euodia ruticarpa (A. Juss.) Benth	China	1.88	1.00	1.00
Cinnamomum cassia Presl	Vietnam	1.88	0.67	0.67
Zingiber officinale Roscoe	Ulsan, Gyeongbuk, Korea	3.75	0.33	0.33
Sum	–	45.01	12.02	12.02

2. Sample preparation of OGT WE and commercial granules

A mixture of crude herbal drugs consisting of OGT was extracted with a 10-fold volume of distilled water (w/v) at 100 ℃ for 2 h under pressure (1 kgf/㎠) using an electric extractor (COSMOS-660; Kyungseo Machine, Incheon, Korea). The extracted decoction was filtered through a standard sieve (no. 270, 53 ㎛; Chunggyesangongsa, Seoul, Korea) and then freeze-dried to obtain an OGTWE powder.

Accurately weighed OGTWE (100 mg) and commercial OGT granules (300 mg) were dissolved in 10 mL distilled water, and the solutions were filtered through a 0.2 ㎛ syringe filter (SmartPor®; Woongki Science, Seoul, Korea) prior to injection into the HPLC apparatus.

3. Preparation of standard solutions of marker compounds

The stock solutions were prepared by dissolving accurately weighed standard compounds in methanol at concentrations of 1000 ㎍/mL. Working solutions were produced by diluting the stock solutions containing standard compounds. Diluted working solutions were used to construct calibration curves.

4. Chromatographic conditions

The HPLC-PDA system comprised a LC-20A (Shimadzu, Kyoto, Japan) equipped with a solvent delivery unit, autosampler, column oven, degasser, and PDA. The acquired data were processed using LabSolutions software (v. 5.3; Shimadzu). The 18 marker compounds were separated on a Gemini C$_{18}$ column (4.6 × 250 ㎜, 5 ㎛ ; Phenomenex, Torrance, CA, USA). The column oven was maintained at 40 ℃. The mobile phase consisted of water containing 0.1 % formic acid (A) and acetonitrile (B), with gradient elution (see Table 2). The flow rate was 1.0 mL/min and the injection volume was set to 10 ㎕. The detection wavelengths were optimized according to the maximum absorption wavelengths of the standard compounds.

Table 2. Mobile phase gradient conditions (A:B)

Time (min)	A (%)	B (%)
0	92	8
10	83	17
15	83	17
25	80	20
28	80	20
32	72	28
35	72	28
45	44	56
47	44	56
55	23	77
57	23	77
65	92	8
72	92	8

A, 0.1% formic acid in water; B, acetonitrile.

5. Method validation

1) Linearity

Accurately weighed standard compounds were dissolved in methanol to prepare stock solutions in concentrations of 1000 ㎍/mL. Stock solutions of the 18 marker compounds were diluted to construct calibration curves. The diluted concentrations of marker compounds were plotted against the peak area on the calibration curves and the linearity was determined using correlation coefficient.

2) The limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined as follows: LOD = $3.3 × SD/S$ and LOQ = $10 × SD/S$, where SD is the standard deviation of the response and S is the slope of the calibration curve.

3) Precision

The precision was evaluated by four analyses in one day (intra-day precision) and by analysis over four consecutive days (inter-day precision. Precision was calculated by analysing sample extracts containing

low, medium, and high concentrations of marker compounds. The precision value was represented as the relative standard deviation (RSD) [(standard deviation / mean) × 100].

4) Recovery

The recovery test was performed to evaluate the accuracy of the method used. The three known amounts of marker compounds (low, medium, and high) were added to the samples and the recovery was calculated as follows: recovery (%) = [(detected concentration – initial concentration) / spiked concentration) × 100].

5) Reproducibility

The reproducibility was determined by calculating the RSD values of the retention times and the absolute areas of marker compounds ($n = 6$).

6) Stability

The stability test was conducted by analysing the extracted solution after storage under refrigeration at 10 ℃ for 4, 8, 12, and 16 h

6. Statistical analysis

One-way analysis of variance (ANOVA) using the Tukey test, Pearson correlation efficient, and the principal component analysis (PCA) were performed for multiple group comparisons using open-source software R (v. 3.0.2). Differences were considered significant at $P < 0.05$, $P < 0.01$, or $P < 0.001$.

Results

1. Optimization of chromatographic conditions

The mobile phase consisted of water (A) and acetonitrile (B), and a modifier. Two different modifiers, such as 0.1% trifluoroacetic acid and 0.1% formic acid, were compared for the optimal separation of the 18 marker compounds. Better resolution and peak shapes between marker compounds were obtained when 0.1% formic acid was used as modifier. Various ratios of the components of the mobile phase (A:B) were tested using gradient elution for optimal separation of marker compounds. Selected gradient elution conditions used are listed in TableII. The optimal UV wavelength for each marker compound was tested in the range 190-400 nm: paeoniflorin and benzoylpaeoniflorin at 230 nm; ononin and glycyrrhizin at 250 nm; oxypaeoniflorin at 255 nm; gallic acid at 270 nm; (+)-catechin, liquiritin, coumarin, cinnamic acid,

and paeonol at 275 nm; 1,2,3,4,6-pentagalloylglucose and 6-gingerol at 280 nm; cinnamaldehyde at 290 nm; chlorogenic acid, caffeic acid, and ferulic acid at 325 nm; and nodakenin at 335 nm. The 18 marker compounds were reasonably well separated for quantitative analysis on a chromatogram without severe overlapping or interception of adjacent peaks(Fig. 2).
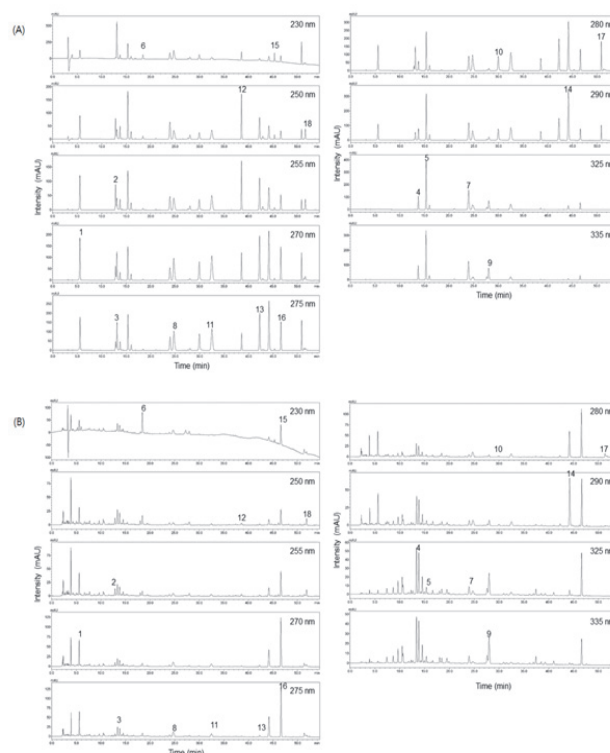

Fig. 2. Chromatograms of the 18 standard compounds (A) and OGT water extract (B). (1) Gallic acid, (2) oxypaeoniflorin, (3) (+)-catechin, (4) chlorogenic acid, (5) caffeic acid, (6) paeoniflorin, (7) ferulic acid, (8) liquiritin, (9) nodakenin, (10) 1,2,3,4,6-pentagalloylglucose, (11) coumarin, (12) ononin, (13) cinnamic acid, (14) cinnamaldehyde, (15) benzoylpaeoniflorin, (16) paeonol, (17) 6-gingerol, and (18) glycyrrhizin.

2. Method validation

1) System suitability

The system suitability was measured in terms of the capacity factor (k), relative retention (a), resolution (Rs), theoretical plate number (N), and symmetry factor (S) of the 18 marker compounds. The capacity factor ranged from 0.81 to 15.60. The relative retention was $1 < a < 4$ and the resolutions of marker compounds were > 1.2, which indicate that the marker peaks were not severely overlapped by adjacent peaks and interference from other components. The number of theoretical plates was > 8944. The symmetry factor was $0.9 < S < 1.6$, which demonstrated that peak fronting or tailing was not found(Table 3).

Table 3. System suitability data

Compound	k	α	Rs	N	S
Gallic acid	0.81	3.94	33.73	8944.47	0.98
Oxypaeoniflorin	3.18	1.03	1.22	66669.90	1.07
(+)-Catechin	3.26	1.03	1.22	57929.90	1.04
Chlorogenic acid	3.48	1.06	3.10	75069.98	1.11
Caffeic acid	3.99	1.15	7.64	79454.31	1.07
Paeoniflorin	4.94	1.25	12.25	65062.78	1.52
Ferulic acid	6.80	1.04	1.82	57503.58	1.02
Liquiritin	7.05	1.04	1.82	47476.51	1.29
Nodakenin	8.14	1.15	8.18	96048.90	0.99
1,2,3,4,6-Pentagalloylglucose	8.75	1.08	5.11	98953.30	1.13
Coumarin	9.59	1.10	5.35	50320.04	0.99
Ononin	11.41	1.21	15.28	503128.80	1.10
Cinnamic acid	12.77	1.10	13.58	268351.87	1.02
Cinnamaldehyde	13.39	1.05	5.89	293251.85	1.04
Benzoylpaeoniflorin	13.60	1.03	4.66	1234168.88	1.41
Paeonol	14.19	1.03	6.52	631799.59	1.07
6-Gingerol	15.57	1.02	3.65	1553984.63	1.10
Glycyrrhizin	15.60	1.02	3.65	655683.08	1.09

k, capacity factor; a, relative retention; Rs, resolution; N, theoretical plate; S, symmetry factor.

2) Linear regression, LOD, and LOQ

The linearity was represented as the correlation coefficient (r^2) of compounds: it ranged from 0.9998 to 1.0000. The LOD and LOQ values were 0.000-0.016 µg/mL and 0.001-0.047 µg/mL, respectively(Table 4).

Table 4. Linear equation, correlation coefficients (r^2), LOD ,and LOQ fo rthe marker compounds in OGT

Compound	Detection wavelength (nm)	Linear equation	r^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Gallic acid	270	y = 37527x - 9073.9	0.9999	0.94-60	0.002	0.007
Oxypaeoniflorin	255	y = 13639x - 1395.9	1.0000	0.63-40	0.010	0.030
(+)-Catechin	275	y = 6309.1x - 102.97	0.9999	0.08-5	0.016	0.047
Chlorogenic acid	325	y = 37099x - 8987.9	0.9999	0.94-60	0.002	0.005
Caffeic acid	325	y = 50288x - 1834.7	0.9999	0.16-10	0.001	0.004
Paeoniflorin	230	y = 13648x - 5626.6	1.0000	3.91-250	0.005	0.015
Ferulic acid	325	y = 44083x - 4028.1	1.0000	0.47-30	0.001	0.004
Liquiritin	275	y = 20603x - 3948.9	1.0000	1.17-75	0.005	0.014
Nodakenin	335	y = 31107x - 8810.6	1.0000	1.56-100	0.003	0.009
1,2,3,4,6-Pentagalloylglucose	280	y = 24679x - 4035.9	0.9998	0.31-20	0.004	0.011
Coumarin	275	y = 53552x - 8072.9	1.0000	0.78-50	0.002	0.006
Ononin	250	y = 31487x - 661.44	1.0000	0.12-7.5	0.002	0.007
Cinnamic acid	275	y = 64369x - 1459.6	1.0000	0.12-7.5	0.002	0.005
Cinnamaldehyde	290	y = 140371x - 9931.9	1.0000	0.49-31.5	0.000	0.001
Benzoylpaeoniflorin	230	y = 24942x - 4605.7	0.9999	0.39-25	0.003	0.008
Paeonol	275	y = 54045x - 1984.1	1.0000	1.95-125	0.002	0.006
6-Gingerol	280	y = 6131.2x + 31.083	0.9998	0.08-2.5	0.014	0.044
Glycyrrhizin	250	y = 7812.5x - 246.21	1.0000	1.56-100	0.009	0.028

LOD, limit of detection; LOQ, limit of quantification; y, peak area (mAU); x, concentration of compound (µg/mL).

3) Precision, recovery, reproducibility, and stability

The intra- and inter-day precisions were represented as RSD values < 4.0% in three concentration levels (Table 5). The recoveries of the 18 marker compounds were in the range 90.63-107.57 %, with RSD values < 4.0%, at three different spiked concentrations(Table 6).

The reproducibility of the marker compounds was also represented as RSD values; < 0.2% for the retention time and < 2.5% for absolute area. The stability did not exceed 3.5 % of the RSD value (Table 7). These results indicate that the analytical method that was developed was precise, reproducible, and stable for the analysis of the 18 marker compounds in OGT samples.

Table 5. Intra- and inter-day precision of the 18 marker compounds in OGT

Compound	Spiked conc. (µg/mL)	Intra-day (n=4)			Inter-day (n=4)		
		Detected conc. (µg/mL)	RSD (%)	Accuracy (%)	Detected conc. (µg/mL)	RSD (%)	Accuracy (%)
Gallic acid	2.50	2.46	0.89	97.40	2.48	1.15	98.16
	5.00	4.90	0.84	98.15	4.90	0.84	97.74
	10.00	10.06	0.20	100.63	10.06	0.20	100.68
Oxypaeoniflorin	2.00	1.92	1.75	97.86	1.92	1.89	97.86
	4.00	4.07	0.43	102.11	4.06	0.45	102.11
	8.00	7.99	0.18	99.60	7.99	0.20	99.60
(+)-Catechin	0.50	0.50	1.64	100.99	0.50	1.05	100.99
	1.00	0.97	1.56	94.85	0.96	1.29	94.85
	2.00	2.02	0.28	101.23	2.02	0.25	101.23
Chlorogenic acid	2.50	2.49	0.55	99.57	2.47	1.00	99.57
	5.00	4.87	0.48	97.68	4.86	0.72	97.68
	10.00	10.07	0.10	100.61	10.08	0.23	100.61
Caffeic acid	1.00	0.98	0.65	98.02	0.99	0.75	98.02
	2.00	1.90	1.34	96.24	1.89	2.21	96.24
	4.00	4.05	0.32	101.06	4.06	0.47	101.06
Paeoniflorin	15.00	15.05	2.27	100.99	14.87	1.35	98.36
	30.00	29.89	1.09	100.31	30.59	3.81	107.70
	60.00	60.04	0.25	99.86	59.74	0.96	98.17
Ferulic acid	1.00	1.07	0.71	107.57	1.07	1.63	109.72
	2.00	2.08	0.83	103.11	2.06	2.36	99.41
	4.00	3.94	0.20	98.75	3.95	0.51	99.54
Liquiritin	3.00	3.00	1.47	100.40	3.00	1.78	100.40
	6.00	5.97	0.87	98.45	5.98	0.86	98.45
	12.00	12.02	0.26	100.36	12.01	0.23	100.36
Nodakenin	4.00	3.79	0.82	95.36	3.77	1.30	95.36
	8.00	7.89	0.22	98.39	7.89	0.18	98.39
	16.00	16.11	0.07	100.69	16.11	0.08	100.69
1,2,3,4,6-Pentagalloylglucose	0.50	0.51	1.97	99.22	0.50	2.00	99.22
	1.00	1.05	1.21	105.33	1.06	1.69	105.33
	2.00	1.97	0.23	98.72	1.97	0.36	98.72
Coumarin	1.00	1.07	0.64	107.47	1.07	0.73	107.47
	2.00	2.10	0.28	105.23	2.11	0.29	105.23
	4.00	3.93	0.09	98.23	3.93	0.04	98.23
Ononin	0.50	0.51	0.40	102.08	0.52	1.86	102.08
	1.00	0.99	0.16	99.49	0.99	0.35	99.49
	2.00	2.00	0.03	100.00	2.00	0.05	100.00
Cinnamic acid	0.50	0.49	1.86	98.84	0.50	2.09	98.84
	1.00	1.01	0.65	101.77	1.01	1.02	101.77
	2.00	2.00	0.15	99.63	2.00	0.19	99.63
Cinnamaldehyde	1.05	0.95	1.08	89.89	0.95	1.31	89.89
	2.10	2.19	0.50	104.44	2.19	0.35	104.44
	4.20	4.18	0.09	99.52	4.18	0.07	99.52
Benzoylpaeoniflorin	1.00	0.95	2.10	94.37	0.96	1.37	94.37
	2.00	2.10	2.00	102.71	2.08	3.17	102.71
	4.00	3.96	0.63	99.68	3.97	0.87	99.68
Paeonol	4.00	3.95	1.02	98.12	3.94	1.51	98.12
	8.00	7.68	0.31	96.32	7.70	0.32	96.32
	16.00	16.17	0.11	101.04	16.16	0.03	101.04
6-Gingerol	0.40	0.41	1.17	102.42	0.41	1.11	102.77
	0.80	0.79	3.71	96.17	0.81	3.80	97.99
	1.60	1.60	0.91	100.81	1.59	0.96	100.33
Glycyrrhizin	4.00	3.94	2.05	98.07	3.93	2.46	98.07
	8.00	7.79	0.16	97.54	7.77	0.41	97.54
	16.00	16.12	0.12	100.74	16.13	0.22	100.74

Conc., concentration; SD, standard deviation; RSD, relative standard deviation (%) = (SD/mean) × 100.

Table 6. Recovery and repeatability of the 18 marker compounds in OGT (*n*=5)

Compound	Initial conc. (μg/mL)	Spiked conc. (μg/mL)	Detected conc. (μg/mL)	Recovery (%)	RSD (%)
Gallic acid	9.77	2.50	12.25	99.17	1.77
		5.00	14.75	99.69	0.91
		10.00	20.02	102.52	1.06
Oxypaeoniflorin	6.32	2.00	8.19	93.95	2.84
		4.00	10.30	99.64	1.10
		8.00	14.15	97.92	0.37
(+)-Catechin	0.94	0.50	1.44	99.11	2.67
		1.00	1.94	99.69	1.96
		2.00	3.04	104.72	1.25
Chlorogenic acid	9.84	2.50	12.21	94.79	1.29
		5.00	14.54	94.04	1.61
		10.00	19.63	97.92	1.11
Caffeic acid	1.17	1.00	2.16	98.88	1.10
		2.00	3.17	99.97	0.42
		4.00	5.48	107.57	0.95
Paeoniflorin	67.60	15.00	82.57	99.82	1.85
		30.00	97.81	100.70	1.36
		60.00	128.08	100.80	1.52
Ferulic acid	2.95	1.00	4.03	108.26	1.06
		2.00	4.98	101.42	1.63
		4.00	6.72	94.20	0.55
Liquiritin	12.41	3.00	15.31	96.69	1.81
		6.00	18.14	95.49	1.44
		12.00	23.92	95.97	2.30
Nodakenin	15.97	4.00	19.70	93.38	2.09
		8.00	23.95	99.75	1.18
		16.00	32.51	103.35	0.88
1,2,3,4,6-Pentagalloylglucose	1.94	0.50	2.47	104.49	2.62
		1.00	2.99	104.30	0.77
		2.00	3.89	97.35	0.71
Coumarin	3.60	1.00	4.66	105.64	0.49
		2.00	5.59	99.31	0.61
		4.00	7.23	90.63	0.58
Ononin	0.94	0.50	1.46	105.33	0.87
		1.00	1.95	101.35	0.20
		2.00	2.97	101.56	0.20
Cinnamic acid	0.92	0.50	1.43	101.13	2.42
		1.00	1.97	105.07	1.35
		2.00	2.99	103.57	0.41
Cinnamaldehyde	6.56	1.05	7.52	91.58	1.24
		2.10	8.81	107.03	0.67
		4.20	10.87	102.59	0.44
Benzoylpaeoniflorin	2.75	1.00	3.70	94.89	0.62
		2.00	4.80	102.63	1.67
		4.00	6.73	99.66	2.65
Paeonol	23.68	4.00	27.54	96.55	0.94
		8.00	31.35	95.89	0.90
		16.00	40.00	102.01	0.73
6-Gingerol	0.13	0.40	0.51	96.66	2.47
		0.80	0.87	93.10	1.56
		1.60	1.67	96.42	0.36
Glycyrrhizin	13.42	4.00	17.43	100.16	3.78
		8.00	21.40	99.75	1.34
		16.00	30.07	104.05	1.14

Conc., concentration; RSD, relative standard deviation (%) = (SD/mean) × 100.

Table 7. Reproducibility and stability of the 18 marker compounds (RSD, %)

Compound	Reproducibility (*n*=6)		Stability (≤ 16 h)
	Retention time	Absolute area	
Gallic acid	0.04	0.79	1.10
Oxypaeoniflorin	0.06	0.75	0.85
(+)-Catechin	0.06	1.11	2.02
Chlorogenic acid	0.05	0.64	0.44
Caffeic acid	0.05	0.91	0.59
Paeoniflorin	0.06	1.06	0.42
Ferulic acid	0.06	0.95	0.58
Liquiritin	0.06	0.83	0.87
Nodakenin	0.07	0.97	0.55
1,2,3,4,6-Penta galloylglucose	0.08	1.64	3.28
Coumarin	0.06	1.18	0.18
Ononin	0.04	0.88	0.58
Cinnamic acid	0.04	1.09	0.60
Cinnamaldehyde	0.03	1.09	0.85
Benzoylpaeoniflorin	0.02	0.89	0.25
Paeonol	0.03	0.86	0.50
6-Gingerol	0.02	2.43	1.66
Glycyrrhizin	0.11	0.87	1.57

RSD, relative standard deviation (%) = (standard deviation/mean) × 100.

3. Quantification of the 18 marker compounds in OGT samples

The validated method was successfully applied to determine and quantify the 18 marker compounds in OGTWE and two commercial OGT granules (OGT01 and OGT02)(Fig. 3).
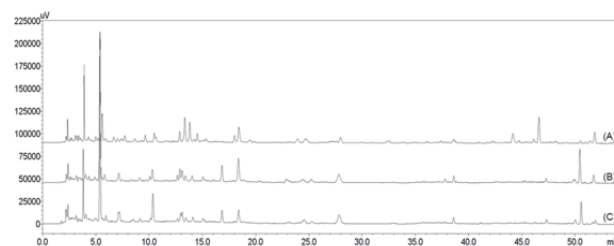

Fig. 3. Chromatograms of OGT water extract and OGT commercial granules at 250 nm. (A) OGT water extract, (B) OGT01 (commercial OGT granules), and (C) OGT02 (commercial OGT granules).

In OGTWE, the amount of paeoniflorin was highest, followed by paeonol, while the amounts of 6-gingerol, (+)-catechin, ononin, and cinnamic acid were lowest. The amount of paeoniflorin was more than 500-fold compared to that of 6-gingerol. Furthermore, paeoniflorin and gallic acid were present in higher amounts, while ferulic acid and paeonol were present in lower amounts, in two commercial OGT granules in which (+)-catechin, caffeic acid, coumarin, cinnamaldehyde, and paeonol were not detected (and hence it was not possible to quantify these compounds). In addition, 1,2,3,4,6-pentagalloylglucose was not detected in the OGT01(Table 8).

Table 8. Quantification of the 18 marker compounds in OGT samples

Compound	Content (mg/g)[a]		
	OGTWE	OGT01	OGT02
Gallic acid	0.985 ± 0.007	1.332 ± 0.007	1.649 ± 0.005
Oxypaeoniflorin	0.632 ± 0.005	0.095 ± 0.004	0.047 ± 0.001
(+)-Catechin	0.094 ± 0.002	ND	ND
Chlorogenic acid	0.987 ± 0.001	0.064 ± 0.001	0.054 ± 0.000
Caffeic acid	0.117 ± 0.000	ND	ND
Paeoniflorin	6.783 ± 0.023	2.818 ± 0.043	1.629 ± 0.067
Ferulic acid	0.295 ± 0.001	0.008 ± 0.000	0.006 ± 0.000
Liquiritin	1.250 ± 0.006	0.237 ± 0.004	0.281 ± 0.002
Nodakenin	1.599 ± 0.010	0.078 ± 0.001	0.106 ± 0.002
1,2,3,4,6-Pentagalloylglucose	0.191 ± 0.004	ND	0.016 ± 0.000
Coumarin	0.361 ± 0.000	ND	ND
Ononin	0.094 ± 0.001	0.042 ± 0.000	0.041 ± 0.000
Cinnamic acid	0.093 ± 0.001	0.012 ± 0.000	0.012 ± 0.000
Cinnamaldehyde	0.661 ± 0.004	ND	ND
Benzoylpaeoniflorin	0.275 ± 0.001	0.102 ± 0.001	0.046 ± 0.000
Paeonol	2.377 ± 0.003	0.006 ± 0.000	0.002 ± 0.000
6-Gingerol	0.013 ± 0.000	ND	ND
Glycyrrhizin	1.342 ± 0.023	0.195 ± 0.002	0.103 ± 0.006

[a]Content is represented as 'average ± standard deviation'.
ND, not detected; OGTWE, OGT water extract; OGT01 and OGT02, commercial granules from Korean manufacturers.

Variations in the amounts of the marker compounds were also found between samples. Gallic acid was the only compound that was present in a significantly higher amount in the commercial granules than in OGTWE, while the amounts of most detectable compounds in the commercial samples were significantly lower than those of OGTWE. The difference in the amounts of the marker compounds between OGT samples was about 2-1200-fold(Fig. 4).
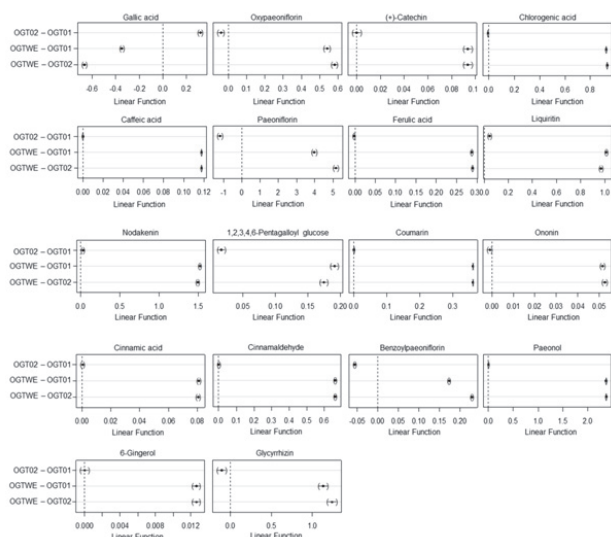

Fig. 4. One-way ANOVA using the Tukey test of the 18 marker compounds among samples with 95% family-wise confidence level.

4. Pearson correlation efficient and PCA of OGT samples

The relationship between OGT samples was represented by the Pearson correlation coefficient, using the quantification results. In the box plot, the median value of the coefficient of OGTWE was low compared with the values of the two commercial OGT granules(Fig. 5).
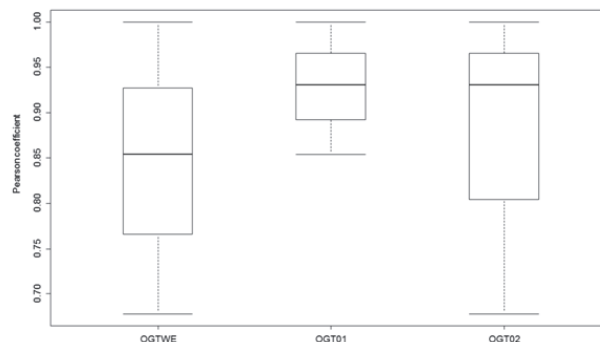

Fig. 5. Pearson coefficient of OGT samples. OGTWE, OGT water extract; OGT01-OGT02, commercial OGT granules

PCA results showed a distinct relationship between OGT samples. In the score plot, OGTWE was clearly separated from two commercial OGT samples by principal component 1 (PC1) with high total variance (98.3%). Principal component 2 (PC2) score further divided the commercial granules into positive and negative scores, with an insignificant effect on total variance (1.7%) (Fig. 6A). Of the 18 marker compounds on loading plot, only gallic acid was positioned at a positive PC1 score, while the 17 compounds were all positioned at a negative PC1 score(Fig. 6B).
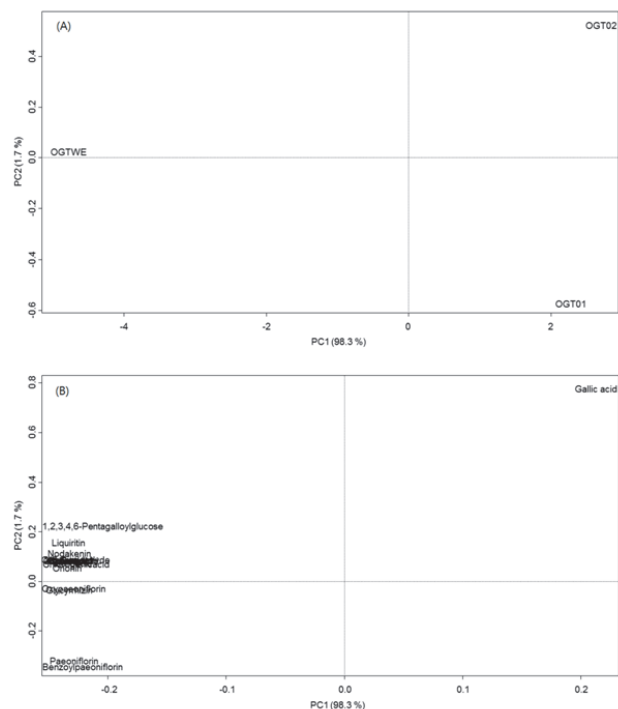

Fig. 6. Score plot (A) and loading plot (B) of principal components (PC1 vs. PC2) on the variables (amounts of the 18 marker compounds) with the objectives (OGT samples). PC1 and PC2 contributed to 98.3% and 1.7% of total variance, respectively. OGTWE, OGT water extract; OGT01-OGT02, commercial OGT granules.

Discussion

The HPLC conditions for the analysis of OGTWE were established; these included the mobile phase ratio, modifier, and UV wavelength of PDA. The reversed-phase C_{18} is the most frequently used stationary phase in herbal medicine analysis using a HPLC system[28-30]. We, therefore, employed a C_{18} column for the simultaneous determination of the 18 marker compounds in OGTWE.

There were noticeable variation in the amounts of the 18 marker compounds observed between OGTWE and two commercial granules. Of the marker compounds of which amounts were significantly higher in OGTWE, the amount of gallic acid was solely higher in the commercial granules than in OGTWE. This is because cinnamaldehyde and paeonol are volatile; therefore, so they can be easily vaporized at high temperature, without causing the vapour valve of the extractor to be closed and (+)-catechin is degraded at high temperature[31-33]. The other compounds, except for gallic acid, were not extracted efficiently because of the possible interference from other components, or an excessive amount of excipient.

The result from Pearson correlation coefficient showed that the median value of the coefficient of OGTWE was apparently different from those of the two commercial OGT granules. This result indicates that OGTWE was not closely correlated with the two commercial samples. The principal component analysis (PCA) result also supported the result of Pearson correlation coefficient. In the score plot, principal component 1 (PC1), which is most influential to sample clustering, significantly contributed to separation of OGTWE from two commercial OGT samples. Principal component 2 (PC2) score, next influential variable of sample distribution orthogonal to PC1, also divided two commercial granules with 1.7% of total variance, although influence was not significant. This means that the two commercial samples are closely related, while OGTWE is not associated with those samples-closer PC scores means a closer relationship[34]. In loading plot, only gallic acid was conglomerated far from 17 compounds, which indicates that the compounds located at each PC score contributed to the classification of OGT samples and gallic acid is most potential compound for separation of OGTWE and commercial OGT samples.

Taken together, the results from the quantification of 18 marker compounds, Pearson correlation coefficient, and PCA, the commercial OGT samples were significantly different from laboratory-produced OGT water extract. Moreover, there is variations in the amounts of several compounds within commercial OGT samples. These are presumably ascribed to the difference of manufacturing process among companies or herbal resources used, which led to variations in the amounts of marker compounds. It is the reason why a similar processing method of herbal preparation among the companies is much needed to maintain constant quality of OGT preparations.

Conclusions

In the present study, the analytical methods of HPLC-PDA, using reversed-phase C_{18} column, was developed for the simultaneous determination of the 18 marker compounds in OGTWE.

1. Validation methods included system suitability, precision, recovery, reproducibility, and stability. The developed method was precise, reproducible and reliable, and successfully applied to the quantification of OGTWE and commercial OGT granules.

2. Variation in the amounts of the 18 marker compounds were observed among OGT samples.

3. The relationship between OGTWE and commercial granules, as evaluated by the Pearson correlation coefficient and PCA, was not close.

The results of this analytical study provide chemical information on OGT preparations. The quantitative analysis developed will be useful for further quality assessment.

Conflict of interest

The authors declare no conflict of interest in this work

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