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Simultaneous Determination of Seven Compounds in *Houttuynia cordata* using UPLC-PDA

Hyo Seon Kim[1], A Yeong Lee[1], Jun Yeon Park[1], Ji Eun Jo[1], Byeong Cheol Moon[1], Jin Mi Chun[1], ki Jung Kil[2], and Ho Kyoung Kim[3,*]

[1]Basic Herbal Medicine Reesarch Group, Korea Institute of Oriental Medicine, Yuseong-daero 1672, Yuseong-gu, Daejeon 305-811, Republic of Korea
[2]Department of Oriental Pharmaceutical Science, Joongbu University, Geumsan 312-702, Republic of Korea
[3]Herbal Material Management Team, Korea Institute of Oriental Medicine, Yuseong-daero 1672, Yuseong-gu, Daejeon 305-811, Republic of Korea

Abstract – A UPLC method for the simultaneous determination of seven compounds was established for the quality control in *H. cordata*. The UPLC was performed on a C_{18} HSS T3 2.1 × 100 mm, 1.8 μm column during a 13 minute gradient elution of 0.2% aqueous acetic acid and acetonitrile with the flow rate of 0.2 mL/min at 30 °C. The UPLC method was validated according to the ICH guideline of analytical procedures with respect to precision, accuracy, and linearity. The limit of determination and quantitation for the seven compounds were 0.01-0.09 and 0.03-0.28 μg/mL, respectively. The calibration curves of all seven compounds showed good linearity ($r^2 > 0.999$). The intra-day and inter-day the RSD values used to evaluate the precision of analysis were less than 0.9%. The recoveries of quantified compounds ranged from 98.63 to 103.85%. The developed UPLC method was found to be effective, convenient and sensitivity for quantitative analysis of seven compounds in *H. cordata*. This work could be provided a baseline source for quality control of *H. cordata.*
Keywords – *Houttuynia cordata*, UPLC-PDA, Quantitative analysis

Introduction

Houttuynia cordata Thunb. (Saururaceae) is a traditional medicinal herb with febrifugal, uretic and detoxifying effects that is used for the treatment of inflammatory diseases such as ureteritis, cervicitis, and atopy in China and Korea (Lu *et al*., 2006; Kim *et al*., 2007). The chemical constituents of *H. cordata* belong to the six active classes of volatile oils, flavonoids, alkaloids, fatty acids, sterols and polyphenolic acid (Bansiddhi *et al*., 2003; Ch *et al*., 2007; Meng *et al*., 2007; Toda, 2005; Nitra *et al*., 2009). Most previous studies of *H. cordata* focused on the chemistries of its essential oils by Gas Chromatography Mass Spectrometry (GC-MS) (Kwon *et al*., 1996). However, GC-MS has only been used to analyze volatile components in *H. cord*ata (Meng *et al*., 2005). In recent reports, phenolic, alkaloid and flavonoids components were analyzed by high performance liquid chromatography (HPLC). However, These HPLC analysis studies of *H. cordata* are time consuming; simultaneous quantification of eight major bioactive chemical constituents of *H. cordata* requires an analysis time of 90 min and quantification of only four or five flavonoids requires an analysis time of 50-70 min (Meng *et al*., 2008; Wu *et al*., 2009; Meng *et al*., 2009).

To address this problem, techniques such as ultra performance liquid chromatography (UPLC) have been used for pharmaceutical and biomedical analysis. UPLC is characterized by high performance, good separation, short analysis time and high sensitivity (Cheng *et al*., 2010). However, previous study on UPLC analysis of *H.codata* is not reported yet.

Therefore, simultaneous quantitative analysis of seven compounds was performed by using UPLC/PDA. In this study, among total seven standard compounds, three flavonoids was isolated from *H. cordata.* The method developed in this paper is suitable for the quality control of *H. cordata* samples from different cultivation regions. This report is the first quantitative analysis of *H. cordata* using UPLC-PAD and presents an effective method for evaluating herbal products that offers high sensitivity, low retention time and requires minimal solvent.

*Author for correspondence
Tel: +82-42-868-9502; E-mail: hkkim@kiom.re.kr

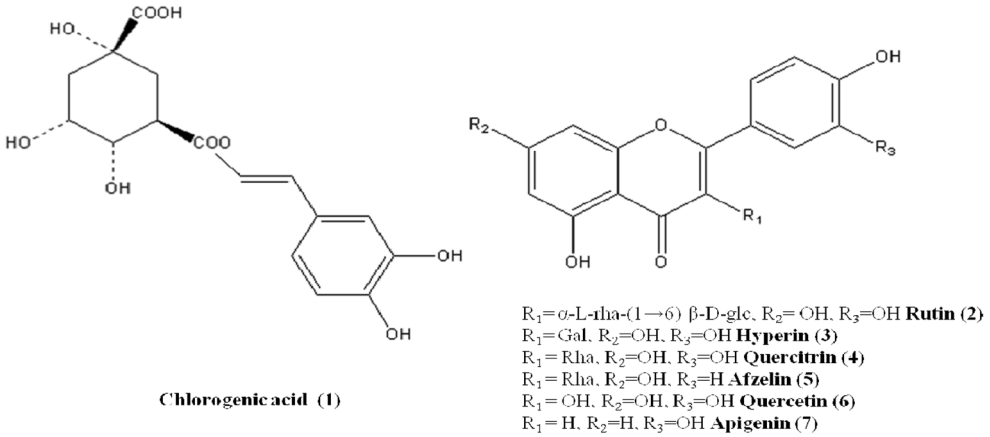

Fig. 1. Chemical structures of seven compounds.

Materials and methods

Chemicals and reagents – HPLC grade methanol, ethanol, 2-propanol, *n*-hexane, dichloromethane, ethyl acetate, acetonitrile, acetic acid and distilled water were obtained from J. T. Baker Inc. (Phillipsburg, NJ, USA). Standard compounds of chlorogenic acid and quercetin were obtained from Sigma-Aldrich (Steinheim, Germany), rutin and hyperin were obtained from ChromaDEX (Laguna Hills, CA, USA) and ROTICHROM (Roth AG, Germany), respectively. Other compounds including quercitrin, afzelin, and apigenin were isolated and purified from dichloromethane and ethyl acetate fractions of *H. cordata*. The structures of compounds 1 - 7 are shown in Fig. 1. The isolated compounds structures were identified as ^{1}H NMR, ^{13}CNMR, MS data and were compared with spectroscopic data drawn from the literature (Agrawal, 1989; Almeida *et al*., 1998; Han, 2008; Miyazawa and Hisama, 2003).

Plant materials – Samples of *H. cordata* (H1-H12) were directly supplied from major cultivation regions: Gyeongsangdo (H1-H6), Jeolla-do (H7-11) and Chungcheongdo (H12) of Korea. The samples were identified by professor Ki Jung Kil. All voucher specimens have been deposited in the herbarium of the Center of Resources Research at the Korea Institute of Oriental Medicine (KIOM), Korea.

HPLC-PAD analysis – The HPLC equipment used in this study included a Waters Alliance 2695 system coupled with a 2996 Photodiode Array Detector. Data were processed using Empower software (Waters Co., Milford, MA, USA). Separation was carried out with a Luna 5 μ C18(2) 100A (4.6 × 250 mm, 5 μm, no. 00G-4252-E0, Phenomenex Co., Torrance, CA, USA). The mobile phase consisted of 0.2% aqueous acetic acid (A) and acetonitrile (B). The gradient elution was: initial 0 - 20 min, linear change from A-B (95 : 5, v/v) to (80 : 20, v/v); 20 - 40 min, linear change from (80 : 20, v/v) to (79.5 : 20.5, v/v); 40 - 50 min, linear change from (79.5 : 20.5, v/v) to (65 : 35, v/v); 50 - 65 min, linear change from (65 : 35, v/v) to (40 : 60, v/v). The flow rate was 0.6 mL/min and the injection volume was 10 μL. The UV wavelength was between 190 ~ 400 nm. The detection set was recorded at 280 nm. Sample compounds were identified by comparing peak retention times and the UV spectra of the seven standard compounds in chromatograms.

UPLC-PAD analysis – UPLC analyses were performed on a Waters Acquity system (Waters Co., Milford, MA, USA) that consisted of a binary solvent manger, auto sampler and PAD. Chromatographic separation was performed on four types of columns: Waters Acquity® Acquity HSS T3 (2.1 × 100 mm, 1.8 μm), HSS T3 (2.1 × 150 mm 1.8 μm), BEH C_{18} (2.1 × 100 mm, 1.7 μm) (Waters Co., MA, USA), and Kinetex XB-C_{18} 100A (4.6 × 100 mm, 2.6 μm, Phenomenex Co., Torrance, CA, USA). Column temperature was maintained at 30 °C. The mobile phase consisted of (A) 0.2% aqueous acetic acid and (B) acetonitrile. The gradient program was: initial 0 -13 min, linear change from (90 : 10, v/v) to (58 : 42, v/v). The UV wavelength was scanned at 190 ~ 400 nm and recorded at 280 nm. The flow rate of 0.2 ml/min was applied and the sample injection volume was 1.0 μL. The compounds peaks were identified according to retention time and the UV spectra of the seven standard compounds in the chromatogram.

Sample and standard preparation – The dried powders of *H. cordata* (3 g), were extracted by refluxing twice in 25 mL of 80% methanol at 60 °C for 60 min each

time and filled up to 50 mL. For each stock solution, 1 mg of each standard was dissolved with 5 mL of methanol. The working standard solutions were prepared by dilution at five different concentrations to establish calibration curves. The sample extract and standard solutions was filtered through a 0.20 μm membrane filter (PALL Corporation, Ann Arbor, MI, USA) before the sample solution was analyzed with UPLC and HPLC.

Validation of the UPLC method – The newly UPLC method was validated in terms of linearity, precision, accuracy and according to ICH guidelines (ICH Topic Q2B, 1996). The LOD and LOQ for the seven compounds were calculated with 3.3*σ/s and 10*σ/s, where σ is the standard deviation and s is the slope of the regression equation (Park *et al*., 2009). The accuracy of this method was evaluated by a recovery test of standards in each sample. The precision of the developed method was determined using measurements different analysts on three different days. Three different quantities (low, medium, high) of the authentic standards were added to the known real sample.

Results and Discussion

Optimization of extraction conditions – To obtain satisfactory extraction, optimization of extraction condition was done as: to obtain satisfactory extraction method, optimization of extraction condition was carried out. Extraction was carried out by ultrasonication extraction method and reflux extraction method separately. A comparative study on difference in solvents (methanol, ethanol, 2-propanol, n-hexane, dichloromethane, ethyl acetate and acetonitrile) for extraction was carried out with reflux connected. As the methanol extract showed high content of chemical constituents, difference in methanol ratio (40,60 and 80%) was chosen as variant for extraction. 80% methanol showed comparatively high content of chemical constituents so extraction time (30 min 1, 2 and 3 hours) was taken as variant for extraction (Fig. 2). Reflux was shown to be an efficient method for the total extraction of all seven compounds. Chlorogenic acid and rutin were not detected in acetonitrile and ethyl acetate extract. All seven compounds were found at high concentrations in ethanol extract. Methanol extract exhibited the highest concentrations of the seven compounds at approximately 0.30 mg/g. Tests using different ratios of solvent found that 40% methanol has lowest content of seven compounds compared with other. The content of all seven compounds began increasing from 60% methanol extract and showed the highest content in 80% methanol extract. In particular, quercitrin content differed considerably according to the solvent ratio. These results agree with those of previous studies indicating that methanol and water mixtures are effective extraction solvents for herbal medicines because chlorogenic acid and flavonoid glycosides are soluble in polar solvents (Zhou *et al*., 2004; Guo and Xiao, 1996). Therefore, the optimal extraction conditions for *H. cordata* were determined to be two-hours of reflux extraction with 80% methanol.

Suitability, analytical column and temperatures – To develop the UPLC method described in this study, the separation was performed on four differential RP_{18} columns: HSS T3 (length of column 100 mm), HSS T3 (length of column 150 mm), BEH C_{18} and Kinetex XB-C_{18} 100A. We compared these columns according to

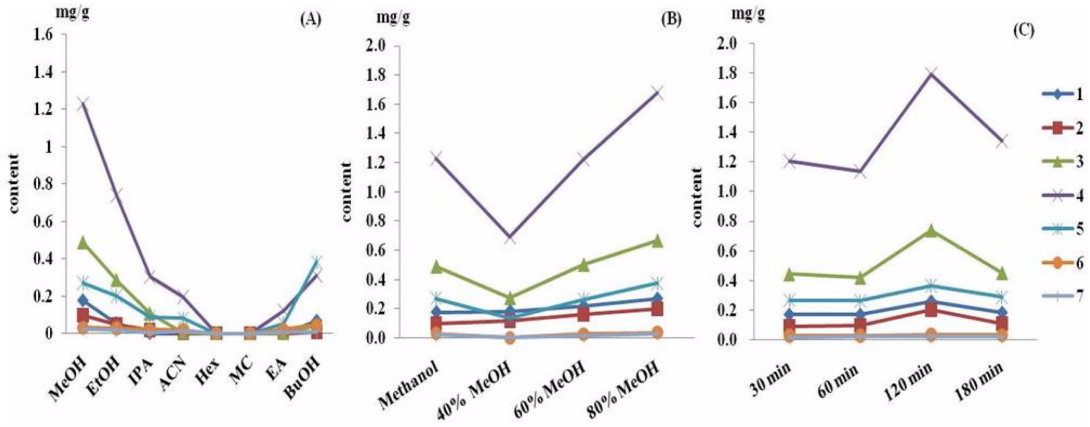

Fig. 2. Optimization of extraction conditions: contents of seven compounds by various extraction solvents (A), extraction solvent concentration (B) and extraction time (C): methanol (MeOH), ethanol (EtOH), 2-propanol (IPA), acetonitrile (ACN), *n*-hexane (Hex), dichloromethane (MC), ethyl acetate (EA), buthanol (BuOH).

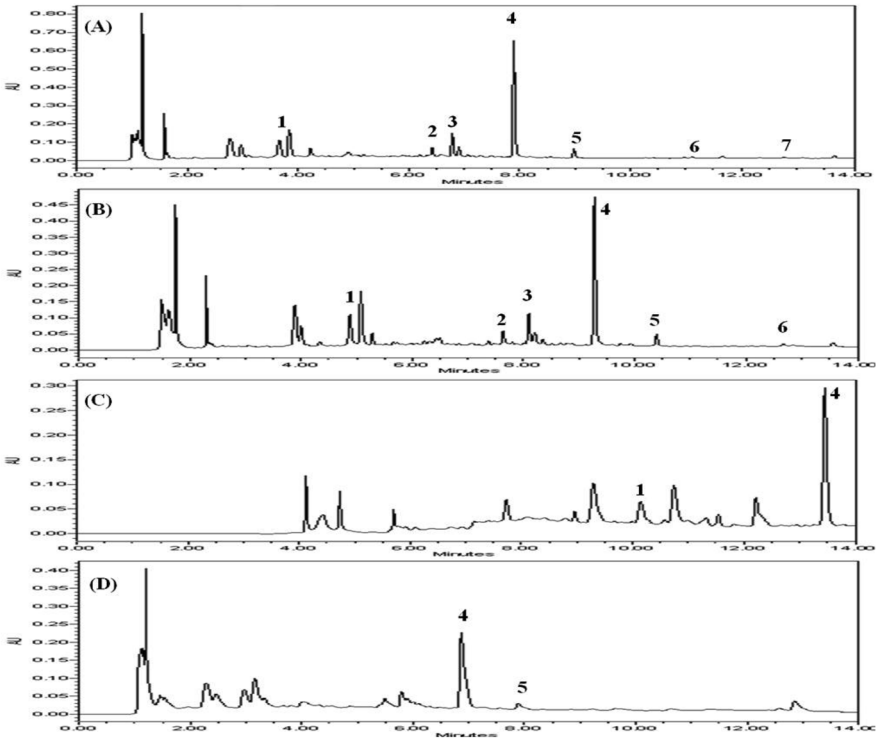

Fig. 3. UPLC chromatograms with different columns of seven compounds in *H. cordata*: HSS T3 (2.1 × 100 mm, 1.8 μm) (A), HSS T3 (2.1 × 150 mm, 1.8 μm) (B), BEH C_{18} (4.6 × 250 mm, 1.7 μm) (C), Kinetex XB-C_{18} (4.6 × 100 mm, 2.6 μm) (D).

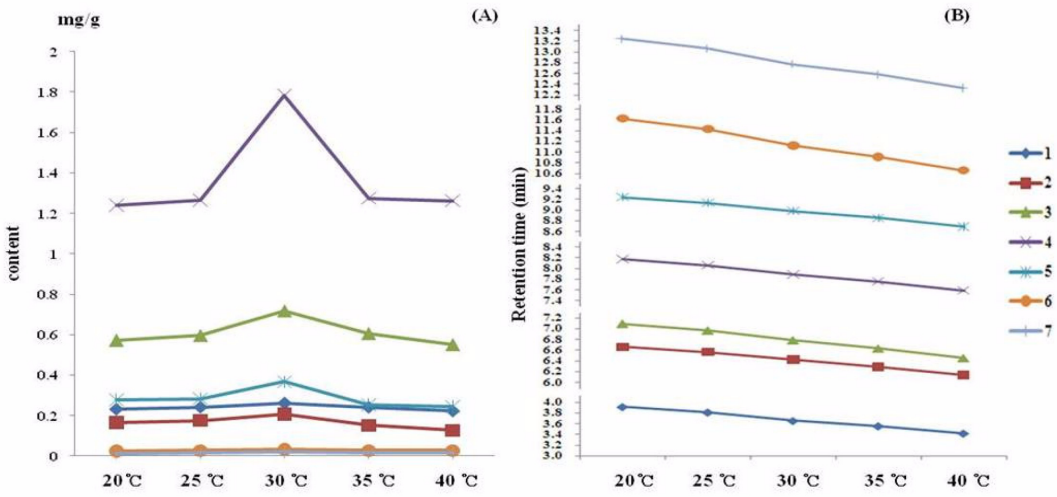

Fig. 4. Contents (A) and retention time (B) according to column temperature in UPLC analysis.

comparative chromatograms of retention times, separations, resolutions and shapes of the seven compounds peaks to identify the best column for use in our analyses (Fig. 3). Therefore, we selected the 100 mm HSS T3 column, which achieved ideal separation of the seven compounds and reduced the analytical time required to 13 min.

To identify the optimal column temperatures, were analyzed at five different column temperatures (Fig. 4). With increases in column temperature, the retention times gradually decreased. The content of each compound declined above 30 ºC, indicating that column temperature is an important factor in quantitative analysis and helps to determine analytical time. In summary, satisfactory separation of *H. cordata* was obtained on an HSS T3

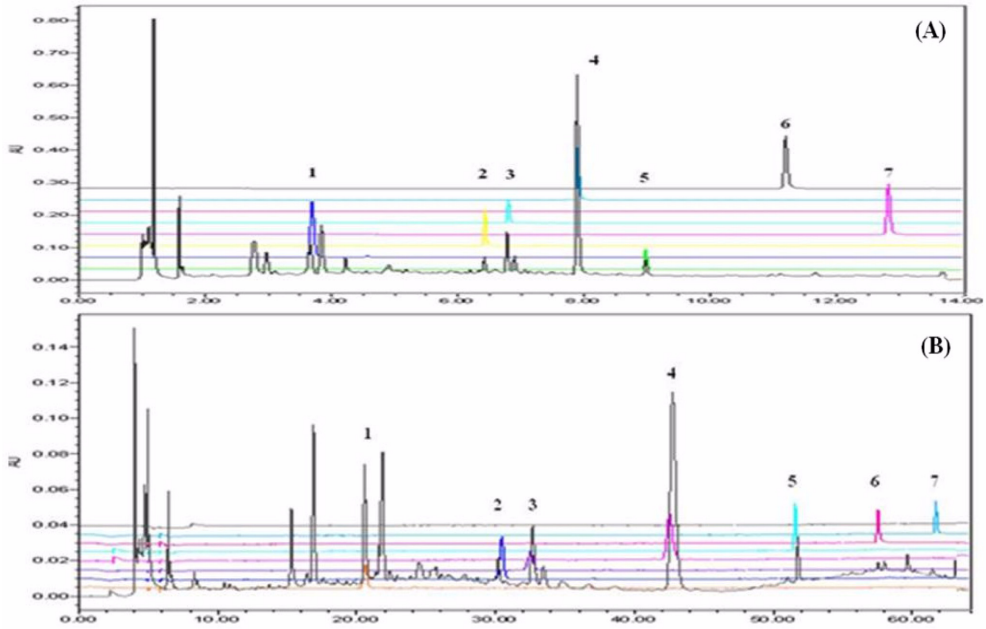

Fig. 5. Comparison of chromatograms of seven compounds in *H. cordata* according to UPLC (A) and HPLC (B).

Table1. Calibration curves, LOD and LOQ data for seven compounds in *H. cordata*

Analytes[a]	Regression equation[b]	Correlation coefficient (R^2)	Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
1	Y = 28730.218x – 5387.609	1.000	0.05 ~ 50	0.01	0.03
2	Y = 11016.478x – 264.657	1.000	0.05 ~ 50	0.03	0.10
3	Y = 7439.117x + 2732.893	1.000	0.5 ~ 150	0.05	0.14
4	Y = 17740.876x + 3943.613	1.000	5 ~ 200	0.09	0.28
5	Y = 7011.289x + 201.333	1.000	0.05 ~ 50	0.03	0.10
6	Y = 28124.994x – 29032.633	0.999	0.05 ~ 100	0.06	0.20
7	Y = 23162.985x + 1356.515	1.000	0.05 ~ 50	0.05	0.15

[a] The analytes are shown in Fig. 1. [b] y is the peak area, x is the concentration.

column (2.1 × 100 mm, 1.8 μm) at a temperature of 30 ºC.

Comparison of UPLC and HPLC – Comparative data on of a performance the HPLC and UPLC techniques using the same sample of *H. cordata* (H-1) are presented in Fig. 5. The contents of two compounds, afzelin (**5**) and apigenin (**7**) were similar in HPLC and UPLC. The contents of chlorogenic acid (**1**), rutin (**2**), hyperin (**3**) and quercetin (**6**) were detected to be 0.01, 0.04, 0.02 and 0.02 mg/g respectively higher in UPLC when compared with HPLC analysis. The content of quercitrin, the main compound, was 0.15 mg/g higher than HPLC in the analysis by UPLC. The retention times of the seven compounds were in the range of 20.346 to 62.364 minutes according to the HPLC analysis, but were in the range of 3.657 to 12.825 minutes according to UPLC analysis. The UPLC analysis required only a quarter of the run-time required for HPLC. The reason could be that the UPLC provided higher peak capacity, better sensitivity and higher analysis speed (Wilson *et al*., 2005). Therefore, we recommend the use of UPLC to simultaneously determine the seven components in *H. cordata*.

Validation of the UPLC method – We performed analyses of seven compounds to investigate the linearity, precision and accuracy of our novel UPLC method. Standard stock solutions of chlorogenic acid (**1**). rutin (**2**), afzelin (**5**) and apigenin (**7**) were diluted to produce a test range from 0.05 - 50 μg/mL and hyperin (**3**), quercitrin (**4**) and quercetin (**6**) were diluted from 0.5 - 150, 5 - 200, and 0.05 - 100 μg/mL, respectively. All of the analyzed compounds presented good linearity ($r^2 > 0.999$) in the concentration ranges examined. The LOD and LOQ of the seven compounds were 0.01 - 0.09 and 0.03 - 0.28 μg/mL, respectively (Table 1). The intra-day and inter-day

Table 2. Intra-day and inter-day variability for seven compounds of *H. cordata*

Analytes[a]	Content (μg/mL)	Intra-day			Inter-day		
		Found (μg/mL)	RSD[b] (%)	Accuracy[c] (%)	Found (μg/m)	RSD (%)	Accuracy (%)
1	5	4.98	0.04	99.82	4.98	0.07	99.81
	25	25.04	0.83	100.07	25.12	0.05	100.23
	50	49.63	0.04	99.63	49.62	0.03	99.62
2	5	5.05	0.17	100.45	5.05	0.19	100.47
	25	25.02	0.11	101.50	25.71	0.82	101.90
	50	50.42	0.08	100.42	50.35	0.18	100.35
3	5	5.02	0.09	100.15	5.02	0.23	100.25
	25	25.40	0.29	100.76	25.37	0.25	100.74
	50	50.60	0.12	100.60	50.61	0.07	100.61
4	5	5.30	0.30	103.04	5.33	0.65	103.33
	25	25.78	0.05	101.56	25.79	0.05	101.59
	50	50.84	0.92	100.24	50.09	0.52	100.09
5	5	5.11	0.08	101.11	5.12	0.20	101.16
	25	25.71	0.03	101.42	25.72	0.10	101.46
	50	51.36	0.16	101.36	51.29	0.14	101.29
6	5	5.34	0.50	103.40	5.35	0.37	103.46
	25	24.81	0.39	99.62	24.85	0.24	99.73
	50	49.30	0.03	99.30	49.26	0.22	99.26
7	5	5.03	0.48	100.28	5.04	0.30	100.40
	25	25.54	0.11	101.08	25.51	0.16	100.97
	50	50.61	0.54	100.61	50.48	0.41	100.48

[a] The analytes are shown in Fig. 1. [b] RSD (%) = 100 × SD/mean, [c] Accuracy (%) = {1-(nominal – mean of measured concentration)/nominal concentration} × 100

Table 3. Recovery of seven compounds by the UPLC method

Analytes[a]	Contents (μg/mL)	Added (μg/mL)	Theoretical amount (μg/mL)	Recorded amount (μg/mL)	Recovery (%)	RSD (%)
1	4.07	1.39	5.27	5.25	99.56	0.35
	4.07	6.34	10.23	10.18	99.51	0.13
	4.07	12.55	16.43	16.42	99.91	0.14
2	2.85	1.28	4.11	4.11	100.14	0.13
	2.85	6.37	9.20	9.08	98.63	0.10
	2.85	12.62	15.45	15.40	99.68	0.05
3	9.90	1.25	11.52	11.58	100.56	0.10
	9.90	6.22	16.48	16.64	101.01	0.42
	9.90	12.37	22.63	23.10	102.08	0.71
4	26.14	1.56	27.48	27.59	100.40	0.33
	26.14	6.61	32.53	33.05	101.58	1.43
	26.14	12.86	38.78	39.23	101.16	0.46
5	5.92	2.34	8.29	8.31	100.27	0.27
	5.92	11.54	17.49	17.51	100.14	1.13
	5.92	23.22	29.17	29.73	101.93	2.92
6	1.26	2.11	2.34	2.43	103.85	2.62
	1.26	6.99	7.22	7.23	100.25	0.29
	1.26	13.10	13.32	13.24	99.41	0.63
7	0.22	1.22	1.50	1.50	100.05	0.58
	0.22	6.35	6.63	6.58	99.28	1.27
	0.22	12.58	12.86	12.79	99.42	0.53

[a] The analytes are shown in Fig. 1.

Table 4. Contents (mg/g) of seven compounds in *H. cordata* from different regions of Korea

Sample NO.	Analytes[a]						
	1	2	3	4	5	6	7
H-1	0.26 ± 0.92	0.20 ± 2.11	0.74 ± 2.95	1.79 ± 4.88	0.37 ± 1.53	0.04 ± 0.51	0.01 ± 1.50
H-2	0.76 ± 3.19	0.31 ± 1.86	2.25 ± 0.64	3.40 ± 2.54	0.49 ± 0.81	0.03 ± 0.68	0.001 ± 0.49
H-3	0.24 ± 1.88	0.16 ± 1.54	0.54 ± 3.45	1.32 ± 3.83	0.27 ± 3.34	0.02 ± 0.21	0.006 ± 1.71
H-4	0.74 ± 4.40	0.24 ± 2.52	0.63 ± 2.60	2.00 ± 3.67	0.37 ± 3.04	0.87 ± 0.30	0.001 ± 4.45
H-5	0.38 ± 1.04	0.16 ± 4.86	0.38 ± 2.65	1.50 ± 4.19	0.27 ± 3.93	0.03 ± 0.62	0.007 ± 3.98
H-6	0.22 ± 1.63	0.20 ± 4.76	0.74 ± 2.05	1.72 ± 4.39	0.30 ± 1.69	0.73 ± 4.85	0.02 ± 3.23
H-7	0.68 ± 1.77	0.18 ± 2.29	0.80 ± 4.45	1.70 ± 4.22	0.28 ± 2.01	0.02 ± 0.17	0.001 ± 4.06
H-8	0.47 ± 1.67	0.21 ± 3.27	0.49 ± 3.19	1.87 ± 4.53	0.42 ± 4.33	0.04 ± 1.42	0.002 ± 1.01
H-9	0.10 ± 4.30	0.07 ± 0.71	0.47 ± 3.17	0.88 ± 0.66	0.16 ± 1.10	0.04 ± 0.38	0.02 ± 1.06
H-10	0.66 ± 3.52	0.28 ± 1.37	0.19 ± 0.65	3.05 ± 2.06	0.49 ± 3.30	0.03 ± 0.68	0.001 ± 1.34
H-11	0.24 ± 2.39	0.09 ± 1.02	0.17 ± 0.52	0.67 ± 1.30	0.13 ± 0.77	0.02 ± 0.03	0.007 ± 2.69
H-12	0.25 ± 1.33	0.13 ± 0.30	0.50 ± 2.78	1.34 ± 4.70	0.27 ± 2.58	0.02 ± 1.09	0.004 ± 0.46

[a]The analytes are shown in Fig. 1.

precisions were determined from the stock solutions in triplicate on three different days, and the RSD of all seven compounds were less than 1.0% (Table 2). The accuracy of the UPLC method was evaluated using the recovery test. The recoveries of seven compounds with RSD of less than 3% were within 98.63 - 103.85% (Table 3). Therefore, our UPLC method exhibits good linearity, precision, and accuracy for determining the seven compounds in *H. cordata.*

Applications – The newly developed UPLC method was used for the simultaneous quantification of seven compounds in *H. cordata* harvested from different regions of Korea. The contents of the seven compounds, chlorogenic acid (**1**), rutin (**2**), hyperin (**3**), quercitrin (**4**), afzelin (**5**), quercetin (**6**) and apigenin (**7**) were within ranges of 0.10 - 0.76, 0.07 - 0.31, 0.17 - 2.25, 0.67 - 3.40, 0.13 - 0.49, 0.02 - 0.87 and 0.001 - 0.02 mg/g, respectively. Results for 12 samples were summarized in Table 4.

H. cordata is now included in the Korean Herbal Pharmacopeia (KHP), however, standard compound for quantitative analysis has not been set. In this study, among the seven compounds, the content of quercitrin was the highest, being greater than those of all other compounds in all samples. Therefore, this experiment suggests that quercitrin as the major standard compound. Among the analyzed samples, the highest contents of quercitrin was found in samples from H-2 (Gyeongsangdo), which contained 0.34% quercitrin, while the lowest was from H-11 (Jeolla-do), which contained 0.07%. The content of each compound represents a large difference, which could be differences in climatic conditions, harvest time, and soil conditions for each cultivation area. Therefore, there is a need of quality control of *H. cordata* on the basis of quantitative analysis of standard compounds cultivated in many different localities.

Conclusions

In this study, we developed a UPLC method for the simultaneous determination of seven compounds in *H. cordata*. This method proved to be easy, efficient and fast compared with previous HPLC analyses. The UPLC method is suitable for validating linearity, precision and accuracy. In addition, this technique is more environmentally friendly than other analytical techniques due to its low solvent requirements. Our results constitute a baseline source of quality control data for *H. cordata*.

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